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RESEARCH ARTICLE

Functional interplay between E2F1 and chemotherapeutic drugs defines immediate E2F1 target genes crucial for cancer cell death

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Abstract The E2F1 transcription factor enhances apoptosis by DNA damage in tumors lacking p53. To elucidate the mechanism of a potential cooperation between E2F1 and chemotherapy, whole-genome microarrays of chemoresistant tumor cell lines were performed focusing on the identification of cooperation response genes (CRG). This gene class is defined by a synergistic expression response upon endogenous E2F1 activation and drug treatment. Cluster analysis revealed an expression pattern of CRGs similar to E2F1 mono-therapy, suggesting that chemotherapeutics enhance E2F1-dependent gene expression at the transcriptional level. Using this approach as a tool to explore E2F1-driven gene expression in response to anticancer drugs, we identified novel apoptosis genes such as the tumor suppressor TIEG1/KLF10 as direct E2F1 targets. We show that TIEG1/KLF10 is transcriptionally activated by E2F1 and crucial for E2F1-mediated chemosensitization of cancer cells. Our results provide a broader picture of E2F1-regulated genes in conjunction with cytotoxic treatment that allows the design of more rational therapeutics.

Keywords E2F1 · Chemotherapy · Cooperation response genes · TIEG1/KLF10 · Cell death · Cancer

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Introduction

A critical determinant of the efficacy of antineoplastic therapy is the ability of malignant cells to undergo apoptosis in response to DNA damage induced by chemotherapeutic agents. The success of genotoxic treatments is attributed, at least in part, to the fact that DNA damage induces cell death more readily in cancer cells than in normal cells. The molecular mechanism(s) underlying this enhanced sensitivity of cancer cells to chemotherapy-induced apoptosis are not fully understood.

In this regard, the cellular transcription factor E2F1, which is frequently upregulated in neoplastic cells, appears to be of particular significance. E2F regulates cell cycle progression by coordinating genes that promote G1- to S-phase transition [1]. Beside its well-established function as a proliferative factor contributing to malignant transformation of primary rodent cells and tumorgenesis in mice (reviewed in [2]), ectopic expression of E2F1 leads to high levels of apoptosis [3]. E2F1 knockout mice exhibit defects in thymocyte apoptosis and have a high incidence of tumor development [4, 5], providing evidence that E2F1 acts as a tumor suppressor in vivo. Apoptosis related to E2F1 is mediated in a p53-dependent and p53-independent manner (reviewed in [6]). In most cases, induction of cell death by E2F1 occurs via direct transcriptional activation of proapoptotic genes, including p14ARF [7], p73 [8, 9], Apaf-1 [10], BH3-only proteins [11], and caspases [12], or through inhibition of survival and antiapoptotic signaling mediated by NF-κB [13], Bcl-2 [14], and GRP78 [15]. Although E2F2 and E2F3 also induce apoptosis, this effect is modest compared to E2F1, and in the case of E2F3, largely attributed to its ability to transactivate the E2F1 gene [12].

Like p53, E2F1 is a crucial determinant of the cellular response to DNA damage. Its apoptotic activity is induced

by DNA damage [16], and aberrant expression of the transcription factor has been shown to increase the sensitivity of certain neoplastic cell types to apoptosis when treated with ionizing radiation or chemotherapeutic agents [17–24]. In response to genotoxic stress, the E2F1 protein is stabilized through direct phosphorylation by ATM/ATR and Chk2 [25, 26]. Because E2F1 also functions upstream of these kinases by transcriptionally regulating their expression, DNA damage triggers a positive-feedback loop between E2F1 and ATM/Chk2 [27–29]. Consistent with the selective accumulation of E2F1 protein during DNA damage, ATM/ATR do not phosphorylate E2F2 or E2F3 [25]. In addition, E2F1 stabilization during DNA damage occurs by binding of 14-3-3 proteins to phosphorylated E2F1, which inhibits its ubiquitination [30]. E2F1 activation upon genotoxic stress is also caused by acetylation. DNA damage triggers P/CAF (p300/CREB-binding protein associated factor) to bind and acetylate E2F1 [31]. This modification specifically influences the DNA-binding activity and transactivation potential of E2F1, and has been suggested to affect its target promoter selectivity, favoring induction of proapoptotic genes such as p73 [31–33]. Furthermore, Chk1 and Chk2 are required for p73 accumulation after DNA damage, suggesting their potential role in p53-independent sensitization of cancer cells to cytotoxic drugs induced by deregulated E2F1 [34]. Based on recent findings, microcephalin (MCPH1) cooperates with E2F1 by complex formation on the Chk1 and p73 promoter, and regulates expression and induction of both genes during DNA damage and E2F1-dependent apoptosis [35].

With respect to our previous studies demonstrating a positive correlation between E2F1 overexpression, sensitivity to clinically relevant drugs, and reduction of p53-deficient tumors in vivo [18], this emphasizes the therapeutic potential of downstream mediators of E2F1-induced chemosensitization. However, the molecular mechanisms underlying the cooperative effect are largely unknown. Considering that E2F1 controls the transcription of several genes, we elucidated alterations in gene expression profiles of chemoresistant osteosarcoma and pancreatic cancer cells exposed to increased endogenous E2F1 and in drug treatment focusing on cooperation response genes (CRGs).

Materials and methods

Cell lines, genotoxic treatments and inhibition of protein de novo synthesis

Maintenance of Saos-2 and Saos-2.ER-E2F1 cells has been described previously [8]. The SK-Mel-147 cell line was kindly provided by Dr. M.S. Soengas (Department of Dermatology, University of Michigan, Comprehensive Cancer Center, Ann Arbor, MI, USA) and cultured as indicated [36]. MZA pancreas adenocarcinoma cells (obtained from D.I. Smith, Mayo Clinic, Rochester, MN, USA) were maintained in Dulbecco's modified Eagle medium (high glucose, 4.5 g/l) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/ml streptomycin, and 1.15 µg/ml amphotericin B (PAA). The inducible cell line MZA.ER-E2F1 was established by transfection of parental MZA with pLPC-ER-E2F1 using Effectene transfection reagent (Qiagen). Cells were selected in DMEM containing 10% FCS supplemented with 0.25 µg/ml puromycin (Sigma-Aldrich) and cloned by limiting dilution. For starvation conditions, cells were grown in DMEM with 0.5% FCS for 24 h. ER-E2F1 translocation was induced via addition of 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) at a final concentration of 1 µM. For chemotherapeutic treatment, cells were exposed to 0.5 µM doxorubicin (DOX), 35 µM cisplatin (cDDP), and 20 µM gemcitabine (GEM). Cycloheximide (10 µg/ml; Sigma-Aldrich) was added for 4 h prior to harvesting.

RNA isolation and microarray hybridization

Cell lines were cultured under starvation conditions prior to genotoxic treatment and/or E2F1 activation. At 24 and 48 h after treatment, total RNA was isolated by standard methods using RNeasy Mini Kit (Qiagen). Five micrograms of total RNA was used to prepare biotinylated cRNA probes which were hybridized to GeneChip® Human Genome U133 Plus 2.0 Array according to the supplier's instructions (Affymetrix). Microarrays were analyzed by laser scanning (Affymetrix GeneChip Scanner 3000).

Microarray data processing and analysis

Three independent GeneChip® expression analyses were performed for each treatment including untreated cells. Background-corrected signal intensities were determined and processed using MAS5 function of the R/Bioconductor *affy* package (http://www.r-project.org/http://www.bioconductor.org). All calculations including normalization of microarray data, statistical tests, clustering, and further filtering methods were accomplished by the gene expression analysis software, GeneSpring GX 9.0 (Agilent). Genes whose transcripts were not detected in any of the investigated conditions were excluded from statistical analysis to reduce the number of false positive genes. To determine differentially expressed genes, expression data were grouped according to treatment and statistically analyzed in reference to untreated cells using *t* test and multiple testing correction (Benjamini and Hochberg False Discovery Rate). Cut-offs were set at twofold and $P \leq 0.05$. Sets of

co-regulated genes identified from microarray analysis were analyzed using the WEB-based Gene Set Analysis Toolkit at bioinfo.vanderbilt.edu/webgestalt to predict their biological function.

Quantitative RT-PCR

After DNase I treatment, 1 μg of total RNA was reverse transcribed using Omniscript RT (Qiagen) and Oligo-dT primer. cDNA samples were mixed with iQ™ SYBR® Green Supermix (Bio-Rad) and analyzed on iQ™5 Multicolor Real-Time PCR Detection System using 1/20 volume of the RT reaction. Relative gene expression was calculated using iQ™5 Optical System Software. All specific primer pairs used are available upon request.

Chromatin immunoprecipitation

ChIP assay was performed on Saos-2.ER-E2F1 cells grown in presence or absence of 4-OHT. The proteins bound to DNA were cross-linked using formaldehyde at a final concentration of 1.42% for 15 min at room temperature. The reaction was stopped by adding glycine to a final concentration of 125 μM. Following sonication protein–DNA complexes were immunoprecipitated using primary antibody for E2F1 (#554213; BD Pharmingen) or appropriate control IgG antibodies overnight at 4°C. Immunoprecipitated chromatin was eluted from the beads in 10% Chelex100 and boiled for 10 min. The precipitated DNA was analyzed by PCR using the following primer sequences: (KLF10-promoter) sense, 5′-CGAGCTCAA ACTCGATTCCGCA-3′ and antisense, 5′-TGTCACGGA GCCGACACCT-3′; (Apaf1-promoter) sense, 5′-GCCCCG ACTTCTTCCGGCTCTTCA-3′ and antisense, 5′-GGAG CTGGCAGCTGAAAGACTC-3′, and (neg. ctrl.) sense, 5′-ACCCAGCCGAGCTGTTTAACAA-3′ and antisense, 5′-TGCCCTGCCTTCCAAACAAACA-3′. The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

Plasmid construction and luciferase reporter assay

The human KLF10 promoter fragment comprising the putative E2F1 binding site (−909 to −659, 251 bp) was amplified from genomic DNA isolated from Saos-2 cells by PCR using the primers 5′-CGAGCTCAAACTCGAT TCCGCA-3′ (forward) and 5′-TGTCACGGAGCCGACA CCT-3′ (reverse), and cloned into the TOPO-TA vector (Invitrogen). The promoter sequence was inserted into the *Xho*I and *Kpn*I restricted pGL3-Basic (Promega) to allow transcription of firefly luciferase gene under its control. The KLF10 promoter fragment with the mutated putative E2F1 site (GTCGGCGC to ATCATCAT) was generated from KLF10P-luc through site-directed mutagenesis using the QuikChange II XL kit (Stratagene). The following primer pairs were used: 5′-CTCAAACTCGATTCCGCAGCCGA GTATCATCATCAGAGAAGGATAAAAACTCGGG-3′ (forward) and 5′-CCCGAGTTTTTATCCTTCTCTGA TGATGATACTCGGCTGCGGAATCGAGTTTGAG-3′ (reverse). All promoter constructs were verified by sequencing. Expression plasmids for wild-type E2F1, the DNA-binding defective E2F1-mutant E132, the E(-TA) mutant lacking the transactivation domain and the control *TP73* promoter reporter construct have been described previously [8]. Saos-2 cells were transfected using Effectene (Qiagen). Luciferase activity was measured 16 h post-transfection using a premanufactured Luciferase Reporter Assay System (Promega) and normalized to total protein concentration in cell extracts.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were carried out essentially as described [15]. Briefly, 1 μg nuclear extract from Saos-2.ER-E2F1 cells treated with 4-OHT were incubated with biotin end-labeled (BrightStar Bio-Detect Kit, Ambion) double-stranded oligonucleotides corresponding to the E2F1 binding site (−880 to −873) in the KLF10 promoter in appropriate buffer for 30 min at room temperature. For competition assays, the competitor DNA was added 10 min prior to addition of labeled probe. Samples were evaluated by electrophoresis on an 8% non-denaturating acrylamide gel. The gels were blotted on nylon transfer membrane and exposed to X-ray film. Double-strand DNA probes are listed below (*sense*). KLF10 E2F1 site (wt), 5′-GCAGCCGAGT**GTCGGCGC** CAGAGAAGG-3′; KLF10 E2F1 site (mt), 5′-GCAGCC GAGT**ATCATCAT**CAGAGAAGG-3′.

Western blotting

Cells were lysed in RIPA buffer and total protein concentration was quantified by Bradford assay. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes (Amersham Biosciences) and probed with E2F1 (#554213) or p73 (#558785) from BD Pharmingen, TIEG1 (sc-67062) and actin (sc-1615; Santa Cruz Biotechnology). The corresponding HRP-labeled secondary antibody was detected using ECL western blotting reagents (Amersham Biosciences).

RNA interference and adenovirus production

Adenovirus expressing shRNA against E2F1 has been described previously [37]. The Ad vector encoding shRNA against TIEG1/KLF10 (Ad.shKLF10) was generated with

the BLOCK-iT System (Invitrogen). Specific oligonucleotides were designed using online software (rnaidesigner.invitrogen.com/rnaiexpress): The target sequence for shKLF10 is GCTAAATGACATTGCTCTACC (mRNA bp 1521–1541). A control shRNA oligo (GTCAAATGGGAAACGTAGACA), which does not match any known human coding cDNA, was used as control. Synthesized oligos (Metabion) were ligated into pENTR/U6 (Invitrogen), and a recombination reaction with pAd/BLOCK-iT-DEST adenoviral backbone plasmid was performed using the Gateway LR Clonase II enzyme mix. Following propagation in *Escherichia coli* (One shot TOP10, Invitrogen) plasmids were PacI digested and transfected into 293 cells. Viruses were purified by CsCl buoyant density centrifugation and titrated using the Adeno-X Rapid Titer Kit (BD Biosciences Clontech). Adenoviral infections were carried out at MOIs that allow 100% transduction of cells.

FACS analysis, Hoechst staining, and caspase 3 assay

Treated cells were harvested with trypsin–EDTA, washed once with ice-cold phosphate-buffered saline (PBS) and incubated overnight at −20°C with 1 ml of 70% ice-cold ethanol. Subsequently, cells were washed with PBS, resuspended in 500 µl of propidium iodide/RNase A solution (PBS, 100 mg/ml RNase A and 100 mg/ml propidium iodide), incubated for 30 min at room temperature, and subjected to flow cytometry using FACSCalibur (Becton-Dickinson). Data were analyzed by CellQuest software. The subdiploid population (sub-G1) was calculated as an estimate of the apoptotic cell population. Hoechst 33342 was added to the cell culture medium at 1 µg/ml 24 h after treatment. Cells were incubated for 15 min at 37°C and subjected to fluorescence microscopy. Apoptotic cells with condensed or fragmented nuclei were easily distinguished from normal cells with intact nuclei. Quantification of apoptosis was determined by counting the number of apoptotic cells from five randomly chosen fields of view per condition with a minimum number of 500 cells scored in each. Caspase 3 activity was assayed using the ApoAlert kit (Takara Bio) according to the protocol. Absorbance was measured at 405 nm in a spectrophotometer. The caspase 3 specific inhibitor DEVD-fmk (Calbiochem) was added at a final concentration of 50 mM.

Results

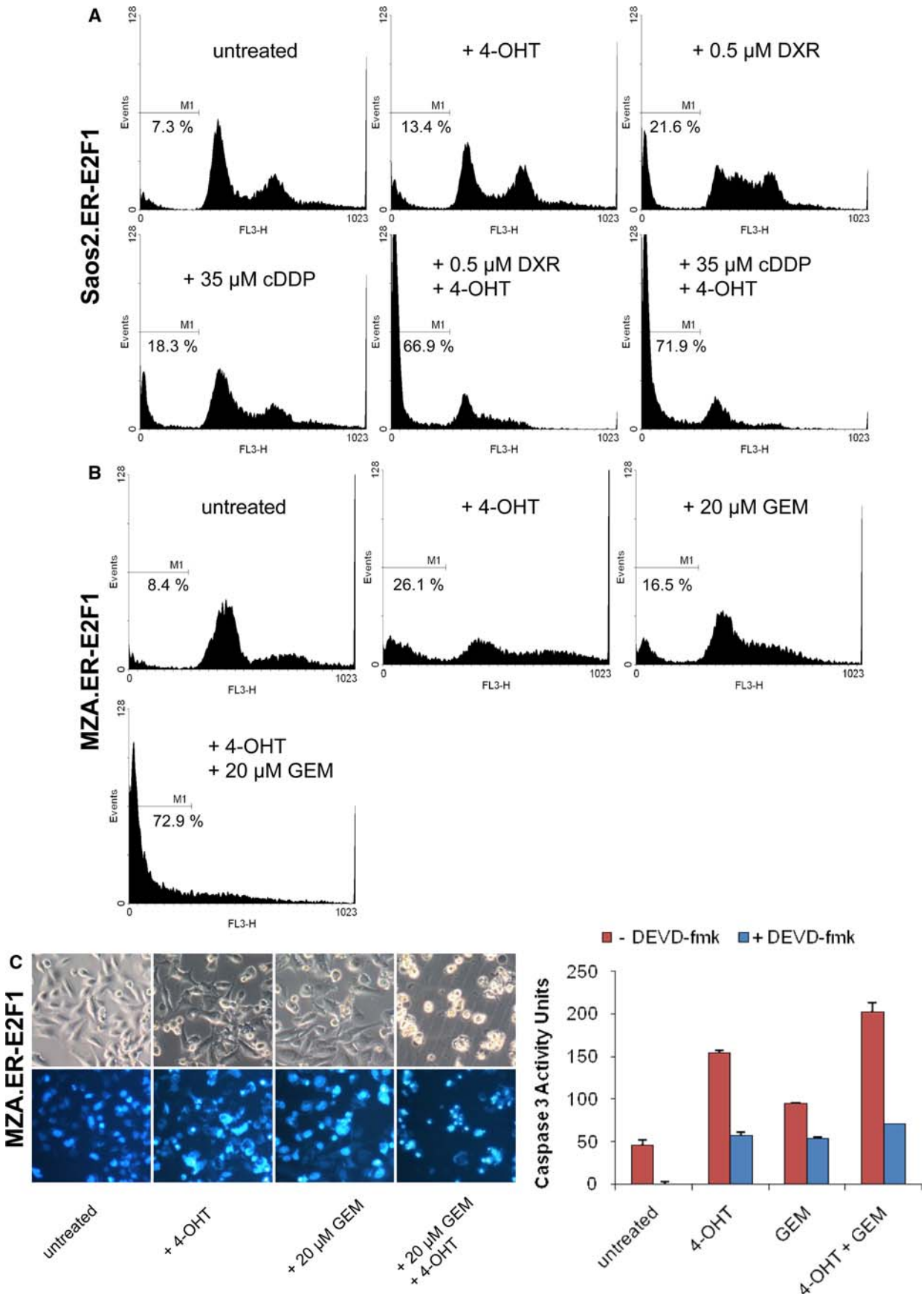
Combined treatment with E2F1 and chemotherapy induces a synergistic effect on cancer cell apoptosis

In order to ascertain E2F1's ability to sensitize chemoresistant cancer cells to genotoxic stress, we treated p53 deficient human Saos-2 osteosarcoma and MZA pancreatic adenocarcinoma cells with sub-lethal doses of DNA-damaging agents in combination with a moderate E2F1 activation. Both cell lines stably express the ER-E2F1 fusion protein to conditionally regulate E2F1 activation through 4-OHT (4-hydroxytamoxifen) administration [38]. Saos-2.ER-E2F1 were treated with doxorubicine (DXR) and cisplatin (cDDP), which are commonly used in current chemotherapy regimens of osteosarcoma [39]. As first line therapy for patients with pancreas cancer, gemcitabine (GEM) was used to induce DNA damage of MZA.ER-E2F1 cells [40]. To assess apoptotic death resulting from E2F1 induction or chemotherapy, and both combined, treated cells were analyzed by FACS analysis of propidium iodide (PI)-stained cells. E2F1 activation alone leads to 13.4 and 26.2% apoptotic Saos-2 (Fig. 1a) and MZA cells (Fig. 1b) 24 and 48 h after addition of 4-OHT, respectively. Similarly low levels of apoptosis were induced by individual chemotherapy (DXR, 21.6%; cDDP, 18.3%; GEM, 16.5%). Compared to either treatment alone, quantification of sub-G1 populations clearly revealed substantially higher apoptotic rates after combination therapy. This synergistic effect occurred with Saos-2 (Fig. 1a; 66.9 and 71.9%) as well as with MZA cells (Fig. 1b; 72.9%) and was not drug specific. Consistent with the FACS data, enforced E2F1 activity together with chemotherapy in these cells produced pronounced apoptotic features with striking changes in the nuclear morphology, characterized by intense staining of condensed chromatin and nuclear fragmentation (Fig. 1c, left). The combined treatment was accompanied by increased caspase-3 activity, which can be significantly blocked by the caspase-3 specific inhibitor DEVD-fmk (Fig. 1c).

Fig. 1 E2F1 sensitizes p53 negative cancer cells to chemotherapy. ▶ Apoptosis analysis in human osteosarcoma and pancreatic cancer cell lines stably expressing ER-E2F1 fusion protein. Flow cytometry DNA profiles of Saos-2 (**a**) and MZA cells (**b**) 24 and 48 h following treatment with *4-OHT*, genotoxic agent, and the combination of 4-OHT plus drug compared to untreated cells. Saos-2.ER-E2F1 were treated with 0.5 µM doxorubicin (*DXR*) or 35 µM cisplatin (*cDDP*), and MZA.ER-E2F1 with 20 µM gemcitabine (*GEM*). The percentage of apoptotic cells with sub-G1 DNA content is labeled as M1. **c** Hoechst 33342 staining of MZA.ER-E2F1 cells 48 h after therapy. Apoptotic cells show the typical features of membrane blebbing, cell shrinkage, and nuclear condensation (*left panel*). Detection of caspase-3 activity in MZA.ER-E2F1 cells treated with 4-OHT, GEM, and 4-OHT + GEM combination in the absence and presence of caspase-3 specific inhibitor DEVD-fmk (*right*). Means ± SD of three independent experiments are shown

Microarray-based identification of cooperation response genes in cancer cells chemosensitized by E2F1

Next, we tested whether significant cell death visible at 24 and 48 h of E2F1 activation plus chemotherapy impairs


A
Saos2.ER-E2F1
untreated
7.3 %
+ 4-OHT
13.4 %
+ 0.5 μM DXR
21.6 %
+ 35 μM cDDP
18.3 %
+ 0.5 μM DXR
+ 4-OHT
66.9 %
+ 35 μM cDDP
+ 4-OHT
71.9 %
B
MZA.ER-E2F1
untreated
8.4 %
+ 4-OHT
26.1 %
+ 20 μM GEM
16.5 %
+ 4-OHT
+ 20 μM GEM
72.9 %
Events
FL3-H
M1
C
MZA.ER-E2F1
untreated
+ 4-OHT
+ 20 μM GEM
+ 20 μM GEM
+ 4-OHT
- DEVD-fmk
+ DEVD-fmk
Caspase 3 Activity Units
250
200
150
100
50
0
untreated
4-OHT
GEM
4-OHT + GEM

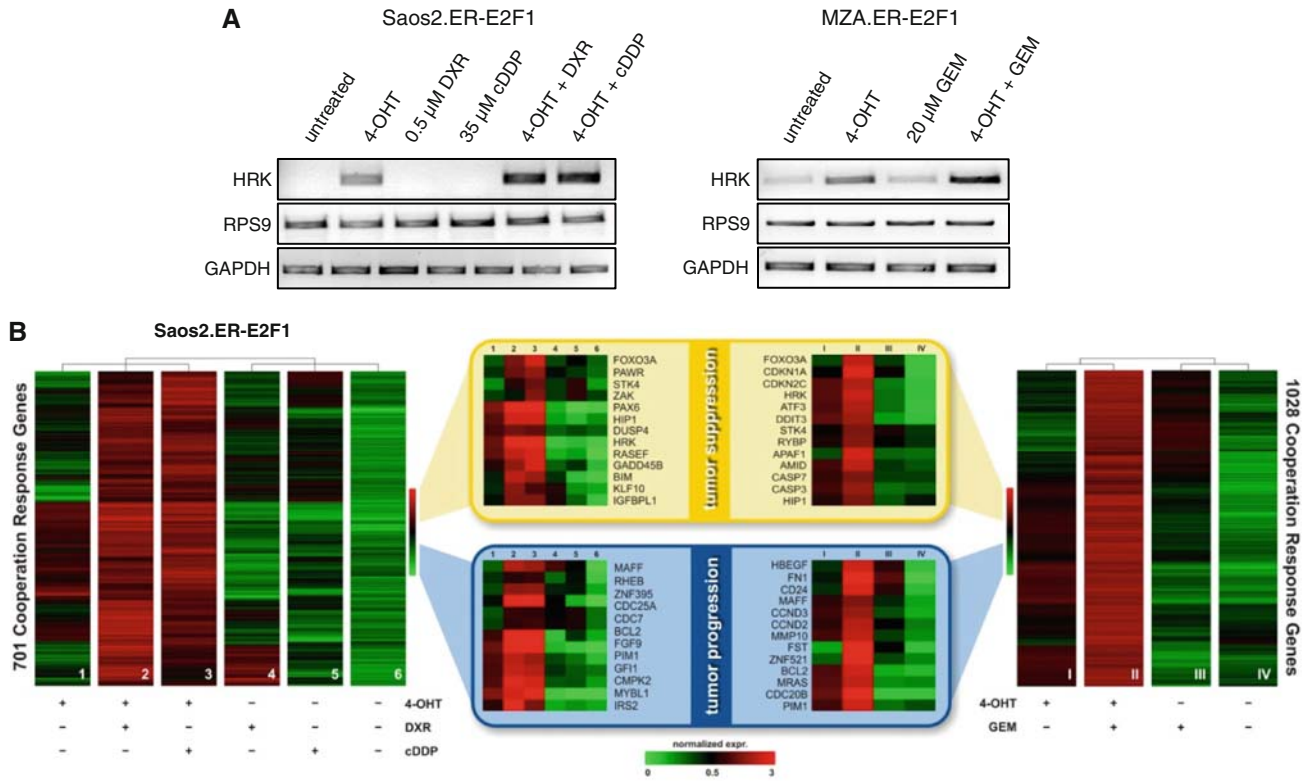


Fig. 2 DNA damage enhances E2F1-driven global gene expression. **a** Semiquantitative RT-PCR analysis of *HRK* expression in Saos-2 and MZA.ER-E2F1 cells treated as outlined above. *GAPDH* and *RPS9* transcripts are shown as control. **b** Equal amounts of RNA from treated cells were subjected to microarray analysis. Affymetrix gene expression data were analyzed using hierarchical clustering. *Colors* represent normalized expression levels. Treatment procedures are clustered in *columns* and corresponding gene clusters are displayed in *rows*. *Green shading* indicates low gene expression, whereas *red shading* indicates higher expression. Two-dimensional clustering revealed enhanced expression of a specific subset of genes (cooperation response genes, CRGs) following E2F1 activation in combination with genotoxic treatment. Only CRGs displaying a minimum twofold induction by 4-OHT plus drug were included for clustering. Subsets of CRGs involved in tumor suppression and tumor progression are displayed enlarged in the *middle*

expression of known E2F1 target genes. As shown in Fig. 2a, increased transcript levels of the E2F1 cell death mediator harakiri (*HRK*) were detectable in both cell lines after combination treatment that perfectly match the observed synergistic apoptosis effect, demonstrating that they are suitable for further analysis. To explore the possibility that the observed cooperative apoptosis-inducing effect correlates with gene expression, treated cells were subjected to whole-genome Affymetrix GeneChip array analyses using RNA isolated at the same time point as apoptosis assays were performed. Genes that displayed at least a twofold increase in expression between treated (single and combination treatments) and untreated cells were assigned as differentially regulated. Subsequently, a subset of 701 genes in Saos-2.ER-E2F1 and 1028 genes in MZA.ER-E2F1 cells was extracted whose gene expression was either induced or enhanced in the presence of 4-OHT and genotoxic drug compared to each individual treatment. Clustering of this gene category confirmed the filtering strategy, indicating that cooperative response genes (CRGs) exhibit their maximum expression when E2F1 synergistically interacts with chemotherapeutics (Fig. 2b). While cells exclusively exposed to genotoxic agents are in the same cluster as untreated cells (lanes 4, 5, 6, and lanes III, IV), the gene expression pattern of both Saos-2 and MZA with activated E2F1 clustered together with chemosensitized (E2F1 plus drug) cells (lanes 1, 2, 3, and lanes I, II), suggesting that the majority of genes are E2F1-induced target genes. Classification of these CRGs using GO annotations and literature survey indicated that they are overwhelmingly associated with tumor suppressor function, and some of them are well-known direct apoptotic targets of E2F1, such as *HRK*, bcl-2 interacting mediator of cell death (*BIM*), apoptotic peptidase activating factor (*APAF1*), caspase 7 (*CASP7*), forkhead box O3A (*FOXO3A*), dual specificity phosphatase 4 (*DUSP4*), and cyclin-dependent kinase inhibitor 1A (*CDKN1A*) [11, 41–45] (Fig. 2, central upper panel; Table 1). Importantly, the results also show that chemotherapy combined with deregulated E2F1 activity considerably enhanced expression of genes that positively regulate survival and tumor progression (Fig. 2b, central lower panel; Table 2). This

Table 1 List of proapoptotic/anti-proliferative cooperation response genes (fold change: E2F1 + drug vs untreated)

Genbank ID	Gene symbol	Saos2 fold change	MZA fold change	Description
NM_005157	ABL1	2.3	–	v-abl Abelson murine leukemia viral oncogene homolog 1
AI922797	AIFM2	–	5.9	Apoptosis-inducing factor (AIF)-like, mitochondrion-associated2
AF248734	APAF1	–	7.7	Apoptotic peptidase activating factor
AB037797	ARRDC3	3.1	–	Arrestin domain containing 3
NM_001674	ATF3	–	22.9	Activating transcription factor 3
AL031177	ATG4A	–	2.1	ATG4 autophagy related 4 homolog A (*S. cerevisiae*)
NM_004849	ATG5	–	2.0	ATG5 autophagy related 5 homolog (*S. cerevisiae*)
BE857425	BHLHB3	–	2.3	Basic helix-loop-helix domain containing, class B, 3
AK027160	BIM	13.0	6.6	Bcl-2 interacting mediator of cell death
AL535380	BTG1	–	2.1	B-cell translocation gene 1, anti-proliferative
BC028229	BTG3	–	15.8	BTG family, member 3
NM_004346	CASP3	–	4.2	Caspase 3, apoptosis-related cysteine peptidase
NM_001227	CASP7	–	2.8	Caspase 7, apoptosis-related cysteine peptidase
NM_000389	CDKN1A	–	47.7	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
N95363	CDKN1C	–	40.6	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
U17074	CDKN2C	–	16.4	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
NM_004394	DAP	–	2.4	Death-associated protein
BC003637	DDIT3	–	15.3	DNA damage-inducible transcript 3
AI129699	DNASE1	–	2.7	Deoxyribonuclease I
AI939563	DPF1	10.4	3.9	D4, zinc and double PHD fingers family 1
NM_007026	DUSP14	–	3.0	Dual specificity phosphatase 14
NM_001394	DUSP4	71.2	–	Dual specificity phosphatase 4
U16996	DUSP5	–	3.2	Dual specificity phosphatase 5
BC003143	DUSP6	3.5	–	Dual specificity phosphatase 6
NM_004420	DUSP8	–	4.4	Dual specificity phosphatase 8
U16797	EFNB2	2.8	10.1	Ephrin-B2
BE302085	FDX1	–	3.3	Ferredoxin 1
AI056872	FOXO3A	9.4	28.3	Forkhead box O3A
AF087853	GADD45B	–	5.4	Growth arrest and DNA damage-inducible 45, beta
NM_002048	GAS1	3.6	–	Growth arrest-specific 1
NM_015895	GMNN	2.4	–	Geminin, DNA replication inhibitor
AW193511	HEXIM1	7.2	–	Hexamethylene bis-acetamide inducible 1
AU145049	HIP1	2.2	2.5	Huntingtin interacting protein 1
NM_003806	HRK	9.6	20.2	Harakiri, BCL2 interacting protein (contains only BH3 domain)
AL522781	IGFBPL1	93.4	–	Insulin-like growth factor binding protein-like 1
NM_019071	ING3	–	2.8	Inhibitor of growth family, member 3
M13981	INHA	4.0	–	Inhibin, alpha
NM_001567	INPPL1	–	2.5	Inositol polyphosphate phosphatase-like 1
NM_005655	KLF10	14.1	–	Kruppel-like factor 10
BF514079	KLF4	–	10.0	Kruppel-like factor 4 (gut)
AF274972	LRDD	7.9	–	Leucine-rich repeats and death domain containing
AB050468	LRIG1	5.7	–	Leucine-rich repeats and immunoglobulin-like domains 1
N21184	LZTS1	–	3.3	Leucine zipper, putative tumor suppressor 1
AA541479	MAP3K1	2.8	–	Mitogen-activated protein kinase kinase kinase 1
AK024029	MOAP1	–	4.1	Modulator of apoptosis 1
AL096842	MTUS1	5.7	27.1	Mitochondrial tumor suppressor 1
AW071793	MXD1	–	8.0	MAX dimerization protein 1
AI336206	PAWR	2.2	–	PRKC, apoptosis, WT1, regulator

Table 1 continued

Genbank ID	Gene symbol	Saos2 fold change	MZA fold change	Description
AW088232	PAX6	68.9	–	Paired box gene 6
AJ251830	PERP	–	2.8	PERP, TP53 apoptosis effector
BG547855	PLAGL1	2.2	2.7	Pleiomorphic adenoma gene-like 1
AK023188	PPP1R13B	–	3.0	Protein phosphatase 1, regulatory (inhibitor) subunit 13B
AV724783	PRDM2	–	4.3	PR domain containing 2, with ZNF domain
AF035594	PRKCA	–	2.3	Protein kinase C, alpha
AK025152	PRKCE	6.1	6.5	Protein kinase C, epsilon
J03580	PTHLH	7.1	52.1	Parathyroid hormone-like hormone
AI129941	PURA	–	3.1	Purine-rich element binding protein A
AA826324	RASEF	32.1	–	RAS and EF-hand domain containing
NM_007182	RASSF1	–	4.6	Ras association (RalGDS/AF-6) domain family 1
BC004270	RASSF5	–	3.5	Ras association (RalGDS/AF-6) domain family 5
AY009093	RHOBTB2	–	11.0	Rho-related BTB domain containing 2
W84482	RYBP	2.2	2.8	RING1 and YY1 binding protein
AB018333	SASH1	2.9	4.8	SAM and SH3 domain containing 1
U38276	SEMA3F	–	3.8	Semaphorin 3F
AW131754	SMARCA2	–	2.8	SWI/SNF related, subfamily a, member 2
Z25430	STK4	4.3	3.2	Serine/threonine kinase 4; serine/threonine kinase 4
NM_005426	TP53BP2	2.4	2.2	Tumor protein p53 binding protein, 2
NM_006034	TP53I11	4.7	2.5	Tumor protein p53 inducible protein 11
NM_021158	TRIB3	–	3.7	Tribbles homolog 3 (Drosophila)
AK022859	UNC5B	–	6.9	Unc-5 homolog B (*C. elegans*)
AL833307	ZAK	3.5	2.2	Hypothetical protein LOC339751
AI356398	ZFP36L2	–	2.1	Zinc finger protein 36, C3H type-like 2
AU147613	ZNF346	2.8	–	Zinc finger protein 346

includes oncogenes like cell division cycle 25A (*CDC25A*), fibroblast growth factor 9 (*FGF9*), v-myb myeloblastosis viral oncogene homolog (*MYBL1*) in Saos-2 cells, heparin-binding EGF-like growth factor (*HBEGF*) in MZA, and B-cell CLL/lymphoma 2 (*BCL2*) or pim-1 oncogene (*PIM1*) in both cell types that are in part known as E2F1 target genes [42, 46].

Cooperative expression pattern of known and putative E2F1 target genes in response to genotoxic agents

We verified the expression pattern of several of the proapoptotic genes including *BIM*, *HRK*, *DUSP4*, leucine-rich repeats and death domain containing (*LRDD*), Krüppel-like factor 10 (*KLF10*), cyclin-dependent kinase inhibitor 2C (*CDKN2C*), *FOXO3A*, *CDKN1A*, caspase 3 (*CASP3*), *CASP7*, growth arrest and DNA damage-inducible 45 beta (*GADD45B*), apoptosis-inducing factor, mitochondrion-associated, 2 (*AIFM2*), and Huntingtin interacting protein 1 (*HIP1*) classified as CRGs in Saos-2 and MZA cells, respectively, by quantitative real-time PCR using gene specific primers. Each of the three genes in the left panel is induced by E2F1 in Saos-2 cells, whereas extremely low or negligible transcript levels were detected in response to doxorubicin or cisplatin treatment alone (Fig. 3a). Additional administration of cDDP and DXR following E2F1 stimulation significantly increased target gene expression ranging in fold inductions from 18 to 100 for *DUSP4* and from 33 to 110 for *HRK*, suggesting that E2F1 exhibits its maximum transcriptional activity for this subset of genes only after DNA damage. Other putative death genes, such as *LRDD* and *KLF10* expressed at comparable high levels after E2F1 activation or chemotherapy, are synergistically upregulated when Saos-2 cells were treated with the combination of both (Fig. 3a, right panel). Verification of a representative choice of apoptosis-related CRGs in MZA pancreatic carcinoma cells revealed a similar activation pattern as shown for *LRDD* and *KLF10*, demonstrating that the highest gene expression levels can be achieved only by the cooperation of E2F1 with gemcitabine, while each single treatment is less effective (Fig. 3b). As indicated for GEM, drug treatment alone induced a substantial increase (stabilization) of endogenous E2F1 protein (Fig. 3c), which was also detectable when Saos-2 cells were exposed to

Table 2 List of tumor-progression-related cooperation response genes (fold change: E2F1 + drug vs untreated)

Genbank ID	Gene symbol	Saos2 fold change	MZA fold change	Description
AU146963	BCL2	23.0	17.6	B-cell CLL/lymphoma 2
NM_001760	CCND3	–	8.6	Cyclin D3
AF112857	CCNE2	–	6.9	Cyclin E2
AA761181	CD24	–	22.3	CD24 antigen (small cell lung carcinoma cluster 4 antigen)
NM_001789	CDC25A	2.1	–	Cell division cycle 25A
BC037547	CDC20B	–	56.6	Cell division cycle 20 homolog B
NM_003503	CDC7	2.1	–	CDC7 cell division cycle 7 (*S. cerevisiae*)
NM_002010	FGF9	13.0	–	Fibroblast growth factor 9 (glia-activating factor)
AK026737	FN1	–	277.3	Fibronectin 1
BF438173	FST	–	448.0	Follistatin
NM_005263	GFI1	11.7	–	Growth factor independent 1
M60278	HBEGF	–	23.5	Heparin-binding EGF-like growth factor
AF073310	IRS2	325.1	–	Insulin receptor substrate 2
AI742057	CPMK2	141.1	–	Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial
AL021977	MAFF	55.4	6.4	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
BG170541	MET	70.1	–	Met proto-oncogene (hepatocyte growth factor receptor)
NM_002425	MMP10	–	8.3	Matrix metallopeptidase 10 (stromelysin 2)
BF343625	MRAS	–	8.1	Muscle RAS oncogene homolog
AW592266	MYBL1	12.7	–	v-myb myeloblastosis viral oncogene homolog (avian)-like 1
M24779	PIM1	6.1	6.1	Pim-1 oncogene; pim-1 oncogene
D78132	RHEB	8.9	–	Ras homolog enriched in brain
AI797677	ZNF395	7.1	–	Zinc finger protein 395
AF141339	ZNF521	–	27.5	Zinc finger protein 521

DXR (Fig. 6c) or cDDP (data not shown). In line with the microarray data, these results indicate that genotoxic agents influence transcription of E2F1-dependent target genes, suggesting that genes induced by chemotherapy in cancer cells with aberrant E2F1 are directly regulated by E2F1.

Identification of novel apoptosis genes directly activated by E2F1

Differential gene expression analysis and qPCR-based array verification experiments revealed enhanced transcriptional activity of E2F1 under genotoxic stress. Assuming that the set of CRGs is enriched of direct E2F1 targets, we focused on identifying proapoptotic genes that are activated by E2F1 in a direct manner. Real-time RT-PCR was performed on ER-E2F1-expressing Saos-2 and MZA grown in the presence of the protein synthesis inhibitor cycloheximide (CHX) plus 4-OHT or 4-OHT alone. The results from a subset of transcripts known to mediate cell death [47–49] are shown in Fig. 4. As indicated for the primary E2F1 target HRK, a significant stimulation of RNA expression was observed for *STK4, KLF10*, and *HIP1* by addition of 4-OHT (black bars) even in the presence of CHX (gray bars) in both cell lines with a peak between 8 and 16 h after E2F1 activation (Fig. 4). Based on these data, de novo protein synthesis is not required for E2F1-induced upregulation of these genes, underscoring that they are direct targets of E2F1.

E2F1 directly binds to the KLF10 promoter in vivo

The transforming growth factor-β (TGF-β)-inducible early gene-1/Krüppel-like factor 10 (TIEG1/KLF10) is a member of a subfamily of TGF-β inducible Sp1-like proteins. Overexpression of this transcription factor was recently shown to induce apoptosis in cancer cells in part through the mitochondrial pathway [49]. Although the promoter misses typical transcription start elements like a TATA box or initiation sequence, the region from -130 to -41 is critical for basal gene transcription [50]. Enforced by the finding of substantially enhanced KLF10 mRNA levels in response to direct E2F1 activity, we then ascertained whether its promoter contains E2F-binding sites. Sequence analysis revealed a putative motif in the region between -880 and -873 upstream of the transcriptional start site. Chromatin immunoprecipitation assays were performed to determine the in vivo binding of E2F1 to the KLF10

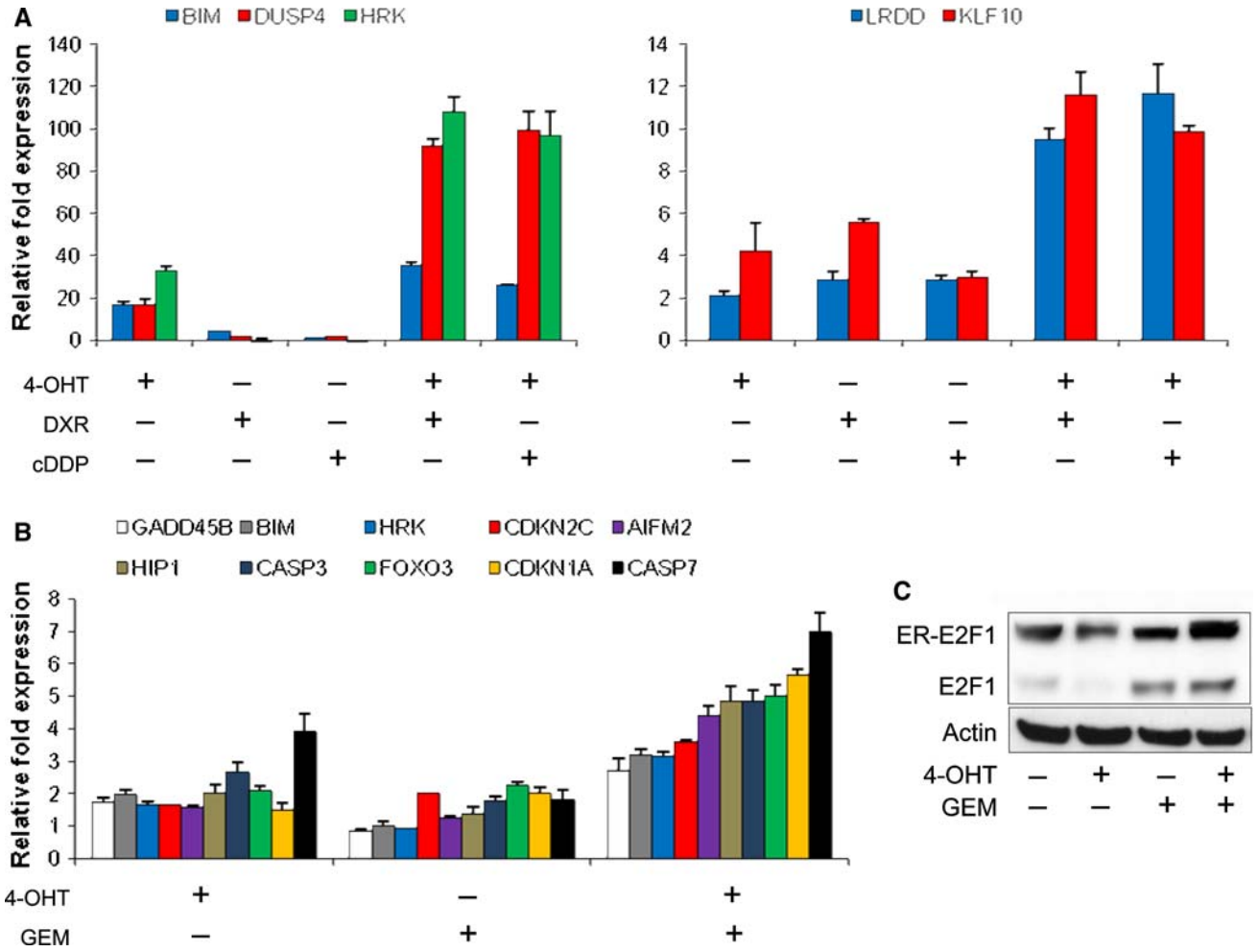


Fig. 3 Activation of selected CRGs in response to E2F1 in combination with chemotherapy verified by real-time PCR. Endogenous gene expression levels were quantitated in Saos-2.ER-E2F1 (**a**) and MZA.ER-E2F1 cells (**b**) treated either with 4-OHT or each drug (DXR, cDDP, GEM) alone, and the combination. Fold expression was calculated after normalization with GAPDH relative to untreated cells (set as 1). Averages and standard deviations of at least three independent experiments are shown. **c** Western blot showing the protein levels of E2F1 and ER-E2F1 in the presence or absence of 4-OHT and the therapeutic drug (GEM) in MZA.ER-E2F1 cells. Actin was used for equal loading

promoter. Saos-2 cells that stably express the ER-E2F1 fusion protein were used to conditionally regulate E2F1 activation. After synchronization of cells by serum starvation, nuclear translocation of E2F1 was induced by the addition of 1 μM 4-OHT for 24 h. DNA–protein complexes were immunoprecipitated with antibodies against E2F1. IgG was used as unspecific antibody. Purified DNA was analyzed by PCR using primer pairs that amplified a 251-bp region containing the putative E2F1 binding site, or, as a positive control, the E2F1 binding region of the Apaf-1 promoter [10] (Fig. 5a, left panel). The ChIP assay showed a strong increase in E2F1 binding to this particular region of the KLF10 promoter comparable with its binding to the Apaf-1 consensus sequence. In contrast, there was no binding of E2F1 in untreated cells (mock), when using an unspecific antibody, or a random genomic sequence lacking potential E2F binding sites (neg. ctrl.). A specific interaction of E2F1 with the KLF10 promoter was also detectable in SK-Mel-147 melanoma cells expressing high amounts of E2F1, while binding was completely abolished after infection with an adenoviral (Ad) vector encoding gene-specific shRNA shown to efficiently knockdown endogenous E2F1. Moreover, treatment of parental Saos-2 cells with DNA-damaging agents such as doxorubicin resulted in a significant binding of endogenous E2F1 to this promoter region (Fig. 5a). To validate the ChIP data, we analyzed E2F1 binding to the KLF10 promoter by real-time PCR (Fig. 5a, right). While *KLF10* and *Apaf-1* exhibit a clear binding (25- to 45-fold, respectively) when E2F1 is activated, a random genomic region without E2F motif used as negative control did not. In order to determine the specificity of the DNA–protein interaction, we carried out EMSAs using a synthetic oligonucleotide corresponding to the putative E2F1 site between −880 and −873. As

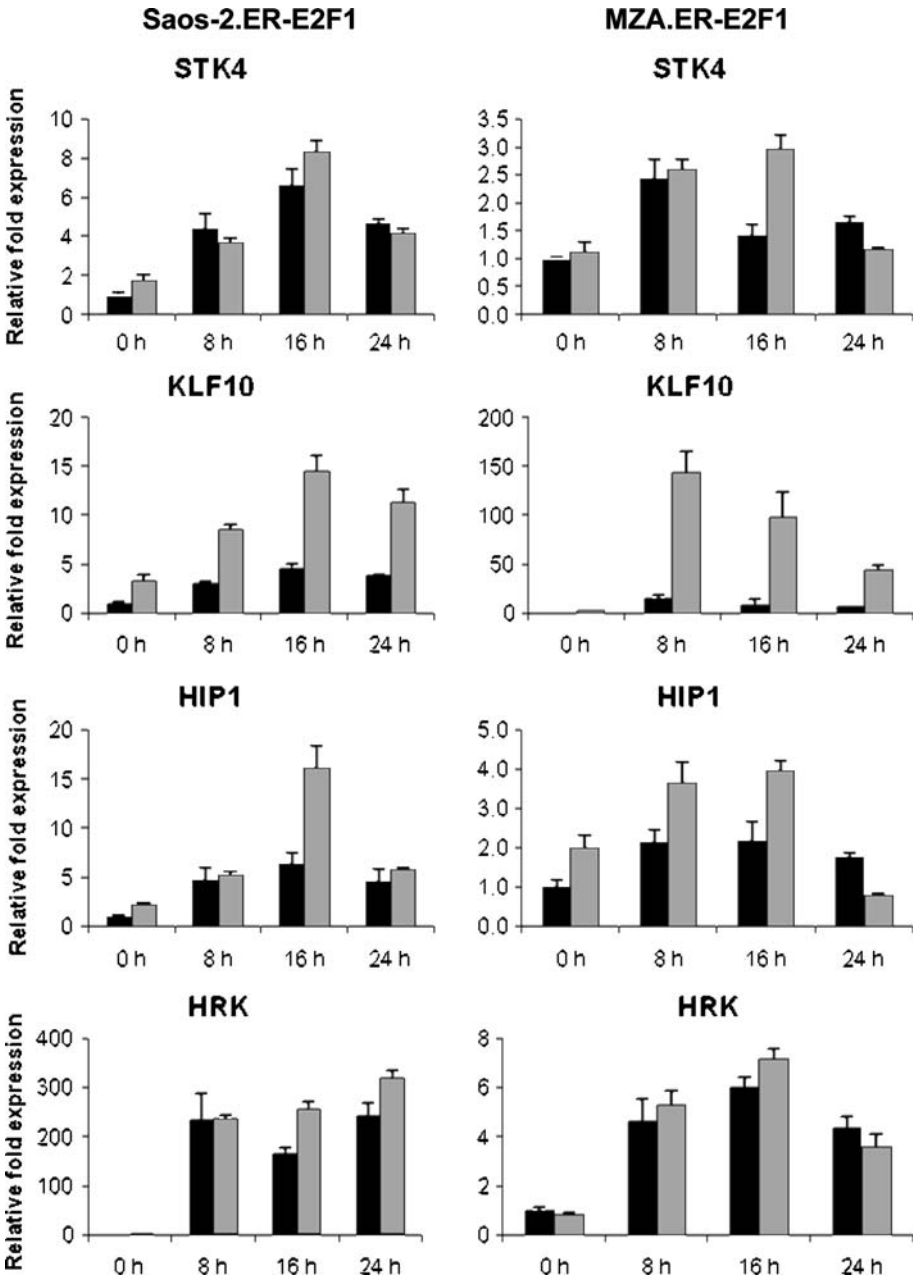


Fig. 4 Identification of direct proapoptotic E2F1 target genes from CRGs. E2F1 was activated by addition of 4-OHT with (*gray bars*) and without (*black bars*) de novo protein synthesis inhibitor cycloheximide (10 μg/ml, added 4 h prior to harvesting). mRNA expression levels of *STK4*, *HIP1*, and *KLF10* were analyzed at indicated time points after E2F1 induction in Saos-2.ER-E2F1 (*left panel*) and MZA.ER-E2F1 (*right panel*) by quantitative PCR. Data are illustrated as fold expression after normalization with GAPDH. Levels of untreated cells were set as 1. Transcript levels of the direct E2F1 target gene *HRK* served as positive control

illustrated in Fig. 5b, the KLF10 DNA probe gave retarded bands of strong intensities with nuclear extracts from Saos-2.ER-E2F1 cells after E2F1 activation (lane 2). Competition experiments revealed that the predominant band A′ of the KLF10 E2F site binding complex was efficiently displaced by an excess of homologous cold probe (lane 3, 50-fold), whereas other faster migrating complexes were not reduced. In contrast, the KLF10 DNA–protein complex was not affected by a cold probe of the mutated KLF10 oligonucleotide (lane 4, 50-fold), stressing the sequence specificity of this interaction. In addition, formation of a smaller complex B′ was competed to some extent by the KLF10 E2F site (lane 3) but not by DNA carrying the mutated motif (lane 4), assuming that this band also contains E2F1. While complex A′ most likely contains the ER-E2F1 fusion protein, band B′ indicates binding of endogenous E2F1 to the KLF10 E2F motif. No binding was found in the absence of nuclear extract (lane 1). Analog to the E2F1-dependent TP73 promoter [8], transfection of asynchronously growing Saos-2 cells with a KLF10 promoter luciferase reporter containing the putative E2F1-binding site (−880 to −873) demonstrated a strong (approximately 10-fold) induction of luciferase activity by cotransfection of wild-type E2F1, whereas the E2F1-mutant E132 (defective for DNA binding) and the E(-TA) mutant (deletion of TA domain) did not significantly enhance the

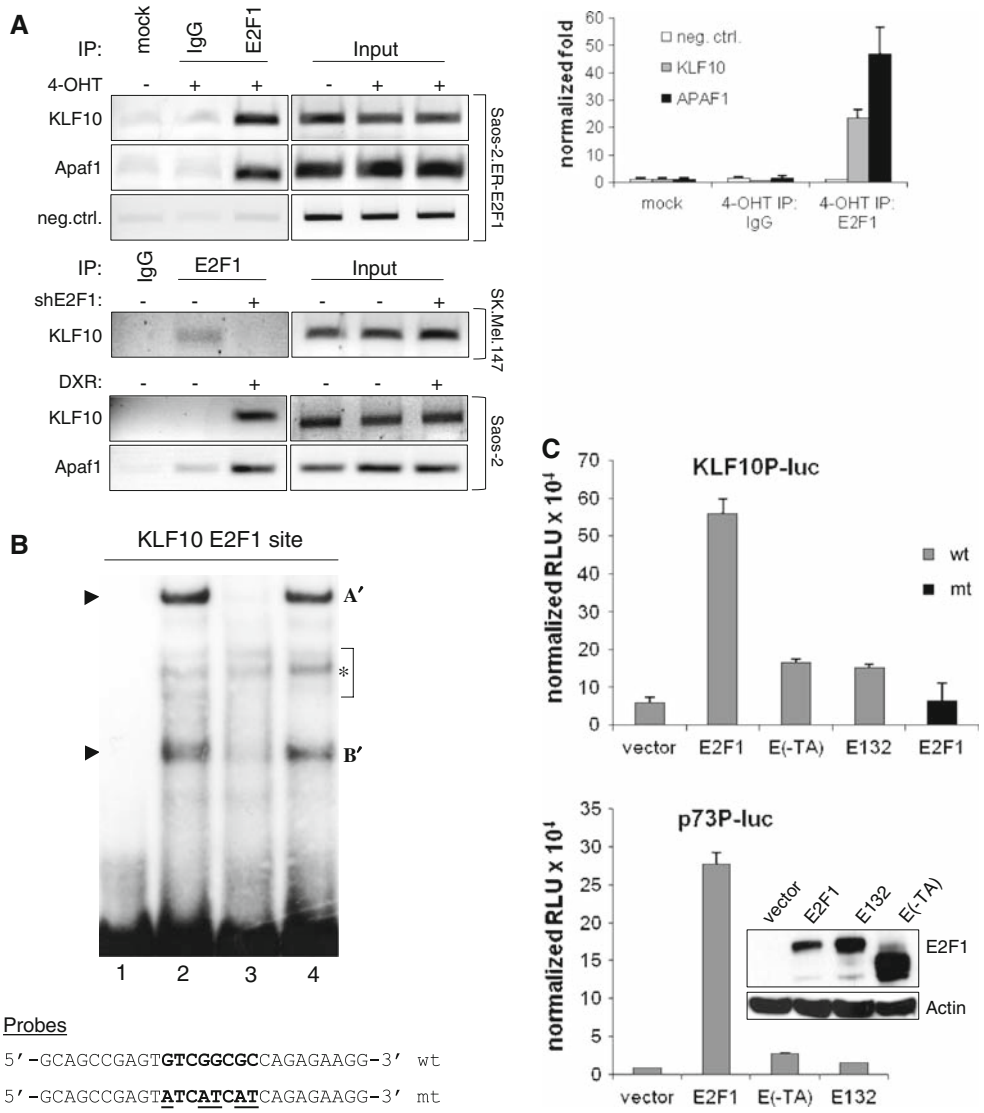


Fig. 5 Direct interaction of E2F1 with the KLF10 promoter in vivo. **a** Chromatin immunoprecipitation of serum-starved Saos-2 cells stably expressing ER-E2F1 grown in the presence or absence of 4-OHT for 24 h, SK-Mel-147 melanoma cells infected with Ad.shE2F1 for 48 h, and parental Saos-2 cells following 24 h DXR treatment. ChIP was performed using either a control IgG antibody or antibody against E2F1. PCR primers were designed to amplify a region containing an E2F1 binding site (bp −880 to bp −873) spanning from −909 to −659 bp. PCR primers for the Apaf-1 promoter were used as positive control. Input lane represents 10% of total chromatin used in ChIP assay (*left panel*). Bar graphs show quantitative PCR results of E2F1 promoter binding in Saos-2.ER-E2F1 cells normalized to input (*right panel*). Primers amplifying a random non-E2F-binding site were used as negative control (neg. ctrl.). Mock DNA-amplicon levels were set to 1. **b** Biotin-labeled double-stranded oligonucleotide corresponding to the KLF10 promoter E2F1 binding sequence (*lanes 1–4*) was incubated with nuclear extract from Saos-2.ER-E2F1 cells treated with 4-OHT for 24 h (*lanes 2–4*) and a 50-fold molar excess of cold specific competitor for the KLF10 motif (*lane 3*) or mutant KLF10 E2F1 site (*lane 4*). *Arrows* indicate a predominant band A′ and complex B′ specific for E2F1. Unspecific bands are marked, *asterisk lane 1*, binding without nuclear extract. **c** Parental Saos-2 cells were cotransfected with 0.5 µg of KLF10P-luc plasmid (containing the wild-type E2F1 binding site) or mutated KLF10P-luc vector and 0.5 µg of expression plasmid encoding E2F1, DNA-binding defective mutant E132, E(-TA), lacking the transactivation domain, or empty pcDNA3.1 as mock control (vector). The E2F1-responsive *TP73* promoter reporter construct (p73P-luc [8]) was used as positive control. Luciferase activity (*RLU*) was measured 16 h after transfection. *Error bars* SD of three independent experiments. E2F1 and actin protein expression was verified by western blotting

basal promoter activity (Fig. 5c). These data indicate that the DNA binding and transactivation domain of E2F1 are required for the full stimulatory effect on the KLF10 promoter. In addition, the sequence of the E2F responsive element was mutated (from GTCGGCGC to ATCATCAT) and luciferase reporter gene assays were performed with this mutant in the presence of E2F1. As shown in Fig. 5c, the mutation inhibited the ability of E2F1 to stimulate

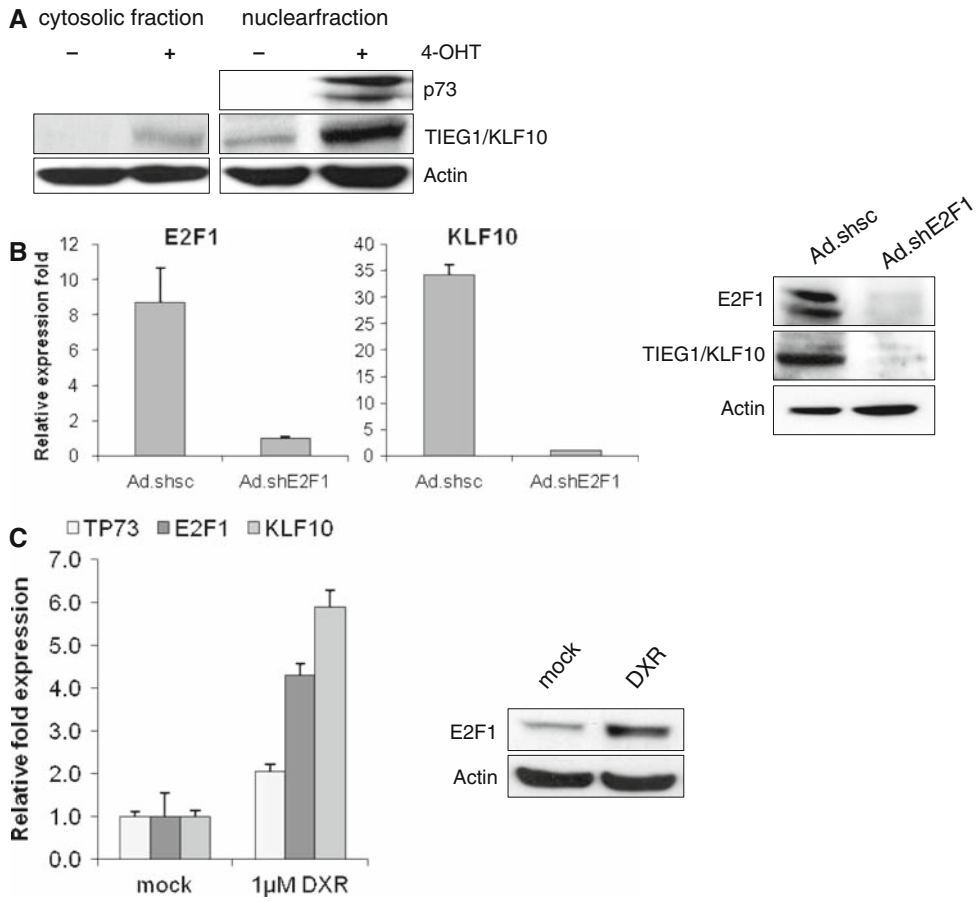


Fig. 6 E2F1 stimulates KLF10 expression on RNA and protein level. **a** Western blot analysis of serum-starved Saos-2.ER-E2F1 treated for 24 h with 4-OHT. Equivalent amounts of total cell proteins and nuclear protein fractions were separated by SDS-PAGE and probed with antibodies specific for TIEG1/KLF10 and p73. Equal loading was confirmed by probing the blots with β-actin antibody. Detection was achieved by chemiluminescence. **b** Expression of E2F1 and KLF10 examined by qPCR (*upper panel*) and immunoblotting (*lower panel*) in SK-Mel-147 cells 48 h after infection with adenovirus expressing scrambled-shRNA or shRNA against E2F1. mRNA levels of cells expressing E2F1-specific shRNA were set as 1. Actin was used as control. **c** Relative mRNA levels of KLF10, TP73, and E2F1 were measured in parental Saos-2 cells after 24 h of DXR monotherapy (1 μM) by quantitative RT-PCR (*left panel*). Expression was normalized to GAPDH. Fold changes are relative to mock controls (set as 1). *Bars* indicate mean values ± SD of three independent experiments. The E2F1 protein level after doxorubicin treatment is shown in the *right panel* using actin as control

promoter activity in a manner comparable to that of the negative controls (empty vector and E-(TA) or E132 on wild-type promoter). This result strongly argues for an E2F1-dependent regulation of KLF10 promoter activity via the GTCGGCGC site.

To assess TIEG1/KLF10 protein expression levels following E2F1 promoter binding, Saos-2.ER-E2F1 cells were treated with 4-OHT and analyzed by western blot (Fig. 6a). As previously described [51, 52], TIEG1/KLF10 protein was mainly localized in the nuclei of untreated cells. In accordance with the upregulation of KLF10 mRNA by E2F1, the amount of TIEG1/KLF10 protein significantly increased when E2F1 was activated. Although the strongest enhancement appeared in the nuclear fraction (comparable to the direct E2F1 target p73), TIEG1/KLF10 protein was also upregulated in the cytosol. Interestingly, several transcriptional target genes of TIEG1/KLF10 such as SMAD family member 2 (*SMAD2*), secreted phosphoprotein 1 (*SPP1*), runt-related transcription factor 2 (*RUNX2*), and serpin peptidase inhibitor, clade E, member 1 (*SERPINE1*) were found upregulated in Saos-2 and MZA cells in response to E2F1 as detected by our microarrays, whereas osteoprotegerin (*OPG*) repressed by KLF10 is downregulated (data not shown). To determine whether KLF10 is modulated by endogenous E2F1, we examined the expression of KLF10 in E2F1-knockdown cells using qPCR and western blot (Fig. 6b). In accordance with the ChIP data, silencing of E2F1 resulted in a complete inhibition of KLF10 expression on mRNA and protein level compared to cells transduced with control shRNA that does not cause a reduction of E2F1 expression, suggesting that KLF10 is strictly E2F1 regulated. A strong stimulating effect on KLF10 transcript level was also visible in parental Saos-2 cells when they were exposed to genotoxic

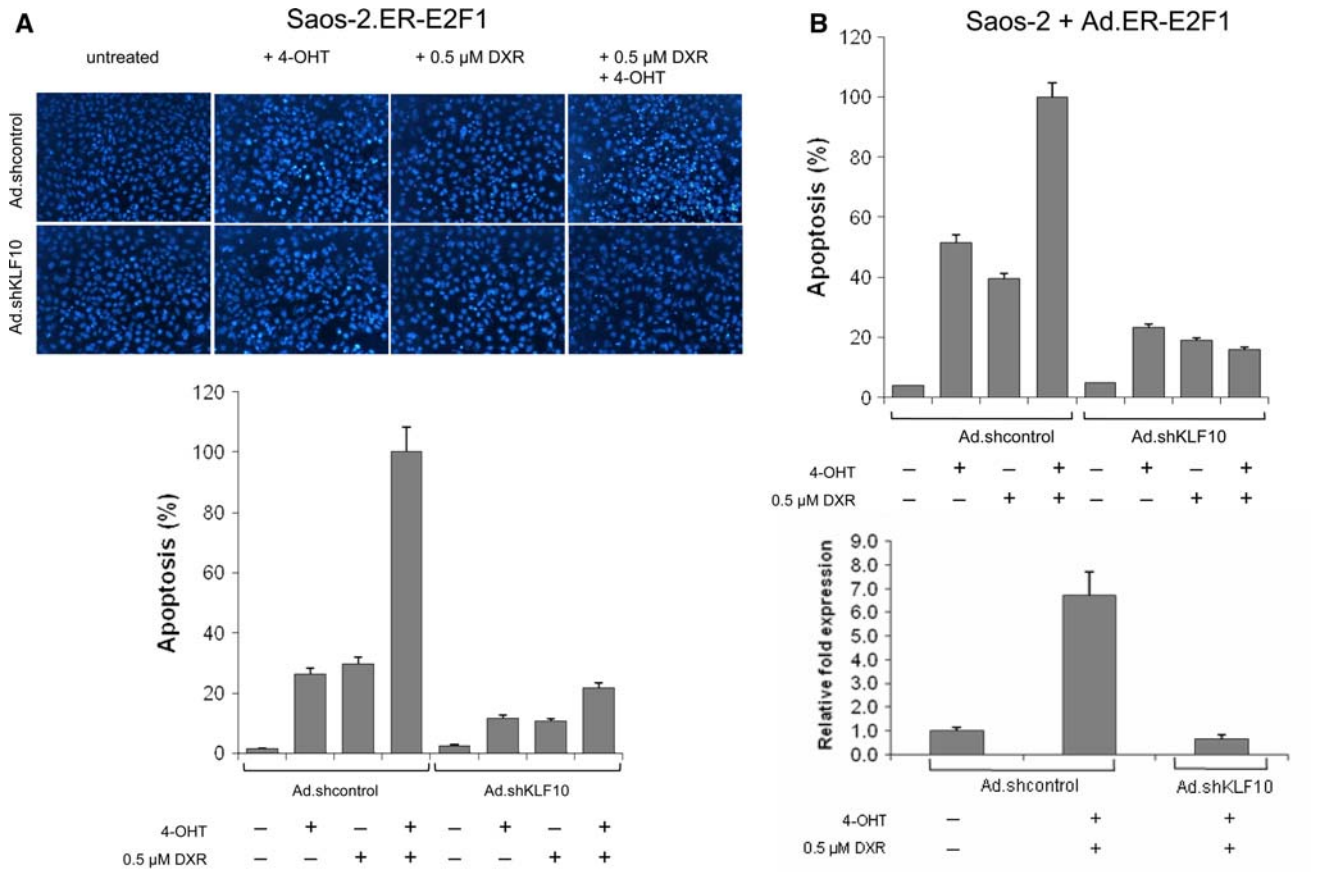


Fig. 7 Upregulation of TIEG1/KLF10 by E2F1 accounts for E2F1-associated chemosensitization of cancer cells. **a** Saos-2.ER-E2F1 cells and **b** Ad.ER-E2F1 infected parental Saos-2 cells were subjected to Ad.shKLF10 or Ad.shcontrol virus infection, after 24 h infected cells were treated with 4-OHT and/or 0.5 µM DXR. Apoptotic cells were measured 24 h after induction by counting the cells after staining with Hoechst 33342 (**a**,**b**, *top panel*). Knockdown effects on KLF10 transcript levels in Ad.ER-E2F1 infected cells treated with 4-OHT plus doxorubicin (1 µM DXR) were quantified by real-time PCR (**b**, *bottom panel*). Fold expression was calculated after normalization with GAPDH relative to untreated cells. Averages and SD of at least three independent experiments are shown

mono-therapy. As shown in Fig. 6c (left panel), doxorubicin treatment resulted in a sixfold increase of KLF10 mRNA, while both chemotherapy inducible targets *TP73* and E2F1 itself showed a 2- to 4.5-fold induction in response to DNA damage. A clear upregulation of endogenous E2F1 protein in Saos-2 cells following DXR exposure is indicated in the right panel.

The tumor suppressor TIEG1/KLF10 is required for E2F1-induced chemosensitization

Finally, we analyzed whether TIEG1/KLF10 activity is required for E2F1-induced chemosensitization. DNA damage stabilizes E2F1 with subsequent induction of apoptosis [25, 26, 31]. The data described above showed that activated E2F1 or chemotherapeutic drugs only increased KLF10 levels, and both cooperated in inducing its synergistic upregulation. We thus hypothesized that TIEG1/KLF10 is a critical effector for E2F1 to induce apoptosis in association with DNA damage. To test this, the Saos-2.ER-E2F1 cell line was treated with 4-OHT, DXR, and the combination of both following infection with adenoviral vector expressing shRNA against KLF10 or control-shRNA. Alternatively, parental Saos-2 cells were infected with Ad.ER-E2F1 prior to drug treatment. Hoechst apoptosis assays consistently demonstrated that depletion of endogenous KLF10 rendered osteosarcoma cells resistant to E2F1- and/or DNA damage-induced cell death (Fig. 7a, b, top panel). In both cases, knockdown of KLF10 was associated with a 50% (E2F1 activation alone and DXR treatment alone) to 80% (4-OHT plus DXR) reduction of apoptosis compared to cells with the non-specific shRNA. QRT-PCR analysis revealed that shKLF10 severely impaired KLF10 transcript elevation in response to combination therapy (Fig. 7b, bottom panel), as well as either treatment alone (data not shown). In view of these findings, we conclude that TIEG1/KLF10 expression has a major impact on E2F1- and DNA damage-induced

apoptosis, and plays a central role in E2F1-mediated sensitization of cancer cells to chemotherapy.

Discussion

Understanding the molecular mechanism(s) underlying the collaboration of E2F1 activity with DNA-damaging agents in inducing apoptosis is of critical importance to the development of targeted cancer intervention strategies. The observations that ectopic E2F1 causes increased sensitivity of malignant cells to chemotherapy suggests a p53-independent cooperative process involving synergy at multiple levels of regulation including gene expression. The data described here indicate that the cooperative nature of this phenomenon depends, to a considerable degree, on forced transcriptional upregulation of E2F1-dependent effector genes in response to distinct genotoxic drugs. We show that the CRGs contain a large fraction of genes that are important for the execution of the cell death program, some of which are known to be primarily regulated by E2F1. In addition to a recent report, suggesting that inactivation of antiapoptotic NF-κB is associated with tumor cell apoptosis induced by E2F1 and chemotherapy [53], this provides a link between enhanced proapoptotic gene activity and chemosensitivity of cancer cells. Synergistic behavior found in the microarray data thus seems highly informative for the identification of direct apoptotic target genes of E2F1, and provides a rational path to the discovery of both cancer cell-specific vulnerabilities and targets for intervention in neoplastic cells haboring multiple tumor suppressor gene defects including the loss of p53 function.

We noted that there were two groups of genes regulated through the combination of E2F1 activation with doxorubicin and cisplatin treatment in Saos-2 cells, one consisted of genes encoding direct apoptotic E2F1 targets such as the BH3-only proteins *BIM* and *HRK* [11], that were upregulated by elevated E2F1 but did not respond to drug therapy alone, compared to the group where each genotoxic agent per se had an expression-stimulating effect similar to individual E2F1 treatment. These genes included, for example, *KLF10* which was identified in our study as a downstream apoptosis effector of E2F1. Considering previous reports indicating that endogenous E2F1 induction correlates with tumor cell sensitivity to some DNA-damaging agents [54, 55] and our own data, this suggests that endogenously deregulated E2F1 in these osteosarcoma and pancreatic cancer cell lines contributes to the selective activation of its target genes during chemotherapy, and drug-induced stabilization of E2F1 is sufficient for the observed synergistic effects. In addition, consistent with the proposed mechanism by which E2F1 and the chemotherapeutic drugs converge on regulating gene expression, and the bimodal behavior of E2F1 favoring either apoptosis or proliferation depending on the cellular context, the microarray data interestingly demonstrated a synergism in the upregulation of genes that are related to cell survival and tumor progression. Based on these results, it is possible that DNA-damaging agents also promote oncogene activation in the context of E2F1 signaling. This assumption is supported by the observation that conditional E2F1 activation in transgenic mice targeted to the testes, which results in the activation of E2F1 target genes and p53-independent apoptosis (testicular atrophy) caused premalignant changes resembling carcinoma in situ cells in humans [56]. Although the ability of genotoxic compounds to transcriptionally induce oncogenes in association with aberrant E2F1 activity did not affect the net outcome of enhanced cell death in Saos-2 and MZA cells treated with the combination, this potentially carcinogenic side effect might reduce sensitivity to therapy, and should therefore be determined in each type of tumor.

Among the genes differentially expressed in a synergistic manner in response to E2F1 activation plus chemotherapeutics, we found several genes that are involved in the regulation of apoptosis but have not yet been recognized as targets of E2F1. One of these novel E2F1-induced genes is the tumor suppressor TGFβ-inducible early gene 1/Krüppel-like transcription factor 10. TIEG1/KLF10 was initially identified in normal human fetal osteoblasts following TGFβ treatment [57], and encodes a three zinc-finger Krüppel-like transcription factor, whose overexpression has been shown to functionally mimic the effects of TGFβ in human osteosarcoma and pancreatic carcinoma cells [58–60]. Relevant to our study, enforced expression of TIEG1/KLF10 decreases cellular proliferation [61, 62] and induces apoptosis in normal and cancer cells [63], whereas in support of its tumor suppressor activities, expression is lost during cancer progression [51, 64]. Normal breast tissues, for example, displayed a high expression of TIEG mRNA and protein, while in situ carcinoma showed less than one-half of the levels, and invasive carcinoma a complete absence. TIEG1/KLF10 has been shown to promote apoptosis induced by oxidative stress [65] and homoharringtonine or velcade, similar to p53 through the mitochondrial pathway involving caspase 3 activation [49]. Recently, TIEG/KLF10 was implicated in the regulation of T regulatory cell suppressor function and Treg activation through targeting TGFβ1 and Foxp3 expression [52, 62].

We have shown here that E2F1 strongly upregulates the expression of KLF10 in both Saos-2 and MZA cells through a direct transcriptional mechanism. Sequence scanning of the KLF10 promoter revealed a potential binding motif for the E2F1 transcription factor. Indeed,

using this particular region as a probe, we have demonstrated by ChIP that it specifically bound to E2F1 in vivo. Similar to TGFβ-treated cells [51], the levels of TIEG1/KLF10 protein in the cell nuclei increased when E2F1 was activated. Furthermore, perturbation experiments performed with short hairpin RNA (shRNA)-dependent knockdown of KLF10 expression inhibited cell death mediated by E2F1. Notably, apoptosis was dramatically reduced after E2F1 stimulation in cooperation with chemotherapy when Saos-2 cells (where *KLF10* was identified as CRG) were simultaneously treated with adenovirus expressing shKLF10, underscoring the relevance of TIEG1/KLF10 as a key mediator of E2F1-induced chemosensitization.

Taken together, these experiments indicate the importance of CRGs for identification of functional apoptotic networks in a variety of cellular backgrounds and genetic contexts as a basis for cancer-destroying therapies. In turn, the data provide new arguments not to use E2F1 overexpression in combination with conventional chemotherapeutics as a treatment option for cancer. In particular, we identified the tumor suppressor TIEG/KLF10 as a valuable tool to potentiate death of p53 deficient cancer in association with low dose chemotherapy.

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