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

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11 Running title: SC35 is a new E2F1 target gene

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23

24 **Abstract**

25

26 The transcription factor E2F1 plays a key role during S phase progression and apoptosis. It has
27 been well-demonstrated that the apoptotic function of E2F1 involves its ability to transactivate

pro-apoptotic target genes. Alternative splicing of pre-mRNAs also plays an important role in the regulation of apoptosis. In this study, we identify the splicing factor SC35, a member of the Ser-Rich Arg (SR) proteins family, as a new transcriptional target of E2F1. We demonstrate that E2F1 requires SC35 to switch the alternative splicing profile of various apoptotic genes such as *c-flip*, *caspases-8*, *-9* and *Bcl-x*, towards the expression of pro-apoptotic splice variants. Finally, we provide evidence that E2F1 upregulates SC35 in response to DNA damaging agents and show that SC35 is required for apoptosis in response to these drugs. Taken together, these results demonstrate that E2F1 controls pre-mRNA processing events to induce apoptosis and identify the SC35 SR protein as a key E2F1-direct target in this setting.

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

Introduction

Pre-mRNA splicing is an essential step for the expression of most genes in higher eukaryotic cells. This process has emerged as an important mechanism of genetic diversity since about 74% of human genes undergo alternative splicing, leading to the production of various protein isoforms {Smith, 2000 #79}. SC35 belongs to the serine/arginine-rich (SR) protein family, one of the most important class of splicing regulators. Members of the SR family have a modular structure consisting of one or two copies of an N-terminal RRM (RNA-recognition

motif) followed by a C terminus rich in serine and arginine residues known as the RS domain. They act at multiple steps of spliceosome assembly and participate in both constitutive and alternative splicing {Sanford, 2005 #57}. Together with most other splicing factors, SR proteins localize to nuclear subregions termed nuclear speckles {Spector, 1991 #47}. Extensive serine phosphorylation of the RS domain plays an important role in the regulation of both the localization and the activities of SR proteins {Sanford, 2003 #71}. While the splicing functions of SR proteins have been well documented *"in vitro"*, less is known about their roles and physiological targets *"in vivo"*. However, based on gene targeting experiments demonstrating that they are required for cell viability and/or animal development, SR proteins undoubtedly control essential biological functions.

Apoptosis is one of the cellular process in which alternative splicing plays an important regulatory role. Indeed, a remarkable number of transcripts that encode proteins involved in the apoptotic pathway are subjected to alternative splicing. This usually drives the expression of proteins with opposite functions, either pro- or anti-apoptotic {Schwerk, 2005 #68}. Interestingly, changes in SR protein phosphorylation have been observed upon apoptotic stimulation following activation of the Fas receptor {Utz, 1998 #53}. In addition, *"in vitro"* and overexpression experiments have suggested a potential role for SR proteins in the control of the splicing of pre-mRNAs encoding apoptotic regulators {Jiang, 1998 #19 ; Li, 2005 #140}. Moreover, depletion of the ASF/SF2 SR protein has been reported to induce apoptosis {Li, 2005 #140 ; Wang, 1996 #70}. Nevertheless, whether individual SR proteins are necessary to modulate alternative splicing of mRNAs encoding apoptotic factors remains largely unknown, as well as the factors that control expression and/or activity of SR proteins in this context.

The E2F1 transcription factor belongs to the E2F family encompassing eight members involved in a diverse array of essential cellular functions {DeGregori, 2006 #90}. E2F1 is best-known for its role in driving cell cycle progression in S phase. In addition, E2F1 can induce apoptosis by mechanisms involving or not its transcriptional function. We previously demonstrated the ability of E2F1 to trigger apoptosis through caspase-8 activation at the death-

1 inducing signaling complex and showed that E2F1 acts through specific downregulation of the
2 cellular FLICE-inhibitory protein short isoform, c-FLIP$_{short}$ {Salon, 2006 #144}. As *c-flip*
3 predominantly encodes two isoforms arising from alternative splicing, namely c-FLIP$_{short}$ and c-
4 FLIP$_{long}$, we postulated that E2F1 could control the expression and/or activity of some splicing
5 factors. In this study, we identify the SC35 splicing factor as a direct transcriptional target of
6 E2F1 and show that SC35 is involved in the ability of E2F1 to trigger apoptosis through
7 downregulation of c-FLIP$_{short}$. Importantly, we demonstrate that E2F1 and SC35 also cooperate to
8 switch the alternative splicing pattern of *caspases-8*, *-9* and *Bcl-x* towards the expression of pro-
9 apoptotic isoforms. Finally, we provide evidence that E2F1 promotes the accumulation of SC35
10 in cells treated with DNA damaging agents, and show that SC35 is required for induction of
11 apoptosis in this setting. Taken together, these results demonstrate that E2F1 and SC35 proteins
12 control pre-mRNA processing events to promote apoptosis.
13
14 ## Results
15
16 ### E2F1 upregulates SC35 protein expression
17 We previously established a model of stable E2F1-inducible clones in the H358 cell line
18 derived from a human lung adenocarcinoma {Salon, 2006 #144}. To test whether E2F1 could
19 control the expression pattern of some splicing factors, we first studied in this model the
20 expression of three members of the SR proteins family, one of the most important class of
21 splicing regulators, namely SC35, SRp20 and SF2/ASF. Immunoblotting experiments
22 demonstrated a 3-4 fold increased expression of SC35 in cells overexpressing E2F1, whereas the
23 total level of SRp20 and SF2/ASF was not affected (Figure 1A, left panel). This effect required
24 the DNA binding activity of E2F1 since overexpression of an E2F1(E132) DNA-binding
25 defective mutant did not affect SC35 protein level (Figure 1A, right panel).
26 To confirm these data, we knocked-down E2F1 expression by using small interfering
27 RNAs (siRNAs) in the H69 and H810 neuroendocrine lung carcinoma cell lines that

1 physiologically express high levels of E2F1, and analyzed SC35, SRp20 and SF2/ASF expression
2 by western blotting. As shown in figure 1B, the silencing of E2F1 was accompagnied by a strong
3 downregulation of the endogenous SC35 protein in both cell lines as compared to cells
4 transfected with *mismatch* siRNA. In contrast, the expression of SRp20 and SF2/ASF proteins
5 was not affected. In addition, we observed that SC35 protein level was strongly reduced in E2F1
6 knock-out MEF as compared to wild-type MEF, whereas those of SRp20 and SF2/ASF did not
7 change (Figure 1C). Taken together, these data demonstrate that SC35 expression is positively
8 regulated by E2F1.
9
10 **SC35 is a direct transcriptional target of E2F1**
11 To test whether E2F1 controlled SC35 expression at the transcriptional level, we
12 performed RT-PCR experiments using primers localized at the 5'-end of the *sc35* ORF. The data
13 showed that expression of *sc35* mRNA was induced by E2F1 but not by the mutant E2F1(E132)
14 (Figure 2A). To go further, we performed Chloramphenicol Acetyl Transferase (CAT)
15 experiments using a pR264-CAT plasmid that contains the 1kb human *sc35* promoter upstream
16 of CAT cDNA {Sureau, 1992 #2}. Co-transfection of H1299 (Figure 2B) or SAOS2 (data not
17 shown) cells with pR264-CAT vector and increasing amounts of an E2F1 expression vector
18 resulted in a dose–dependent increase of CAT activity. These results indicated that E2F1 can
19 transactivate the promoter of *sc35*. To confirm these data "*in vivo*", we performed chromatin
20 immunoprecipitation (ChIP) experiments in the H358/Tet-On/E2F1 cells cultured in the presence
21 of doxycylin (Figure 2C, upper panel). As a positive control of ChIP assays, we used the
22 proximal human *Skp2* promoter that was recently reported as a target of E2F1 {Reichert, 2007
23 #126}. In our conditions, binding of E2F1 to the proximal *Skp2* gene promoter was clearly
24 detected whereas no binding was observed with the *Gapdh* promoter (Figure 2C, upper panel).
25 Importantly, the *sc35* promoter fragment (-296/-79) that encompasses two putative E2F1 binding
26 sites was precipitated by an anti-E2F1 antibody (Figure 2C, upper panel). In order to confirm that
27 SC35 was a direct target of E2F1, endogenous E2F1 was immunoprecipitated from H1299 cells

and ChIP experiments were performed. As shown (Figure 2C, lower panel), endogenous E2F1 clearly bound to the *sc35* promoter in these cells. Altogether, these results identify *sc35* as a direct transcriptional target of E2F1.

E2F1 promotes SC35-dependent apoptosis through modulation of $\text{FLIP}_{long}/\text{FLIP}_{short}$ ratio

As we previously demonstrated the ability of E2F1 to induce apoptosis through downregulation of the c-FLIP_{short} protein isoform {Salon, 2006 #144}, we next analyzed whether SC35 played a role in this setting. By the use of *sc35* siRNAs and Hoechst staining, we firstly observed that neutralization of SC35 strongly reduced the number of apoptotic cells in response to E2F1 (Figure 3A). Therefore, these results indicated that SC35 is required for E2F1-induced apoptosis. Importantly, RT-PCR (Figure 3B, left panel) and immunoblot analyses (Figure 3B, right panel) revealed that downregulation of both $flip_{short}$ mRNA and protein levels was also prevented in these conditions. A high level of both c-FLIP protein isoforms has been found in many tumor cells and was correlated with resistance to FAS- and TRAIL-induced apoptosis, two death receptor ligands {Tschopp, 1998 #119}. Consistently, we previously reported that the downregulation of c-FLIP_{Short} by E2F1 was sufficient to restore the sensitivity of tumor cells to these ligands {Salon, 2006 #144}. In this study, we showed that neutralization of *sc35* expression overrided the ability of E2F1 to sensitize H358 cells to FasL (Figure 3C) as well as to TRAIL (data not shown) treatments. Collectively, our data demonstrate that E2F1 induces apoptosis through SC35-dependent modulation of the $\text{FLIP}_{Long}/\text{FLIP}_{Short}$ ratio, at the expense of the FLIP_{Short} protein isoform.

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

As the expression of numerous apoptotic genes is regulated by pre-mRNA alternative splicing {Schwerk, 2005 #68}, we undertook a series of experiments to test whether E2F1 and

SC35 could also affect the ratio of other apoptotic splice variants. Various *caspases* are subjected to alternative splicing. Alternative splicing of *casp-2* proceeds through selective insertion or removal of exon 9 giving rise to anti-apoptotic caspase-2S and pro-apoptotic caspase-2L isoforms respectively {Jiang, 1998 #19}. The use of a distant splice donor site at the 3'-end of exon 8 of human *caspase-8* pre-mRNA leads to the synthesis of an alternative spliced variant, *caspase-8L*, a competitive inhibitor of caspase-8 {Eckhart, 2001 #22 ; Himeji, 2002 #23}. The inclusion or exclusion of an exon cassette in *caspase-9* causes the expression of two splice variants, namely the proapoptotic *caspase-9a* and antiapoptotic *caspase-9b* {Chalfant, 2002 #25 ; Massiello, 2006 #24}. To assess whether E2F1 induces changes in the alternative splicing profile of these *caspases* pre-mRNAs, RNAs recovered from uniduced or induced H358/Tet-On/E2F1 cells were analyzed by RT-PCR using primers specific for each caspase splice variant (Figure 4A). The results showed that E2F1 increased the expression of pro-apoptotic *caspases-2L*, *-8a* and *-9a* mRNA levels and decreased those of anti-apoptotic *caspases-8L* and *-9b* (Figure 4B). In contrast, these effects were not observed with mutant E2F1(E132). Of note, we were unable to detect the *caspase-2S* transcript in our cells. Altogether, these data indicate that E2F1 can switch the splicing pattern of *caspases-8* and *–9* in favor of pro-apoptotic isoforms. Therefore, besides its ability to transactivate *caspases-8* and *–9* genes {Nahle, 2002 #27}, E2F1 also controls their alternative splicing.

Bcl-x is a member of the *bcl-2* gene family that also plays a key role in apoptosis. Several spliced isoforms of *Bcl-x* have been reported. The use of a 5' proximal site generates the Bcl-x_L large isoform which protects cells against apoptosis. In contrast, the use of a 5' distal site results in the synthesis of a short proapoptotic Bcl-x_S isoform {Boise, 1993 #14}. To analyze whether E2F1 regulates the splicing pattern of Bcl-x, we performed RT-PCR analysis with specific primers in H358/Tet-On/E2F1 cells (Figure 4A). The results showed that expression of E2F1 induced a concomittant decrease of *Bcl-x_L* and increase of *Bcl-x_S* mRNA levels (Figure 4C, upper panel). Similarly to *caspases* regulation, the mutant E2F1(E132) had no effect on *bcl-x* splicing. Western blotting with Bcl-x antibodies specific for each isoform confirmed the RT-PCR results

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

3 Finally, we questionned 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 $Bcl\text{-}x_L$ or $Bcl\text{-}x_S$. By contrast,
8 treating cells with doxycyclin strongly prevented the ability of E2F1 to affect the splicing pattern
9 of these genes (Figure 4D and Supplementary Figure 1). Furthermore, when we performed RT-
10 PCR analyses in A549 cells transiently transfected with a vector encoding SC35, we found that
11 overexpression of SC35 affected the splicing profile of *caspase-8*, *-9* and *Bcl-x* pre-mRNAs in a
12 similar way than did E2F1 (compare Figure 4E to Figures 4B and C) and induced apoptosis (data
13 not shown). Altogether, these results demonstrate that E2F1 requires SC35 to regulate the pre-
14 mRNA alternative splicing of apoptotic genes.

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

17 Our results so far demonstrated the ability of E2F1 and SC35 to modify the splicing
18 pattern of various apoptotic genes in a model of overexpression. Thus, we attempted to identify
19 in which physiological context both proteins could cooperate to induce apoptosis. It is now well-
20 known that DNA damaging agents stabilize E2F1 and induce its transcriptional activity towards
21 apoptotic genes thereby causing apoptosis {Stevens, 2003 #29 ; Wang, 2006 #30}. In agreement
22 with previous reports, treatment of H358 cells with either methylmethanesulfonate (MMS) or
23 cyclophosphamide, two alkylating agents that create interstrand DNA crosslinks, significantly
24 increased E2F1 expression as detected by immunoblotting (Figure 5A, upper panel). In these
25 conditions, upregulation of E2F1 was accompagnied by an increase of SC35 protein and mRNA
26 expression (Figure 5A, upper panels), as well as by the induction of apoptosis (Figure 5A, lower
27 panel). In cyclophosphamide-treated cells, the neutralization of E2F1 using siRNAs prevented

the accumulation of SC35 (Figure 5B), indicating that E2F1 was involved in SC35 induction. In addition, as detected by ChIP experiments, the binding of endogenous E2F1 to the *sc35* promoter was increased following drug treatment, indicating that SC35 is a direct target of E2F1 in response to DNA damaging agents (Figure 5C). Furthermore, inhibiting SC35 expression strongly repressed apoptosis following cyclophosphamide treatment, as detected by immunoblotting of pro- or cleaved caspase-3 and Hoechst 33342 staining (Figure 5D). Taken together, these results demonstrate that E2F1 requires SC35 to induce apoptosis in response to genotoxic stresses.

Finally, we analyzed whether E2F1 and SC35 acted through regulation of the alternative splicing of apoptotic genes and studied the expression pattern of *caspases -2*, *-8*, *-9* and *Bcl-x* splice variants by RT-PCR following cyclophosphamide treatment. As shown in Figure 6A (left panel), an increase of pro-apoptotic *caspase-9a* and $Bcl\text{-}x_S$ mRNA levels was observed in cyclophosphamide-treated cells together with a decrease of anti-apoptotic *caspase-9b* and $Bcl\text{-}x_L$ splice variants. Again, immunoblotting experiments confirmed the downregulation and upregulation of $\mathrm{Bcl\text{-}x_L}$ and $\mathrm{Bcl\text{-}x_S}$ proteins, respectively (Figure 6A, right panel). In contrast, accumulation of *caspase-2L* and of both anti- and pro-apoptotic *caspase-8L* and *–8a* transcripts was detected in cyclophosphamide-treated cells (Figure 6A, left panel). Therefore, these results demonstrate that cyclophosphamide modifies the alternative splicing pattern of *Bcl-x* and *caspase-9* pre-mRNAs in favor of pro-apoptotic splice variants. Importantly, the neutralization of either *e2f1* or *sc35* before cyclophosphamide cell treatment prevented these modifications, as detected by RT-PCR (Figure 6B, upper panel) and western blotting (Figure 6B, lower panel). Overall, these data strongly suggest that E2F1 and SC35 proteins act together during apoptosis of DNA damaged cells by controlling *Bcl-x* and *caspase-9* pre-mRNAs alternative splicing.

Discussion

1 E2F1 is a transcription factor that plays a critical role in cell cycle progression by favoring
2 entry into S phase. Besides its role in cell cycle control, E2F1 is also widely accepted as an
3 inducer of apoptosis. It has been well-demonstrated that E2F1 promotes apoptosis through both
4 transcription-dependent and -independent mechanisms. So far, numerous apoptotic genes whose
5 transcription is enhanced by E2F1 have been identified {DeGregori, 2006 #90}. In this study, we
6 show that E2F1 switches the alternative splicing pattern of key apoptotic genes in favor of their
7 pro-apoptotic splice variants, and identify the SC35 protein, a member of the SR family of
8 splicing regulators, as a key direct target of E2F1. Interestingly, two high throughput studies
9 using ChIP on ChIP analysis {Ren, 2002 #172} or DNA microarrays {Muller, 2001 #171}
10 previously suggested that SC35 is an E2F1-regulated gene. In this study, we provide the first
11 evidence of a direct functional link between E2F1 and SC35 proteins to regulate cellular
12 apoptosis. Therefore, besides its ability to transactivate pro-apoptotic target genes, E2F1 is also
13 able to control pre-mRNA processing events to induce apoptosis.
14 It has now emerged from the litterature that splicing not only depends on the interaction
15 of splicing factors with their target pre-mRNAs, but is also coupled to transcription {Kornblihtt,
16 2005 #138}. Indeed, variations of pol II promoter structure can lead to differences in alternative
17 splicing of the transcript {Cramer, 1997 #133 ; Cramer, 1999 #60} and components of the
18 spliceosome such as the p54nrb (p54 nuclear RNA binding protein) and PSF (polypyrimidine
19 tract-binding protein-associated splicing factor) RNA binding proteins are involved in both
20 transcription and splicing processes {Auboeuf, 2005 #129} (for review). In addition,
21 transcriptional coregulators of the nuclear receptors family recruited at the promoter level not
22 only enhance the transcriptional activity of this promoter, but also affect the nature of the spliced
23 variants produced {Auboeuf, 2004 #65 ; Auboeuf, 2004 #128 ; Auboeuf, 2005 #129}. Moreover,
24 some transcription factors have been reported to bind to proteins of the spliceosome and/or
25 display dual functions in splicing and transcription {Chansky, 2001 #62 ; Guillouf, 2006 #46}.
26 Taken together, these results indicate a role for proteins controlling transcription in splicing
27 regulation. In this study, we demonstrate that the transcription factor E2F1 transactivates the

1 expression of *sc35*, a component of the spliceosome and that both proteins regulate pre-mRNA
2 processing events. Altogether, these results reinforce the connection between the transcriptional
3 and splicing machineries. We show that E2F1 alters the splicing pattern of some of its
4 transcriptional targets such as *c-flip* (Figure 3B), *caspases-8* and *-9* (Figures 4B & 4D). As an
5 increased level of *caspases–8* and *–9* transcripts was also detected in this context, it is possible
6 that the transactivating functions of E2F1 cooperate with SC35 accumulation to regulate pre-
7 mRNA processing events. Interestingly, the expression of *c-flip* mRNA was apparently not
8 affected by E2F1 in the same conditions (Figure 3A). It was recently shown that the Spi-1/PU.1
9 transcription factor could modify alternative splicing of a transcriptional target gene, without
10 modulation of its mRNA transcription {Guillouf, 2006 #46}. Therefore, another but not exclusive
11 possibility is that E2F1 acts as a scaffold protein to drive SC35 to the nascent transcribed RNA of
12 some of its target genes, according to the cell-specific promoter occupation model {Kornblihtt,
13 2005 #138}.
14 Apoptosis is one of the cellular processes in which alternative splicing plays important
15 regulatory roles {Schwerk, 2005 #68}. Several components of the splicing machinery have been
16 already involved in apoptotic processes. For example, depletion of SF2/ASF {Li, 2005 #140}
17 induces apoptosis. In addition, overexpression of SC35 alters the splicing of *caspase-2* mRNA, in
18 favor of the pro-apoptotic isoform accumulation {Jiang, 1998 #19}. Furthermore,
19 phosphorylation of SR proteins, which is known to control their sub-cellular localization as well
20 as their activities, have been reported during apoptosis {Utz, 1998 #53 ; Kamachi, 2002 #52}.
21 However, the upstream signaling molecules that regulate the expression and/or activity of SR
22 proteins during apoptosis, as well as the endogenous targets of SR proteins in this context remain
23 largely unknown. In this study, we provide evidence that E2F1 triggers apoptosis through SC35
24 accumulation and demonstrate that both proteins cooperate to affect the splicing pattern of
25 *caspase-8*, *caspase-9*, *flip* and *bcl-x* genes, in favor of pro-apoptotic splice variants. Altogether,
26 our data identify SC35 as a new mediator of E2F1-induced apoptosis and identify some of its
27 endogenous targets in this setting.

Alteration of alternative splicing is believed to contribute to the resistance of tumor cells to chemotherapy, notably through overexpression of anti-apoptotic splice variants {Mercatante, 2000 #37 ; Hayes, 2006 #44}. However, the molecular mechanisms involved in such process remain largely unknown. Importantly, and consistent with a role of SR proteins during the response to DNA damage, it has been previously shown that SC35 accumulates following γ-irradiation {Cardoso, 2002 #41}. In addition, it was recently reported that the expression of SRp55, another member of the SR protein family, is upregulated and required for apoptosis of p53-deficient cells after mitomycin C treatment {Filippov, 2007 #149}. In this study, we demonstrate that SC35 is upregulated by an E2F1-dependent pathway in response to methylmethanesulfonate or cyclophosphamide treatment. Furthermore, we show that the E2F1 and SC35 proteins are required for apoptosis of DNA damaged cells and control *Bcl-x* and *caspase-9* pre-mRNAs alternative splicing at the expense of anti-apoptotic splice variants. Therefore, our data strongly suggest that E2F1 and SC35 are key-determinants of the cellular response to chemotherapeutic agents.

To conclude, we highlight the first functional connection between the transcription factor E2F1 and a component of the splicing machinery, SC35, in the control of cellular apoptosis. It is well-known that abnormalities in E2F signalling pathways contribute to tumorigenesis {Johnson, 2006 #91}. Accordingly, we previously described a differential pattern of E2F1 protein expression in human lung tumors {Eymin, 2001 #31}. Interestingly, some SR proteins are overexpressed in ovarian cancer {Fischer, 2004 #88} and SF2/ASF was recently assessed as a proto-oncogene in human tumors {Karni, 2007 #89}. Therefore, it remains to determine whether alterations of both E2F1 and SC35 proteins could cooperate to promote carcinogenesis.

1

Materials and Methods

3

Cell lines, treatment, apoptotic assay, plasmids and transfection

A549, H358, H1299 and H69 human lung carcinoma cell lines were cultured as previously described {Salon, 2007 #125}. The H810 large cell neuroendocrine lung carcinoma cell line was cultured in 5% CO_2 at 37°C in RPMI-1640 medium supplemented with 5% (v/v) FCS, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-oestradiol and 10 mM HEPES. Murine embryonic fibroblasts (MEF) wild-type and E2F1 -/- were cultured in DMEM (GIBCO) supplemented with 10%(v/v) heat-inactivated FCS. The human lung adenocarcinoma H358/Tet-On/E2F1 and Tet-On/E2F1(E132) inducible clones were obtained as previously described {Salon, 2006 #144}. Apoptosis was evaluated by scoring the percentage of apoptotic cells on 500 cells after Hoechst 33342 staining. Transient transfections were carried out using Fugene 6 (Roche Diagnostic). Plasmids used in transient transfections were pcDNA3, pCMV-E2F-1, pcDNA3-HA-SC35 and pR264-CAT. Recombinant soluble human FLAG-tagged FasL was purchased from Alexis (San Diego, CA, USA). Methanesulfonic acid methyl ester (MMS) and cyclophosphamide monohydrate were all purchased from Sigma (Saint Quentin Fallavier, France).

19

Antibodies

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

26

1 **CAT assays**

2 For CAT assay measurements, 2x10^5 cells per well were seeded in duplicate in 6-wells
3 plates, and transfected with the pR264CAT plasmid in the presence or absence of increasing
4 amounts of pCMV-E2F1 vector. pR364CAT vector contains the 1kb human *sc35* promoter
5 {Sureau, 1992 #2} and encompasses two putative E2F1 binding sites at –170 (TTTGGCCCG)
6 and –236 (TTTCGCGGG) bp upstream of the transcription start site. Transfection was performed
7 using Fugene 6 according to the manufacturer's instructions, and CAT activity was measured 24-
8 48 hours after transfection using CAT ELISA (Roche Diagnostic). CAT activity was then
9 normalized in each sample according to the protein amount.

10

11 **Chromatin immunoprecipitation experiments**

12 Chromatin immunoprecipitation experiments were performed in H358 cells, H1299 cells
13 or H358/Tet-On/E2F1 cells cultured in the presence of 1 μg/ml doxycylin for 24 hours. An equal
14 amount of chromatin (25μg) was precleared and immunoprecipitated with a polyclonal antibody
15 specific for E2F1 (C-20, Santa Cruz) or unrelated rabbit IgG or no antibody, overnight at +4°C.
16 Co-precipitated chromatin was analyzed for the presence of *sc35* promoter DNA between –296
17 and –79 bp upstream of the *sc35* transcription start site by semiquantitative PCR. This fragment
18 encompasses two putative E2F1 binding sites at -170 and –236 bp upstream of the transcription
19 start site. The primers used were as follow: forward 5'-GAGCACCTCCTCTTCCTCCTG-3' and
20 reverse 5'-CCGGAAATGAAACCTTCTGA-3'. PCR conditions were 94°C for 3 min, (94°C 30
21 sec, 55°C 30 sec, 72°C 30 sec) for 36 cycles, and 72°C for 10 min. For Skp2 and glyceraldehyde-
22 3-phosphate dehydrogenase (GAPDH) promoter analysis, the specific primers used were SKP2-
23 95 5'-CTCCCCGCCTACCCCGTGG-3', SKP2-+135 5'-
24 CAGACCCGCTAAGCCTAGCAACG-3', GAPDH forward 5'-
25 AGCTCAGGCCTCAAGACCTT-3' and GAPDH reverse 5'-AAGAAGATGCGGCTGACTGT-
26 3' as previously described {Reichert, 2007 #126}. PCR conditions were 94°C for 3 min, (94°C
27 30 sec, 57°C for gapdh or 63°C for Skp2 30 sec, 72°C 30 sec) for 36 cycles, and 72°C for 10 min.

Signals obtained on ethidium bromide stained gels were quantified using ImageJ software and each ChIP DNA sample was normalized according to the corresponding input DNA sample.

Transfection of siRNA oligonucleotides

The sequences designed to specifically target human *sc35* and *e2f1* RNAs were as follow: sc35(1): 5'-GCGUCUUCGAGAAGUACGGTT-3'; sc35(2): 5'-UCGUUCGCUUUCACGACAATT-3'; e2f-1(1): 5'-GUCACGCUAUGAGACCUCATT-3'; e2f1(2): 5'-ACAAGGCCCGAUCGAUGUUTT-3'. The second siRNA targeting *sc35* was from Invitrogen (Stealth Select RNAi, SFRS2, Invitrogen). The scrambled siRNA oligonucleotides used as control for all RNA interference experiments were as follow: 5'-UCGGCUCUUACGCAUUCAATT-3' and 5'-CAAGAAAGGCCAGUCCAAGTT-3'. Cells were transfected with siRNA oligonucleotides duplex using Oligofectamine reagent according to the manufacturer's instruction (Invitrogen). Doxycyclin (1 μg/ml) was added or not in the culture medium 4 hours after transfection. The cells were analyzed 48 or 72 hours post-transfection. For experiments with cyclophosphamide, cells were transfected for 48 hours with *mismatch*, *sc35* or *e2f-1* siRNAs, then cyclophosphamide (50 μM) was added in the culture medium for 24 additional hours.

RT/PCR analyses of alternative splice transcripts

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

1 **Immunoblotting and immunoprecipitation experiments**

2 Immunoblotting experiments were performed as previously described {Salon, 2006
3 #144}.

4

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19

20 ## References

21

22 ## Titles and legends to figures

23

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

25 **(A)** H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells were incubated for indicated times in
26 the presence (+) or absence (-) of 1μg/ml doxycyclin (Dox). Mutant E2F1(E132) is unable to
27 bind DNA. Expression of E2F1, SC35, SRp20 and SF2/ASF proteins was studied by western

1 blotting. Actin was used as a loading control. **(B)** H69 and H810 neuroendocrine lung carcinoma
2 cell lines were transfected for 72h with *mismatch* or *e2f1* siRNAs as indicated and subjected to
3 western blot analyses for the detection of E2F1, SC35, SRp20 and SF2/ASF proteins. Actin was
4 used as a loading control. **(C)** Western blot analysis of E2F1, SC35, SRp20 and SF2 protein
5 expression in E2F1-deficient (E2F1 -/-) and wild-type control Murine Embryonic Fibroblasts
6 (MEFs). Actin was used as a loading control.
7
8 **Figure 2: SC35 is a direct transcriptional target of E2F1.**
9 **(A)** RT-PCR analysis of *sc35* mRNA. H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells
10 were incubated for 48 or 72 hours in the presence (+) or absence (-) of 1μg/ml doxycyclin (Dox)
11 as indicated. Total RNAs were extracted as described in the material and methods section.
12 Amplified *g3pdh* was used as an internal control. **(B)** Chloramphenicol Acetyl Transferase
13 (CAT) experiments were performed in the H1299 cell line co-transfected for 48 hours with 1μg
14 pR264CAT plasmid, encoding CAT under the control of the *sc35* promoter, and increasing
15 amounts of pCMV-E2F1 as indicated. The CAT activity obtained in cells transfected with
16 pR264CAT alone was normalized to 1 and a relative CAT activity was then calculated for each
17 condition. Representative data of at least three independent experiments performed in duplicate
18 are shown. **(C)** *Upper panel*: H358/Tet-On/E2F1 cells cultured in the presence of doxycyclin for
19 48h were processed for ChIP analysis using C20 antibody for E2F1. The coprecipitated
20 chromatin DNA was analyzed by semiquantitative PCR using pair of primers that amplify the
21 human *sc35*, *skp2* or *gapdh* promoter respectively, as described in the material and methods
22 section. IgG was used as an irrelevant antibody. No Ab means that no antibody was used in this
23 case. *Lower panel*: Similar ChIP analyses were performed in H1299 cells in order to detect the
24 binding of endogenous E2F1 to the *sc35* promoter.
25
26 **Figure 3: SC35 is required for E2F1-induced apoptosis.**

H358 Tet-On/E2F1 cells were cultured in the presence (+) or absence (-) of doxycyclin for 72h. **(A)** H358 Tet-On/E2F1 cells were transfected for 72h either with *mismatch* or *sc35* siRNA. Apoptosis was evaluated using Hoechst staining. Results shown are the mean ± SD of three independent experiments. **(B)** H358 Tet-On/E2F1 cells were transfected for 72h with either *mismatch* or *sc35* siRNA and subjected to RT-PCR (left panel) and western blot (right panel) analyses. **(C)** H358/Tet-On/E2F1 cells were transfected for 48h with either *mismatch* or *sc35* siRNA, cultured with (+) or without (-) doxycyclin as indicated, and treated or not with 25 ng/ml FasL for 20 additional hours. Apoptosis was evaluated as in **(A)**.

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

(A) Alternative splicing patterns of *caspases-2*, *-8*, *–9* and *Bcl-x* primary transcripts. Coding and alternative exons are indicated by ▨ and ■, respectively. The position of primers, the expected sizes (in nucleotides) and the names of the RT-PCR products corresponding to the different mRNA isoforms are indicated. The ability of each transcipt to encode proapoptotic (+) or antiapoptotic (-) protein isoform is depicted on the right. **(B, C)** H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells were cultured for 48 hours in the presence (+) or absence (-) of doxycyclin, as indicated. Total RNAs were extracted and subjected to RT-PCR analyses using the specific primers depicted in **(A)**. Representative agarose gels of RT-PCR products corresponding to *casp-2*, *casp-8*, *casp-9* and *Bcl-x* splicing variants are presented. The position of spliced variants (in bp) is shown on the right, and the various splicing isoforms are named on the left of each panel. *g3pdh* was used as an internal control. **(C)** *Lower panel:* western blot analysis was performed using specific anti-E2F1, anti-Bcl-x_L and anti-Bcl-x_S antibodies. Actin was used as a loading control. Results are representative of three independent experiments. **(D)** H358/Tet-On/E2F1 cells cultured in the presence (+) or absence (-) of 1 μg/ml doxycyclin were transfected for 72 hours with either *mismatch* or *sc35* siRNA and processed as in **(B)**. **(E)** A549 human lung adenocarcinoma cells were transiently transfected for 48 hours with 10 μg pcDNA3.1/SC35 or

control (Mock) vector and processed as in **(B)**. *Upper panel:* immunoblot demonstrating the accumulation of SC35 in cells transfected with pcDNA3.1/SC35 as compared to mock transfected cells. Actin was used as a loading control.

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

(A) H358 cells were treated or not for 24 hours with the indicated cytotoxic agents (50μM, each) and analyzed for E2F1 and SC35 protein levels by western blotting (upper panel). *sc35* mRNA level was studied by RT-PCR in the same conditions (lower panel). Actin and *g3pdh* were used as internal controls for immunoblotting and RT-PCR experiments, respectively. Apoptosis was evaluated after Hoechst 333342 staining. Results shown are the mean ± SD of three independent experiments. **(B)** H358 cells were transfected for 48 hours with either *mismatch* or *e2f1* siRNA and treated (+) or not (-) for additional 24 hours with cyclophosphamide (50 μM). Western blot analyses were performed for E2F1 and SC35 detection. **(C)** H358 cells were treated or not for 24 hours with cyclophosphamide (50μM) and ChIP analyses were performed using C20 antibody for E2F1 and primers specific for the human *sc35* or *gapdh* promoter respectively. IgG was used as an irrelevant antibody. No Ab means that no antibody was used in this case. *Lower panel*: Bands obtained with E2F1 ChIP DNA samples were quantified and normalized relative to the corresponding input DNA sample. *Sc35* promoter occupancy in non-treated cells was arbitrarily set at 1. **(D)** H358 cells were transfected for 48 hours with either *mismatch* or *sc35* siRNA and treated (+) or not (-) for 24 additional hours with cyclophosphamide (50μM). Apoptosis was evaluated after Hoechst 33342 staining and immunoblotting with anti-caspase-3 or cleaved caspase-3 antibody. Results are the mean ± SD of three independent experiments.

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

1 **(A)** H358 cells were treated (+) or not (-) for 24 hours with cyclophosphamide (50 μM). *Left*
2 *panel:* RT-PCR analysis for detection of *caspases -2*, *-8*, *-9* and *Bcl-x* splice variants was
3 performed as previously described in Figures 4B and 4C. *Right panel:* western blot analysis was
4 performed using specific anti-SC35, anti-Bcl-x_L and anti-Bcl-x_S antibodies. Actin was used as a
5 loading control. **(B)** H358 cells were transfected for 48 hours with either *mismatch* or *e2f1* or
6 *sc35* siRNA and treated (+) or not (-) for 24 additional hours with cyclophosphamide (50μM).
7 RT-PCR (upper panel) and western blot (lower panel) analyses of *Bcl-x* or *caspase-9* splice
8 variants were performed as previously described. *G3pdh* and actin were used as internal controls
9 for RT-PCR and immunoblotting experiments, respectively.
10
11 **Supplementary Figure 1:**
12 H358/Tet-On/E2F1 cells cultured in the presence (+) or absence (-) of 1 μg/ml doxycyclin were
13 transfected for 72 hours with either *mismatch* or *sc35* siRNA (Invitrogen). *Upper panel:* Total
14 RNAs were extracted and subjected to RT-PCR analyses using the specific primers depicted in
15 Figure 4A. Representative ethidium bromide stained gels of RT-PCR products corresponding to
16 *casp-8*, *casp-9* and *Bcl-x* splicing variants are presented. The position of spliced variants (in bp)
17 is shown on the right, and the various splicing isoforms are named on the left of each panel.
18 *g3pdh* was used as an internal control. *Lower panel:* western blot analysis was performed using
19 specific anti-E2F1, anti-SC35, anti-Bcl-x_L and anti-Bcl-x_S antibodies. Actin was used as a loading
20 control.