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Apoptosis-stimulating protein of p53-2 (ASPP2/^{53BP2L}) is an E2F target gene

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Abstract

The p53 pathway is a central apoptotic regulator. Deregulation of the Rb/E2F pathway occurs in a majority of tumors, resulting in both unrestrained proliferation and enhanced apoptosis sensitivity via p53-dependent and independent mechanisms. However, the mechanisms coupling the p53 and Rb/E2F pathways remain incompletely understood. We report that ASPP2/^{53BP2L}, a p53/p73-binding protein that promotes p53/p73-dependent apoptosis, is an E2F target gene. The ASPP2/^{53BP2L} promoter was identified and ectopic expression of transcription-competent E2F-1 (E2F-2 and E2F-3) stimulated an ASPP2/^{53BP2L} promoter-luciferase reporter. Mutational analysis of the ASPP2/^{53BP2L} promoter identified E2F-binding sites that cooperate for E2F-1 induction and basal repression of ASPP2/^{53BP2L}. Moreover, endogenous ASPP2/^{53BP2L} levels increased after E2F-1 expression, and E2F-1 bound the endogenous ASPP2/^{53BP2L} promoter after chromatin immunoprecipitation. Typical for an E2F target, ASPP2/^{53BP2L} expression was maximal in early S-phase. Thus, ASPP2/^{53BP2L} is downstream of E2F, suggesting that it functions as a common link between the p53/p73 and Rb/E2F apoptotic pathways.

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Abbreviations: ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; β-gal, β-galactosidase; HU, hydroxyurea; luc, luciferase

Introduction

An important determinant of our ability to treat cancers rests on the observation that tumor cells often have an enhanced apoptotic sensitivity compared to normal cells.[1] The p53 apoptotic pathway is a central mediator of the apoptotic response.[2] Additionally, deregulation of the Rb/E2F pathway also occurs in a majority of human tumors, resulting in both unrestrained proliferation as well as promotion of apoptosis via p53-dependent and p53-independent mechanisms.[3–5] Thus, understanding the molecular linkage between cellular proliferation and sensitivity to apoptotic inputs in cancer cells is one of the most fundamental, yet incompletely understood, issues in oncology today.

The p53 tumor suppressor belongs to a transcription factor family of structurally related proteins including p73 and p63.[2,6] Diverse cellular stresses (such as deregulated oncogenes or genotoxic damage) activate p53; however, what factors favor p53-mediated apoptosis (*versus* other outcomes such as cell cycle arrest) is an incompletely understood and highly complex process dependent on cellular context. p53-induced apoptosis occurs primarily through transactivation of pro-apoptotic target genes,[2] although transcriptional repression as well as transcription-independent functions may also play roles.[7–11] Cell context can influence p53 target gene specificity by affecting p53 covalent modifications or through complex protein–protein interaction networks[2,12,13] (and references within). Moreover, p73 and p63 directly or indirectly cooperate with p53 to trigger apoptosis.[14–17] Understanding the mechanisms that modulate the p53 family will provide important insight into the cellular context favoring the apoptotic response.

Apoptosis Stimulating Protein of p53–2 (ASPP2), also referred to as 53BP2L, encoded by *TP53BP2*,[18–20] enhances damage-induced apoptosis[21,22] through selective stimulation of p53 transactivation of proapoptotic target genes.[19] Recently, ASPP2/^{53BP2L} and its homologue ASPP1 were additionally shown to enhance the apoptotic function of p73 and p63 by selective stimulation of proapoptotic target genes.[23] The carboxy-terminus of ASPP2, originally named 53BP2,[18] interacts with the wild-type, but not mutant, p53 core domain.[24,25] This physical association between ASPP proteins and the conserved core domain of p53 family members appears necessary for full sensitization of cells to apoptotic inputs.[19,23] Given the important functions of ASPP2/^{53BP2L}, it is not surprising that it may be subject to complex regulation dependent on cell context.[22] However, the mechanisms regulating ASPP2/^{53BP2L} remain poorly understood.

The E2F family of transcription factors plays an important role in simultaneously promoting both cell proliferation and cell death. The 'activating' E2Fs (E2F-1, E2F-2, E2F-3) are important for controlling gene expression in late G1/early S-phase in addition to promoting apoptosis – particularly in the case of E2F-1.[3,4,26–29] Entry into the cell cycle is therefore accompanied by sensitization to apoptotic inputs. In tumors that have not completely disabled the apoptotic pathway, this apoptosis sensitization may provide the 'therapeutic window' that allows chemotherapy to selectively kill cancer cells.[1] The Rb/E2F pathway cooperates with the p53 pathway at least in part by transactivation of ARF, although it is not clear how

strictly ARF is required to mediate p53 function.[30–36] E2F-1 also promotes apoptosis via parallel pathways such as by transactivation of p73.[37–39] However, a common link coupling the Rb/E2F and p53/p73 pathways to promote sensitivity to apoptotic signals has not been described.

In this report, we present evidence that ASPP2/53BP2L is a direct E2F target gene. These findings suggest a common link between the p53/p73 and the Rb/E2F apoptotic pathways and provide insight into a possible unifying mechanism of how these pathways cooperate.

Results

ASPP2/53BP2L promoter has features of an E2F target gene

Previous studies have suggested that, through unknown mechanisms, ASPP2/53BP2L expression varies greatly between different cell types,[40] as well as within certain cells in a context-dependent manner.[22] In order to begin understanding the mechanisms controlling ASPP2/53BP2L expression, we examined the genomic sequence encompassing the cDNA start site of ASPP2/53BP2L. Three different computer algorithms independently predicted a promoter region within the 3.0 kB region surrounding the predicted transcription start site (TSS).[41–44] Sequence analysis revealed a dense CpG island (0.98 probability score) (open boxes) encompassing the first exon (green sequence) and the predicted TSS (+1), and multiple putative E2F-binding sites (underlined)[45,46] (Figure 1a). Using the stringent contextual E2F SiteScan algorithm (performed for us by Dr Alexander Kel, Inst. of Cytology & Genetics, Novosibirsk, Russia), two of these E2F sites were further identified as high scoring sites (asterisks).[45] We also found phylogenetic conservation between human and mouse ASPP2 promoters (not shown). Together, the sequence is suggestive that this genomic region contains a promoter and that ASPP2/53BP2L has the features of an E2F target gene.[45]

ASPP2/53BP2L minimal basal promoter

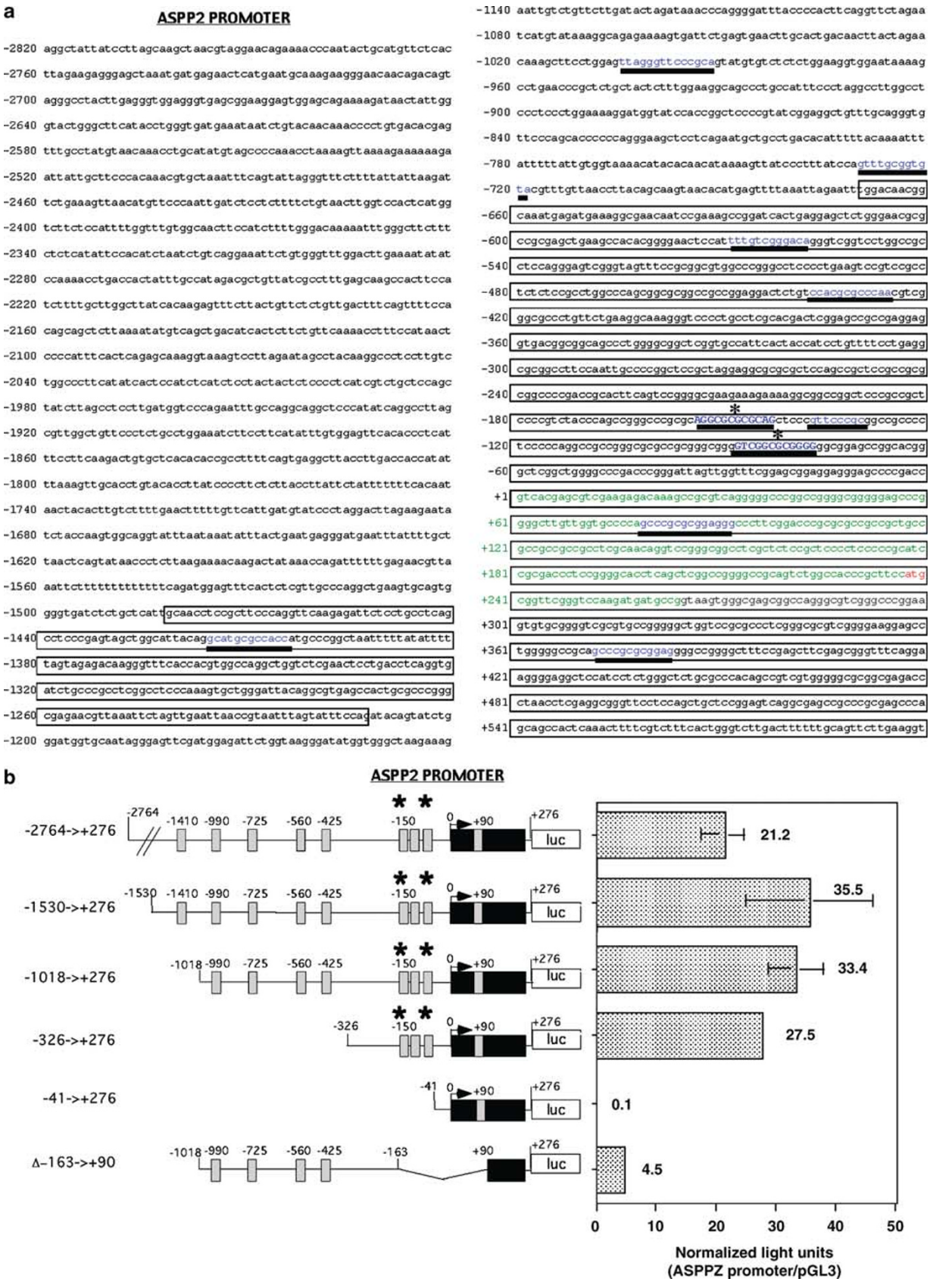
Since the sequence analysis of the region encompassing the ASPP2/53BP2L putative TSS was predicted to contain a promoter region, we wished to functionally interrogate this region for the ability to activate transcription. We first identified a human BAC clone derived from chromosome 1 and containing the *TP53BP2* locus. From this we isolated and sequenced a 3.3 kB genomic fragment from –2764 to +276 relative to the first exon start site, and cloned this into the pGL3-Basic luciferase reporter vector (Promega). After transfection into Saos-2 cells, this region stimulates the luciferase reporter at least a 20-fold relative to the empty vector – suggestive that an active promoter region is present within this region (Figure 1b). To identify the minimal promoter region that could activate transcription, we generated a series of deletion mutants as shown schematically on the left-hand side of Figure 1b. Sequential deletions from the 5′ direction to nucleotide –326 did not attenuate luciferase reporter activation, suggesting that regions downstream of –326 were capable of promoter activation. However, a 5′ deletion to nucleotide –41 eliminates luciferase reporter activation. These results suggest that a region contained within nucleotides –326 to –41 is important for promoter activity. To test the extent to which other upstream or downstream elements cooperate with this region, we deleted nucleotides –163 to +90 from an ASPP2/53BP2L promoter spanning –1018 to +276 and likewise found significant attenuation of luciferase activity. Together, these results are consistent with the notion that a minimal basal ASPP2/53BP2L promoter is contained within this region although the possibility of control elements outside the genomic region used for these studies cannot be excluded.

The ASPP2/53BP2L promoter-luciferase reporter is stimulated by E2F-1 and serum

Since the ASPP2/53BP2L promoter sequence was typical of an E2F-regulated gene (Figure 1), we wished to determine whether our functional ASPP2/53BP2L promoter-reporter was stimulated by E2F as well as serum. Co-transfection of increasing amounts of E2F-1 expression vector with an ASPP2/53BP2L promoter-luciferase reporter (−1018 to +276) results in a dose-dependent increase in luciferase activation (Figure 2a). However, neither transactivation-incompetent E2F-1 (1–368) nor DNA-binding-incompetent E2F-1 (132E) could activate the ASPP2/53BP2L promoter-reporter. We also investigated the ability of other E2F family members to stimulate the ASPP2/53BP2L promoter by co-transfecting E2F-1, E2F-2, E2F-3, and E2F-4 and DP1 expression vectors with the ASPP2/53BP2L promoter-reporter (Figure 2b) and found that the activating E2Fs (E2F-1–3)[28] could stimulate the ASPP2/53BP2L promoter compared to E2F-4 (Figure 2b). Equivalent expression of E2F-4 was confirmed by immunoblot (not shown). Consistent with these findings, after transfection of the ASPP2/53BP2L-promoter-reporter, increased luciferase activity is detected in cells grown in 10% serum, as compared to cells grown in 0.1% serum (Figure 2c). Together, these results suggest that the ASPP2/53BP2L promoter is regulated by E2F.

E2F-1 expression stimulates endogenous ASPP2/53BP2L expression

Since E2F-1 most strongly stimulated the ASPP2/53BP2L promoter-reporter (Figure 2), we wondered if E2F-1 could activate endogenous ASPP2/53BP2L expression. To test this, we utilized a Saos-2 cell line containing an E2F-1 cDNA expression vector under the control of a tetracycline-regulated promoter.[30,47] Addition of the tetracycline analogue doxycycline induced the expression of E2F-1 and an increase in endogenous ASPP2/53BP2L protein and mRNA levels (Figure 3a). To demonstrate that E2F-1 transcription function is required to induce endogenous ASPP2/53BP2L, we utilized tetracycline-regulated Saos-2 cell lines containing either a DNA-binding incompetent mutant E2F-1 (132E), or a mutant E2F-1 lacking the *trans*-activation domain (1–374).[47] Neither E2F-1 (132E) nor E2F-1 (1–374) was able to increase endogenous ASPP2/53BP2L (Figure 3b and c). To determine if ASPP2/53BP2L could be stimulated in different cell types, we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ASPP2 PROMOTER
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-1530->+276
-1018->+276
-326->+276
-41->+276
Δ-163->+90
-2764
-1530
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+90
+276
luc
21.2
35.5
33.4
27.5
0.1
4.5
0
10
20
30
40
50
Normalized light units
(ASPPZ promoter/pGL3)

infected HCT116p53−/− colorectal cancer cells with an adenovirus-E2F-1 expression vector and induced endogenous ASPP2/53BP2L mRNA over two-fold compared with infection with an adenovirus-GFP expression vector (data not shown). These results suggest that the endogenous ASPP2/53BP2L promoter is stimulated by E2F-1.

ASPP2/53BP2L expression is maximal in S-phase

Since ASPP2/53BP2L expression was controlled by E2F (Figures 2 and 3) and the activating E2Fs play roles in controlling late G1/early S-phase genes,[28,48] we reasoned that ASPP2/53BP2L levels would vary throughout the cell cycle. To explore this possibility, we cultured HT1080 cells in 0.1% serum-containing media and found that ASPP2/53BP2L expression declined as cells exited the cell cycle and arrested at G0/G1 (Figure 4a and b). However, upon release of the G0/G1 arrest and re-entry into the cell cycle by culturing in 10% serum, we observed that ASPP2/53BP2L levels increased as cells transitioned from late G1 into early S-phase (Figure 4a and b). Consistent with these findings, increases in ASPP2/53BP2L levels were observed in a public microarray data set (http://genome-www5.stanford.edu/) generated from serum and mitogen-stimulated normal human lymphocytes compared to resting/unstimulated normal human lymphocytes (Figure 4c).[49] Since serum factors might also stimulate ASPP2/53BP2L expression independent of the cell cycle, it was necessary to also examine changes in ASPP2/53BP2L levels throughout the cell cycle independent of serum stimulation. Cells were synchronized at the G1 boundary with hydroxyurea (HU) and, upon removal, ASPP2/53BP2L levels increased as cells entered S-phase (Figure 5). Similar results were obtained in normal human diploid GM38 fibroblasts (not shown). These results suggest that ASPP2/53BP2L expression is cell cycle regulated with maximal expression in early S-phase, and are consistent with our findings that E2F regulates ASPP2/53BP2L. However, the existence of additional pathways that modulate ASPP2/53BP2L levels through serum or mitogenic factors remains possible.

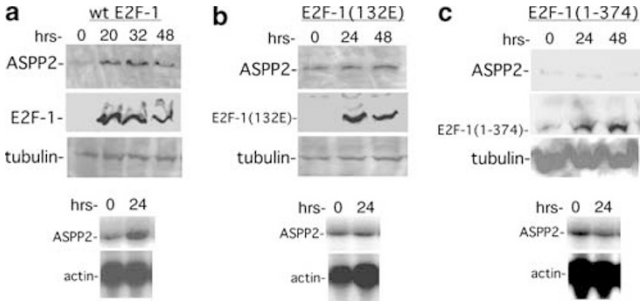

Figure 3 E2F-1 expression stimulates endogenous ASPP2/53BP2L. Western blots (top panels) and Northern blots (bottom panels) for ASPP2/53BP2L on Saos2 cell lines that conditionally express (**a**) wt E2F-1, (**b**) E2F-1(132E), or (**c**) E2F-1 (1–374). Prior to induction, cell lines were maintained in 0.5% FBS containing media for 30 h and then induced with 2.0 μg/ml doxycycline, followed by collection at the indicated timepoints

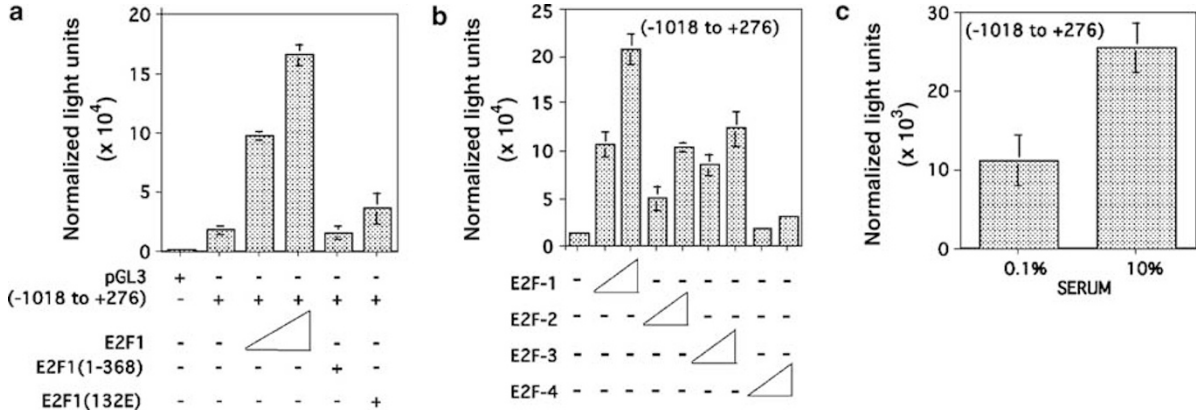

Figure 2 E2F and serum stimulate the ASPP2/53BP2L promoter-luciferase reporter. (**a**) Relative luciferase light units, normalized to β-gal signal, of pGL3 (−1018 to +276) promoter (0.1 μg) co-transfected with increasing amounts of wtE2F-1 (0.05–0.3 μg), or indicated mutant E2F-1 (0.3 μg) expression plasmids, into Saos2 cells. (**b**) Relative luciferase light units, normalized to β-gal signal, of pGL3(−1018 to +276) co-transfected with the indicated HA-tagged E2F (0.05–0.3 μg), and DP1, expression vectors into HCT116p53−/− cells. (**c**) Normalized luciferase light units of pGL3 (−1018 to +276) after transfection into Saos2 cells for 6 h, followed by culturing in 0.1% serum or 10% serum for 24 h. S.D. of triplicate experiments

Figure 1 ASPP2/53BP2L promoter sequence and deletion analysis to define the minimal basal promoter. (**a**) Genomic sequence from nucleotides −2820 to +581 (numbered relative to the predicted transcription start site/cDNA 5′ end indicated as +1). Exon 1 indicated in green type. Open boxes indicate CpG islands. Underlined sequences in blue type denote putative E2F-binding sites. Asterisks denote E2F sites predicted by the SiteScan program (http://compel.bionet.nsc.ru/FunSite/SiteScan.html). (**b**) (Left hand): Schematic representation of a series of promoter deletion mutants in pGL3-Basic-luciferase reporter. Gray boxes denote putative E2F-binding sites, with asterisks denoting sites predicted by the SiteScan program. Black box represents exon 1, open box with [luc] represents luciferase reporter. Bent arrow represents predicted transcription start site. (Right hand): Luciferase readout of indicated promoter-reporters after transfection into Saos2 cells. Light units normalized to β-gal signal and expressed relative to empty pGL3-Basic vector. In all, 0.1 μg of indicated promoter-reporter and pRSV β-gal plasmids were used for all transfections. S.D. of triplicate experiments

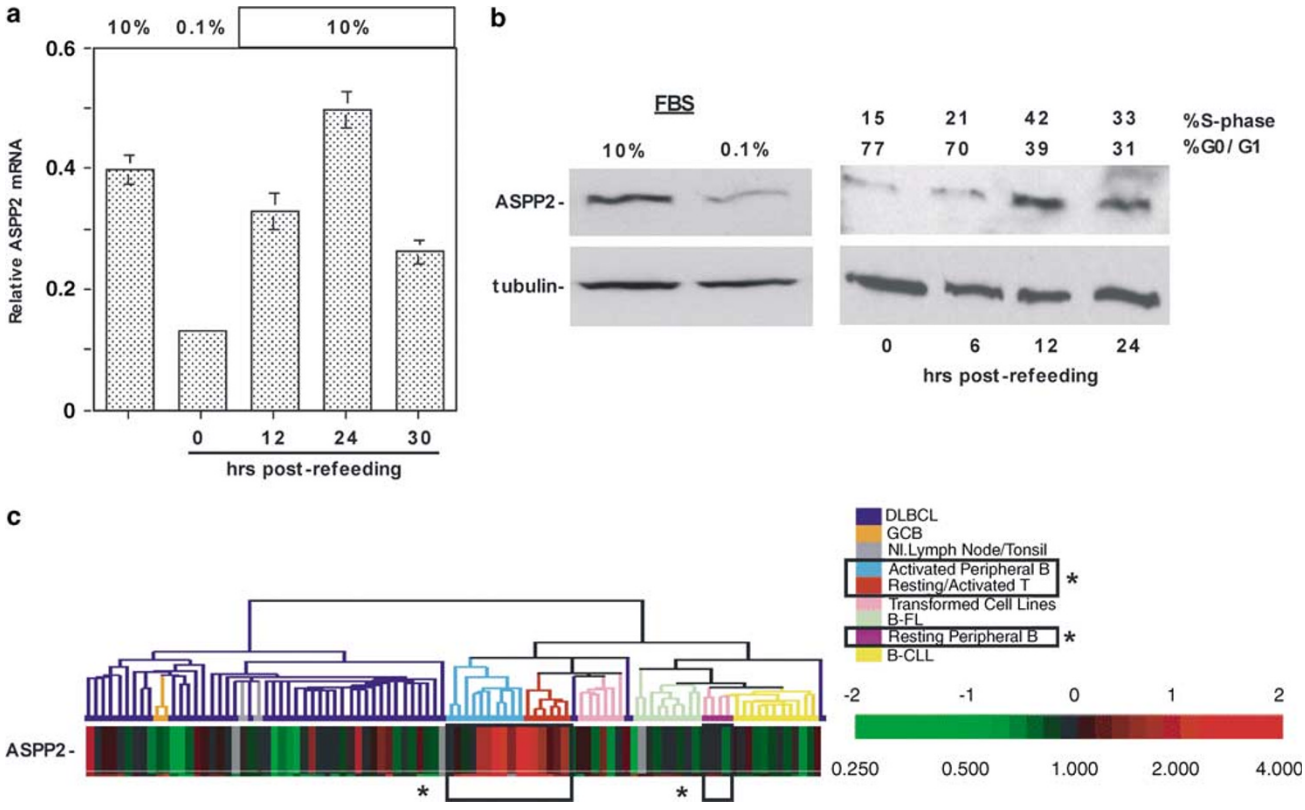

Figure 4 Endogenous ASPP2/^{53BP2L} expression stimulated by serum release. HT1080 cells were cultured in 0.1% FBS containing media for 30 h and then re-fed with 10% FBS containing media followed by collection at indicated timepoints. (**a**) Relative ASPP2/^{53BP2L} mRNA expression measured by quantitative RT-PCR in exponentially growing cells in 10% FBS (left hand column), or at the indicated timepoints after starvation and re-feeding. S.D. of triplicate experiments. (**b**) ASPP2/^{53BP2L} Western blot on lysates prepared from cells at the indicated conditions. Percentages of S-phase and G0/G1-phase obtained at indicated timepoints determined by flow cytometry and cell cycle analysis. (**c**) Microarray expression profile of ASPP2/^{53BP2L} in a diffuse large-cell lymphoma data set (http://genome-www5.stanford.edu/)[49] that includes serum/mitogen-stimulated normal human peripheral lymphocytes demonstrating four-fold greater ASPP2/^{53BP2L} expression relative to resting peripheral lymphocytes (blue/red *versus* purple dendrogram arms)

E2F-binding site mutants attenuate ASPP2/^{53BP2L} promoter activation

Sequential deletions from the 5′ direction suggest that a minimal promoter is contained within −326 to −41. Moreover, a dropout deletion from −163 to +90 implicates control elements possibly to +90, relative to the first ASPP2/^{53BP2L} exon (Figure 1b). Sequence analysis from −326 to +90 reveals four putative E2F-binding sites, denoted as M1, M2, M3, and M4 in Figure 6a. Two sites, M1 and M3, were identified using the contextual E2F SiteScan algorithm and are denoted with asterisks.[45] In order to interrogate these putative E2F-binding sites for ability to influence ASPP2/^{53BP2L} promoter activation, we changed the core nucleotide CG motif to AT in each binding site alone, or in combination (M1–4), and determined the differences in ASPP2/^{53BP2L} promoter-reporter basal luciferase activity (Figure 6b). Relative to basal promoter activity contained within the –326 to +276 ASPP2/^{53BP2L} promoter-reporter, mutation at the M3 E2F site alone results in a greater than 10-fold stimulation of basal promoter activity. In contrast, mutagenesis at other individual putative E2F sites does not appreciably alter basal promoter activity, although mutation of all four sites modestly attenuates basal promoter activity. Increases in basal promoter activity after mutation at specific E2F sites have been described with other E2F-regulated genes.[28,48,50–52] To determine if mutations at these E2F sites affect E2F-inducibility of the ASPP2/^{53BP2L} promoter, we co-transfected increasing amounts of E2F-1 expression vector with the different E2F-binding site mutant ASPP2/^{53BP2L} promoter-reporters (Figure 6c). ASPP2/^{53BP2L} promoter-reporters harboring individual binding site mutations remained sensitive to E2F-1-induction in a dose-dependent manner, although the absolute induction of the M3 site mutant promoter was significantly higher because of the enhanced basal levels. In contrast, mutation of all four putative E2F sites in this promoter region (M1–4, Figure 6c) significantly attenuates E2F-1 induction. Similarly, when the ASPP2/^{53BP2L} promoter-reporter M1–4 (with all four putative E2F sites mutated) was assayed after serum starvation and release, luciferase activation was significantly attenuated compared to the wild-type ASPP2/^{53BP2L} promoter-reporter (Figure 6d). Together, these results support the notion that ASPP2/^{53BP2L} is a direct E2F target gene, but that ASPP2/^{53BP2L} regulation by E2F is complex, with different E2F sites potentially playing different roles – alone or in combination.

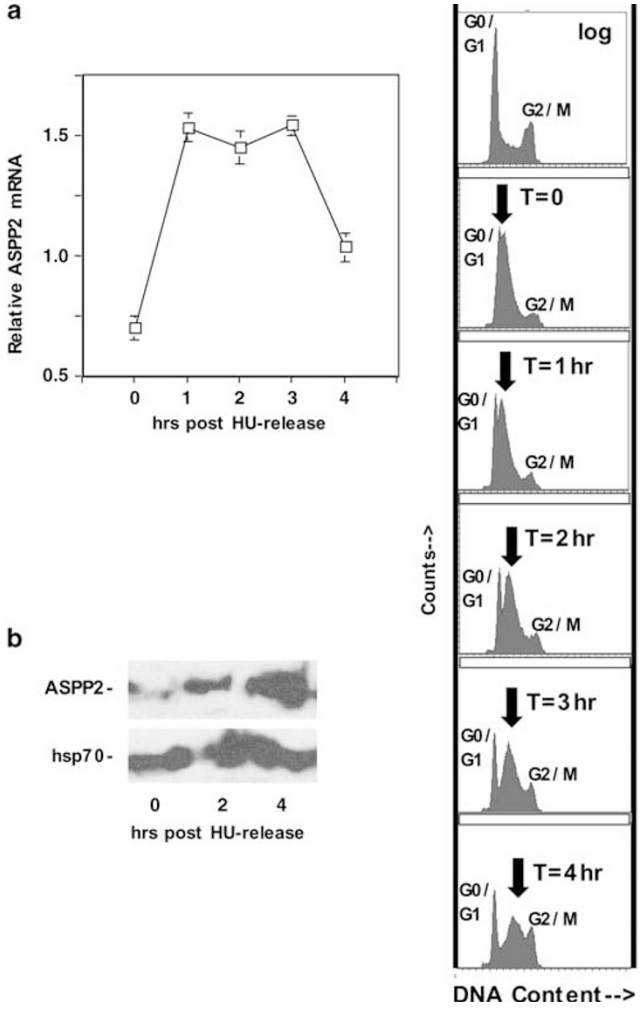

Figure 5 Endogenous ASPP2/53BP2L expression stimulated upon S-phase entry independent of serum factors. HCT116p53−/− cells were synchronized at the G1/S boundary with 1.3 mM HU for 24 h, followed by PBS washout and analysis at the indicated timepoints. (**a**) Relative ASPP2/53BP2L mRNA expression measured by quantitative RT-PCR at the indicated times after HU washout. S.D. of triplicate experiments. (**b**) ASPP2/53BP2L Western blot on lysates prepared from cells at the indicated times after HU washout. (Right-hand panels) Cell cycle profiles of exponentially growing cells (log), cells after 24 h in HU ($T=0$), and cells at the indicated times after HU removal. Downward arrow denotes S-phase

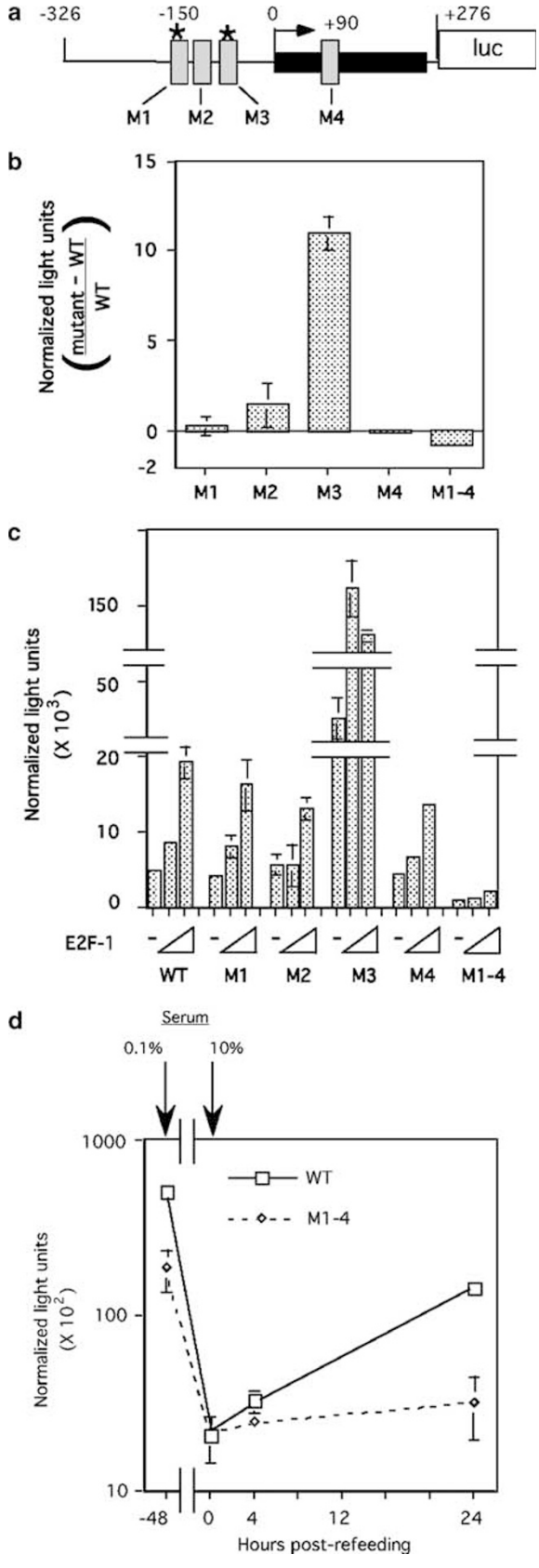

Figure 6 E2F-binding site mutations alter ASPP2/53BP2L promoter luciferase expression. (**a**) Schematic representation of putative E2F-binding sites (gray boxes) mutated with CG to AT core site changes in the pGL3 (−326 to +276) promoter-reporter. (**b**) Basal relative luciferase light units, normalized to β-gal signal, of the indicated mutant ASPP2/53BP2L promoter-reporters transfected into HCT116p53−/− cells. Fold change of the indicated mutant basal promoter activity expressed relative to nonmutated (WT) pGL3 (−326 to +276): ((*mutant−WT)/WT*). M1–4 denotes all four sites mutated; single-site annotations indicate single-site mutants. (**c**) β-gal normalized luciferase light units of the indicated mutant ASPP2/53BP2L promoter-reporters co-transfected with E2F-1 expression vector (0.05–0.3 μg). Discontinuous *Y*-axis scaled to permit representation of M3 mutant (with high basal activity) with other mutants. (**d**) β-gal normalized luciferase light units of pGL3 (−326 to +276) (WT) and M1–4 ASPP2/53BP2L promoter-reporters, transfected into HCT116p53−/− cells in 10% FBS, switched to 0.1% FBS for 48 h, and then re-fed with 10% FBS, and assayed at the indicated timepoints. S.D. of triplicate experiments

Endogenous ASPP2/53BP2L promoter binds E2F-1 protein

Since ASPP2/53BP2L expression is induced by E2F (Figures 2 and 3) and site mutations at E2F-binding sites in the ASPP2/53BP2L promoter can attenuate E2F-1 induction (Figure 6c), we reasoned that E2F-1 would bind the endogenous ASPP2/53BP2L promoter in its native chromatin context. To examine this, we performed a chromatin immunoprecipitation (ChIP) assay on the proximal ASPP2/53BP2L promoter region (Figure 7). ASPP2/53BP2L promoter-specific PCR primers amplify this region after chromatin IP with an anti-E2F-1-specific antibody, but not an irrelevant control antibody (anti-caspase 12), as shown in Figure 7a. To further demonstrate that this was a specific interaction, ASPP2/53BP2L-specific primers that amplify an upstream region that does not contain E2F-binding sites (control primers) cannot generate a PCR product after chromatin IP with anti-E2F-1-specific antibody (Figure 7a). To further demonstrate the specificity of the E2F-1 DNA-binding interaction on the ASPP2/53BP2L endogenous promoter, we also performed chromatin IP with an anti-HA antibody on HCT116p53−/− cells transfected with an HA-tagged wild-type E2F-1 expression vector and successfully PCR amplified the ASPP2/53BP2L proximal promoter region (Figure 7b). In contrast, no PCR product could be amplified after chromatin IP with anti-HA antibody from cells transfected with an HA-tagged DNA-binding incompetent mutant E2F-1 (132E), nor from cells transfected with an HA-empty vector. Equivalent expression of transfected E2F-1 is shown on an anti-HA immunoblot in Figure 7b, bottom panel. These findings suggest that the ASPP2/53BP2L promoter, in its native chromatin context, can specifically bind E2F-1 protein, and is consistent with our data demonstrating that ASPP2/53BP2L is a direct E2F target gene.

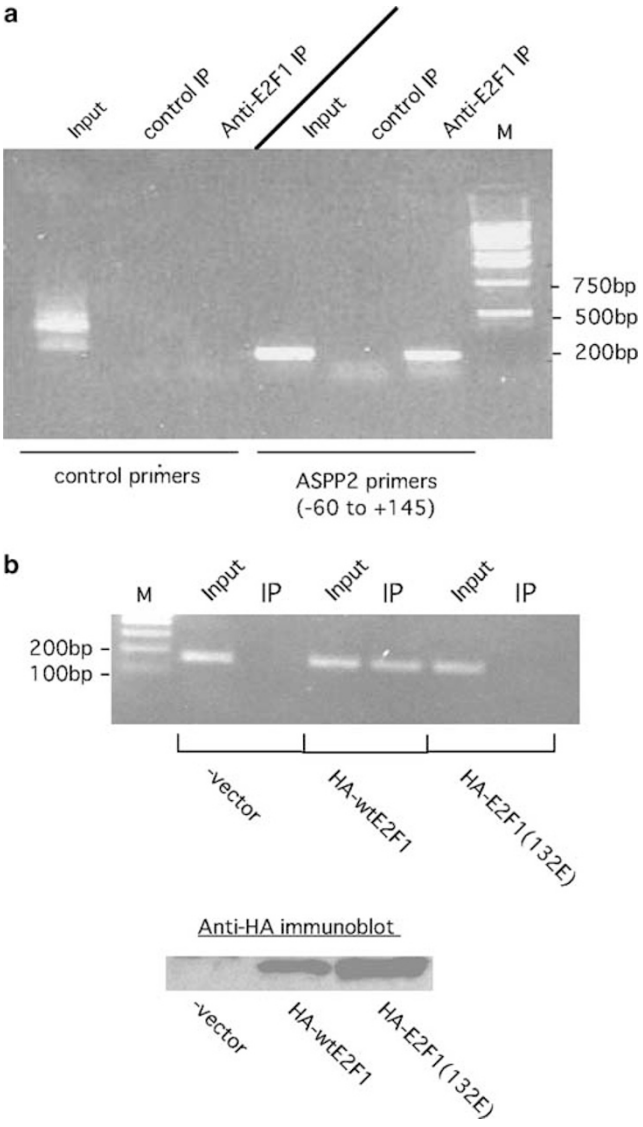

Figure 7 ChiP of E2F-1 at the ASPP2/53BP2L promoter. Ethidium bromide-stained agarose gels of PCR products. (**a**) ChIP was performed using an anti-E2F-1 antibody or irrelevant control antibody (anti-caspase 12), followed by PCR amplification with ASPP2/53BP2L primers specific for the proximal promoter (right hand) or ASPP2/53BP2L-specific primers 11 kB upstream of the transcription start site (control primers). (**b**) Top panel: PCR products after anti-HA immunoprecipitation of HCT116p53−/− cells transfected with empty vector, HA-E2F-1, or DNA-binding incompetent HA-E2F-1 (132E). Bottom panel: Anti-HA immunoblot on cell lysates from cells transfected in parallel with indicated expression vectors. All input chromatin at 1 : 1000 dilution of IP reactions

Discussion

We have demonstrated that ASPP2/53BP2L is a direct E2F target gene utilizing complex mechanisms within the proximal promoter region. Given that ASPP2/53BP2L may play an important role in modulating sensitivity to apoptotic signals at least in part through stimulation of p53 family member apoptotic function,[19,21–23] our findings that ASPP2/53BP2L is downstream of the Rb/E2F pathway suggests a previously undescribed link between these two important tumor suppressor pathways.

Our data underscore the complex nature of ASPP2/53BP2L regulation that has been previously suggested,[22] and now provides mechanistic insight into an upstream pathway controlling ASPP2/53BP2L transcription. First, the ASPP2/53BP2L promoter has typical features suggestive of an E2F target (Figure 1). Second, expression of E2F family members 1, 2, and 3 can stimulate an ASPP2/53BP2L promoter-luciferase reporter (Figure 2) as well as endogenous ASPP2/53BP2L levels, at least when tested with E2F-1 in the latter example (Figure 3). Third, mutation of the four E2F-binding sites in the proximal promoter region (–326 to +276) significantly attenuates E2F-1 induction of the ASPP2/53BP2L promoter-luciferase reporter (Figure 6c), as well as serum induction (Figure 6d). This suggests that these E2F sites cooperate for full E2F-1-induced activation at the ASPP2/53BP2L promoter since individual mutations at these sites do not attenuate E2F-1 induction. We also noted that mutation at E2F site M3 results in a dramatic increase in ASPP2/53BP2L basal promoter activity (Figure 6b), as has been described for many E2F-regulated genes. This suggests de-repression, for example, due to loss of E2F-4/5-p130 repressor complexes,[28,48,50–52] although whether such regulation also occurs with ASPP2/53BP2L remains to be experimentally tested. Finally, we found that endogenous

E2F-1 could bind the endogenous ASPP2/53BP2L proximal promoter in its native chromatin context, and that wild-type E2F-1, but not DNA-binding incompetent E2F-1(132E), binds the ASPP2/53BP2L promoter (Figure 7). Taken together, these results strongly suggest that ASPP2/53BP2L is an E2F target gene and that the E2F DNA-binding sites contained within –326 to +276 play an important role in regulating ASPP2/53BP2L. However, the precise contributions of the different E2F sites contained within this region – alone or in combination – and which specific roles the various E2F family members may play in controlling ASPP2/53BP2L induction remain to be elucidated. Moreover, we cannot exclude that other E2F response elements may also be important for regulating ASPP2/53BP2L outside the –326 to +276 promoter region.

Our observations also imply that other non-E2F-mediated upstream pathways are involved in regulating ASPP2/53BP2L transcription. Although ASPP2/53BP2L expression is cell cycle regulated with maximal expression at early S-phase as is typical for E2F-regulated genes (Figures 4 and 5), we cannot exclude that additional mitogen-induced regulation by serum factors could also be important, since the ASPP2/53BP2L promoter is stimulated by serum (Figures 2c and 6d). Mutation of all four E2F sites in the ASPP2/53BP2L minimal promoter (−326 to +276) significantly attenuates serum-induced promoter activation, suggesting that the combination of these E2F sites is important for mediating serum responsiveness (Figure 6d). However, the basal activity of this mutated minimal promoter in the presence of 10% serum still remains significantly elevated compared to empty vector control, implying that other *cis*-acting control elements are also involved in regulating ASPP2/53BP2L. Studies interrogating the ASPP2/53BP2L promoter to further define other upstream regulatory pathways are underway.

In this report, we focus on the transcriptional control of ASPP2/53BP2L by E2F as this places ASPP2/53BP2L in a central role linking the Rb/E2F and p53 family member pathways. However, given that ASPP2/53BP2L is a potent modulator of cellular apoptotic thresholds,[19,22,23] it is likely that multiple mechanisms are important for controlling ASPP2/53BP2L levels. Interestingly, the proteins encoded by the two *TP53BP2* mRNA splice variants, ASPP2 or 53BP2L (long), and BBP or 53BP2S (short),[20,53] have different abilities to promote p53-mediated apoptosis: ASPP2/53BP2L has more potent proapoptotic function than BBP/53BP2S.[19] Thus, mechanisms regulating these different splice variants may have a profound influence on biologic outcomes. Likewise, post-translational mechanisms may also play important roles in controlling ASPP2/53BP2L function.[22,54] However, details of such potential mechanisms remain to be explored. Nevertheless, the paradigm that both transcriptional and post-transcriptional mechanisms can play cell context-dependent roles in controlling proapoptotic gene products has been described, such as is the case for E2F-1.[4,29,55–57] To what extent cell context influences E2F regulation of ASPP2/53BP2L remains to be determined. Intriguingly, as described in the accompanying report, ASPP1 is also regulated by E2F. Although both ASPP2/53BP2L and ASPP1 can stimulate transcription function of the p53 family members,[23] they also appear to have distinct functions.[19] Moreover, ASPP2/53BP2L knockout mice are not viable, suggesting that ASPP1 does not have completely overlapping function with ASPP2/53BP2L (Lopez *et al*., unpublished data; Lu *et al*., unpublished data). The functional implication of E2F regulating both ASPP2/53BP2L and ASPP1 remains to be elucidated but underscores the complexity of the E2F-ASPP pathway.

In the majority of human tumors, deregulation of the Rb/E2F pathway (e.g. such as by loss of Rb, overexpression of D-type cyclins, or loss of $p16^{INK4}$) releases E2F activity, resulting in both unrestrained proliferation as well as promotion of apoptosis via p53-dependent and p53-independent mechanisms – at least in tumors that have not disabled the apoptotic pathway.[3–5] E2F-1 protein is also stabilized in response to damage to promote apoptotic function through complex and incompletely understood mechanisms.[29,55,57–60] E2F-1 can lower the apoptotic threshhold by directly activating death machinery genes such as caspases, Apaf-1 and BH3-only proteins[61–64] in addition to directly activating p73.[37,38] Our finding that ASPP2/53BP2L is an E2F target gene provides the basis for a working model of a common linkage between the Rb/E2F and p53/p73 apoptotic pathways independent of the ARF–MDM2 axis (Figure 8). Consistent with the notion that ASPP2/53BP2L may play a role in tumorigenesis and chemotherapy sensitivity, ASPP2/53BP2L (and ASPP1) expression is frequently silenced in breast cancers,[19] and low ASPP2/53BP2L expression trends towards a poor clinical outcome in patients with diffuse large B-cell lymphoma treated with anthracycline-based chemotherapy.[65] Although the precise role of the E2F-ASPP2/53BP2L pathway in mediating cell death sensitivity remains to be elucidated, our results nevertheless open new avenues for investigation into the molecular mechanisms linking two important tumor suppressor pathways. Therapies designed to exploit these pathways by enhancing E2F-ASPP2/53BP2L apoptosis may ultimately prove useful in the oncology clinic.

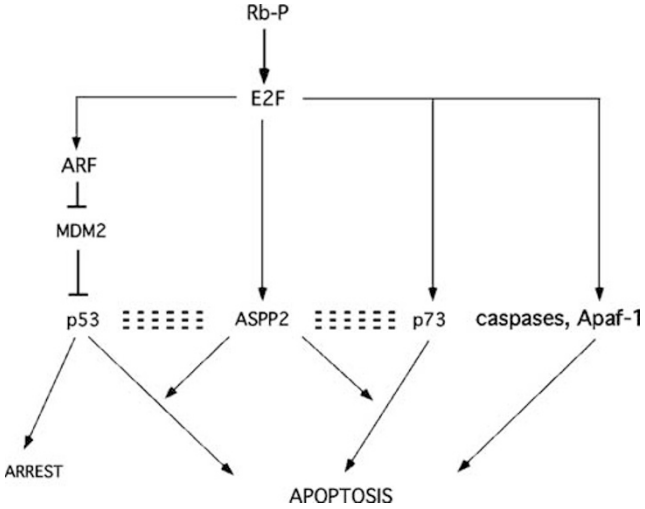

Figure 8 Working model of ASPP2/53BP2L linking the p53/p73 and Rb/E2F pathways. Lines with arrowheads denote positive regulation, slashed lines denote negative regulation, dashed lines represent physical interaction between ASPP2/53BP2L and p53/p73

Materials and Methods

Cloning of the ASPP2/53BP2L promoter and plasmids

A human BAC clone (RP11-332L18) derived from chromosome 1 containing the *TP53BP2* locus (BACPAC Resources) was used to PCR amplify different regions from −2764 to +276 relative to the ASPP2/53BP2L cDNA start site.[19,20] PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen) and then cloned into the pGL3-Basic luciferase vector (Promega) using standard restriction endonuclease digestion and DNA ligation. Primer sequences used were: (−2764 to +276) forward 5′-gaagagggagctaaatgatgag-3′; reverse 5′-tcgcccacttaccggcatcatc-3′. (−1530 to +276) Forward 5′-ctctcgttgcccaggctgaagtg-3′; reverse 5′-tcgcccacttaccggcatcatc-3′. (−326 to +276) Forward 5′-gtgacggcggcagccctggggcgg-3′; reverse 5′-tcgcccacttaccggcatcatc-3′. pGL3 (−1018 to +276) was generated by *Hind*III digest and religation of pGL3 (−1530 to +276). pGL3 (Δ−163 to +90) was generated by *Sma*I digest and religation of pGL3 (−1018 to +276). pGL3 (−41 to +276) was generated by *Apa*I digest and religation of pGL3 (−1018 to +276). The different site mutations were generated in the pGL3 (−326 to +276) vector with the Promega GeneEditor system according to manufacturer-specified protocols. All resultant constructs were sequence verified. The mutation oligonucleotide sequences were as follows:

M1: 5′-cccgcgcaggcgatcgcagctcccgt-3′. M2: 5′-agctcccgttccatcggcc gcccctc-3′.

M3: 5′-gggcggggtcggatcgggggcgggag-3′. M4: 5′-ccccagcccgcatggag ggcccttc-3′.

Various E2F family member expression constructs were gifts from Drs. William Kaelin (Harvard University Dana Farber Cancer Institute, Boston, MA, USA), WC Lin (University of Alabama, Birmingham, AL, USA), and Rosalie Sears (Oregon Health & Science University, Portland, OR, USA). DP1 and pRSV β-galactosidase (β-gal) expression vectors were gifts from Dr. Rosalie Sears.

Luciferase-reporter assays

Cells grown to 60% confluence in six-well plates were transfected with the specified ASPP2/53BP2L luciferase reporter plasmid, together with specified E2F expression vector at the indicated amount, pRSV β-gal expresssion vector (0.1 μg), as well as with the appropriate amount of empty plasmid vector, to give a total of 1 μg plasmid DNA per transfection. Transfections were performed with FuGENE 6 reagent per manufacture protocol (Roche). At 36 h post-transfection, cells were harvested for luciferase assays as described previously.[66] Briefly, cells were collected into ice-cold 0.1 M K_2PO_4, 1 mM DTT (pH 7.8) buffer, freeze/thawed three times in liquid nitrogen, centrifuged at 10 000 × g × 20 min at 4°C, and supernatants collected. Luciferase activity was assayed in 10 mM luciferin, glygly buffer (25 mM glygly, 15 mM $MgSO_4$, 10 mM ATP, pH 7.5), with an AutoLumatB953. Relative luciferase light units were normalized to β-gal activity determined by incubating 20 μl lysate with 20 μl β-gal reaction buffer (1 mg/ml CPRG, 50 mM K_2PO_4 (pH 7.8), 1 mM $MgCl_2$) for 20 min at 37°C and reading absorbance at 570 nm.

Western blotting

Total cellular lysates were prepared and quantitated as described previously,[22] resolved on SDS-6% SDS-PAGE, transferred to PVDF membrane, blocked with 5% nonfat milk, probed with the specified primary antibody, followed by HRP-conjugated secondary antibody, and then visualized with enhanced chemiluminescence (Pierce SuperSignal) and X-ray film using standard techniques. Anti-ASPP2/53BP2L mouse monoclonal antibody was from BD-BioSciences, anti-E2F-1 antibodies were from Santa Cruz (C-20) or from NeoMarkers (KH20), anti-HSP70 antibody was a gift from Dr. Louie Naumovski (Stanford University), anti-tubulin and anti-HA antibodies were from Sigma. HRP-conjugated secondary antibodies were from Jackson.

Quantitative RT-PCR

TaqMan real-time PCR was performed as described previously[65] with minor modifications. Briefly, total RNA was isolated with the RNAeasy kit (Qiagen) and reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) as reported.[67] Expression of mRNA for ASPP2/53BP2L and 18S endogenous control was measured in each specimen with real-time PCR using the Applied Biosystems Assays-on-Demand™ Gene Expression Product and 18S endogenous control kit (P/N 4319413) on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For calibration and generation of standard curves, we used HCT116 cDNA. All reactions were carried out in triplicate with minimal Ct variability seen, with a variance of 0.14. ASPP2/53BP2L signal was normalized to an 18S signal and the relative expression of ASPP2/53BP2L across samples was determined by the ASPP2/53BP2L/18S ratio from each sample relative to the calibrator RNA sample derived from HCT116 cells. Northern blots were performed as described previously.[22]

Chromatin immunoprecipitation (ChIP)

ChIP analysis on HCT116p53−/− cells was performed as described,[66] with minor modifications. Briefly, cells were crosslinked with 1% formaldehyde solution in PBS × 15 min and then stopped with 0.125 M glycine × 5 min. Cells were then lysed in 5 ml of lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 5 mM EDTA) with protease inhibitors (10 μg/ml each of PMSF, leupeptin, and pepstatin) and lysates sonicated on ice to yield chromatin fragments of approximately 600–1000 nucleotides. Debris was removed by centrifugation at 13 000 × g × 10 min and extract pre-cleared with 50 μl of protein A-sepharose/salmon sperm DNA slurry (1 : 1) for 30 min at 4°C. In all, 2 μg of anti-E2F1 antibody (Santa Cruz), 2 μg of control antibody (anti-caspase 12), or 2 μg of anti-HA antibody was incubated with precleared supernatants overnight at 4°C. A volume of 50 μl of protein A/G-agarose slurry was added and incubated for 2 h at 4°C. Beads were washed twice with ice-cold RIPA buffer, four times with ice-cold IP wash buffer (100 mM Tris (pH 8.0), 500 mM LiCl, 1% NP-40, 1% deoxycholic acid), and twice more with ice-cold RIPA buffer. IP elution buffer (150 μl; 50 mM $NaHCO_3$, 1% SDS, freshly made) was added and rocked for 15 min at RT, and the beads spun out and supernatant collected. Another 150 μl IP elution buffer was added to the beads as before and supernatants combined. To reverse the formaldehyde crosslinking, 1 μl of 10 mg/ml RNase A and 12 μl of 5 M NaCl were added and incubated at 65°C × 6 h. DNA fragments were purified by phenol–chloroform extraction, ethanol precipitation, and dissolved in 100 μl of $n$$H_2O$. PCR amplification of specific ASPP2/53BP2L promoter sequence was performed with 25 cycles (94°C for 15 s; 65°C for 45 s), and the product analyzed on a 2% TAE-agarose gel stained with ethidium bromide. ASPP2/53BP2L-specific primers used were: (−60 to +145) forward primer: 5′-gctcggctggggcccgacccgg-3′; reverse primer: 5′-gacctgttgcgaggcggcggcggc-3′. ASPP2/53BP2L control primers used were: (−110096 to −110440) forward primer: 5′-gcatgcgcctatagtcccagc-tac-3′; reverse primer: 5′-caggtttgcaccacacatgg-3′. For ChIP assays on

transfected cells, 4 μg each of HA-E2F1, HA-E2F1(132E), or empty plasmid expression vectors was transfected into HCT116p53−/− cells in 10 cm plates and harvested for analysis 24 h later.

Cell culture

Cells were grown in DMEM or McCoy's media supplemented with 10% heat-treated fetal bovine serum (FBS) and 290 μg of L-glutamine, 100 U penicillin, and 100 μg of streptomycin per ml and maintained in logarithmic growth at 37°C in 5% CO_2. Saos2 cells stably transfected with the various E2F-1 expression vectors under control of tetracycline-regulated promoters (a gift from Dr. Karen Vousden, Beatson Institute, UK) were maintained in certified tetracycline-free FBS (Clontech). HCT116p53−/− cells were a gift from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD, USA). All other cell lines were obtained from the American Type Culture Collection (ATCC).

Flow cytometry

Cells were fixed and stained with propidium iodide and analyzed on a Becton Dickinson FACScan as described previously.[22] Cell cycle analysis was performed using ModFitLT Software (Verity).

Acknowledgements

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Note added in proof

For this report, we designated the 5′-end of the published ASPP2/53BP2L cDNA (Samuels-Lev *et al*.[19]) as the putative transcription start. After our manuscript went to press, it was pointed out to us that Fogal and Lu (this issue) designated the putative transcription start site 55 bp upstream of the published sequence based on an unpublished EST in the database (gl 30774493). Therefore, although the nomenclature of these two reports differs slightly, the conclusions remain completely unchanged.

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