



HAL
open science

E2F1 controls alternative splicing pattern of genes involved in apoptosis through upregulation of the splicing factor SC35

Galina Merdzhanova, Valérie Edmond, De Serano, Arnaud van Der Broeck, Laurent Corcos, Christian Brambilla, Elisabeth Brambilla, Sylvie Gazzeri, Béatrice Eymin

► **To cite this version:**

Galina Merdzhanova, Valérie Edmond, De Serano, Arnaud van Der Broeck, Laurent Corcos, et al.. E2F1 controls alternative splicing pattern of genes involved in apoptosis through upregulation of the splicing factor SC35. *Cell Death and Differentiation*, 2008, 15 (12), pp.1815-1823. inserm-02337615

HAL Id: inserm-02337615

<https://inserm.hal.science/inserm-02337615>

Submitted on 29 Oct 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **E2F1 and SC35 proteins cooperate to trigger apoptosis through generation of**
2 **mRNAs species encoding apoptotic isoforms.**

3 Galina Merdzhanova^{1,2}, Valérie Edmond^{1,2}, Arnaud Van der Broeck^{1,2}, Laurent Corcos³,
4 Christian Brambilla^{1,2}, Elisabeth Brambilla^{1,2}, Sylvie Gazzeri^{1,2} and Beatrice Eymin^{1,2*}.

5 ¹ Equipe Bases Moléculaires de la Progression des Cancers du Poumon, Centre de Recherche
6 INSERM U823, Institut Albert Bonniot, 38042 Grenoble Cedex, France.

7 ² Université Joseph Fourier, 38041 Grenoble Cedex 09, France.

8 ³ INSERM U613/ EA948, 29238 Brest Cedex 3, France

9

10 * Corresponding author:

11 Dr. Beatrice Eymin

12 Equipe Bases Moléculaires de la Progression des Cancers du Poumon

13 Centre de Recherche INSERM U823

14 Institut Albert Bonniot, BP170

15 38042 Grenoble Cedex 09, FRANCE

16 Tel: +33 4 76 54 94 76 / Fax: +33 4 76 54 94 13

17 email: Beatrice.Eymin@ujf-grenoble.fr

18 To whom requests for reprints should be addressed

19

20

21

22

23

24

25

26

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. Indeed, a single pre-mRNA precursor can generate various transcripts
7 encoding proteins with sometimes opposite functions. In this study, we identify the splicing
8 factor SC35, a member of the Ser-Rich Arg (SR) proteins family, as a new transcriptional target
9 of E2F1. Furthermore, we show that E2F1 directly interacts with SC35 and stimulates its
10 phosphorylation by an AKT-dependent signaling pathway. Importantly, we further demonstrate
11 that E2F1 and SC35 cooperate to switch the alternative splicing profile of various apoptotic genes
12 such as *caspases-8, -9* and *Bcl-x*, towards the expression of pro-apoptotic splice variants. Finally,
13 we provide evidence that E2F1 and SC35 are upregulated and required for cellular apoptosis in
14 response to DNA damaging agents. Taken together, these results demonstrate for the first time
15 that E2F1 controls pre-mRNA processing events to induce apoptosis, and identify the SC35 SR
16 protein as a key E2F1-direct target in this setting.

17

18

19

20

21

22

23

24

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 ((Black, 2000 ; Smith and Valcarcel, 2000) for review ; Johnson, 2003 #81}).
7 SC35 belongs to the serine/arginine-rich (SR) protein family, one of the most important class of
8 splicing regulators. Members of the SR family have a modular structure consisting of one or two
9 copies of an N-terminal RRM (RNA-recognition motif) followed by a C terminus rich in serine
10 and arginine residues known as the RS domain. They act at multiple steps of spliceosome
11 assembly and participate in both constitutive and alternative splicing ((Sanford et al., 2005), for
12 review). Together with most other splicing factors, SR proteins localize to nuclear subregions
13 termed nuclear speckles (Spector et al., 1991). Extensive serine phosphorylation of the RS
14 domain plays an important role in the regulation of both the localization and the activities of SR
15 proteins (Sanford et al., 2003). While the splicing functions of SR proteins have been well
16 documented "*in vitro*", less is known about the roles and the physiological targets of these
17 proteins "*in vivo*". However, based on gene targeting experiments demonstrating that they are
18 required for cell viability and/or animal development, SR proteins undoubtedly control essential
19 biological functions.

20 Apoptosis is one of the cellular process in which alternative splicing plays an important
21 regulatory role. Indeed, a remarkable number of transcripts that encode proteins involved in the
22 apoptotic pathway are subjected to alternative splicing. This usually drives the expression of
23 proteins with opposite functions, either pro- or anti-apoptotic (Jiang and Wu, 1999 ; Schwerk and

1 Schulze-Osthoff, 2005; Shin and Manley, 2004). Interestingly, changes in SR protein
2 phosphorylation have been observed upon apoptotic stimulation following activation of the Fas
3 receptor (Utz et al., 1998). In addition, "in vitro" and overexpression experiments have
4 suggested a potential role for SR proteins in the control of the splicing of pre-mRNAs encoding
5 apoptotic regulators (Jiang et al., 1998 ; Li et al., 2005). Moreover, depletion of the ASF/SF2 SR
6 protein has been reported to induce apoptosis (Li et al., 2005; Wang et al., 1996). Nevertheless,
7 whether individual SR proteins are necessary to modulate alternative splicing of mRNAs
8 encoding apoptotic factors remains largely unknown, as well as the factors that control
9 expression and/or activity of SR protein in this context.

10 The E2F1 transcription factor belongs to the E2F family encompassing eight members
11 involved in a diverse array of essential cellular functions (DeGregori, 2002 ; DeGregori and
12 Johnson, 2006). E2F1 is best-known for its role in driving cell cycle progression in S phase. In
13 addition, E2F1 can induce apoptosis by mechanisms involving or not its transcriptional function.
14 In human lung tumors, we previously described an altered pattern of E2F1 expression suggesting
15 an important function of this protein during bronchial carcinogenesis (Eymin et al., 2001).
16 Accordingly, we further demonstrated the ability of E2F1 to trigger apoptosis in various lung
17 adenocarcinoma cell lines through caspase-8 activation at the death-inducing signaling complex
18 (Salon et al., 2006). In this setting, we also provided evidence that E2F1 acted through the
19 specific downregulation of the cellular FLICE-inhibitory protein short isoform. *c-flip*
20 predominantly encodes two isoforms, namely c-FLIP_{short} and c-FLIP_{long}, that arise from alternative
21 splicing. Aiming at deepen the molecular mechanisms by which E2F1 specifically affects c-
22 FLIP_{short} expression, we postulated that E2F1 could control the expression and/or activity of some
23 splicing factors. In this study, we identify *sc35* as a direct transcriptional target of E2F1. In

1 addition, we show that E2F1 interacts directly with SC35 and stimulates its phosphorylation by
2 an AKT-dependent signaling pathway. Moreover, we provide evidence that SC35 is required for
3 the downregulation of c-FLIP_{Short} by E2F1 and further apoptosis, but also that both proteins
4 cooperate to switch the alternative splicing profile of *caspases-8, -9* and *Bcl-x*, towards the
5 expression of pro-apoptotic splice variants. Finally, at a physiological level, we demonstrate that
6 both E2F1 and SC35 accumulate in response to genotoxic stresses and cooperate to induce
7 apoptosis. Taken together, these results demonstrate for the first time that E2F1 controls pre-
8 mRNA processing events to induce apoptosis, and identify the SC35 SR protein as a key E2F1-
9 direct target in this setting.

10

11 **Material and methods**

12

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

14 A549, H358 and H1299 human lung carcinoma cell lines were cultured in 5% CO₂ at
15 37°C in RPMI-1640 medium (GIBCO, Cergy Pontoise, France), supplemented with 10% (v/v) of
16 heat-inactivated FCS. The H69 small cell lung carcinoma cell line was cultured in 5% CO₂ at
17 37°C in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2.5 mg/ml
18 glucose, 10 mM HEPES and 1 mM sodium pyruvate. The H810 large cell neuroendocrine lung
19 carcinoma cell line was cultured in 5% CO₂ at 37°C in RPMI-1640 medium supplemented with
20 5% (v/v) FCS, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM
21 hydrocortisone, 10 nM β-oestradiol and 10 mM HEPES. Murine embryonic fibroblasts (MEF)
22 wild-type and E2F1^{-/-} were cultured in DMEM (GIBCO) supplemented with 10%(v/v) of heat-
23 inactivated FCS. H358/Tet-On/E2F1 and Tet-On/E2F1(E132) inducible clones were obtained as

1 previously described (Salon et al., 2006). Expression of E2F1 or the mutant E2F1 (E132) unable
2 to bind DNA was induced when cells were cultured in presence of 1 $\mu\text{g/ml}$ doxycyclin. Apoptosis
3 was evaluated by scoring the percentage of apoptotic cells on 500 cells after Hoechst 33342
4 staining. Transient transfections were carried out using Fugene 6 (Roche Diagnostic). Plasmids
5 used in transient transfections were pcDNA3, pCMV-E2F-1, pcDNA3-HA-SC35 and pR264-
6 CAT. Wortmanin and AKT inhibitor VIII were purchased from Calbiochem. Recombinant
7 soluble human FLAG-tagged FasL was purchased from Alexis (San Diego, CA, USA).
8 Melphalan, methanesulfonic acid methyl ester (MMS), cyclophosphamide monohydrate and
9 etoposide were all purchased from Sigma (Saint Quentin Fallavier, France).

10

11 **Antibodies**

12 The anti-E2F1 (C-20), anti-SC35 (H-55), anti-Bcl-x_L (H5) and anti-GST (B14) antibodies
13 were purchased from Santa Cruz, the anti-Bcl-x_s (Ab-1) from Oncogene Research, the anti-
14 phospho-AKT(Thr308) and anti-AKT from Cell Signaling, the anti-E2F1 (KH95) and anti-
15 procaspase-3 from Pharmingen, the anti-FLIP (NF6) from Alexis, the anti-actin (20-33) and anti-
16 phospho-SC35 from Sigma, the anti-phospho-SC35 from Abcam, the anti-SC35 (4F-11) from
17 Euromedex and the anti-SRp20 (7B4) and anti-SF2/ASF from Zymed.

18

19 **CAT assays**

20 For CAT assay measurements, 2×10^5 cells per well were seeded in duplicate in 6-wells
21 plates, and transfected with the pR264CAT plasmid in the presence or absence of increasing
22 amounts of pCMV-E2F1 vector. pR364CAT vector contains the 1kb human *sc35* promoter
23 (Sureau et al., 1992) and encompasses two putative E2F1 binding sites at -170 (TTTGGCCCG)

1 and -236 (TTTCGCGGG) bp upstream of the transcription start site. Transfection was performed
2 using Fugene 6 according to the manufacturer's instructions, and CAT activity was measured 24-
3 48 hours after transfection using CAT ELISA (Roche Diagnostic). CAT activity was then
4 normalized in each sample according to the protein amount.

6 **Chromatin immunoprecipitation experiments**

7 Chromatin immunoprecipitation experiments were performed in H358/Tet-On/E2F1 cells
8 cultured in the presence of 1 $\mu\text{g/ml}$ doxycyclin for 24 hours. Cells were cross-linked for 5 min by
9 adding formaldehyde drop-wise directly in the media to a final concentration of 0.75%. Cross-
10 linking was stopped by adding 0.125M glycine for 5 min at RT, then cells were scraped, rinsed
11 with cold PBS1X and collected by centrifugation. Pellet was resuspended in FA lysis buffer
12 (50mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8, 1% Triton X-100, 0.1%
13 Sodium Deoxycholate, 0.1% SDS) and sonicated to shear DNA to an average fragment size of
14 500 to 1000 bp. For input, 50 μl of each sonicated sample were diluted in 400 μl elution buffer
15 (1% SDS, 100mM NaHCO₃) and incubated overnight at 65°C with 100 μg proteinase K. DNA
16 was recovered by phenol-chloroform extraction, ethanol precipitation and taken up in 100 μl H₂O.
17 For immunoprecipitation, 25 μg of chromatin were precleared and immunoprecipitated with a
18 polyclonal antibody specific for E2F1 (C-20, Santa Cruz) or unrelated rabbit IgG or no antibody,
19 overnight at +4°C. Immune complexes were recovered with blocked protein A/G beads. The
20 beads were washed thoroughly, then the complexes were eluted, cross-links were reversed, and
21 material was recovered by phenol-chloroform extraction and ethanol precipitation. DNA was
22 resuspended in 100 μl H₂O and coprecipitated chromatin was analysed by PCR for the presence
23 of *sc35* promoter DNA between -296 and -79 bp upstream of the *sc35* transcription start site.

1 This fragment encompasses two putative E2F1 binding sites at -170 and -236 bp upstream of the
2 transcription start site. The primers used were as follow: forward 5'-
3 GAGCACCTCCTCTTCCTCCTG-3' and reverse 5'-CCGGAAATGAAACCTTCTGA-3'. PCR
4 conditions were 94°C for 2 min, (94°C 30 sec, 55°C 30 sec, 72°C 30 sec) for 35 cycles, and 72°C
5 for 10 min.

6

7 **Transfection of siRNA oligonucleotides**

8 The sequences designed to specifically target human *sc35* and *e2f1* RNAs were as follow:

9 *sc35*(1): 5'-GCGUCUUCGAGAAGUACGGTT-3'; *sc35*(2): 5'-
10 UCGUUCGCUUUCACGACAATT-3'; *e2f-1*(1): 5'-GUCACGCUAUGAGACCUCATT-3';
11 *e2f1*(2): 5'-ACAAGGCCCGAUCGAUGUUTT-3'. The scrambled siRNA oligonucleotides used
12 as control for all RNA interference experiments were as follow: 5'-
13 UCGGCUCUUACGCAUUCAATT-3' and 5'-CAAGAAAGGCCAGUCCAAGTT-3'. Cells
14 were transfected with siRNA oligonucleotides duplex using Oligofectamine reagent according to
15 the manufacturer's instruction (Invitrogen). Doxycyclin (1 µg/ml) was added or not in the culture
16 medium 4 hours after transfection. The cells were analyzed 48 or 72 hours post-transfection. For
17 experiments with cyclophosphamide, cells were transfected for 48 hours with *mismatch*, *sc35* or
18 *e2f-1* siRNAs, then cyclophosphamide (50 µM) was added in the culture medium for 24
19 additional hours.

20

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

22 Total cellular RNAs were isolated using Trizol reagent (Invitrogen). In all condition, 1 µg
23 of total RNA was reversed transcribed using oligo(dT) primer and MMLV reverse transcriptase

1 (Invitrogen) according to the manufacturer's protocol. The different primer sequences used in this
2 study as well as the PCR conditions are recapitulated in the Supplementary Table 1.
3 Amplification of a fragment of the cDNA of G3PDH (Invitrogen) was performed in the same
4 PCR reaction as an internal control. PCR products were run on a 1-2% agarose gel and visualized
5 by ethidium bromide staining.

6

7 **Immunoblotting and immunoprecipitation experiments**

8 For immunoblotting, lung tumor cell lines were lysed in ice-cold lysis buffer (5 mM
9 EDTA, 150 mM NaCl, 100mM Tris, pH 8, 0.5% sodium deoxycholate, 0.5% Nonidet-P40, 0.5%
10 sodium dodecyl sulfate (SDS), 0.1% aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, and 1 mM
11 PMSF) for 30 min at +4°C and centrifugated for 30 min at 10000rpm. Supernatants were
12 collected and frozen at -80°C until use. Proteins (40 μ g) were denatured in Laemmli buffer (60
13 mM Tris-HCl pH 6.8, 20% glycerol, 10% β -mercaptoethanol, 4.6% SDS, 0.003% bromophenol
14 blue), separated by 7-10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and
15 electroblotted on PVDF membrane (Hybond P, Amersham, Les Ulis, France). Non specific
16 binding sites were blocked by incubating the membranes in 5% non-fat milk TPBS (Tween 0.1%,
17 PBS 1X) or 5% BSA/TTBS (Tween 0.1%, TBS1X) for P-SC35 and P-AKT, for 1 hour at room
18 temperature. Then, membranes were incubated overnight at +4°C with primary antibody in 2%
19 non-fat milk TPBS, washed three times in TPBS, incubated with secondary horseradish
20 peroxydase-conjugated goat anti-mouse or anti-rabbit antibodies (The Jackson Laboratory, West
21 Grove, PA) for 30 minutes and revealed using enhanced chemoluminescence detection kit (ECL,
22 Amersham). To ensure equal loading and transfer of proteins, the membranes were subsequently
23 probed with an anti-actin antibody.

1 For immunoprecipitation experiments, cells were lysed in ice-cold TNE buffer (120mM
2 NaCl, 50mM Tris pH 7.4, 0.5% NP40, 1mM EDTA) supplemented just before use with a cocktail
3 of protease inhibitors, and incubated for 30 min on ice. The supernatants were cleared by
4 centrifugation at 10000rpm at +4°C for 30 min. Equal amounts of proteins (200µg) were
5 precleared using protein A/protein G sepharose beads and SC35 or E2F-1 protein was
6 immunoprecipitated by using appropriate antibodies. For co-precipitation of endogenous SC35
7 and E2F1 proteins, nuclear extracts of H69 cells were prepared by passing cells several times
8 through a 25G needle in ice-cold buffer containing 250 mM sucrose, 20 mM HEPES-KOH, 1.5
9 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. Nuclei were pelleted
10 by centrifugation and further lysed for 30 min at +4°C in 1 ml ice-cold TNE buffer supplemented
11 just before use with protease and phosphatase inhibitors. After a 15 min centrifugation step at
12 10000 rpm, supernatants containing nuclear proteins were recovered. One mg of total nuclear
13 extracts were then subjected to immunoprecipitation with the anti-SC35 antibody (H-55; Santa-
14 Cruz). An irrelevant rabbit IgG was used as a negative control for immunoprecipitation.

15

16 **Indirect immunofluorescence**

17 Cells were fixed in 2% paraformaldehyde/0.2% Triton 100X/PBS 1X for 10 min at room
18 temperature, washed one time in PBS and permeabilized in 100% acetone for 5 min at -20°C.
19 After extended washes, non specific binding sites were saturated for 45 min at RT in the presence
20 of 1% BSA, 5% goat serum in PBS and incubation was carried out for 2h at RT in PBS, 2% BSA
21 with rabbit anti-E2F-1 (C-20, 1/2000) and mouse anti-phospho-SC35 (1/2000, Sigma) primary
22 antibodies. After three PBS washes, AlexaTM 488 goat anti-mouse and AlexaTM 568 goat anti-
23 rabbit IgG (H+L) conjugates (2 mg/ml, 1/1000, Interchim, Montluçon, France) were added and

1 cells were further incubated for 30 min in dark at 37°C. Cells were then washed three times in
2 PBS, counterstained with Hoechst 33342 (1/5000) and observed using an Olympus microscope (x
3 40-60 magnification). Images were captured with a Coolview CCD camera (Photonic Science)
4 and digitally saved using Visilog software.

5

6 **GST pull-down assay**

7 GST-fusion proteins encoding SC35 or E2F1 as well as their truncated forms were
8 prepared according to the manufacturer's protocol (Bulk GST Purification Module, Pharmacia
9 Biotech) and GST pull-down assays were performed as previously described (Eymin et al.,
10 2001). Briefly, beads coated with either GST, GST-E2F1 or its truncated mutants fusion proteins
11 were incubated with equivalent amounts of "*in vitro*" translated wild type SC35 protein. Beads
12 were washed three times in 20mM Tris, pH 7.5, 150mM NaCl, 0.1% Tween 20 and separated
13 onto a 10% SDS-PAGE gel. The interaction between SC35 and the GST-E2F1-coated beads was
14 detected by immunoblotting using an anti-SC35 antibody (4F-11, Euromedex). Conversely,
15 beads coated with either GST, GST-SC35 or its truncated mutants fusion proteins were incubated
16 with equivalent amounts of "*in vitro*" translated wild type E2F1 protein and binding of E2F1 was
17 detected by immunoblotting using an anti-E2F1 antibody (KH95, Pharmingen). In order to ensure
18 the equal amount of GST-fusion proteins used in each case, immunoblotting experiment was
19 performed in parallel using an anti-GST antibody.

20

21

22

23

1 **Results**

2

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

4 We previously established a model of stable E2F1-inducible clones in the H358 cell line
5 derived from a human lung adenocarcinoma (Salon et al., 2006). In these cells, we demonstrated
6 that expression of E2F1 by doxycyclin induced apoptosis by a mechanism involving the
7 downregulation of the anti-apoptotic c-FLIP_{short} protein isoform (Salon et al., 2006). Interestingly,
8 these results were not observed when using an E2F1(E132) DNA-binding defective mutant
9 inducible clone, indicating that the DNA binding activity of E2F1 was required in this context.
10 Because c-FLIP isoforms arise from pre-mRNA alternative splicing, we wanted to determine
11 whether E2F1 could control this process notably by modifying the expression pattern of SR
12 proteins, one of the most important class of splicing regulators. To do so, we first studied the
13 expression of three major SR proteins SC35, SRp20 and SF2/ASF, in our E2F1-inducible cells.
14 Immunoblotting experiments demonstrated an increased expression of SC35 in cells
15 overexpressing E2F1, whereas the total level of SRp20 and SF2/ASF was not affected (Figure
16 1A, left panel). This effect required the DNA binding activity of E2F1 since overexpression of
17 E2F1(E132) did not affect SC35 protein level (Figure 1A, right panel). As measured by semi-
18 quantitative RT-PCR, we showed that expression of *sc35* mRNA was also induced by E2F1 but
19 not by E2F1(E132) (Figure 1B), suggesting that E2F1 stimulates the transcription of *sc35*. To test
20 this hypothesis, we performed Chloramphenicol Acetyl Transferase (CAT) experiments using the
21 pR264-CAT plasmid that contains the 1kb human *sc35* promoter (Sureau et al., 1992). Co-
22 transfection of H1299 (Figure 1C) or SAOS2 (data not shown) cells with the pR264-CAT vector
23 and increasing amounts of an E2F1 expression vector resulted in a dose-dependent increase of

1 CAT activity. Therefore, these results indicated that E2F1 can transactivate the promoter of *sc35*.
2 To confirm these results “*in vivo*”, we performed chromatin immunoprecipitation (ChIP)
3 experiments in the H358/Tet-On/E2F1 cells cultured in the presence of doxycyclin. As depicted in
4 Figure 1D, the results clearly showed that the *sc35* promoter DNA was precipitated by an anti-
5 E2F1 antibody. Altogether, these results identify *sc35* as a transcriptional target of E2F1.

6 Finally, we wanted to confirm that SC35 is a target of E2F1 in other cellular models. We
7 knocked-down E2F1 expression by using small interfering RNAs (siRNAs) in the H69 and H810
8 neuroendocrine lung carcinoma cell lines that express high levels of E2F1, and analysed SC35,
9 SRp20 and SF2/ASF expression by western blotting. As shown in figure 1E, the silencing of
10 E2F1 was accompanied by a strong downregulation of the endogenous SC35 protein in both cell
11 lines as compared to cells transfected with *mismatch* siRNA. In contrast, the expression of SRp20
12 and SF2/ASF was not affected. Moreover, SC35 protein level was strongly reduced in E2F1
13 knock-out MEF as compared to wild-type MEF, whereas those of SRp20 and SF2/ASF did not
14 change (Figure 1F). Taken together, these data confirm in various cellular models that SC35 is a
15 specific target of E2F1.

16

17 **E2F1 modifies the splicing pattern of *sc35***

18 It has been previously shown that SC35 autoregulates its own expression by promoting
19 splicing modifications of its mRNA (Sureau et al., 2001). Therefore, having demonstrated that
20 E2F1 induces SC35 accumulation, we asked whether E2F1 could affect *sc35* splicing events. To
21 detect the various splicing isoforms of *sc35*, the 3' untranslated region of *sc35* was amplified by
22 RT-PCR using specific primers as previously reported (Pilch et al., 2001) (Figure 2A). In cells
23 cultured in the absence of doxycyclin, only a PCR product corresponding to the 2.0 kb mRNA

1 transcript was detected (Figure 2B). Interestingly, overexpression of E2F1 but not E2F1(E132)
2 led to the appearance of an additional PCR product corresponding to the 1.7 kb mRNA (Figure
3 2B). Importantly, this transcript was previously found to preferentially accumulates in cells
4 overexpressing SC35 (Sureau et al., 2001). Therefore, these results imply that E2F1 is able to
5 modify *sc35* splicing events, through SC35 upregulation.

6

7 **E2F1 stimulates SC35 phosphorylation by AKT signaling pathway**

8 It is well-known that the activity and subcellular localization in nuclear speckles of SR
9 proteins are regulated by extensive phosphorylation of their RS domain (Graveley, 2000).
10 Therefore, having demonstrated that E2F1 triggers SC35 accumulation, we asked whether it
11 could also activate signaling pathways involved in the phosphorylation of SC35. To answer, we
12 performed immunoblotting experiments in the H358/Tet-On/E2F1 cells using a mouse
13 monoclonal antibody recognizing a phospho-epitope in SC35. As shown in Figure 3A,
14 accumulation of a phospho-SC35 product was found upon E2F1 expression. Furthermore, by
15 using immunofluorescence studies, we also observed that the phospho-SC35 protein localized in
16 nuclear speckles of larger size in E2F1-expressing cells as compared to control cells (Figure 3B).
17 Therefore, these results demonstrated that E2F1 stimulates the phosphorylation of SC35 as well
18 as its accumulation into nuclear speckles.

19 To date, several kinases have been reported to phosphorylate SR proteins on serine
20 residues, including SRPK (Gui et al., 1994), Clk (Colwill et al., 1996) and DNA topoisomerase I
21 (Rossi et al., 1996). However, which kinase phosphorylate SR proteins in a particular setting
22 remains largely unknown. It was recently shown that the AKT kinase was also able to
23 phosphorylate SR proteins (Blaustein et al., 2005). In addition, activation of AKT by E2F1 has

1 been previously described (Chaussepied and Ginsberg, 2004). Therefore, we postulated that
2 E2F1-mediated SC35 phosphorylation could require AKT signaling pathways. Using a specific
3 antibody recognizing the active phospho-threonine 308 form of AKT, we first confirmed by
4 western blotting in our cellular model the activation of AKT upon E2F1 expression (Figure 3C).
5 Then, to test whether AKT was involved in SC35 phosphorylation by E2F1, we treated our cells
6 with wortmanin or Akt inhibitor VIII, two potents cell-permeable pharmacological inhibitors of
7 AKT, and analyzed SC35 and P-SC35 expression by western blot. The results showed that
8 phosphorylation of AKT was efficiently inhibited by these agents, as reflected by the absence of
9 P-AKT(Thr308) induction (Figure 3C). Furthermore, inactivating AKT strongly inhibited the
10 phosphorylation of SC35 (Figure 3C) as well as its accumulation into nuclear speckles (Figure
11 3D) following E2F1 induction. Of note, such treatments did not affect the total SC35 expression
12 level in cells overexpressing E2F1. Taken together, these data demonstrate that E2F1 stimulates
13 an AKT-dependent phosphorylation of SC35.

14

15 **E2F1 and SC35 proteins directly interact**

16 When performing the immunofluorescence studies, we repeatedly noticed that E2F1 and
17 SC35 colocalized in our cells (Figure 3D). These observations led us to investigate whether both
18 proteins could interact. We first performed co-immunoprecipitation in H1299 cells transiently
19 transfected with a plasmid encoding E2F1. As shown, E2F1 was clearly detected in SC35
20 immunoprecipitates (Figure 4A). When the same immunoprecipitation experiment was
21 performed from nuclear extracts of H69 cells that physiologically express high levels of E2F1,
22 the E2F1 protein was also recovered from SC35 immunoprecipitates (Figure 4B). Taken together,
23 these data demonstrated that E2F1 and SC35 proteins interact “*in vivo*”. Next, we examined

1 whether this interaction was direct. To do so, we performed a GST pull-down assay in which “*in*
2 *vitro*” translated SC35 was tested for its ability to interact with recombinant GST-E2F1. As
3 Figure 4C illustrates, SC35 was efficiently retained on GST-E2F1 beads. Furthermore, using
4 various mutant GST-E2F1 fusion proteins, we showed that the 120-191 residues encompassing
5 the DNA binding domain of E2F1 were involved in SC35 interaction (Figure 4C). Interestingly,
6 we noticed that SC35 was also retained, but to a lesser extent, on GST-E2F1(284-437) beads
7 suggesting the existence of another binding site in the carboxy-terminal domain of E2F1. Overall,
8 these results provide the first evidence of a direct interaction between E2F1 and a component of
9 the spliceosome machinery.

10

11 **SC35 is required for E2F1-mediated apoptosis**

12 We previously demonstrated the capacity of E2F1 to induce apoptosis in a model of lung
13 adenocarcinoma cell lines (Salon et al., 2006). In this setting, we further provided evidence that
14 the downregulation of the c-FLIP_{short} protein isoform was necessary and sufficient for apoptosis
15 induction in response to E2F1 (Salon et al., 2006). Both c-FLIP_{long} and c-FLIP_{short} isoforms arise
16 from alternative splicing. Therefore, having demonstrated a direct link between E2F1 and SC35
17 proteins, we first tested whether SC35 could be implicated in the decrease of c-FLIP_{short} upon
18 E2F1 induction. As shown by RT-PCR and immunoblot analyses, the neutralization of SC35 by
19 siRNA prevented the downregulation of both *flip_{short}* mRNA and protein levels in response to
20 E2F1 expression (Figure 5A). These results identified *c-flip_{short}* as a target of SC35 and suggested
21 that SC35 could be involved in E2F1 apoptotic functions. Consistently, as compared to a control
22 *mismatch* siRNA, the use of *sc35* siRNAs strongly reduced the number of apoptotic cells in
23 response to E2F1, as detected after Hoechst staining (Figure 5B). Overall, these results indicated

1 that SC35 contributes to E2F1-dependent apoptosis likely by modifying the $\text{flip}_{\text{long}}/\text{flip}_{\text{short}}$ ratio.
2 Moreover, treating H358/Tet-On/E2F1 cells with wortmanin or AKT inhibitor VIII prevented
3 both c-FLIP_{Short} downregulation (Figure 5C) and apoptosis (data not shown) induced by E2F1
4 suggesting that AKT-dependent phosphorylation of SC35 could be involved in this process.

5 A high level of the c-FLIP protein has been found in many tumor cells and was correlated
6 with resistance to FAS- and TRAIL-induced apoptosis, two death receptor ligands (Tschopp et
7 al., 1998). Consistently, we previously reported that the downregulation of c-FLIP_{Short} by E2F1
8 was sufficient to restore the sensitivity of tumor cells to these ligands (Salon et al., 2006).
9 Therefore, we investigated whether SC35 could play a role in this process. As measured by
10 Hoechst staining, we showed that neutralization of *sc35* expression overrode the capacity of
11 E2F1 to sensitize H358 cells to FasL treatment (Figure 5D).

12 Collectively, these data demonstrate that SC35 is involved in E2F1-mediated apoptosis
13 and affects the $\text{FLIP}_{\text{Long}}/\text{FLIP}_{\text{Short}}$ ratio, at the expense of the $\text{FLIP}_{\text{Short}}$ protein isoform.

14

15 **E2F1 and SC35 cooperate to regulate the splicing pattern of *caspase-8*, *caspase-9* and** 16 ***Bcl-x* pre-mRNAs in favor of splice variants encoding pro-apoptotic isoforms**

17 The expression of numerous apoptotic genes is regulated by alternative splicing that
18 triggers the synthesis of various mRNA species encoding proteins with sometimes opposite
19 functions (Alnemri et al., 1995; Boise et al., 1993; Papoff et al., 1996; Shaham and Horvitz,
20 1996; Wang et al., 1994). Having demonstrated that E2F1 modify the $\text{flip}_{\text{Short}}/\text{flip}_{\text{Long}}$ ratio through
21 SC35 upregulation, we undertook a series of experiments aiming at investigate whether the ratio
22 of other apoptotic splice variants could be affected. Various *caspases* are subjected to alternative
23 splicing. Alternative splicing of *casp-2* proceeds through two mutually exclusive splicing events

1 that selectively include or exclude exon 9, and give rise to anti-apoptotic caspase-2S and pro-
2 apoptotic caspase-2L isoforms respectively (Figure 6A) (Jiang and Wu, 1999; Jiang et al., 1998).
3 The use of a distant splice donor site at the 3'-end of exon 8 of the human *caspase-8* pre-mRNA
4 leads to the synthesis of an alternative spliced variant, *caspase-8L*, a competitive inhibitor of
5 caspase-8 (Eckhart et al., 2001; Himeji et al., 2002; Horiuchi et al., 2000) (Figure 6A). The
6 inclusion or exclusion of an exon cassette in *caspase-9* causes the expression of two splice
7 variants, namely the proapoptotic *caspase-9a* and antiapoptotic *caspase-9b* (Chalfant et al., 2002;
8 Massiello and Chalfant, 2006; Srinivasula et al., 1999) (Figure 6A). To determine whether E2F1
9 induces changes in the alternative splicing profile of these *caspases* pre-mRNAs, RNA recovered
10 from uninduced or induced H358/Te-On/E2F1 cells were analyzed by RT-PCR using primers
11 specific for each caspase splice variant (Figure 6A). The results showed that the expression of
12 E2F1 concomittantly increased pro-apoptotic *caspases-2L*, *-8a* and *-9a* and decreased anti-
13 apoptotic *caspases-8L* and *-9b* mRNA levels (Figure 6B). In contrast, these effects were not
14 observed with E2F1(E132). Of note, we were unable to detect the *caspase-2S* transcript in our
15 cells. Altogether, these data indicate that E2F1 switches the splicing pattern of *caspases-8* and
16 *-9*, at the expense of transcripts encoding the anti-apoptotic isoforms. Therefore, besides its
17 ability to transactivate *caspases-8* and *-9* genes (Nahle et al., 2002), these results demonstrate
18 that E2F1 also controls their alternative splicing.

19 Then, we asked whether E2F1 could modulate the splicing of genes which it does not
20 transactivate. *Bcl-x* is a member of the *bcl-2* gene family that can either promote or prevent
21 apoptosis (Boise et al., 1993). Several spliced isoforms of *Bcl-x* have been reported. The use of a
22 5' proximal site generates the Bcl-x_L large isoform which protects cells against apoptosis. In
23 contrast, the use of a 5' distal site results in the synthesis of a short proapoptotic Bcl-x_S isoform.

1 By performing RT-PCR analysis with specific primers in H358/Tet-On/E2F1 cells, we showed
2 that expression of E2F1 induced a concomitant decrease of *Bcl-x_L* and increase of *Bcl-x_S* mRNA
3 levels (Figure 6C, upper panel). Similarly to *caspases* regulation, the mutant protein E2F1(E132)
4 had no effect on *bcl-x* splicing. Importantly, western blotting with Bcl-x antibodies specific for
5 each isoform confirmed the RT-PCR results (Figure 6C, lower panel). Altogether, these data
6 demonstrate that E2F1 modifies the splicing pattern of several genes that play a key role during
7 the apoptotic process, and favors the expression of transcripts encoding pro-apoptotic isoforms.
8 Finally, we questioned whether SC35 was involved in these effects. siRNAs targeting *sc35* were
9 transfected in H358/Tet-On/E2F1 cells, and expression of *caspases* and *Bcl-x* splice variants was
10 analyzed by RT-PCR. Knockdown of *sc35* in the absence of E2F1 induction did not significantly
11 alter the level of *caspases -2L, -8L, -8a, -9a* or *-9b* mRNAs, nor that of *Bcl-x_L* or *Bcl-x_S* (Figure
12 6D). In contrast, in doxycyclin treated cells, this strongly prevented the ability of E2F1 to affect
13 the splicing pattern of these genes (Figure 6D). Furthermore, when we performed RT-PCR
14 analyses in A549 cells transiently transfected with a vector encoding SC35, we found that
15 overexpression of SC35 affected the splicing profile of *caspase-8, -9* and *Bcl-x* pre-mRNAs in a
16 similar way than did E2F1 (Figure 6E). Overexpression of SC35 also led to apoptosis in these
17 cells (data not shown). Overall, these results demonstrate that E2F1 cooperates with SC35 to
18 regulate pre-mRNA processing events that control the accumulation/downregulation of specific
19 splice variants.

20

21

22

23

1 **E2F1 and SC35 are upregulated and required for apoptosis in response to genotoxic**
2 **stresses**

3 Our results so far demonstrated the ability of E2F1 and SC35 to modify the splicing
4 pattern of various apoptotic genes in a model of overexpression. Thus, we attempted to identify
5 in which physiological context both proteins could cooperate to induce apoptosis. It is now well-
6 known that DNA damaging agents stabilize E2F1 and induce its transcriptional activity towards
7 apoptotic genes, among which are p73, thereby causing apoptosis (Lin et al., 2001; Stevens et al.,
8 2003; Wang et al., 2006). In agreement with previous reports, treatment of H358 cells with either
9 methylmethanesulfonate (MMS) or cyclophosphamide, two alkylating agents that create
10 interstrand DNA crosslinks, strongly increased E2F1 expression as detected by immunoblotting
11 (Figure 7A, upper panel). In these conditions, upregulation of E2F1 was accompanied by an
12 increase of SC35 protein and mRNA expression (Figure 7A, upper panels), as well as by the
13 induction of apoptosis (Figure 7A, lower panel). These results indicate that SC35 could be
14 involved in the cellular response to DNA damage. Of note, accumulation of phospho-SC35 was
15 also observed in these conditions suggesting that phosphorylation of SC35 plays a role in these
16 settings. To study if E2F1 was required for SC35 induction, cells were transfected with *e2f1*
17 siRNAs, treated by cyclophosphamide and analyzed for SC35 expression by western blotting.
18 The results showed that the knockdown of E2F1 prevented the accumulation of SC35 protein in
19 response to cyclophosphamide (Figure 7B). Therefore, it appears that SC35 accumulation
20 requires E2F1 in this setting. Finally, to analyze whether SC35 was involved in
21 cyclophosphamide-mediated apoptosis, we neutralized its expression by siRNAs before treating
22 cells. As detected by immunoblotting of procaspase-3 and Hoechst 33342 staining, neutralization
23 of SC35 strongly prevented the occurrence of apoptosis following cyclophosphamide treatment

1 (Figure 7C). Taken together, these results identify E2F1 and SC35 proteins as essential
2 components of the apoptotic response following genotoxic stresses.

3

4 **Discussion**

5

6 E2F1 is a transcription factor that plays a critical role in cell cycle progression by favoring
7 entry into S phase. Besides its role in cell cycle control, E2F1 is also widely accepted as an
8 inducer of apoptosis. It has been well-demonstrated that E2F1 induces apoptosis through both
9 transcription-dependent and -independent mechanisms. So far, numerous apoptotic genes whose
10 transcription is enhanced by E2F1 have been identified (DeGregori and Johnson, 2006). In this
11 study, we show that E2F1 switches the alternative splicing pattern of key apoptotic genes in favor
12 of their pro-apoptotic splice variants, and identify the SC35 protein, a member of the SR family
13 of splicing regulators, as a key direct target of E2F1 in these settings. Altogether, these results
14 demonstrate for the first time that besides its ability to transactivate apoptotic genes, E2F1 also
15 controls pre-mRNA processing events to induce apoptosis.

16 It has now emerged from the literature that splicing not only depends on the interaction
17 of splicing factors with their target pre-mRNAs, but is also coupled to transcription (Kornbliht,
18 2005). Indeed, the initial observation that variations of pol II promoter structure can lead to
19 differences in alternative splicing of the transcript has supported such interconnection (Cramer et
20 al., 1999 ; Cramer et al., 1997; Pagani et al., 2003). Furthermore, it has been reported that several
21 components of the spliceosome can act as transcriptional coregulators. For example, the p54nrb
22 (p54 nuclear RNA binding protein) and PSF (polypyrimidine tract-binding protein-associated
23 splicing factor) RNA binding proteins as well as the RNA helicases, p68 and p72, have been

1 involved in both transcription and splicing processes (Auboeuf et al., 2005, for review). In
2 addition, it has been shown that transcriptional coregulators of the nuclear receptors family
3 recruited at the promoter level can not only enhance the transcriptional activity of this promoter,
4 but also affect the nature of the spliced variants produced (Auboeuf et al., 2005; Auboeuf et al.,
5 2004a; Auboeuf et al., 2004b). Moreover, several transcription factors bind with proteins of the
6 spliceosome and/or display dual functions in splicing and transcription (Chansky et al., 2001 ; Ge
7 et al., 1998 ; Guillouf et al., 2006; Kameoka et al., 2004). Taken together, these results indicate a
8 role for proteins controlling transcription in splicing regulation. In this study, we reinforce this
9 connection between transcriptional and splicing machineries by providing evidence that the
10 transcription factor E2F1 transactivates the expression of *sc35*, a component of the spliceosome,
11 and more importantly that both E2F1 and SC35 proteins directly interact and cooperate to trigger
12 apoptosis through regulation of pre-mRNA processing events. We show that E2F1 alters the
13 splicing pattern of some of its transcriptional targets such as *c-flip* (Stanelle et al., 2002),
14 *caspases-8* and *-9* (Nahle et al., 2002). Interestingly, we also observe a strong accumulation of
15 some of these spliced variants (*caspases-8a* and *-9a*, Figure 6). Therefore, it is possible that the
16 transactivation of these genes by E2F1, in cooperation with SC35 accumulation, also controls
17 their alternative splicing. Alternatively, it was recently shown that the Spi-1/PU.1 transcription
18 factor could modify alternative splicing of a transcriptional target gene, without modulation of its
19 mRNA transcription (Guillouf et al., 2006). Since we also provide evidence of a direct interaction
20 between E2F1 and SC35 proteins, another but not exclusive possibility is that E2F1 acts as a
21 scaffold protein to drive SC35 to the nascent transcribed RNA of some of its target genes,
22 according to the cell-specific promoter occupation model (Kornblihtt, 2005). In this setting, it

1 remains to determine whether E2F1 tethers SC35 to the promoters of some of the genes whose
2 pre-mRNA splicing is affected by E2F1 expression.

3 Besides their role in pre-mRNA splicing and splice sites selection (Bourgeois et al.,
4 1999), SR proteins are also involved in enhancement of Nonsense Mediated mRNA Decay
5 (NMD) (Caceres et al., 1998 ; Huang and Steitz, 2005; Sanford et al., 2004 ; Zhang and Krainer,
6 2004). NMD is a rare event among human *caspase* mRNAs and affects only mRNA encoding
7 caspase-2S or -10C (Solier et al., 2005). We performed a rapid survey of eight and four
8 transcripts derived from the *flip* and *Bcl-x* genes respectively and did not identify NMD
9 signatures. Therefore, the control of NMD processes by SC35 might not be required for its
10 effects upon E2F1 expression. However, we also show that E2F1 modifies the splicing pattern of
11 *sc35* itself and leads to the accumulation of a 1.7kb *sc35* mRNA. Since it was previously reported
12 that this transcript preferentially accumulates in cells overexpressing SC35 (Sureau et al., 2001),
13 our results are consistent with an upregulation of SC35 in response to E2F1. Moreover,
14 regulation of this 1.7 kb transcript by NMD has been suggested (Sureau et al., 2001) and it was
15 recently confirmed that NMD controls the homeostasis of SR protein levels (Lareau et al., 2007;
16 Ni et al., 2007). Therefore, our results suggest that E2F1 could increase SC35 protein expression
17 through both a transcriptional activation of its promoter and an inhibition of its mRNA
18 degradation, thereby preventing an autonegative feedback regulation.

19 Apoptosis is one of the cellular processes in which alternative splicing plays important
20 regulatory roles (Jiang and Wu, 1999; Schwerk and Schulze-Osthoff, 2005; Shin and Manley,
21 2004). Several components of the splicing machinery have been already involved in apoptotic
22 processes. For example, depletion of SF2/ASF (Li et al., 2005) induces apoptosis. In addition,
23 overexpression of SC35 alters splicing of mRNA encoding caspase-2 such that increased levels

1 of a pro-apoptotic isoform are produced (Jiang et al., 1998). Furthermore, phosphorylation of SR
2 proteins, which is known to control their sub-cellular localization as well as their activities, have
3 been reported during apoptosis (Kamachi et al., 2002; Utz et al., 1998). However, the upstream
4 signaling molecules that regulate the expression and/or activity of SR proteins during apoptosis,
5 as well as the endogenous targets of SR proteins in this context remain largely unknown. Here,
6 we provide evidence that E2F1 triggers apoptosis through SC35 accumulation. In addition, we
7 show that E2F1 stimulates SC35 phosphorylation by an AKT-dependent signaling pathway and
8 further demonstrate that E2F1 and SC35 cooperate to affect the splicing pattern of *caspase-8*,
9 *caspase-9*, *flip* and *bcl-x* genes. Altogether, our data identify SC35 as a new mediator of E2F1-
10 induced apoptosis. Phosphorylation of SR proteins by Clk or SRPK kinases is sufficient to
11 change their localization in nuclear speckles to a diffuse nuclear localization (Caceres et al.,
12 1998; Colwill et al., 1996; Kuroyanagi et al., 1998). In contrast, and consistent with the results of
13 Blaustein and coll (Blaustein et al., 2005) who demonstrated that AKT overexpression did not
14 lead to nuclear speckles disorganization, phospho-SC35 nuclear speckles never disappeared in
15 cells overexpressing E2F1. As AKT was found to elicit opposite effect on splicing to those
16 evoked by Clk and SRPK overexpression (Blaustein et al., 2005), AKT-dependent subcellular
17 localization of SC35 could differentially affect splice site selection upon E2F1 induction. This
18 could explain why pharmacological inhibition of AKT strongly reverses the splicing
19 modifications at the level of *c-flip* (Figure 5A) or *Bcl-x* (data not shown) observed upon E2F1
20 expression. Alterations in alternative splicing also contribute to resistance to chemotherapy,
21 notably through overexpression of anti-apoptotic splice variants in tumor cells (Hayes et al.,
22 2006; Mercatante and Kole, 2000). So far, only one study has reported accumulation of SC35 in
23 response to DNA damage, namely γ -irradiation (Cardoso et al., 2002). In this study, we

1 demonstrate that SC35 is upregulated by an E2F1-dependent pathway in response to alkylating
2 agents. We also show that SC35 is required for the induction of apoptosis in cells treated by these
3 agents or by Fas ligand. Therefore, our results indicate that SC35 plays a role in the response of
4 tumor cells to chemotherapeutic agents, as well as to death receptor stimuli.

5 To conclude, we provide the first evidence of a functional interplay between E2F1 and
6 SC35 proteins to trigger apoptosis, and identify some of their endogenous targets in this setting.
7 E2F1 plays dual functions in the control of both apoptosis and cellular proliferation (Johnson and
8 Degregori, 2006). Therefore, it remains to determine whether E2F1 and SC35 proteins also
9 cooperate to control cell cycle progression.

10

11 **Acknowledgements:**

12 We thank Patricia Betton, Pascal Perron and Celine Lampreia for technical assistance. We thank
13 Johann Soret and Jamal Tazi (IGBMC, Montpellier) for the pECE-SC35HA and pR264-CAT
14 plasmids. We thank Didier Auboeuf, Martin Dutertre, Johann Soret and Jamal Tazi for
15 encouraging discussions. This work was supported by grants from the region Rhône Alpes
16 (Thématique Prioritaire Cancer and Canceropole 2003: Oncocell, Epimed and INACancer), by
17 the Ligue contre le Cancer (Comité de Savoie), by the Ligue Nationale contre le Cancer (Equipe
18 Labellisée), by INCa (PNES, Programme National d'Excellence Spécialisé) and by the Conseil
19 Scientifique National d'AGIR *à dom*. Galina Merdzhanova and Arnaud van der Broeck were
20 supported by a fellowship from the Research French Ministry. Valerie Edmond was supported
21 by a grant from AGIR *à dom*.

22

23

1 **References**

- 2
- 3 Alnemri, E.S., Fernandes-Alnemri, T. and Litwack, G. (1995) Cloning and expression of four
4 novel isoforms of human interleukin-1 beta converting enzyme with different apoptotic
5 activities. *J Biol Chem*, **270**, 4312-4317.
- 6 Auboeuf, D., Dowhan, D.H., Dutertre, M., Martin, N., Berget, S.M. and O'Malley, B.W. (2005)
7 A subset of nuclear receptor coregulators act as coupling proteins during synthesis and
8 maturation of RNA transcripts. *Mol Cell Biol*, **25**, 5307-5316.
- 9 Auboeuf, D., Dowhan, D.H., Kang, Y.K., Larkin, K., Lee, J.W., Berget, S.M. and O'Malley,
10 B.W. (2004a) Differential recruitment of nuclear receptor coactivators may determine
11 alternative RNA splice site choice in target genes. *Proc Natl Acad Sci U S A*, **101**, 2270-
12 2274.
- 13 Auboeuf, D., Dowhan, D.H., Li, X., Larkin, K., Ko, L., Berget, S.M. and O'Malley, B.W.
14 (2004b) CoAA, a nuclear receptor coactivator protein at the interface of transcriptional
15 coactivation and RNA splicing. *Mol Cell Biol*, **24**, 442-453.
- 16 Black, D.L. (2000) Protein diversity from alternative splicing: a challenge for bioinformatics and
17 post-genome biology. *Cell*, **103**, 367-370.
- 18 Blaustein, M., Pelisch, F., Tanos, T., Munoz, M.J., Wengier, D., Quadrana, L., Sanford, J.R.,
19 Muschietti, J.P., Kornblihtt, A.R., Caceres, J.F., Coso, O.A. and Srebrow, A. (2005)
20 Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. *Nat*
21 *Struct Mol Biol*, **12**, 1037-1044.

1 Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X.,
2 Nunez, G. and Thompson, C.B. (1993) bcl-x, a bcl-2-related gene that functions as a
3 dominant regulator of apoptotic cell death. *Cell*, **74**, 597-608.

4 Bourgeois, C.F., Popielarz, M., Hildwein, G. and Stevenin, J. (1999) Identification of a
5 bidirectional splicing enhancer: differential involvement of SR proteins in 5' or 3' splice
6 site activation. *Mol Cell Biol*, **19**, 7347-7356.

7 Caceres, J.F., Sreaton, G.R. and Krainer, A.R. (1998) A specific subset of SR proteins shuttles
8 continuously between the nucleus and the cytoplasm. *Genes Dev*, **12**, 55-66.

9 Cardoso, R.S., Espanhol, A.R., Passos, G.A. and Sakamoto-Hojo, E.T. (2002) Differential gene
10 expression in gamma-irradiated BALB/3T3 fibroblasts under the influence of 3-
11 aminobenzamide, an inhibitor of parp enzyme. *Mutat Res*, **508**, 33-40.

12 Chalfant, C.E., Rathman, K., Pinkerman, R.L., Wood, R.E., Obeid, L.M., Ogretmen, B. and
13 Hannun, Y.A. (2002) De novo ceramide regulates the alternative splicing of caspase 9 and
14 Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. *J Biol*
15 *Chem*, **277**, 12587-12595.

16 Chansky, H.A., Hu, M., Hickstein, D.D. and Yang, L. (2001) Oncogenic TLS/ERG and EWS/Fli-
17 1 fusion proteins inhibit RNA splicing mediated by YB-1 protein. *Cancer Res*, **61**, 3586-
18 3590.

19 Chaussepied, M. and Ginsberg, D. (2004) Transcriptional regulation of AKT activation by E2F.
20 *Mol Cell*, **16**, 831-837.

21 Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C. and Duncan, P.I. (1996)
22 The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their
23 intranuclear distribution. *Embo J*, **15**, 265-275.

1 Cramer, P., Caceres, J.F., Cazalla, D., Kadener, S., Muro, A.F., Baralle, F.E. and Kornblihtt, A.R.
2 (1999) Coupling of transcription with alternative splicing: RNA pol II promoters
3 modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer. *Mol Cell*, **4**, 251-258.

4 Cramer, P., Pesce, C.G., Baralle, F.E. and Kornblihtt, A.R. (1997) Functional association
5 between promoter structure and transcript alternative splicing. *Proc Natl Acad Sci U S A*,
6 **94**, 11456-11460.

7 DeGregori, J. (2002) The genetics of the E2F family of transcription factors: shared functions and
8 unique roles. *Biochim Biophys Acta*, **1602**, 131-150.

9 DeGregori, J. and Johnson, D.G. (2006) Distinct and Overlapping Roles for E2F Family
10 Members in Transcription, Proliferation and Apoptosis. *Curr Mol Med*, **6**, 739-748.

11 Eckhart, L., Henry, M., Santos-Beneit, A.M., Schmitz, I., Krueger, A., Fischer, H., Bach, J., Ban,
12 J., Kirchhoff, S., Krammer, P.H., Mollinedo, F. and Tschachler, E. (2001) Alternative
13 splicing of caspase-8 mRNA during differentiation of human leukocytes. *Biochem*
14 *Biophys Res Commun*, **289**, 777-781.

15 Eymin, B., Gazzeri, S., Brambilla, C. and Brambilla, E. (2001) Distinct pattern of E2F1
16 expression in human lung tumours: E2F1 is upregulated in small cell lung carcinoma.
17 *Oncogene*, **20**, 1678-1687.

18 Ge, H., Si, Y. and Wolffe, A.P. (1998) A novel transcriptional coactivator, p52, functionally
19 interacts with the essential splicing factor ASF/SF2. *Mol Cell*, **2**, 751-759.

20 Graveley, B.R. (2000) Sorting out the complexity of SR protein functions. *Rna*, **6**, 1197-1211.

21 Gui, J.F., Lane, W.S. and Fu, X.D. (1994) A serine kinase regulates intracellular localization of
22 splicing factors in the cell cycle. *Nature*, **369**, 678-682.

- 1 Guillouf, C., Gallais, I. and Moreau-Gachelin, F. (2006) Spi-1/PU.1 oncoprotein affects splicing
2 decisions in a promoter binding-dependent manner. *J Biol Chem*, **281**, 19145-19155.
- 3 Hayes, G.M., Carrigan, P.E., Beck, A.M. and Miller, L.J. (2006) Targeting the RNA splicing
4 machinery as a novel treatment strategy for pancreatic carcinoma. *Cancer Res*, **66**, 3819-
5 3827.
- 6 Himeji, D., Horiuchi, T., Tsukamoto, H., Hayashi, K., Watanabe, T. and Harada, M. (2002)
7 Characterization of caspase-8L: a novel isoform of caspase-8 that behaves as an inhibitor
8 of the caspase cascade. *Blood*, **99**, 4070-4078.
- 9 Horiuchi, T., Himeji, D., Tsukamoto, H., Harashima, S., Hashimura, C. and Hayashi, K. (2000)
10 Dominant expression of a novel splice variant of caspase-8 in human peripheral blood
11 lymphocytes. *Biochem Biophys Res Commun*, **272**, 877-881.
- 12 Huang, Y. and Steitz, J.A. (2005) SRprises along a messenger's journey. *Mol Cell*, **17**, 613-615.
- 13 Jiang, Z.H. and Wu, J.Y. (1999) Alternative splicing and programmed cell death. *Proc Soc Exp*
14 *Biol Med*, **220**, 64-72.
- 15 Jiang, Z.H., Zhang, W.J., Rao, Y. and Wu, J.Y. (1998) Regulation of Ich-1 pre-mRNA alternative
16 splicing and apoptosis by mammalian splicing factors. *Proc Natl Acad Sci U S A*, **95**,
17 9155-9160.
- 18 Johnson, D.G. and Degregori, J. (2006) Putting the Oncogenic and Tumor Suppressive Activities
19 of E2F into Context. *Curr Mol Med*, **6**, 731-738.
- 20 Kamachi, M., Le, T.M., Kim, S.J., Geiger, M.E., Anderson, P. and Utz, P.J. (2002) Human
21 autoimmune sera as molecular probes for the identification of an autoantigen kinase
22 signaling pathway. *J Exp Med*, **196**, 1213-1225.

1 Kameoka, S., Duque, P. and Konarska, M.M. (2004) p54(nrb) associates with the 5' splice site
2 within large transcription/splicing complexes. *Embo J*, **23**, 1782-1791.

3 Kornblihtt, A.R. (2005) Promoter usage and alternative splicing. *Curr Opin Cell Biol*, **17**, 262-
4 268.

5 Kuroyanagi, N., Onogi, H., Wakabayashi, T. and Hagiwara, M. (1998) Novel SR-protein-specific
6 kinase, SRPK2, disassembles nuclear speckles. *Biochem Biophys Res Commun*, **242**, 357-
7 364.

8 Lareau, L.F., Inada, M., Green, R.E., Wengrod, J.C. and Brenner, S.E. (2007) Unproductive
9 splicing of SR genes associated with highly conserved and ultraconserved DNA elements.
10 *Nature*.

11 Li, X., Wang, J. and Manley, J.L. (2005) Loss of splicing factor ASF/SF2 induces G2 cell cycle
12 arrest and apoptosis, but inhibits internucleosomal DNA fragmentation. *Genes Dev*, **19**,
13 2705-2714.

14 Lin, W.C., Lin, F.T. and Nevins, J.R. (2001) Selective induction of E2F1 in response to DNA
15 damage, mediated by ATM-dependent phosphorylation. *Genes Dev*, **15**, 1833-1844.

16 Massiello, A. and Chalfant, C.E. (2006) SRp30a (ASF/SF2) regulates the alternative splicing of
17 caspase-9 pre-mRNA and is required for ceramide-responsiveness. *J Lipid Res*, **47**, 892-
18 897.

19 Mercatante, D. and Kole, R. (2000) Modification of alternative splicing pathways as a potential
20 approach to chemotherapy. *Pharmacol Ther*, **85**, 237-243.

21 Nahle, Z., Polakoff, J., Davuluri, R.V., McCurrach, M.E., Jacobson, M.D., Narita, M., Zhang,
22 M.Q., Lazebnik, Y., Bar-Sagi, D. and Lowe, S.W. (2002) Direct coupling of the cell cycle
23 and cell death machinery by E2F. *Nat Cell Biol*, **4**, 859-864.

- 1 Ni, J.Z., Grate, L., Donohue, J.P., Preston, C., Nobida, N., O'Brien, G., Shiue, L., Clark, T.A.,
2 Blume, J.E. and Ares, M., Jr. (2007) Ultraconserved elements are associated with
3 homeostatic control of splicing regulators by alternative splicing and nonsense-mediated
4 decay. *Genes Dev*, **21**, 708-718.
- 5 Pagani, F., Stuani, C., Zuccato, E., Kornblihtt, A.R. and Baralle, F.E. (2003) Promoter
6 architecture modulates CFTR exon 9 skipping. *J Biol Chem*, **278**, 1511-1517.
- 7 Papoff, G., Cascino, I., Eramo, A., Starace, G., Lynch, D.H. and Ruberti, G. (1996) An N-
8 terminal domain shared by Fas/Apo-1 (CD95) soluble variants prevents cell death in
9 vitro. *J Immunol*, **156**, 4622-4630.
- 10 Pilch, B., Allemand, E., Facompre, M., Bailly, C., Riou, J.F., Soret, J. and Tazi, J. (2001)
11 Specific inhibition of serine- and arginine-rich splicing factors phosphorylation,
12 spliceosome assembly, and splicing by the antitumor drug NB-506. *Cancer Res*, **61**, 6876-
13 6884.
- 14 Rossi, F., Labourier, E., Forne, T., Divita, G., Derancourt, J., Riou, J.F., Antoine, E., Cathala, G.,
15 Brunel, C. and Tazi, J. (1996) Specific phosphorylation of SR proteins by mammalian
16 DNA topoisomerase I. *Nature*, **381**, 80-82.
- 17 Salon, C., Eymin, B., Micheau, O., Chaperot, L., Plumas, J., Brambilla, C., Brambilla, E. and
18 Gazzeri, S. (2006) E2F1 induces apoptosis and sensitizes human lung adenocarcinoma
19 cells to death-receptor-mediated apoptosis through specific downregulation of c-
20 FLIP(short). *Cell Death Differ*, **13**, 260-272.
- 21 Sanford, J.R., Ellis, J. and Caceres, J.F. (2005) Multiple roles of arginine/serine-rich splicing
22 factors in RNA processing. *Biochem Soc Trans*, **33**, 443-446.

- 1 Sanford, J.R., Gray, N.K., Beckmann, K. and Caceres, J.F. (2004) A novel role for shuttling SR
2 proteins in mRNA translation. *Genes Dev*, **18**, 755-768.
- 3 Sanford, J.R., Longman, D. and Caceres, J.F. (2003) Multiple roles of the SR protein family in
4 splicing regulation. *Prog Mol Subcell Biol*, **31**, 33-58.
- 5 Schwerk, C. and Schulze-Osthoff, K. (2005) Regulation of apoptosis by alternative pre-mRNA
6 splicing. *Mol Cell*, **19**, 1-13.
- 7 Shaham, S. and Horvitz, H.R. (1996) An alternatively spliced *C. elegans* ced-4 RNA encodes a
8 novel cell death inhibitor. *Cell*, **86**, 201-208.
- 9 Shin, C. and Manley, J.L. (2004) Cell signalling and the control of pre-mRNA splicing. *Nat Rev*
10 *Mol Cell Biol*, **5**, 727-738.
- 11 Smith, C.W. and Valcarcel, J. (2000) Alternative pre-mRNA splicing: the logic of combinatorial
12 control. *Trends Biochem Sci*, **25**, 381-388.
- 13 Solier, S., Logette, E., Desoche, L., Solary, E. and Corcos, L. (2005) Nonsense-mediated mRNA
14 decay among human caspases: the caspase-2S putative protein is encoded by an extremely
15 short-lived mRNA. *Cell Death Differ*, **12**, 687-689.
- 16 Spector, D.L., Fu, X.D. and Maniatis, T. (1991) Associations between distinct pre-mRNA
17 splicing components and the cell nucleus. *Embo J*, **10**, 3467-3481.
- 18 Srinivasula, S.M., Ahmad, M., Guo, Y., Zhan, Y., Lazebnik, Y., Fernandes-Alnemri, T. and
19 Alnemri, E.S. (1999) Identification of an endogenous dominant-negative short isoform of
20 caspase-9 that can regulate apoptosis. *Cancer Res*, **59**, 999-1002.
- 21 Stanelle, J., Stiewe, T., Theseling, C.C., Peter, M. and Putzer, B.M. (2002) Gene expression
22 changes in response to E2F1 activation. *Nucleic Acids Res*, **30**, 1859-1867.

1 Stevens, C., Smith, L. and La Thangue, N.B. (2003) Chk2 activates E2F-1 in response to DNA
2 damage. *Nat Cell Biol*, **5**, 401-409.

3 Sureau, A., Gattoni, R., Dooghe, Y., Stevenin, J. and Soret, J. (2001) SC35 autoregulates its
4 expression by promoting splicing events that destabilize its mRNAs. *Embo J*, **20**, 1785-
5 1796.

6 Sureau, A., Soret, J., Vellard, M., Crochet, J. and Perbal, B. (1992) The PR264/c-myb
7 connection: expression of a splicing factor modulated by a nuclear protooncogene. *Proc*
8 *Natl Acad Sci U S A*, **89**, 11683-11687.

9 Tschopp, J., Irmeler, M. and Thome, M. (1998) Inhibition of fas death signals by FLIPs. *Curr*
10 *Opin Immunol*, **10**, 552-558.

11 Utz, P.J., Hottellet, M., van Venrooij, W.J. and Anderson, P. (1998) Association of
12 phosphorylated serine/arginine (SR) splicing factors with the U1-small ribonucleoprotein
13 (snRNP) autoantigen complex accompanies apoptotic cell death. *J Exp Med*, **187**, 547-
14 560.

15 Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress,
16 W.D. and Chen, J. (2006) Interactions between E2F1 and SirT1 regulate apoptotic
17 response to DNA damage. *Nat Cell Biol*, **8**, 1025-1031.

18 Wang, J., Takagaki, Y. and Manley, J.L. (1996) Targeted disruption of an essential vertebrate
19 gene: ASF/SF2 is required for cell viability. *Genes Dev*, **10**, 2588-2599.

20 Wang, L., Miura, M., Bergeron, L., Zhu, H. and Yuan, J. (1994) Ich-1, an Ice/ced-3-related gene,
21 encodes both positive and negative regulators of programmed cell death. *Cell*, **78**, 739-
22 750.

1 Zhang, Z. and Krainer, A.R. (2004) Involvement of SR proteins in mRNA surveillance. *Mol Cell*,
2 **16**, 597-607.

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

1 **Figure legends**

2

3 **Figure 1: SC35 is a direct transcriptional target of E2F1**

4 **(A, B)** H358/Tet-On/E2F1 and H358/Tet-On/E2F1(E132) cells were incubated for 48 hours in
5 the presence (+) or absence (-) of 1 μ g/ml doxycyclin (Dox) as indicated. Mutant E2F1(E132) is
6 unable to bind to DNA. **(A)** Expression of E2F1, SC35, SRp20 and SF2/ASF proteins was
7 studied by western blotting. Actin was used as a loading control. **(B)** RT-PCR analysis of *sc35*
8 mRNA. Amplified *g3pdh* was used as an internal control. **(C)** Chloramphenicol Acetyl
9 Transferase (CAT) experiments were performed in the H1299 cell line co-transfected for 48
10 hours with 1 μ g pR264CAT, encoding CAT under the control of the *sc35* promoter, and
11 increasing amounts of pCMV-E2F1 as indicated. The CAT activity obtained in cells transfected
12 with pR264CAT alone was normalized to 1 and a relative CAT activity was then calculated for
13 each condition. Representative data of at least three independent experiments performed in
14 duplicate are shown. **(D)** H358/Tet-On/E2F1 cells cultured in the presence of doxycyclin for 48h
15 were processed for ChIP analysis using C20 antibody for E2F1. The coprecipitated chromatin
16 DNA was analyzed by PCR using a pair of primers that amplify the -296 to -79 bp region
17 upstream of the transcription start site of the *sc35* promoter. IgG was used as an irrelevant
18 antibody. No Ab means that no antibody was used in this case. **(E)** H69 and H810
19 neuroendocrine lung carcinoma cell lines were transfected for 72h with *mismatch* or *E2f1*
20 siRNAs as indicated and subjected to western blot analyses for the detection of E2F1, SC35,
21 SRp20 and SF2/ASF proteins. Actin was used as a loading control. **(F)** Western blot analysis of
22 E2F1, SC35, SRp20 and SF2 protein expression in E2F1-deficient (E2F1 *-/-*) and wild-type
23 control Murine Embryonic Fibroblasts (MEFs). Actin was used as a loading control.

1 **Figure 2: E2F1 affects the alternative splicing of SC35.**

2 (A) Alternative splicing pattern reported for SC35 (Sureau et al., 2001). Coding and alternative
3 exons are indicated by ▣ and ■, respectively. Alternative intronic sequences within the SC35 3'
4 untranslated region are shown (▤). The position of the primers and expected sizes (in
5 nucleotides) of the RT-PCR products corresponding to the different mRNA isoforms are
6 indicated. (B) Representative agarose gel of RT-PCR products corresponding to *sc35* splicing
7 variants in H358/Tet-On/E2F1 or E2F1(E132) cells cultured for 72h in the presence (+) or
8 absence (-) of 1 μ g/ml doxycyclin. The position of spliced variants (in bp) is shown on the right,
9 and the various splicing isoforms are named on the left of each panel.

10

11 **Figure 3: E2F1 stimulates AKT-dependent SC35 phosphorylation**

12 H358/Tet-On/E2F1 cells were incubated for 72 hours in the presence (+) or absence (-) of 1
13 μ g/ml doxycyclin as indicated. (A) Western blot analysis of E2F1, SC35 and phospho-SC35
14 proteins was performed in nuclear extracts. Phosphorylation of SC35 was revealed using a
15 specific antibody recognizing a phospho-epitope. (B) Immunolocalization of the phospho-SC35
16 protein was visualized by immunofluorescence. Note the appearance of P-SC35 nuclear speckles
17 of larger size in cells overexpressing E2F1 as compared to control cells. *Upper panels:* x40
18 magnification. *Lower panels:* x60 magnification. (C) Cells were co-cultured with (+) or without
19 (-) doxycyclin for 72h in the presence (+) or absence (-) of wortmanin (500nM) or Akt inhibitor
20 VIII (5 μ M) as indicated, and subjected to western blot analyses for detection of the E2F1,
21 phospho-AKT, AKT, SC35 and phospho-SC35 proteins. Phosphorylation of AKT was detected
22 using a specific phospho-antibody directed against P-Thr308. Actin was used as a loading
23 control. (D) Immunolocalization of E2F1 (red) and phospho-SC35 (green) proteins in H358/Tet-

1 On/E2F1 cultured for 72h in the presence (+) or absence (-) of 1 $\mu\text{g/ml}$ doxycyclin and 5 μM Akt
2 Inhibitor VIII. Note the existence of colocalization between both E2F1 and SC35 proteins in
3 merged images as reflected by the appearance of yellow dots.

4

5 **Figure 4: E2F1 and SC35 interact physically.**

6 (A) H1299 cells were transfected with pcDNA3.1-E2F1 vector. Whole cell extracts were
7 prepared and subjected to immunoprecipitation with anti-SC35 antibody (H55) or an irrelevant
8 rabbit serum as a negative control (IgG). Immunoprecipitates were tested for the presence of
9 E2F1 and SC35 using anti-E2F1 and anti-SC35 (4F11) antibodies, respectively. (B) Endogenous
10 SC35 was immunoprecipitated from H69 nuclear extracts with anti-SC35 (H55) antibody or an
11 irrelevant rabbit serum as a negative control (IgG). E2F1 and SC35 were revealed by Western
12 blots using anti-E2F1 and anti-SC35 antibodies, respectively. (C) Mapping of SC35 binding site
13 on E2F1. Beads harbouring bacterially produced GST-E2F1, various GST-E2F1 truncated
14 mutants or control GST proteins were incubated with an «*in vitro*» translated SC35 in a GST
15 pull-down experiment and analyzed by western blot using anti-SC35 (4F11) and anti-GST
16 antibodies. *Upper panel*: schematic representation of E2F1 and various E2F1 truncated versions.
17 Their ability to interact (+) or not (-) with “*in vitro*” translated SC35 is depicted on the right.
18 *Lower panel*: a representative immunoblot of SC35 capture on GST-E2F1 or GST-E2F1 mutants
19 beads using anti-SC35 (4F11) and anti-GST antibodies.

20

21 **Figure 5: SC35 is required for E2F1-induced apoptosis.**

22 H358 Tet-On/E2F1 cells were cultured in the presence (+) or absence (-) of doxycyclin for 72h.
23 (A) H358 Tet-On/E2F1 cells were transfected for 72h with either *mismatch* or *sc35* siRNA and

1 subjected to RT-PCR (left panel) and western blot (right panel) analyses. **(B)** H358 Tet-On/E2F1
2 cells were transfected for 72h either with *mismatch* or *sc35* siRNA. Apoptosis was evaluated
3 using Hoechst staining. Relative increase of apoptosis represents the ratio: percentage of
4 apoptosis in doxycyclin treated cells / percentage of apoptosis in controls cells, with the ratio in
5 control cells being arbitrarily assigned to 1. Results shown are the mean \pm SD of three
6 independent experiments. **(C)** Western blot analysis of c-FLIP_L and c-FLIP_S expression in H358
7 Tet-On/E2F1 cells cultured in the presence (+) or absence (-) of wortmanin (500 nM) or Akt
8 inhibitor VIII (5 μ M), as indicated. Actin was used as a loading control. **(D)** H358/Tet-On/E2F1
9 cells were transfected for 48h with either *mismatch* or *sc35* siRNA, cultured with (+) or without
10 (-) doxycyclin as indicated, and treated or not with 25 ng/ml FasL for 20 additional hours.
11 Apoptosis was evaluated as in **(A)**.

12
13 **Figure 6: E2F1 and SC35 cooperate to affect the alternative splicing pattern of *caspases-8*,**
14 ***caspase-9* and *Bcl-x* pre-mRNAs.**

15 **(A)** Alternative splicing patterns of *caspases-2*, *-8*, *-9* and *Bcl-x* primary transcripts. Coding and
16 alternative exons are indicated by \boxtimes and \blacksquare , respectively. The position of primers, the expected
17 sizes (in nucleotides) and the names of the RT-PCR products corresponding to the different
18 mRNA isoforms are indicated. The ability of each transcript to encode proapoptotic (+) or
19 antiapoptotic (-) protein isoform is depicted on the right. **(B, C)** H358/Tet-On/E2F1 or H358/Tet-
20 On/E2F1(E132) cells were cultured for 48 hours in the presence (+) or absence (-) of doxycyclin,
21 as indicated. Total RNAs were extracted and subjected to RT-PCR analyses using the specific
22 primers depicted in **(A)**. Representative agarose gels of RT-PCR products corresponding to *casp-*
23 *2*, *casp-8*, *casp-9* and *Bcl-x* splicing variants are presented. The position of spliced variants (in

1 bp) is shown on the right, and the various splicing isoforms are named on the left of each panel.
2 *g3pdh* was used as an internal control. (C) *Lower panel*: western blot analysis was performed
3 using specific anti-E2F1, anti-Bcl-x_L and anti-Bcl-x_S antibodies. Actin was used as a loading
4 control. Results are representative of three independent experiments. (D) H358/Tet-On/E2F1
5 cells cultured in the presence (+) or absence (-) of 1 μg/ml doxycyclin were transfected for 72
6 hours 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).

9
10 **Figure 7: E2F1 and SC35 proteins are upregulated in cells undergoing apoptosis in**
11 **response to DNA damaging agents**

12 (A) H358 cells were treated for 24 hours with the indicated cytotoxic agents (50μM, each) and
13 analyzed for E2F1, SC35, phospho-SC35 expression by western blotting (upper panel) and RT-
14 PCR (lower panel). Actin and *g3pdh* were used as internal controls for immunoblotting and RT-
15 PCR experiments, respectively. Apoptosis was evaluated after Hoechst 333342 staining. A
16 relative increase of apoptosis was calculated and the ratio between the percentage of apoptosis in
17 treated versus untreated cells is represented. Results shown are the mean ± SD of three
18 independent experiments. (B) H358 cells were transfected for 48 hours with either *mismatch* or
19 *E2f1* siRNA and treated (+) or not (-) for an additional 24 hours with cyclophosphamide (50 μM).
20 Western blot analyses were performed with the indicated antibodies. (C) H358 cells were
21 transfected for 48 hours with either *mismatch* or *sc35* siRNA and treated (+) or not (-) for 24
22 additional hours with cyclophosphamide (50μM). Apoptosis was evaluated after Hoechst 33342
23 staining and immunoblotting with anti-caspase-3 antibody. Apoptosis of untreated cells was

1 normalized to 1. Relative increase of apoptosis represents the ratio: percentage of apoptosis in
2 treated cells versus untreated cells. Results are the mean \pm SD of three independent experiments.

3

4

5

6

7

8

9

10

11

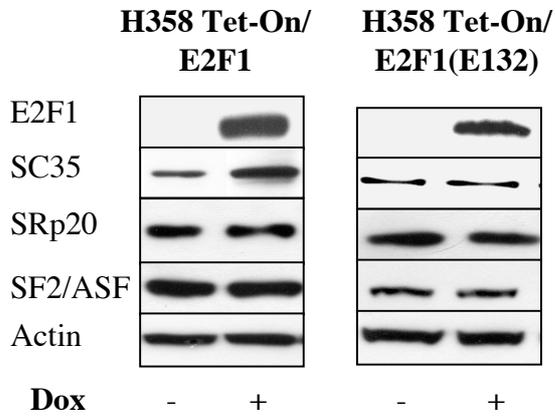
12

13

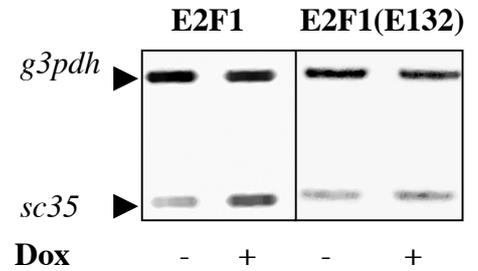
14

Figure 1

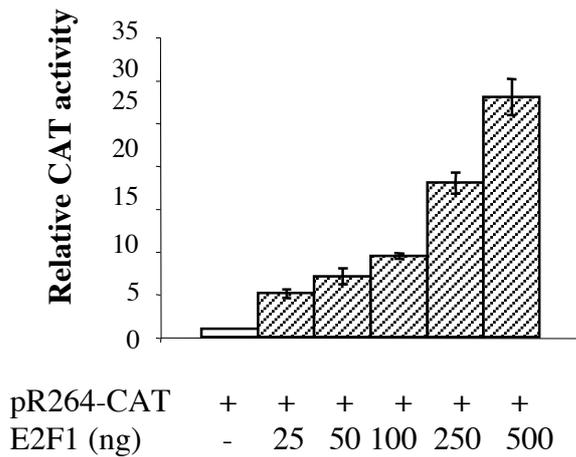
A



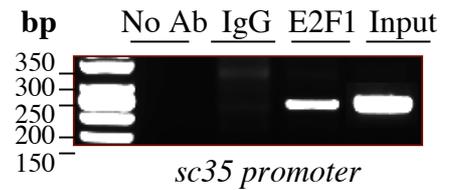
B



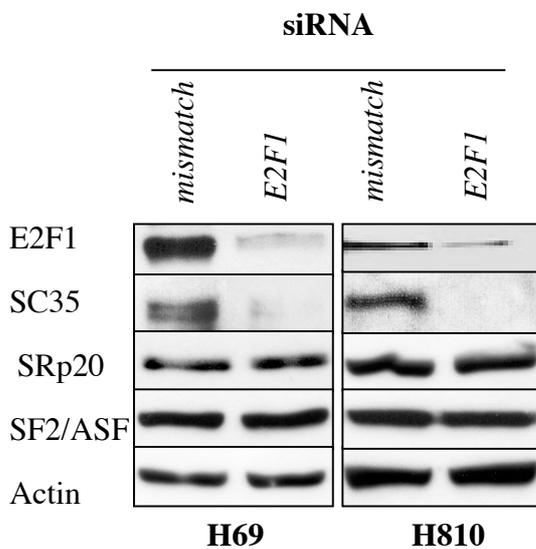
C



D



E



F

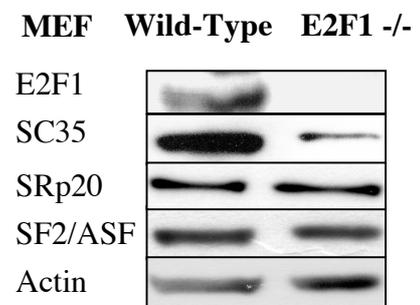
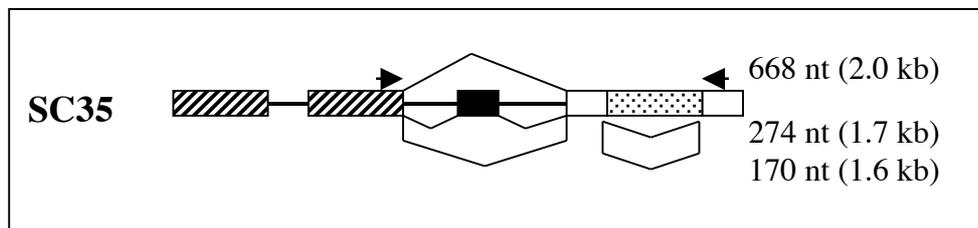


Figure 2

A



B

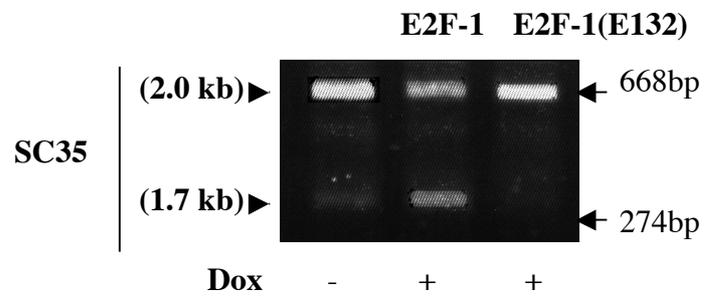
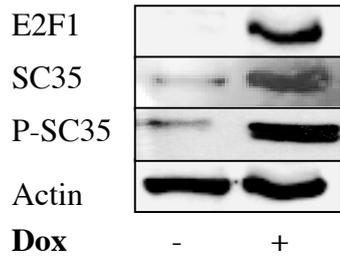
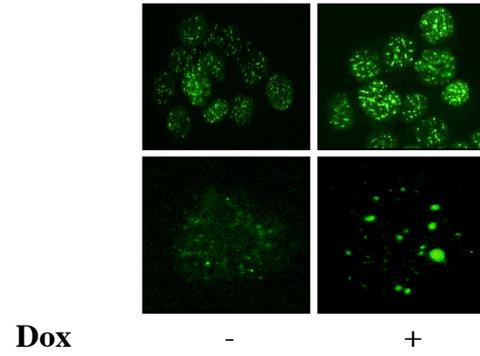


Figure 3

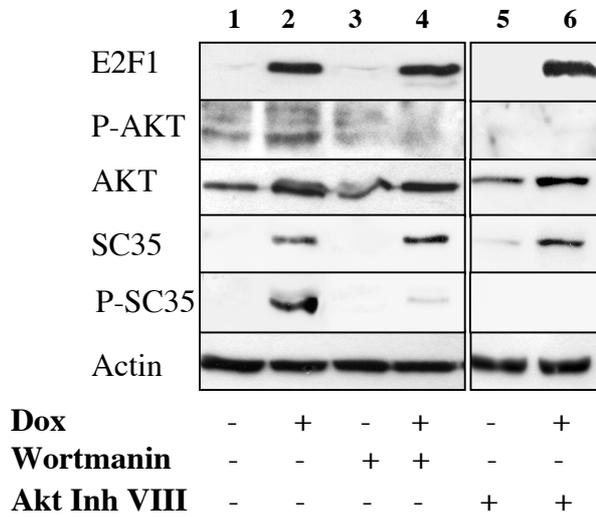
A



B



C



D

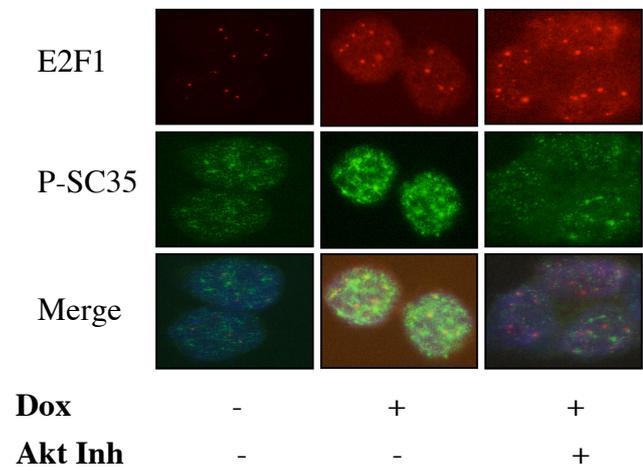
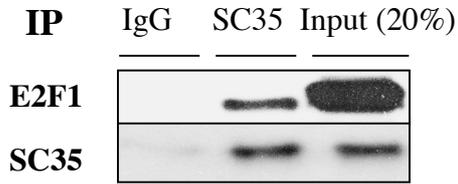
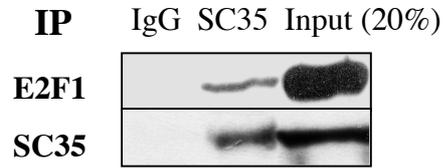


Figure 4

A



B



C

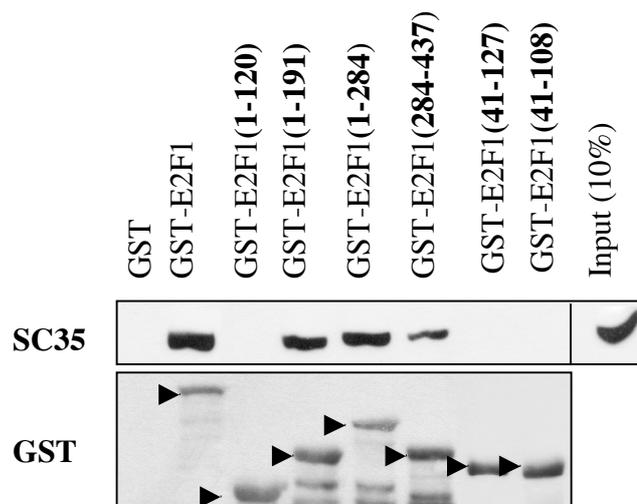
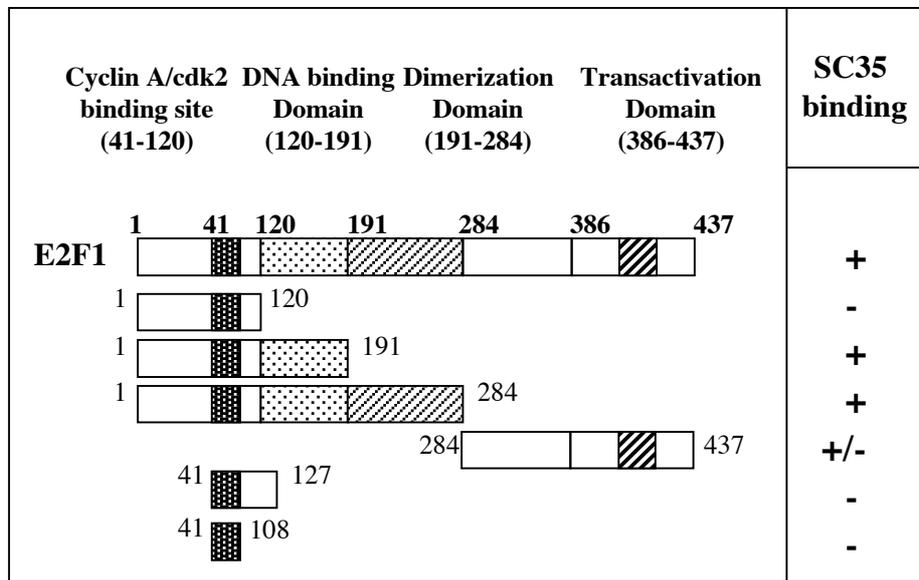
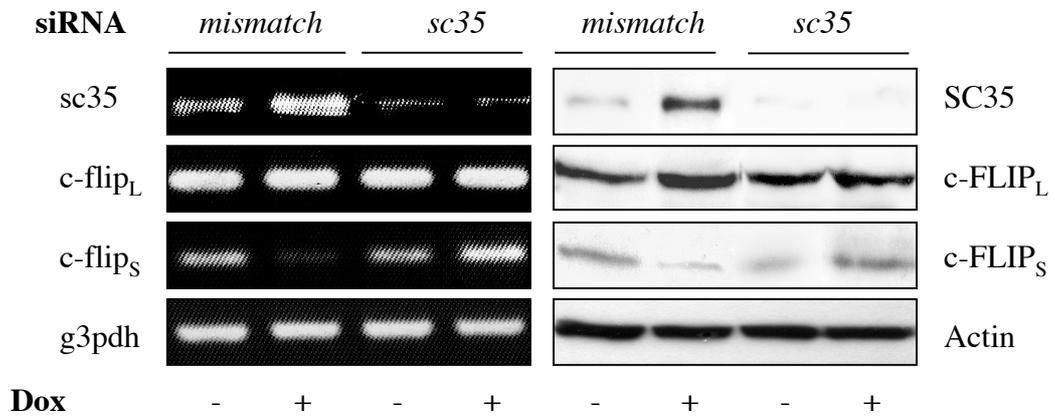
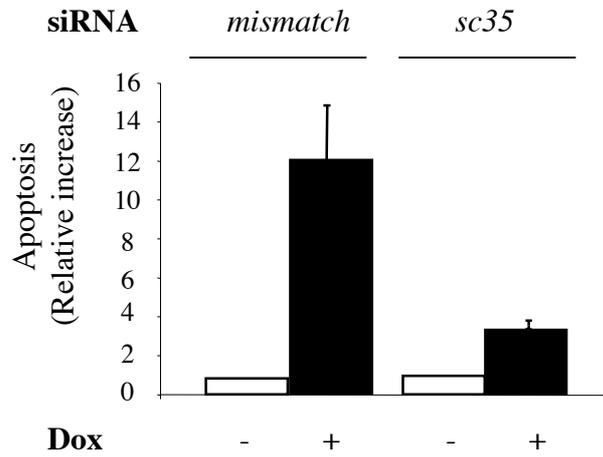


Figure 5

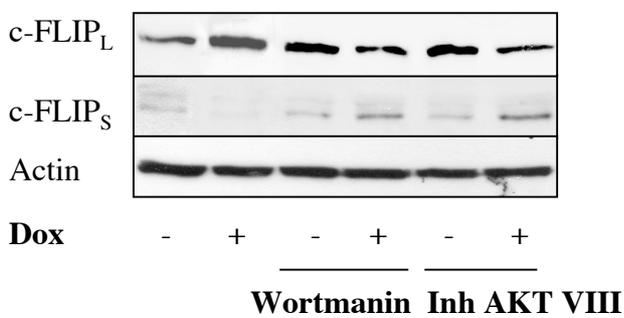
A



B



C



D

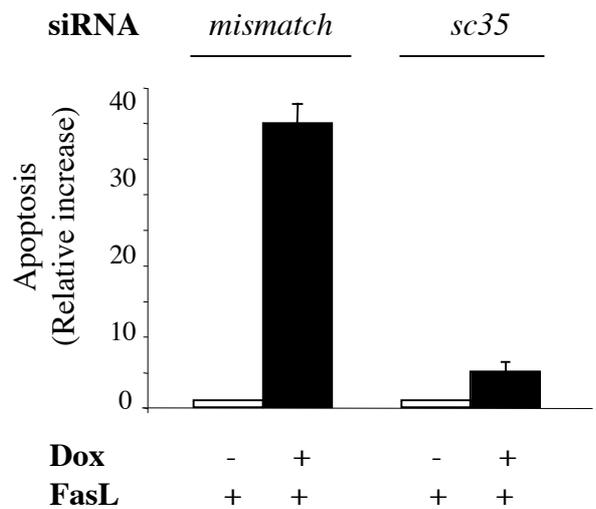
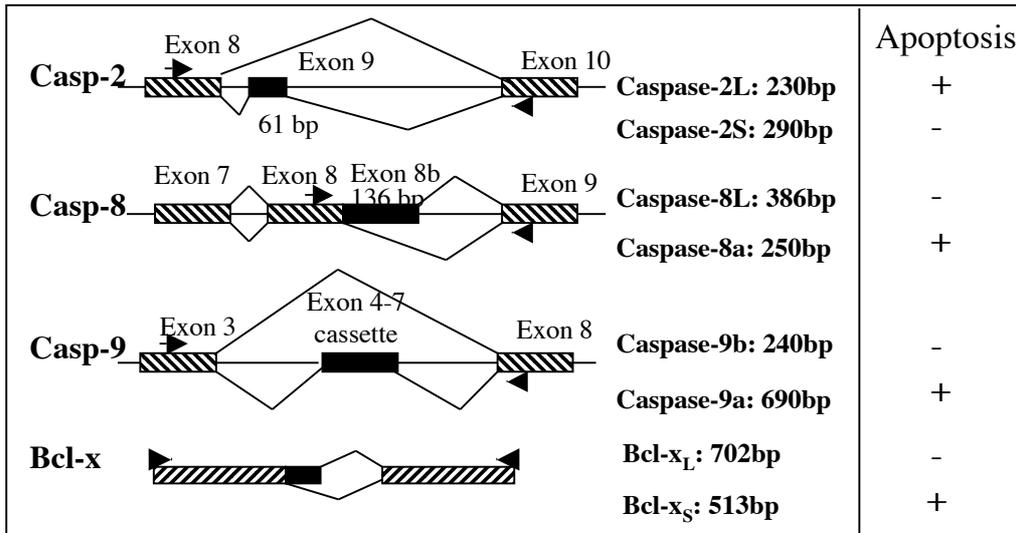
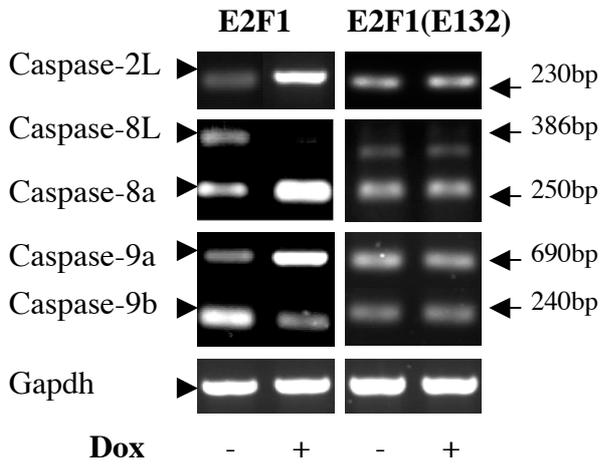


Figure 6

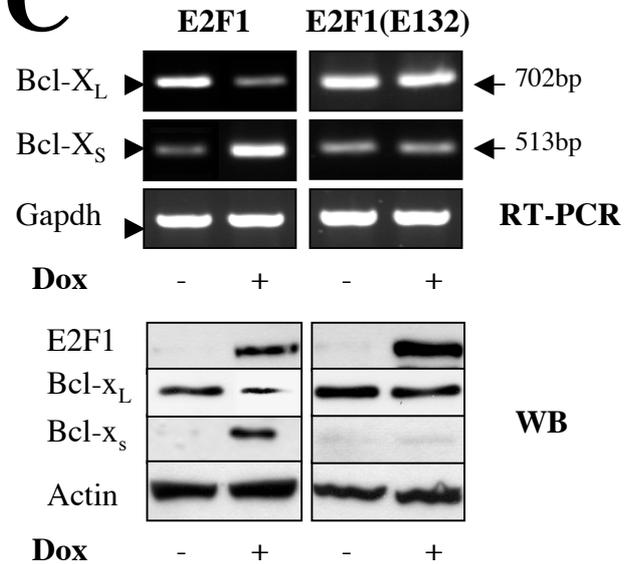
A



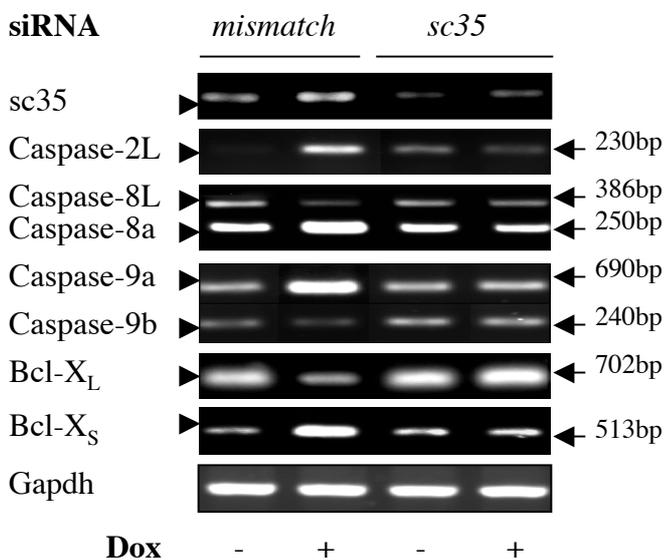
B



C



D



E

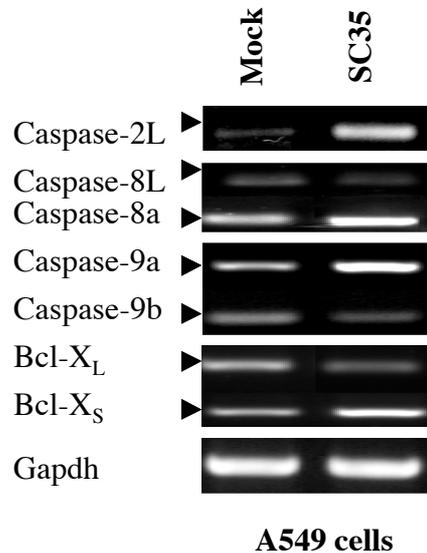
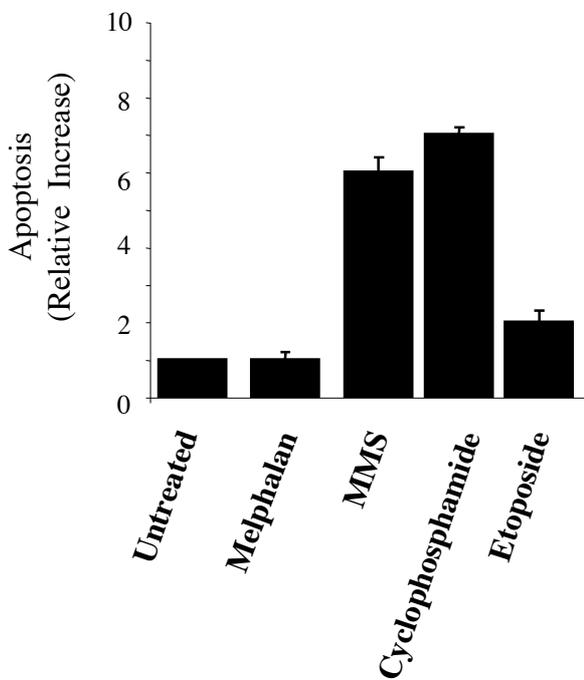
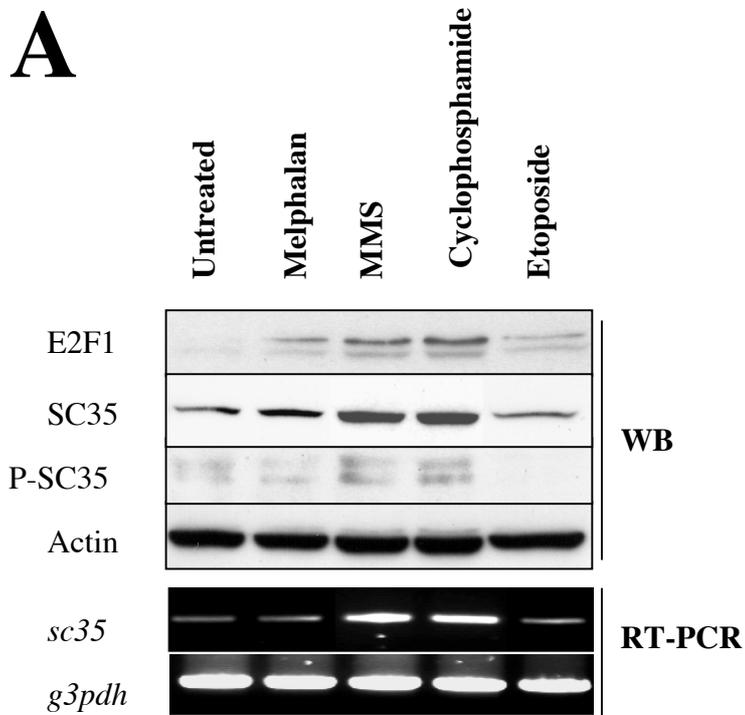
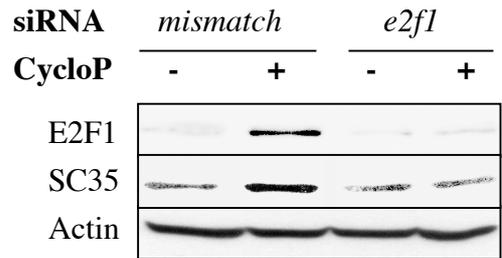


Figure 7

A



B



C

