Selection of novel mediators of E2F1-induced apoptosis through retroviral expression of an antisense cDNA library

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ABSTRACT

The E2F1 transcription factor is an essential mediator of p53-dependent and p53-independent apoptosis as part of an anti-tumour safeguard mechanism. In this study, a functional so-called technical knockout (TKO) approach was applied to Saos-2ERE2F1 cells that conditionally activate E2F1 by the addition of 4-hydroxytamoxifen to search for p53-independent pro-apoptotic E2F1 targets. The approach was based on random inactivation of genes after retroviral transfer of an antisense cDNA library enriched of E2F1-induced genes, followed by the selection of Saos-2ERE2F1 cells that survive in the presence of the apoptotic stimulus. We identified 13 novel E2F1 target genes encoding proteins of known cellular function, including apoptosis and RNA binding. FACS analysis revealed that E2F1-induced apoptosis was significantly attenuated in cell clones containing the antisense cDNA fragments of these genes, demonstrating their participation in E2F1 death pathways. Moreover, inactivation of the target genes resulted in a clear increase of cell viability (>80%) in response to E2F1 activation compared with controls (∼30%). Four genes showed an increase in expression intensity in the presence of cycloheximide, suggesting a direct effect of E2F1 on gene transcription, whereas one gene was identified as an indirect target. Our data provide new insight in the regulation of E2F1-induced apoptosis.

INTRODUCTION

E2F is a transcription factor family that modulates cellular proliferation, differentiation and apoptosis of eukaryotic cells (1–4). It functions as heterodimers with members of the DP family (DP-1 and DP-2). E2F1 transcriptional activity is modulated at multiple levels, including negative regulation by association with the RB tumour suppressor gene product and its related proteins p107 and p130 (5). The interaction between E2F1 and RB is limited by Rb phosphorylation, which is mediated by the cyclin D/cyclin-dependent kinase-4 at the G1/S transition point, thus releasing E2F1 and leading to cell cycle progression (1,5,6). In response to genotoxic stress, E2F1 is stabilized by distinct mechanisms, including direct phosphorylation through the ataxia–telangiectasia mutated (ATM) kinase, the ATM and RAD3-related (ATR) kinase and the Chk2 kinase (7–9), and also by acetylation through p300/CREB-binding protein associated factor (P/CAF) (10).

Among seven E2F family members identified so far, E2F1 is unique in that it efficiently regulates both cellular proliferation and apoptosis. Several recent reports have emphasized that apoptosis induction by E2F1 activation can be both dependent and independent of p53. In the p53-dependent pathway, over-expression of E2F1 induces p53 stabilization and accumulation (3,11), e.g. through direct transactivation of p14ARF/p19ARF tumour suppressor genes (12,13). In the absence of ARF (14–16), E2F1 uses the ATM signalling pathway to induce p53 and Chk2 phosphorylation and thereby apoptosis (9). In the p53-independent pathway, a variety of key pro-apoptotic genes are directly upregulated by E2F1 activation, including p73 (17,18), Apaf-1 (19), and BH3-only proteins, PUMA, Noxa, Hrk/DP5 and Bim (20). All of them contribute to E2F1-induced apoptosis. Nevertheless, the mechanism by which E2F1 induces apoptosis has not been fully understood.

One of the first function-based gene identification systems in mammalian cells was the so-called technical knockout (TKO) method, where an antisense cDNA library in an episomally replicating vector (episomal shuttle vector) was introduced into human cells and subsequently the cells were

screened for the loss of response towards a killing signal. It is assumed that the specific inactivation of a growth inhibitory gene conveys growth advantage in a specific restrictive environment, followed by the selection of phenotypic changes caused by its inactivation (21). This functional genetic approach led to the identification of several mediators of IFN-γ induced cell death, including cathepsin D protease, DAP3 (death-associated protein-3) and a novel serine/threonine kinase (21–24). As the TKO selection requires the transfection of large number of cells with one/few transcription units that express individual antisense cDNAs in the target cells, the maintenance and isolation of these episomally promoted vectors can be difficult to achieve. As retroviruses are the only gene transfer system that efficiently and stably integrates into the genomic DNA of cells thereby readily allowing single-cell cloning, we decided to switch the TKO approach to an oncoretroviral expression system for the introduction of an antisense cDNA library into target cells.

A key element in this approach is a cDNA library, which is enriched in transcripts of genes involved in p53-independent E2F1-mediated apoptosis. In the p53-deficient human osteosarcoma cell line, Saos-2ERE2F1, which constitutively expresses E2F1 fused to the binding domain of the murine oestrogen receptor (ER) (25), the addition of 4-hydroxy-tamoxifen (4-OHT) results in the translocation of E2F1 to the nucleus where it activates its downstream target genes. Using this cell system, we previously identified a number of genes with different cellular functions that are upregulated by E2F1 in a cDNA microarray-based analysis (25). This cell line was utilized here to construct an antisense cDNA library enriched in E2F1-induced genes in a self-inactivating oncoretroviral vector. Following retroviral infection, integrated antisense cDNA fragments inhibited the expression of E2F1-related pro-apoptotic genes, resulting in a reduced susceptibility of the cells to the 4-OHT-induced death signal. With this approach, we identified 13 candidate genes with distinct physiological functions, demonstrating that E2F1-induced apoptosis is regulated at different levels.

MATERIALS AND METHODS

### Cell culture, virus and transfection

Saos-2ERE2F1 cells have been described previously (25). Cells were maintained in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) at 37°C and in 5% CO_2. Media for the maintenance of Saos-2 ERE2F1 cells contained puromycin (Sigma, Steinheim, Germany) at a concentration of 1 μg/ml. E2F1 activity in Saos-2ERE2F1 cells was induced by 1 μM 4-OHT. HEK293T cells were maintained in DMEM medium. All retroviral supernatants were produced into Iscove's Modified Dulbecco's Medium containing HEPES (Sigma). All media were supplemented with 100 μg/ml penicillin and 100 U/ml streptomycin (Invitrogen). The vectors Ad-GFP and Ad-ERE2F1 have been described previously (26,27). Adenoviral infection was carried out at multiplicity of infection (MOI) 35, which allows 100% transduction of the Saos-2ERE2F1 cell line. Transfection was performed by electroporation (18).

Preparation of antisense cDNA library and construction of a retrovirus-derived expression plasmid

Saos-2ERE2F1 cells were induced by the addition of 4-OHT at a final concentration of 1 μM. Eight hours after induction, total RNA was extracted by RNeasy Maxi Kit according to the manual (Qiagen, Hilden, Germany) and poly(A) mRNA was isolated by mRNA Oligotex kit (Qiagen). The cDNA library was constructed mainly based on SUPERSCRIPT™ Plasmid System with GATEWAY™ Technology for cDNA Synthesis and Cloning (Invitrogen). Instead of using pSPORT 1 plasmid provided with the kit, a self-inactivating oncoretroviral plasmid, pCAMS/U3EPac (Figure 1A), was utilized to express the antisense mRNA transcripts. This plasmid was originated from the pCAMS/U3E vector which we previously used in a two-plasmid system to produce GALV-pseudotyped oncoretroviral vectors (28). For construction of the library, the following modifications were made. First, the plasmid backbone was exchanged to include the oriP from the SV40 virus to improve virus production in HEK293T cells. Second, the MluI site 5′ of the cytomegalovirus promotor was destroyed and re-introduced between the NotI site and the 3′ SIN LTR. Third, the enhanced green fluorescent protein (EGFP) sequence 3′ of the SFFV promotor was replaced by a linker (AscI-3′sequence of SFFV–PacI–NotI) using the oligonucleotides 5′-ACAGGCGCGCCAGTCCTCCGATTGACTGA-GTCGCCCGGGTACCGCGG and 5′-TCAGCGGCCGCAT-GATCTTAATTAACGGGCCCGCGGTACCCGGGCGAC. Oligo-(dT) primers with a NotI restriction site were used to synthesize first strand cDNA, afterwards an adaptor oligonucleotide containing MluI was ligated to the double-stranded cDNA. Following digestion with NotI and MluI restriction enzymes, the cDNA was size-fractionated by column chromatography to remove small cDNA fragments, making the cloning of larger inserts more probable. Only the middle and large size fraction of cDNA (⩾200 bp) were ligated in antisense direction into the retroviral vector under control of the SFFV promotor. Ligation products were electrotransformed into XL1-Blue competent cells (Stratagene, Heidelberg, Germany). Colonies from Luria–Bertani agar plates were scraped and pooled; plasmid DNA from primary library was directly extracted with Maxi prep. (MACHEREY-NAGEL, Düren, Germany) without further amplification.

Retrovirus production and transduction of Saos-2 cells

To package the retroviral antisense library, 5 μg plasmid DNA from the primary cDNA library and 5 μg of the envelope plasmids containing VSV-G (28) or RD114 (29) were transfected per 10 cm plate of HEK293T cells using 30 μl FuGENE-6 transfection reagent (Roche, Mannheim, Germany). Forty-eight hours later, retroviruses containing supernatants were harvested fresh, filtered through 0.45 μm and used to infect Saos-2ERE2F1 cells in the presence of 10 μg/ml protamine (Sigma). The gene transfer efficiency of the EGFP expressing VSV-G or RD114 pseudotyped control plasmid into Saos-2ERE2F1 cells was 66 and 59%, respectively, as analysed by flow cytometry on a Becton Dickinson FACSCalibur (Heidelberg, Germany; data not shown). To achieve integration of a single copy of retroviral library vector in the genome, the fresh supernatant was diluted
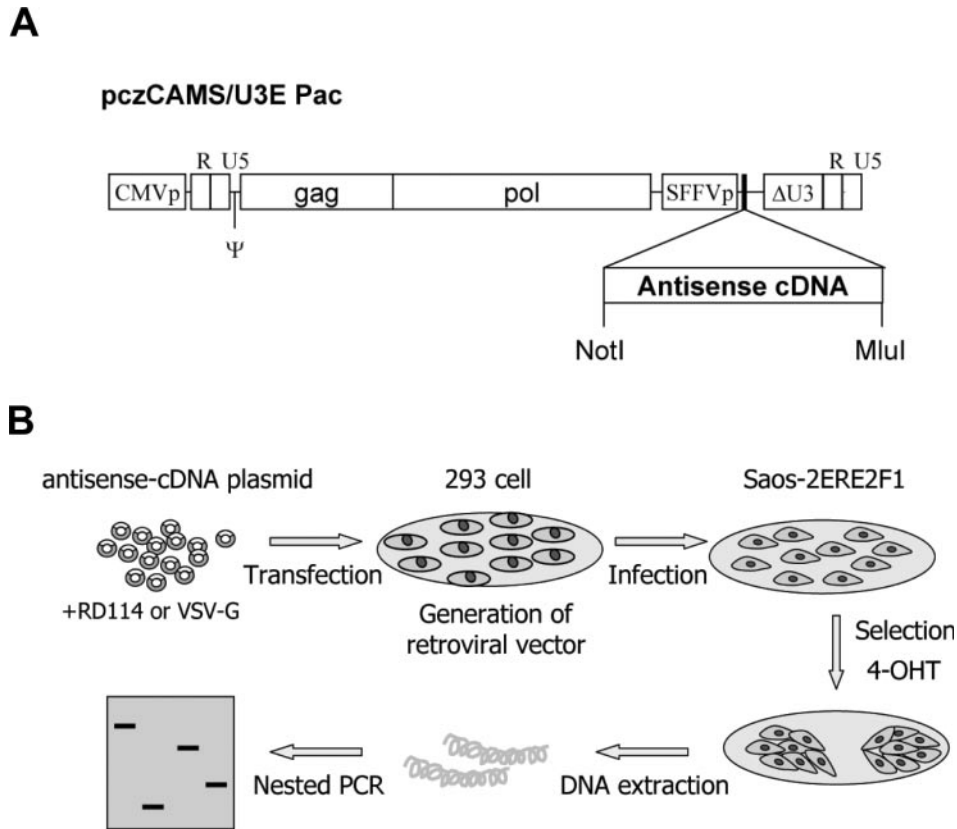
Figure 1. TKO selection. (**A**) Schematic representation of retroviral vector pczCAMS/U3E based on MuLV. The antisense cDNA expression library was ligated into the NotI and MluI restriction sites downstream of the SFFV promoter. (**B**) The strategy is based on random inactivation of genes after retroviral transfer of an antisense cDNA library enriched of E2F1-induced genes, followed by the selection of Saos-2ERE2F1 cells that survive in the presence of the apoptotic stimulus. The antisense cDNA library was cotransfected with RD114 or VSV-G into HEK293T cells to generate retrovirus. Cell supernatant was used to infect Saos-2ERE2F1 cells followed by the addition of 4-OHT. DNA was extracted from growing clones and analysed for integrated genes by PCR.

1:10. Twenty-four hours after infection, fresh medium with 1 µM 4-OHT was added. Cells were cultured in 4-OHT continuously for more than 4 weeks. Thereafter, surviving cells formed single cell clones, which were individually picked and expanded. From these clones, genomic DNA was extracted (Peq lab, Erlangen, Germany) and used as template to characterize integrated genes.

Nested PCR

An aliquot of 100–200 ng genomic DNA was utilized to perform nested PCR to identify integrated genes. The following two primer sets were chosen from the retrovirus vector sequence flanking inserts: outer primers, AGATATGGCCCAACCCT-CAGCAGT (sense) and CTACAGGTGGGGTCTTTCATTCC (antisense); inner primers, TCGCGCGCTTCTGCTTCCCG (sense) and TGGCTGTACTCTATAGGCT (antisense). For the first PCR, the following parameters were used: first 10 cycles, annealing temperature was decreased in 0.5°C each cycle from 66°C, followed by 25 cycles at constant annealing temperature of 61°C. PCR products were purified with PCR purification system (MARLIGEN Bioscience, Heidelberg, Germany) and used as templates for a second PCR under the same conditions over 25 cycles. Reaction products were loaded onto 1% agarose gel, and corresponding bands were cut from gels and subcloned into the pcDNA3.1 V5-His topo vector. cDNA inserts were identified by sequencing (Seqlab, Göttingen, Germany).

Flow cytometry and XTT assay

Cell lines were grown under starvation conditions with 1% BSA for 24 h before 4-OHT was added at a final concentration of 1 µM. Cells were harvested at indicated times after infection, fixed in 70% ethanol and stained for DNA content with propidium iodide. Flow cytometric analysis was carried out in a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA) using CellQuest software. For XTT assay, cells were seeded on 96-well plates and starved for 24 h in 1% BSA before 4-OHT was added at different time points post-induction. TACS™ XTT cell proliferation assay was performed according to the manual (Trevigen Inc., Gaithersburg, MD).

Western blot analysis

Cells were lysed in RIPA buffer [50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100 (v/v), 1% Na-Deoxycholate (v/v) and 0.1% SDS (w/v)] and total protein concentration was quantitated by Bradford assay (Biorad). Samples (100 µg per lane) were separated by SDS–PAGE, transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) and probed with antibodies against actinin α-4 (CHEMICON), melanoma cell adhesion molecule (MCAM) (R&D systems) and small nuclear ribonucleoprotein E (SNRPE) from Santa Cruz Biotechnology (Heidelberg, Germany) according to the manufacturers' guidelines. Primary antibodies were detected using the

appropriate secondary antibody-horseradish peroxidase conjugate (Amersham Pharmacia, Braunschweig, Germany).

Real-time RT–PCR analysis

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and 1 µg RNA was reverse transcribed using Omniscript RT (Qiagen) and Oligo-(dT) primer. The cDNA sample was diluted 1:5. Taqman quantitative PCR was performed using 250 ng cDNA sample, 20× reaction mixture and 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instruction, in conjunction with ABI PRISM 7700 HT Sequence Detection Systems. Gene expression profile was achieved using the comparative CT method of relative quantification (GAPDH as the endogenous control).

Statistical analysis

Statistical significance was calculated by paired Student's *t* test. All statistical tests are two-sided.

RESULTS

Establishment of Saos-2ERE2F1 cell lines that survive in the presence of activated E2F1

A prerequisite for the success of the functional so-called TKO strategy (21) is the stable transduction of a large number of cells with a vector that expresses high levels of an antisense cDNA library in the continuous presence of activated E2F1 and that can be easily rescued from the stable clones. In contrast to the original TKO system, we chose a self-inactivating oncoretroviral vector system for the delivery of the cDNA library into Saos-2ERE2F1 cells, as oncoretroviruses can efficiently transduce human cell lines usually with oligoclonal integration pattern per cell (28). For high level expression of antisense mRNA transcripts, double-stranded cDNA fragments were ligated in antisense direction into the modified oncoretroviral plasmid under the control of the SFFV promoter (Figure 1A), which has been used by us and others for the long-term expression of transgenes in mammalian cells (30). To test the quality of the cDNA library, plasmid DNA from 11 randomly picked clones was extracted and PCR was performed using primers flanking the integration sites. Nine of the eleven clones contained inserts of different size (cloning efficiency of

80%). The library consists of 5×10^6 independent clones per 1 µg vector. VSV-G or RD114-pseudotyped retrovirus vector expressing the antisense cDNA library was rescued from the supernatant of HEK293T cells and used to infect Saos-2ERE2F1 cells. After 4 weeks, single cell clones that survived under continuous activation of E2F1 were picked, genomic DNA was extracted and the antisense cDNAs were rescued (Figure 1B).

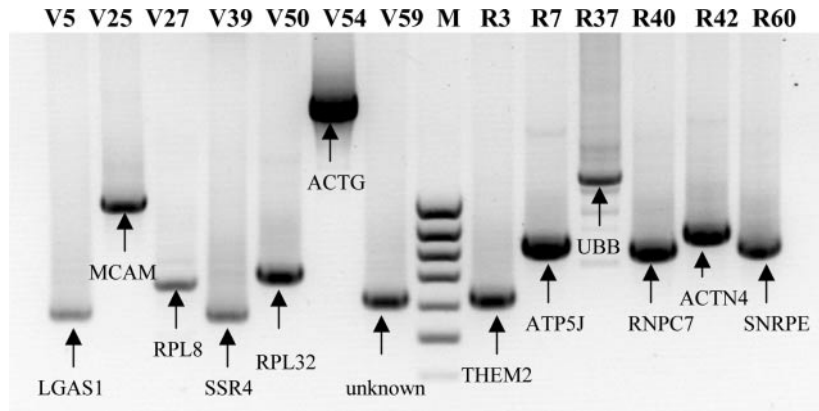### Identification of antisense cDNA inserts

The cDNA inserts contained in the vector that reduced the susceptibility of the clones to E2F1-induced cell death were rescued by PCR. We found 13 cDNA inserts of a size ranging from 300 to 900 bp (Table 1). Sequence analysis revealed that 12 inserts match to genes encoding proteins of known cellular function, including apoptosis (*ACTN4*, *ATP5J*, *LGAS1* and *UBB*), RNA-binding (*RNPC7*, *RPL8* and *RPL32*) and pre-mRNA splicing (*SNRPE*). For example, Ubiquitin B (*UBB*) was shown to be involved in apoptosis via different mechanisms and is required for the degradation of several pro-apoptotic proteins (31,32). The nucleotide sequence of one cDNA was unknown. Eleven cDNA clones were identified as fragments and two appeared to be full-length (Table 1 and Figure 2). The antisense orientation of the cDNA was verified by both restriction and sequence analysis.

Antisense cDNAs rescued from Saos-2ERE2F1 clones in the presence of activated E2F1 confer apoptosis resistance

Previous reports have demonstrated that the TKO strategy is a very efficient method to select for positive mediators of cell death (21). We next performed functional assays to determine whether the isolated antisense fragments could individually convert Saos-2ERE2F1 cells from E2F1 death sensitivity to E2F1 death resistance. In cell clones containing antisense cDNA fragments of three identified genes, *THEM2* (clone R3), *RNPC7* (clone R40) and *ATP5J* (clone R7), E2F1 was stimulated by 4-OHT treatment and the sub-G1 DNA content, indicative for apoptosis, was measured by flow cytometry. As shown in Figure 3, selective inhibition of these genes resulted in a clear decrease of apoptotic cells (increased resistance against E2F1-mediated cell death) at 32 h after induction [between 12% (R7), 23% (R3) and 29% (R40)] compared with stimulated Saos-2ERE2F1 control cells that do not

Table 1. Genes identified by the TKO selection approach

Accession number	Gene name	cDNA	Identified	Locus	Frequency	Function
BC007442	ACTG1	1938	900	17q25	5	Actin G
NM_004924	ACTN4	3893	580	19q13	6	Actinin alpha-4
NM_001003703	ATP5J	1303	540	19q13.2	10	ATP synthase, mitochondrial F0 complex, subunit F6
NM_002305	LGAS1	526	363	22q13.1	3	Lectin, galactoside-binding, soluble, 1 (galectin 1)
NM_006500	MCAM	3583	663	11q23.3	1	Melanoma cell adhesion molecule
BC062440	RNPC7	1087	510	14q24.3	1	RNA-binding motif protein 25
BC000077	RPL8	892	525	8q24.3	1	Ribosomal protein L8
NM_000994	RPL32	521	Full-length	3q25–p24	2	Ribosomal protein L32
NM_006280	SSR4	642	360	Xq28	11	Signal sequence receptor, delta
BC000894	THEM2	616	390	6p22.2	17	Thioesterase superfamily member 2
BC009301	UBB	943	820	17p12–p11.2	2	Ubiquitin B
M37716	SNRPE	491	Full-length	1q25–q43	5	Small nuclear RNA protein E
n.a.	Unknown	n.a.	480	5q33.2	1	n.a.

Figure 2. Identification of integrated genes. A total of 13 genes were identified from indicated clones by NCBI human genomic DNA database search (V = VSV-G; R = RD114).
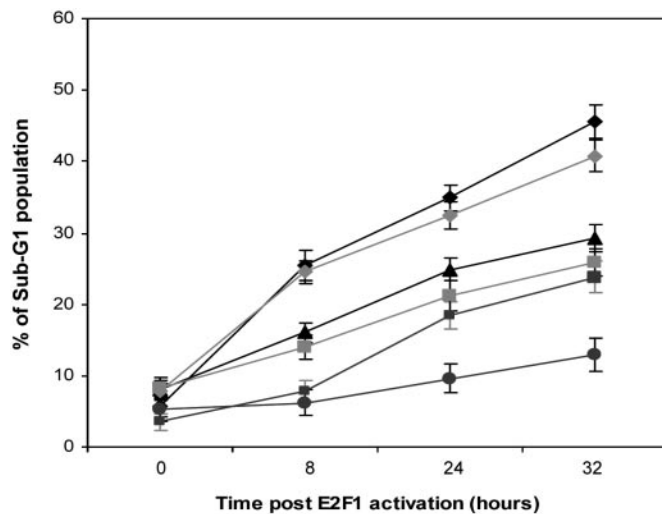
Figure 3. Apoptosis resistance of cell clones containing antisense cDNA fragments. Saos-2ERE2F1 cells were treated with 4-OHT and analysed by flow cytometry at indicated time points. Saos2-ERE2F1 cells transfected with a plasmid expressing antisense against p73 (p73-AS) or GFP (GFP-AS) were used as a positive and negative control, respectively. The percentage of cells with a sub-G1 DNA content is as shown (Saos-2ERE2F, black rhombus; Saos2-ERE2F1 plus GFP-AS, grey rhombus; clone R3_*THEM2*, black triangle; clone R7_*ATP5J*, circle; clone R40_*RNPC7*, black square; Saos2-ERE2F1 plus p73-AS, grey square). Each graph represents the mean ± SD of three independent experiments.

express antisense cDNA fragments (46%). These data suggest that the rescued genes are positive regulators of E2F1-induced apoptosis. Similar results were obtained from cell viability assays shown in Figure 4. We observed a substantial increase of cell viability (>70%) in response to E2F1 activation in those cells lines containing antisense fragments of the genes *LGAS1* (clone V5), *MCAM* (clone V25), *ATP5J* (clone R7), *RPL32* (clone V50), *RPL8* (clone V27) and *SNRPE* (clone R60) at 72 h after induction, whereas only 30% of the control cells lacking antisense fragments survived (Figure 4A). To confirm that the observed phenotype is due to the gene product corresponding to the antisense cDNA, expression plasmids encoding *LGAS1*, *SNRPE* and *ATP5J* antisense fragments were transfected into parental Saos2-ERE2F1 cells. Consistent with the data shown in Figure 4A, ectopic expression of the antisense fragments

resulted in a significantly increased cell viability (>40%) compared with the untransfected control (<25%) at 72 h after E2F1 stimulation (Figure 4B).

Furthermore, changes in the expression of the identified genes in response to E2F1 activation were analysed on protein level. Compared with the expression of, for example, actinin α-4, MCAM and SNRPE in parental Saos-2ERE2F1 cells, cell clones containing the antisense cDNA fragments revealed significantly lower protein levels (Figure 5). These date support the notion that the increased growth advantage of antisense containing cells is acquired owing to the inhibition of E2F1 target gene expression by the antisense cDNA.

Expression profiles of identified genes in response to E2F1 stimulation

As an advantage of the ERE2F1 regulatory system, it enables us to measure the effect of E2F1 activation in the absence of *de novo* protein synthesis, thus allowing the identification of directly activated transcripts (25,33). To correlate changes in the expression of genes corresponding to the identified antisense cDNAs with E2F1 activation, a subset of genes (*LGAS1*, *ACTN4*, *MCAM*, *SSR4* and *UBB*) was further analysed in the presence of cycloheximide (CHX). By the addition of 4-OHT, a significant increase in the expression intensity was observed for all candidate genes between 8 and 24 h. The genes *ACTN4*, *LGAS1*, *MCAM* and *SSR4* were also significantly upregulated in the presence of CHX (*ACTN4*, *LGAS1*, *SSR4* ≤3-fold; *MCAM* >100-fold at 16 h), suggesting that they are directly transactivated by E2F1. In contrast, upregulation of *UBB*, for example, found with 4-OHT alone, could not be observed following the addition of CHX (Figure 6). This result indicates that *UBB* upregulation in response to E2F1 might not be a direct effect. However, since we observed an only moderate increase of *LGAS1*, *SSR4*, and *UBB* in response to the ectopic expression of E2F1, additional experiments are required to unambiguously consider these genes as direct or indirect E2F1 targets.

DISCUSSION

The importance of the E2F1 transcription factor for the induction of apoptosis either in association with p53 or alone is
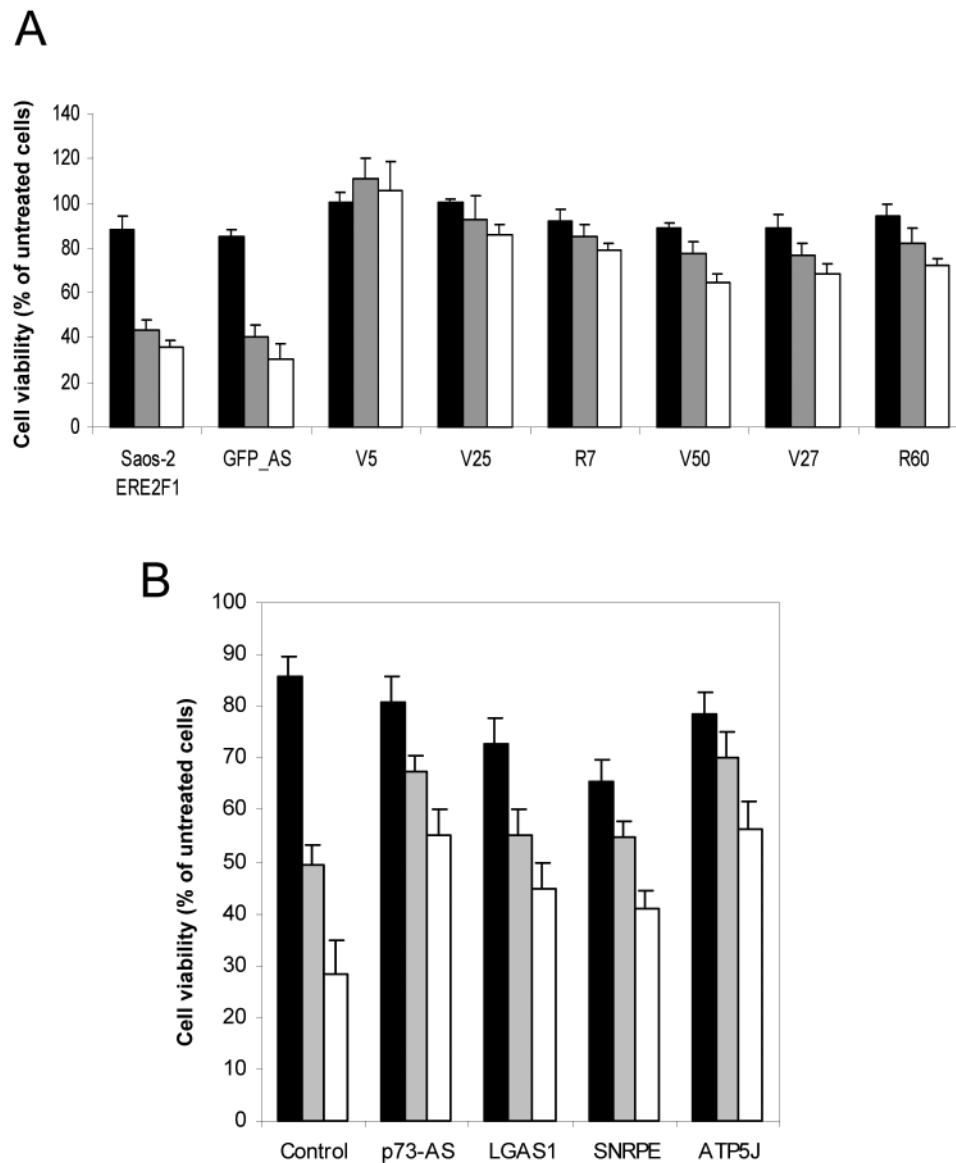
Figure 4. Analysis of cell survival by XTT assay. Cell viability was assayed every 24 h over a period of 3 days after E2F1 activation by 4-OHT. The percentage of viable cells (**A**) from clone V5 (*LGAS1*), clone V25 (*MCAM*), clone R7 (*ATP5J*), clone V50 (*RPL32*), clone V27 (*RPL8*) and clone R60 (*SNRPE*) are shown in relation to parental Saos-2ERE2F1 cells and (**B**) from Saos-2ERE2F1 cells transfected with 10 μg expression plasmid encoding for LGAS1, SNRPE and ATP5J compared with untransfected cells or cells transfected with GFP-AS plasmid. The p73-AS expression plasmid was used as positive control. Data were obtained from three vials per time point measured in duplicate. SD is indicated by error bars; 24 h, black bar; 48 h, grey bar; 72 h, white bar.

evident by a large number of studies. Most of them suggest that E2F1 controls apoptosis through an indirect mechanism by transcriptional activation of downstream factors, which in turn produce secondary changes in gene expression that trigger apoptosis (12,17,18). Although the complete mechanism by which E2F1 induces apoptosis is still not understood, it becomes clear from these studies that E2F1 is involved in many aspects of programmed cell death. In this work, we demonstrate that, consistent with other previous reports (21–24), applying the functional approach of antisense knock-out is a successful strategy for the isolation of novel genes that function as positive mediators of cell death. A growth advantage, conferred by the expression of antisense RNA, serves as a strong positive selection method in an environment restricted to the presence of activated E2F1.
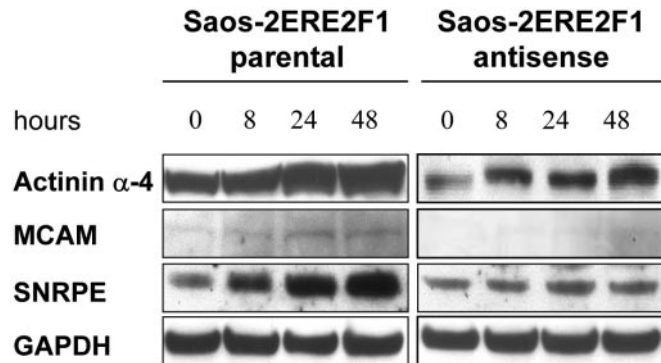
In our study, we identified 12 known genes that are involved in E2F1-induced cell death pathways. Some of these genes have been demonstrated to mediate apoptosis in various cell systems. For example, galectin 1 (*LGAS1*) induced apoptosis in T cell (34) and some other cell lines (35). Actinin α-4 (*ACTN4*) was shown to directly interact with DNase Y and to stabilize its endonuclease activity during apoptosis (36), whereas overexpression of DNase Y alone cannot induce apoptosis. Based on our data, actinin α-4 is directly upregulated by E2F1. Moreover, two ribosomal proteins (RPL32 and RPL8) were identified in this study that belong to a protein family in which other members have been shown to be responsible for cell cycle arrest or apoptosis (37–39) in human and mouse cell lines, and to sensitize them for chemotherapy. However, in contrast to our previous cDNA microarray analysis

(25), none of the positive hits is a well-known component of the apoptotic machinery or an established pro-apoptotic target of E2F1. This is likely due to the fact that the functional TKO approach largely depends on the abundance of antisense cDNA in the library and on its inhibitory efficiency. Because of the lack of a full inhibition of the corresponding genes by the antisense cDNA, the functional approach might not pull out major apoptosis inducers.
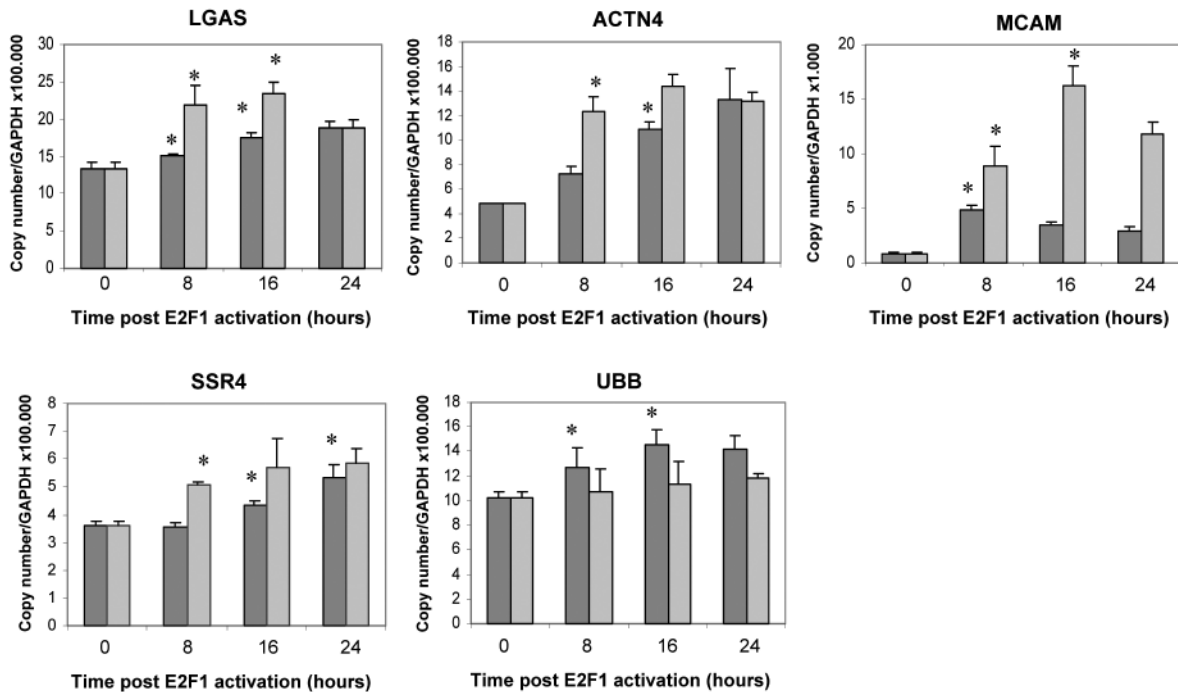
We analysed apoptosis in a representative choice of cell clones containing the identified antisense gene fragments by measuring the sub-G1 DNA content. Following E2F1

Figure 5. Verification of the gene expression profile on protein level. Serum-starved parental Saos-2ERE2F1 cells and Saos-2ERE2F1 cell clones containing the antisense cDNA fragment of actinin α-4, MCAM and SNRPE were induced by the addition of 4-OHT. At indicated time points, equal amounts of whole-cell extracts were assayed for actinin-α4, MCAM and SNRPE protein. GAPDH was used as a loading control.

activation, all cell lines showed a clear resistance against E2F1-mediated apoptosis compared with parental Saos-2ERE2F1. Clone R7, for example, containing the antisense cDNA of the F6 subunit from the ATP synthase F0 sector (*ATP5J*), exhibited the lowest apoptotic rate in response to activated E2F1. The proton–ATP synthase complex is located in the inner mitochondrial membrane where it couples the electrochemical potential gradient generated by the oxidoreduction reactions of the enzyme complexes of the electron transport chain to the synthesis of ATP from ADP and Pi (40). The F0 sector is the membrane sector and is involved in proton translocation. It has previously been shown that the inhibition of ATP synthase suppresses TNF-induced apoptosis in HeLa cells (41). Our data suggest that the mitochondrial ATP synthase might also be involved in the E2F1 death pathway, although the mechanism is unclear. Thus, together with previously published data indicating a direct modulation of the human cytochrome *c*1 promoter (42), and upregulation of the apoptosis-inducing factor in the cytosol and the recently identified mitochondrial death-inducing protein DIP by E2F1 (43), our results support that E2F1 triggers apoptosis by stimulating death-related factors in the mitochondrion.

The same conclusions can be made from the XTT cytotoxicity assay in cells containing the antisense gene fragment of *ATP5J*. Expression of the antisense cDNA clearly protected them from cell death, as judged by an ~60% (at 72 h) enhanced viability in the continuous presence of activated E2F1. Consistent with its role as a promoter of cellular apoptosis, an even better protection against E2F1-induced cell death (>80%) was observed through the inhibition of galectin-1 (*LGAS1*). Galectin-1 has been shown to induce

Figure 6. Classification of E2F1 target genes as direct and indirect. Gene expression was analysed in serum-starved Saos-2ERE2F1 cells containing antisense cDNA in the presence of 4-OHT (dark grey bars) and/or CHX (grey bars) after infection with Ad-ERE2F1 by quantitative RT–PCR. All targets are shown at 0, 8, 16 and 24 h after induction. Bars show the copy number ×100 000 (×1000 for MCAM) after normalization for GAPDH as a housekeeping gene. Significant differences ($P < 0.05$; paired, two-sided *t*-test) between 4-OHT and 4-OHT plus CHX-treated cells are labelled with asterisk. *ACTN4*, *LGAS1*, *MCAM* and *SSR4* are direct targets, whereas *UBB* is an indirect target.

apoptosis of macrophages, thymocytes, T and B cells (44). Pircher and co-workers (45) detected an increase in galectin-1 synthesis after the activation of murine T cells and suggested that galectin-1 may act in an autocrine negative regulatory system to terminate an immune response by killing T cells. In that context, it was demonstrated that E2F1 is required for the apoptosis of autoimmune immature T cells during thymic negative selection *in vivo* (46,47). These results implicate a specific role for galectin-1 in E2F1-mediated T cell apoptosis in the absence of p53.

Applying the method of functional selection has resulted in the identification of genes with a wide spectrum of cellular functions. Actin G (*ACTG1*) and actinin α-4 (*ACTN4*), for example, are cytoskeleton proteins; *RPL32* and *RPL8* belong to the group of ribosomal proteins. *ATP5J* is a component of ATP synthase localized in the mitochondria and sequence receptor delta (*SSR4*) is involved in the secretion of proteins. As the most interesting finding of this screening approach, we identified two genes, *RNPC-7* and the *SNRPE*, one common component of all spliceosomal snRNP core complexes in the nucleus of eukaryotic cells (48). These proteins harbour a RNA-binding motif and participate in pre-mRNA splicing, suggesting that RNA splicing is crucial for mediating E2F1-induced apoptosis. A potential role for alternative splicing in apoptosis was suggested by the discovery of functionally active alternatively spliced variants of major apoptosis regulators (49), such as Bcl-x, Ich-1, caspase-9, Apaf-1 and Tid1 (50–52). In all cases, the alternatively spliced isoforms play an opposing role in apoptosis. For example, the long isoform of Bcl-x (Bcl-Xl) and Ced-4 protect cells against apoptosis, whereas their short isoform (Bcl-Xs) and Cde-4S, respectively, promote cell death. On the other hand, the long isoform of Ich-1 (Ich-1L) induces apoptosis, whereas the short isoform (Ich-1S) inhibits it. Pre-mRNA splicing is a very sophisticated, partially known process, in which over 100 proteins are involved. A potential link between pre-mRNA splicing and E2F1-induced cell death and the underlying mechanism remains to be elucidated.

There is increasing evidence that E2F1 induces p53-independent apoptosis via different cellular pathways, including a variety of pro-apoptotic factors, supporting its role as a key player in an anti-tumour safeguard mechanism. Because of a potential functional redundancy of pro-apoptotic E2F1 targets, it might be possible that some genes are not identified by this approach. Although this study identified several novel E2F1 target genes, which are surely involved in several different pathways, the role of each gene for E2F1-induced cell death remains speculative until further experimental data have been gathered. Of particular interest is the fact that single factors from multifactor complexes, such as ATP synthase or SNRPE, seem to play a role in apoptosis induction even though other components of these complexes could not be identified in our screen. Therefore, it can be anticipated that different complex compositions carry out different functions in distinct cellular contexts. Furthermore, one gene, the MCAM, with a very strong response to E2F1 stimulation but without any currently known relation to apoptosis induction has been identified here. Consequently, follow-up studies analyzing each target gene in detail have to be awaited to further classify particular novel E2F1-regulated genes.

REFERENCES

1. DeGregori,J. (2002) The genetics of the E2F family of transcription factors: shared functions and unique roles. *Biochim. Biophys. Acta*, **1602**, 131–150.
2. Ginsberg,D. (2002) E2F1 pathways to apoptosis. *FEBS Lett.*, **529**, 122–125.
3. Kowalik,T.F., DeGregori,J., Schwarz,J.K. and Nevins,J.R. (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J. Virol.*, **69**, 2491–2500.
4. Muller,H., Bracken,A.P., Vernell,R., Moroni,M.C., Christians,F., Grassilli,E., Prosperini,E., Vigo,E., Oliner,J.D. and Helin,K. (2001) E2Fs regulate the expression of genes involved in differentiation, development, proliferation and apoptosis. *Genes Dev.*, **15**, 267–285.
5. Dyson,N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.*, **12**, 2245–2262.
6. Stevaux,O. and Dyson,N.J. (2002) A revised picture of the E2F transcriptional network and RB function. *Curr. Opin. Cell Biol.*, **14**, 684–691.
7. Lin,W.C., Lin,F.T. and Nevins,J.R. (2001) Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. *Genes Dev.*, **15**, 1833–1844.
8. Stevens,C., Smith,L. and La Thangue,N.B. (2003) Chk2 activates E2F-1 in response to DNA damage. *Nature Cell Biol.*, **5**, 401–409.
9. Powers,J.T., Hong,S., Mayhew,C.N., Rogers,P.M., Knudsen,E.S. and Johnson,D.G. (2004) E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis. *Mol. Cancer Res.*, **2**, 203–214.
10. Ianari,A., Gallo,R., Palma,M., Alesse,E. and Gulino,A. (2004) Specific role for p300/CREB-binding protein-associated factor activity in E2F1 stabilization in response to DNA damage. *J. Biol. Chem.*, **279**, 30830–30835.
11. Hiebert,S.W., Packham,G., Strom,D.K., Haffner,R., Oren,M., Zambetti,G. and Cleveland,J.L. (1995) E2F-1:DP-1 induces p53 and overrides survival factors to trigger apoptosis. *Mol. Cell. Biol.*, **15**, 6864–6874.
12. Bates,S., Phillips,A.C., Clark,P.A., Stott,F., Peters,G., Ludwig,R.L. and Vousden,K.H. (1998) p14ARF links the tumour suppressors RB and p53. *Nature*, **395**, 124–125.
13. Robertson,K.D. and Jones,P.A. (1998) The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol. Cell. Biol.*, **18**, 6457–6473.
14. Rogoff,H.A., Pickering,M.T., Debatis,M.E., Jones,S. and Kowalik,T.F. (2002) E2F1 induces phosphorylation of p53 that is coincident with p53 accumulation and apoptosis. *Mol. Cell. Biol.*, **22**, 5308–5318.
15. Russell,J.L., Powers,J.T., Rounbehler,R.J., Rogers,P.M., Conti,C.J. and Johnson,D.G. (2002) ARF differentially modulates apoptosis induced by E2F1 and Myc. *Mol. Cell. Biol.*, **22**, 1360–1368.
16. Tsai,K.Y., MacPherson,D., Rubinson,D.A., Crowley,D. and Jacks,T. (2002) ARF is not required for apoptosis in Rb mutant mouse embryos. *Curr. Biol.*, **12**, 159–163.
17. Irwin,M., Marin,M.C., Phillips,A.C., Seelan,R.S., Smith,D.I., Liu,W., Flores,E.R., Tsai,K.Y., Jacks,T., Vousden,K.H. *et al.* (2000) Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature*, **407**, 645–648.
18. Stiewe,T. and Putzer,B.M. (2000) Role of the p53-homologue p73 in E2F1-induced apoptosis. *Nature Genet.*, **26**, 464–469.
19. Furukawa,Y., Nishimura,N., Satoh,M., Endo,H., Iwase,S., Yamada,H., Matsuda,M., Kano,Y. and Nakamura,M. (2002) Apaf-1 is a mediator of E2F-1-induced apoptosis. *J. Biol. Chem.*, **277**, 39760–39768.

20. Hershko,T. and Ginsberg,D. (2004) Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. *J. Biol. Chem.*, **279**, 8627–8634.

21. Deiss,L.P. and Kimchi,A. (1991) A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. *Science*, **252**, 117–120.

22. Deiss,L.P., Galinka,H., Berissi,H., Cohen,O. and Kimchi,A. (1996) Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *EMBO J.*, **15**, 3861–3870.

23. Deiss,L.P., Feinstein,E., Berissi,H., Cohen,O. and Kimchi,A. (1995) Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.*, **9**, 15–30.

24. Kissil,J.L., Deiss,L.P., Bayewitch,M., Raveh,T., Khaspekov,G. and Kimchi,A. (1995) Isolation of DAP3, a novel mediator of interferon-gamma-induced cell death. *J. Biol. Chem.*, **270**, 27932–27936.

25. Stanelle,J., Stiewe,T., Theseling,C.C., Peter,M. and Putzer,B.M. (2002) Gene expression changes in response to E2F1 activation. *Nucleic Acids Res.*, **30**, 1859–1867.

26. Putzer,B.M., Stiewe,T., Crespo,F. and Esche,H. (2000) Improved safety through tamoxifen-regulated induction of cytotoxic genes delivered by Ad vectors for cancer gene therapy. *Gene Ther.*, **7**, 1317–1325.

27. Stanelle,J., Stiewe,T., Rodicker,F., Kohler,K., Theseling,C. and Putzer,B.M. (2003) Mechanism of E2F1-induced apoptosis in primary vascular smooth muscle cells. *Cardiovasc. Res.*, **59**, 512–519.

28. Leurs,C., Jansen,M., Pollok,K.E., Heinkelein,M., Schmidt,M., Wissler,M., Lindemann,D., Von Kalle,C., Rethwilm,A., Williams,D.A. *et al.* (2003) Comparison of three retroviral vector systems for transduction of nonobese diabetic/severe combined immunodeficiency mice repopulating human CD34+ cord blood cells. *Hum. Gene Ther.*, **14**, 509–519.

29. Sandrin,V., Boson,B., Salmon,P., Gay,W., Negre,D., Le Grand,R., Trono,D. and Cosset,F.L. (2002) Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. *Blood*, **100**, 823–832.

30. Baum,C., Hegewisch-Becker,S., Eckert,H.G., Stocking,C. and Ostertag,W. (1995) Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. *J. Virol.*, **69**, 7541–7547.

31. Bernassola,F., Salomoni,P., Oberst,A., Di Como,C.J., Pagano,M., Melino,G. and Pandolfi,P.P. (2004) Ubiquitin-dependent degradation of p73 is inhibited by PML. *J. Exp. Med.*, **199**, 1545–1557.

32. Zhang,H.G., Wang,J., Yang,X., Hsu,H.C. and Mountz,J.D. (2004) Regulation of apoptosis proteins in cancer cells by ubiquitin. *Oncogene*, **23**, 2009–2015.

33. Helin,K. (1998) Regulation of cell proliferation by the E2F transcription factors. *Curr. Opin. Genet. Dev.*, **8**, 28–35.

34. Perillo,N.L., Pace,K.E., Seilhamer,J.J. and Baum,L.G. (1995) Apoptosis of T cells mediated by galectin-1. *Nature*, **378**, 736–739.

35. Horiguchi,N., Arimoto,K., Mizutani,A., Endo-Ichikawa,Y., Nakada,H. and Taketani,S. (2003) Galectin-1 induces cell adhesion to the extracellular matrix and apoptosis of non-adherent human colon cancer Colo201 cells. *J. Biochem.*, **134**, 869–874.

36. Liu,Q.Y., Lei,J.X., LeBlanc,J., Sodja,C., Ly,D., Charlebois,C., Walker,P.R., Yamada,T., Hirohashi,S. and Sikorska,M. (2004) Regulation of DNaseY activity by actinin-alpha4 during apoptosis. *Cell Death Differ.*, **11**, 645–654.

37. Khanna,N., Sen,S., Sharma,H. and Singh,N. (2003) S29 ribosomal protein induces apoptosis in H520 cells and sensitizes them to chemotherapy. *Biochem. Biophys. Res. Commun.*, **304**, 26–35.

38. Nishiura,H., Tanase,S., Shibuya,Y., Futa,N., Sakamoto,T., Higginbottom,A., Monk,P., Zwirner,J. and Yamamoto,T. (2004) S19 ribosomal protein dimer augments metal-induced apoptosis in a mouse fibroblastic cell line by ligation of the C5a receptor. *J. Cell. Biochem.*, **94**, 540–553.

39. Shi,Y., Zhai,H., Wang,X., Han,Z., Liu,C., Lan,M., Du,J., Guo,C., Zhang,Y., Wu,K. *et al.* (2004) Ribosomal proteins S13 and L23 promote multidrug resistance in gastric cancer cells by suppressing drug-induced apoptosis. *Exp. Cell Res.*, **296**, 337–346.

40. Breen,G.A., Miller,D.L., Holmans,P.L. and Welch,G. (1986) Mitochondrial DNA of two independent oligomycin-resistant Chinese hamster ovary cell lines contains a single nucleotide change in the ATPase 6 gene. *J. Biol. Chem.*, **261**, 11680–11685.

41. Shchepina,L.A., Pletjushkina,O.Y., Avetisyan,A.V., Bakeeva,L.E., Fetisova,E.K., Izyumov,D.S., Saprunova,V.B., Vyssokikh,M.Y., Chernyak,B.V. and Skulachev,V.P. (2002) Oligomycin, inhibitor of the F0 part of H+-ATP-synthase, suppresses the TNF-induced apoptosis. *Oncogene*, **21**, 8149–8157.

42. Luciakova,K., Barath,P., Li,R., Zaid,A. and Nelson,B.D. (2000) Activity of the human cytochrome c1 promoter is modulated by E2F. *Biochem. J.*, **351**, 251–256.

43. Vorburger,S.A., Pataer,A., Yoshida,K., Liu,Y., Lu,X., Swisher,S.G. and Hunt,K.K. (2003) The mitochondrial apoptosis-inducing factor plays a role in E2F-1-induced apoptosis in human colon cancer cells. *Ann. Surg. Oncol.*, **10**, 314–322.

44. Hernandez,J.D. and Baum,L.G. (2002) Ah, sweet mystery of death! Galectins and control of cell fate. *Glycobiology*, **12**, 127R–136R.

45. Blaser,C., Kaufmann,M., Muller,C., Zimmermann,C., Wells,V., Mallucci,L. and Pircher,H. (1998) Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. *Eur. J. Immunol.*, **28**, 2311–2319.

46. Garcia,I., Murga,M., Vicario,A., Field,S.J. and Zubiaga,A.M. (2000) A role for E2F1 in the induction of apoptosis during thymic negative selection. *Cell Growth Differ.*, **11**, 91–98.

47. Zhu,J.W., DeRyckere,D., Li,F.X., Wan,Y.Y. and DeGregori,J. (1999) A role for E2F1 in the induction of ARF, p53, and apoptosis during thymic negative selection. *Cell Growth Differ.*, **10**, 829–838.

48. Luhrmann,R., Kastner,B. and Bach,M. (1990) Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta*, **1087**, 265–292.

49. Wu,J.Y., Tang,H. and Havlioglu,N. (2003) Alternative pre-mRNA splicing and regulation of programmed cell death. *Prog. Mol. Subcell. Biol.*, **31**, 153–185.

50. Bae,J., Leo,C.P., Hsu,S.Y. and Hsueh,A.J. (2000) MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J. Biol. Chem.*, **275**, 25255–25261.

51. Fu,W.N., Kelsey,S.M., Newland,A.C. and Jia,L. (2001) Apaf-1XL is an inactive isoform compared with Apaf-1L. *Biochem. Biophys. Res. Commun.*, **282**, 268–272.

52. Syken,J., De-Medina,T. and Munger,K. (1999) TID1, a human homolog of the Drosophila tumor suppressor l(2)tid, encodes two mitochondrial modulators of apoptosis with opposing functions. *Proc. Natl Acad. Sci. USA*, **96**, 8499–8504.