AP4 encodes a c-MYC-inducible repressor of *p21*

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In the majority of human tumors, expression of the *c-MYC* oncogene becomes constitutive. Here, we report that c-MYC directly regulates the expression of *AP4* via CACGTG motifs in the first intron of the *AP4* gene. Induction of AP4 was required for c-MYC-mediated cell cycle reentry of anti-estrogen arrested breast cancer cells and mitogen-mediated repression of the CDK inhibitor p21. AP4 directly repressed *p21* by occupying four CAGCTG motifs in the *p21* promoter via its basic region. AP4 levels declined after DNA damage, and ectopic AP4 interfered with p53-mediated cell cycle arrest and sensitized cells to apoptosis induced by DNA damaging agents. AP4 expression blocked induction of *p21* by TGF-β in human keratinocytes and interfered with up-regulation of p21 and cell cycle arrest during monoblast differentiation. Notably, AP4 is specifically expressed in colonic progenitor and colorectal carcinoma cells. In conclusion, our results indicate that c-MYC employs AP4 to maintain cells in a proliferative, progenitor-like state.

colorectal cancer | DNA damage | p53 | progenitor cells | TGF-beta

The proto-oncogene c-*MYC* is commonly activated in human cancer by gene amplification, viral promoter insertion, or chromosomal translocation but also because of mutations of upstream regulators (reviewed in ref. 1). c-MYC is highly expressed in proliferating cells and down-regulated when cells cease to proliferate, e.g., during differentiation. Deregulated c-MYC expression promotes cell proliferation and causes resistance to antimitogenic stimuli (2). Furthermore, constitutive expression of c-MYC sensitizes towards apoptosis (reviewed in ref. 3). The *c-MYC* gene encodes a transcription factor of the basic helix–loop–helix leucine-zipper (bHLH-LZ) class that binds to the E-box motif CACGTG (reviewed in ref. 4). However, the mechanisms that underlie the mitogenicity of c-MYC are only partially understood. It seems likely that the combined actions of multiple genes regulated by c-MYC contribute to the effects of c-MYC on proliferation (5).

The AP4 protein is a member of the bHLH-LZ subgroup of bHLH proteins, exclusively forms homodimers and binds to the E-box motif CAGCTG (6). Initially AP4 was shown to activate transcription (7). More recent studies documented that AP4 also represses viral and cellular genes (8–10). AP4 expression declines during murine brain development (9).

Here, we identified the *AP4* gene as a direct transcriptional target of c-MYC, characterized the central cell cycle regulator *p21* as an AP4 target gene and determined the cellular effects of *AP4* activation.

Results

To identify genes regulated by c-MYC in human epithelial cells, we performed a microarray-based gene expression analysis 12 h after activation of *c-MYC* in MCF-7 breast cancer cells that had been arrested in the G_1 phase by treatment with the anti-estrogen ICI182,780/Fulvestrant (ICI) (P.J. and H.H., unpublished results). Using this approach, we detected a 3.4-fold ($P = 0.0027$) induction of *AP4* mRNA (data not shown), which was confirmed by quantitative real-time PCR (qPCR) (Fig. 1*A*). The increase in AP4 expression was also observed at the protein level [supporting information (SI) Fig. S1]. *AP4* mRNA and protein were also induced after activation of a fusion protein consisting of c-MYC and the hormone-binding domain of the estrogen receptor (ER) in serum-starved human diploid fibroblasts (HDF) (Fig. 1 *B* and *C*). Furthermore, c-MYC-ER activation induced *AP4* mRNA in the presence of the translation inhibitor cycloheximide, indicating that *AP4* is directly transactivated by c-MYC (Fig. 1*D*). The regulation of *AP4* by c-MYC is conserved among species, because AP4 expression was induced after activation of a c-MYC-ER fusion protein in serum-deprived RAT1 fibroblasts (Fig. S2). The first genomic intron of human *AP4* contains a cluster of four canonical c-MYC-binding sites (CACGTG), two of which are conserved in mouse and rat (Fig. 1*E*). Stimulation of MCF-7 cells with serum, which increased c-MYC levels (see Fig. S3*C*), enhanced binding of c-MYC to a region containing three of the four E-boxes in the first intron of *AP4* (ampA+B), as determined in a quantitative ChIP (qChIP) analysis (Fig. 1*F*). A minor binding of c-MYC to this region was detected in serum-starved MCF-7 cells, which express low levels of c-MYC (Fig. 1*F*; see also Fig. 3*C*), whereas a region (ampC) located ≈13 kbp downstream of the transcriptional start site in intron 6 of *AP4* did not display occupation by c-MYC. Taken together, these findings establish that *AP4* is an evolutionarily conserved direct c-MYC target gene.

It was reported that ectopic expression of c-MYC abrogates a cell cycle arrest induced by the anti-estrogen ICI in MCF-7 cells (11). We could reproduce this observation, because *c-MYC* activation increased the fraction of cells in S-phase from ≈7% to ≈16% in the presence of ICI (Fig. 2*A*). When AP4 expression was down-regulated by RNA interference (RNAi) concomitantly with activation of *c-MYC*, the number of cells reentering the cell cycle and performing complete cell divisions was reduced (Fig. 2*A*, Fig. S3). Therefore, the induction of *AP4* contributes to the c-MYC-mediated proliferation in the presence of ICI. Because the CDK inhibitor p21 is known to mediate G_1-arrest induced by ICI (12), we determined whether expression of p21 is modulated by AP4. Indeed, down-regulation of AP4 by RNAi resulted in increased p21 levels in MCF-7 cells (Fig. 2*B*). Furthermore, serum stimulation of MCF-7 cells rapidly induced the expression of endogenous c-MYC followed by an increase in AP4, whereas expression of p21 was strongly suppressed (Fig. 2*C*). RNAi-mediated down-regulation of AP4 prevented the repression of p21 by serum addition (Fig. 2*D*). Therefore, induction of AP4, which is presumably mediated by c-MYC, is required for the down-regulation of p21 expression after serum stimulation of MCF-7 cells.

Interestingly, the induction of a conditional *AP4* allele decreased the amount of *p21* mRNA and protein (Fig. 2 *E* and *F* and Fig. S4). The *p21* promoter contains four E-boxes (5′-CAGCTG-3′) in the vicinity of its transcriptional start site, which

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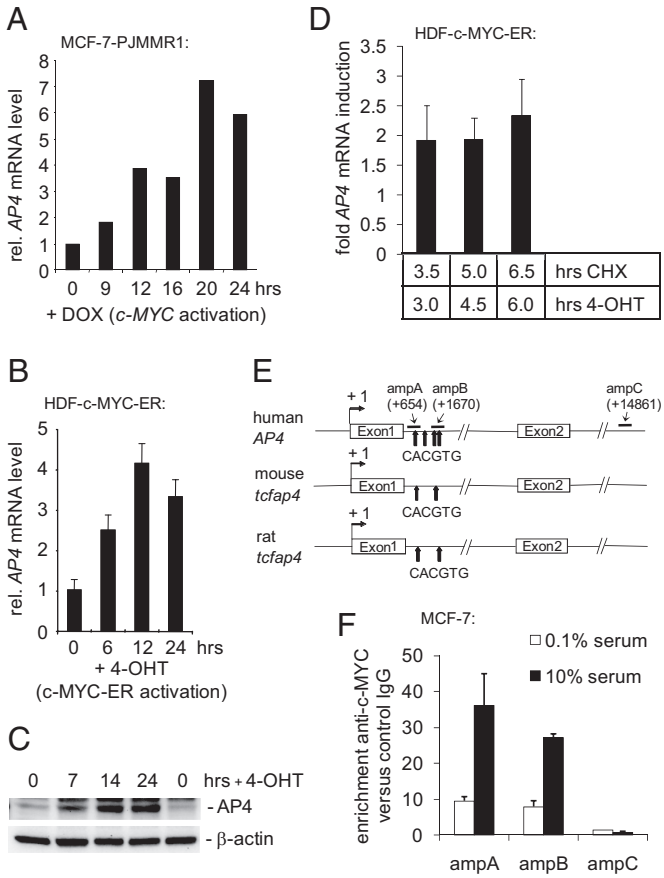

Fig. 1. Characterization of AP4 as a direct c-MYC target gene. (*A*) Quantification of *AP4* mRNA after activation of *c-MYC*. MCF-7-PJMMR1 cells were treated with ICI (1 μM) for 60 h before activation of c-*MYC* by addition of doxycycline (DOX, 1 μg/ml) for the indicated periods, and RNA was subjected to qPCR analysis. (*B*) Quantification of *AP4* mRNA after c-MYC activation. HDF-MYC-ER cells were serum-deprived for 48 h. After addition of 4-OHT (200 nM), total RNA was isolated at the indicated time points from biological triplicates. *AP4* mRNA expression was determined by qPCR analysis. Error bars indicate standard deviations. (*C*) AP4 protein expression after c-MYC activation. Protein lysates were prepared from HDF-MYC-ER cells at the indicated time points. Expression of AP4 and β-actin was determined by immunoblotting. (*D*) HDF-MYC-ER cells were grown to confluence and treated with 4-OHT (200 nM) and CHX (70 nM), as indicated. The expression level of *AP4* after combined CHX/4-OHT treatment was normalized to cells treated with CHX alone. Expression of *AP4* and, for normalization, β-actin mRNA was determined by qPCR. Analyses were performed in triplicates. Error bars indicate standard deviations. (*E*) Comparison of the mouse, rat, and human *AP4* promoter regions. ''+1'' indicates the transcription start site. ''amp'' indicates PCR amplicons used for qChIP analysis with their positions relative to the transcription start site. Arrows indicate the approximate positions of canonical c-MYC-binding sites (CACGTG). The positions of these sites relative to the transcription start site (''+1'') are +660, +1262, +1645, and +1766 for human *AP4*; +560 and +1620 for the mouse *tcfap4*; and +666 and +1725 for the rat *tcfap4*, respectively. (*F*) Detection of c-MYC at the *AP4* promoter. MCF-7 cells were serum-starved (0.1% serum) for 48 h or restimulated (10% serum) for 12 h. Chromatin was cross-linked and subjected to qChIP analysis with a c-MYC-specific antibody and, as a control, rabbit IgG. qPCR analysis was performed with primers flanking three of the four canonical E-boxes in the first *AP4* intron (''ampA'' and ''ampB''; see also Fig. 1*C*) or a control primer pair (''ampC'') localized in the last intron of *AP4*. For normalization, a fragment not containing E-boxes from chromosome 16q22 was used.

were occupied by AP4 *in vivo* (Fig. 2 *G* and *H*). Expression of AP4 drastically reduced the activity of a wild-type *p21* reporter construct, whereas mutation of the two proximal AP4-binding sites A3 and A4 was sufficient to largely alleviate the repressive effects of AP4 (Fig. 3 *A* and *B*). Mutation of all four binding sites completely prevented repression by AP4. Interestingly, mutation of putative Miz1-binding sites within a c-MYC-responsive *p21* promoter region, which was reported to largely alleviate the positive effect of Miz1 on *p21* expression (13), did not affect repression of *p21* by AP4 (Fig. 3*C*, Fig. S5*A*). A *p21* promoter construct containing one E2F and four Sp1/3-binding sites but no E-box (13), was not responsive to AP4 (Fig. 3*C*). Moreover, deletion or mutation of the AP4 basic region (Fig. 3*D*) rendered AP4 unable to repress a *p21* reporter (Fig. 3*E*, Fig. S5*B*) and endogenous p21 protein in U-2OS cells (Fig. 3*F*). These results show that repression of *p21* by AP4 occurs directly via the E-box motifs in the *p21* proximal promoter and is not mediated by Miz1 or Sp1/3.

Because *p21* is a central mediator of cell cycle inhibition by p53, we studied the potential involvement of AP4 in the DNA damage response. Treatment of MCF-7 cells with the topoisomerase II inhibitor etoposide reduced c-MYC and AP4 protein levels, whereas p53 and p21 levels increased (Fig. 4*A*). Induction of ectopic AP4 strongly interfered with p53-mediated transactivation of *p21* after DNA damage and, presumably as a consequence of the diminished CDK inhibition, prevented the formation of hypophosphorylated pRb (Fig. 4*B*, Fig. S6*A*), which inhibits cell cycle progression by binding to E2F family members. Furthermore, AP4 suppressed the p53-mediated induction of a *p21* reporter construct (Fig. 4*C*). This inhibitory effect of AP4 was alleviated by mutation of the two putative AP4-binding sites A3 and A4 in the *p21* promoter (Fig. 4*C*), demonstrating that the suppression of p53-mediated induction of *p21* by AP4 involves direct binding to E-boxes. Simultaneous treatment with etoposide and ectopic AP4 expression sensitized cells to apoptosis, as evidenced by the accumulation of sub-G_1 cells (Fig. 4*D*, Fig. S6*B*). Furthermore, AP4 expression allowed cells to enter S phase in the presence of etoposide (Fig. 4*D*). Interestingly, these cells displayed extensive γ-H2AX staining after treatment with etoposide (data not shown). Therefore, the AP4-mediated repression of p21 presumably allowed cells to continue DNA replication in the presence of DNA damage. It was previously shown that loss of *p21* allows cells to continue DNA replication in the presence of DNA damage (14–16), which could explain the sensitization of cells to apoptosis observed here. These results show that down-regulation of AP4 is a requirement for a coordinated DNA damage response.

Constitutive expression of c-MYC blocks the induction of *p21* by all members of the TGF-β superfamily (17). Therefore, we determined whether AP4 interferes with the TGF-β/Smad-mediated induction of *p21*. In HaCAT cells ectopic AP4 efficiently suppressed the increase of p21 protein and mRNA after exposure to TGF-β, whereas the induction of the CDK inhibitor $p15^{Ink4b}$ was not affected (Fig. 5 *A* and *B*). Similar results were obtained with an AP4-ER fusion protein (Fig. S7). Therefore, AP4 represents a candidate mediator of resistance to TGF-β caused by oncogenic activation of *c-MYC*.

Ectopic *v-myc* expression prevents 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of the myelomonoblast cell line U-937 (18). Interestingly, c-MYC has been reported to block differentiation by interfering with the induction of *p21* expression (13). Treatment of U-937 cells with TPA reduced the expression level of endogenous AP4 (Fig. 5*C*), presumably by down-regulation of c-MYC expression (*SI Text* and Fig. S8*A*). Simultaneously, the expression of p21 was induced. Interestingly, ectopic expression of an *AP4* allele under control of a zinc inducible metallothionein-1 promoter (19) in U-937 cells interfered with TPA-mediated induction of p21 (Fig. 5*D*). However, at later time points a minor induction of p21 was observed (Fig. 5*D*). U-937 cells ectopically expressing AP4 failed to stably arrest in the G_1-phase after TPA treatment and instead underwent apoptosis to a larger extent than control cells (Fig.

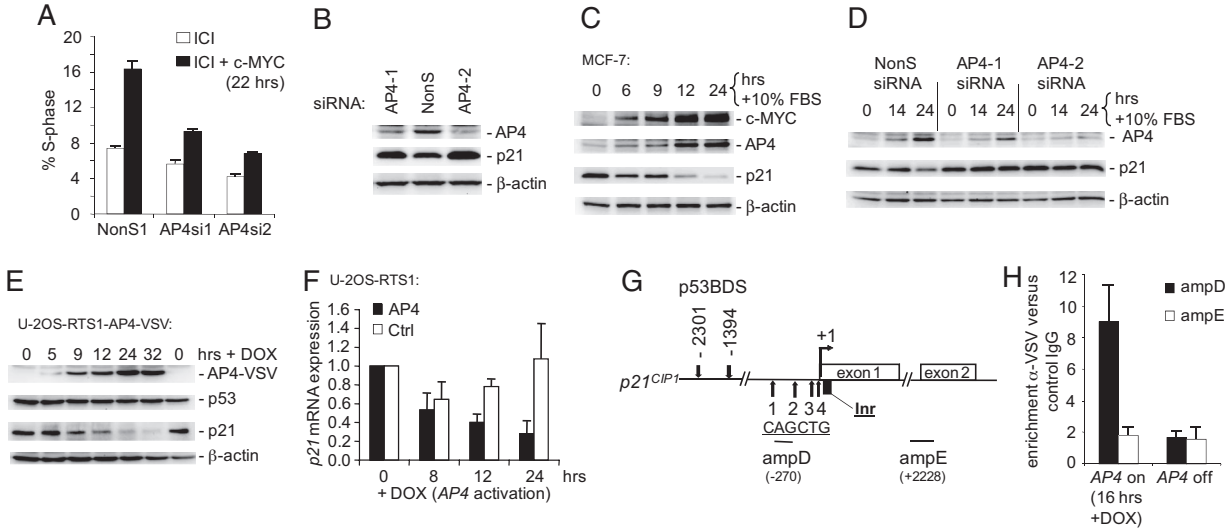

Fig. 2. Effects of AP4 on cell cycle progression and p21 expression. (*A*) Flow cytometric analysis of ICI (1 μM)-treated MCF-7-PJMMR1 cells after siRNA-mediated down-regulation of *AP4.* c-MYC was activated by addition of DOX (1 μg/ml) for 22 h. The depicted diagram shows the percentage of cells in S-phase after treatment with ICI alone or in combination with c-MYC overexpression (ICI+MYC). The experiment was performed in duplicate. The standard error is depicted. (*B*) MCF-7 cells were transfected with two different siRNAs targeting *AP4* or a nonsilencing control siRNA. Expression of AP4, p21 and β-actin was detected by immunoblot analysis. (*C*) MCF-7 cells were serum-deprived for 48 h, restimulated with 10% serum for the indicated periods, and analyzed by immunoblotting. (*D*) MCF-7 cells were transfected with siRNAs targeting AP4 or a nonsilencing siRNA. Thirty-six hours later, cells were serum-starved for 30 h. After restimulation with 10% FBS-containing medium for the indicated periods, cell lysates were subjected to immunoblot analysis. (*E*) Expression of AP4-VSV, p53, p21, and β-actin proteins was detected by immunoblot analysis after induction of a conditional VSV-tagged *AP4* allele in U-2OS cells. (*F*) Quantification of *p21* mRNA after activation of *AP4* in U-2OS cells by qPCR analysis. Expression of *p21* was normalized to β-actin expression. (*G*) The proximal promoter region of the human *p21* gene contains four AP4 binding sites (CAGCTG). ''+1'' indicates the transcription start site. ''amp:'' PCR amplicons used for qChIP analysis with their position relative to the transcription start site. Positions of two p53-binding sites (p53BDS) are indicated. The approximate positions of four putative AP4-binding sites (arrows) and the initiator (Inr) element (TCAGTTCCT) (filled square) are indicated (their precise positions relative to the transcription start site of *p21* is depicted in Fig. 3*A*). (*H*) qChIP analysis of AP4 at the *p21* promoter. A conditional VSV-tagged *AP4* allele was induced by addition of DOX (100 ng/ml) for 16 h in U-2OS cells. Genomic DNA coprecipitated with an anti-VSV or mouse IgG antibody was analyzed by qPCR. For normalization, a fragment on chromosome 16q22, not containing E-boxes was used.

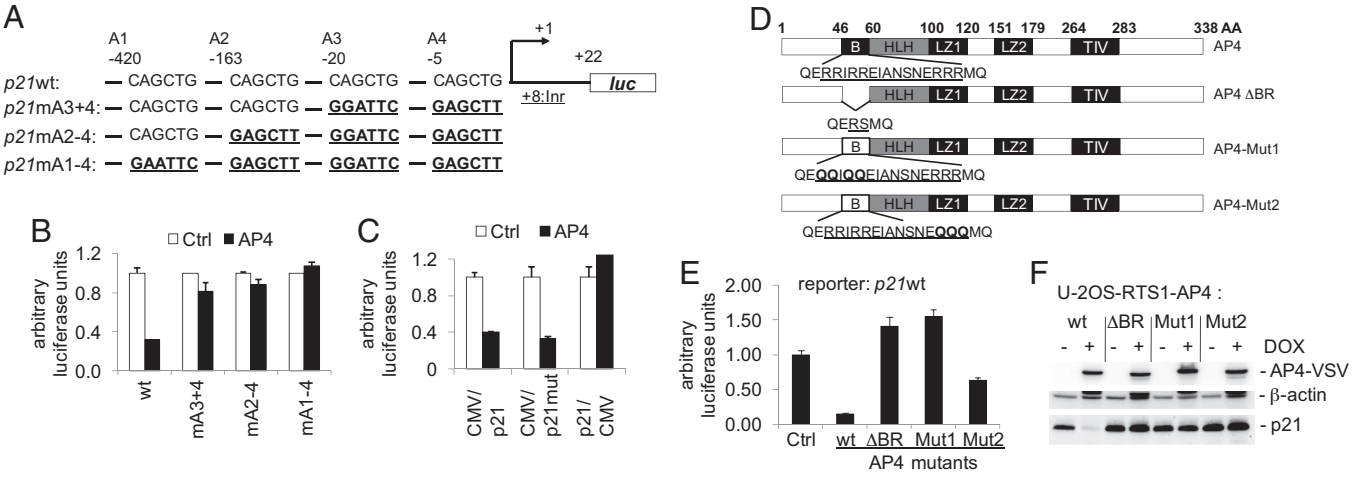

Fig. 3. AP4 directly represses *p21* expression via E-box motifs. (*A*) Schematic presentation of putative AP4-binding sites (A1-A4) and their position in the *p21* promoter region relative to the transcriptional start site (+1). Wild-type and mutant *p21* promoter constructs used in transient reporter assays are depicted. Mutated AP4-binding sites are represented in bold and underlined. The initiator (Inr) element (TCAGTTCCT) localizes to position + 8 to + 16 relative to the transcription start site (''+1'') (13). *luc*: ORF encoding the firefly luciferase. (*B*) Determination of *p21* reporter activity in H1299 cells. Cells were transfected in 12-well plates with wild type or the indicated mutant *p21* reporter plasmids, pcDNA3-AP4-VSV plasmid or equimolar amounts of pcDNA3-VSV backbone. Shown are the median expression values and standard errors of two independent transfection experiments. *p21* prom. wt, mA3 + 4, mA2–4, and mA1–4: reporter plasmids encoding for the *p21* promoter sequence with wild-type or mutant AP4-binding sites (see Fig. 3*A*). (*C*) The CMV/p21 reporter (nucleotides −49 to +16 of *p21*), a mutant version (CMV/p21mut) harboring substitutions of two nucleotides within three potential Miz1-binding sequences, or the p21/CMV reporter (nucleotides −94/-50 of *p21*) containing one E2F and four Sp1/3-binding sites (13) were cotransfected with pcDNA3-AP4-VSV plasmid or equimolar amounts of pcDNA3-VSV backbone in H1299 cells. Shown are the median expression values and standard errors of two independent transfection experiments. (*D*) Schematic representation of AP4 mutants. B: Basic region, HLH: helix–loop–helix, LZ1/2: leuzine zipper motif 1 and 2, TIV: conserved motif of unknown function containing the amino acid sequence TIV. The amino acid sequence of the basic region (underlined) and flanking residues are indicated for the wild type and mutant AP4 versions. Altered residues are represented in bold. (*E*) Effect of AP4 variants on p21 reporter activity in H1299 cells. Cells were transfected in 12-well plates with wild-type *p21* reporter plasmid, pcDNA3-AP4-VSV plasmids (wild type or mutant AP4 versions) or equimolar amounts of pcDNA3-VSV backbone. Shown are the median expression values and standard deviations of three independent experiments. (*F*) Effect of AP4 variants on endogenous p21 expression. Expression level of AP4-VSV, p21, and β-actin proteins was detected by immunoblot analysis 24 h after induction of conditional wild-type or mutant *AP4* alleles in U-2OS cells.

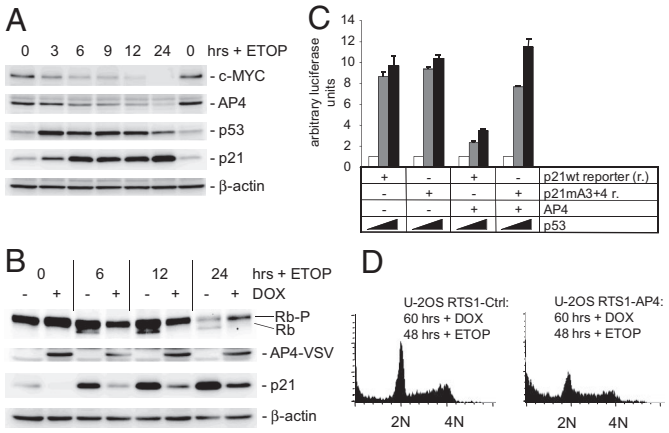

Fig. 4. Role of AP4 in the DNA damage response. (*A*) Effect of DNA damage on AP4 expression. MCF-7 cells were treated with etoposide (ETOP, 20 μg/ml) and cell extracts were obtained at the indicated time points. Expression of the indicated proteins was determined by immunoblotting. (*B*) Ectopic *AP4-VSV* was induced in U-2OS cells for 12 h by addition of DOX (100 ng/ml). Then ETOP (20 μg/ml) was added for the indicated periods. Expression of the differentially phosphorylated retinoblastoma protein (Rb-P/Rb), AP4-VSV, p21 or β-actin was detected by immunoblotting. (*C*) *p21* reporter activity was determined in H1299 cells transfected with the indicated plasmids. Increasing p53 expression was achieved by transfection of 0, 50 or 200 ng of plasmids (indicated as ◢). Shown are the median expression values and standard errors of two independent transfection experiments. *p21* mA3 + 4: see Fig. 3*A*. (*D*) AP4 was induced by DOX for 12 h before treatment of cells with ETOP (20 μg/ml) for 48 h. Then cells were analyzed by flow cytometry. Depicted are exemplary histograms representing 10,000 cells. 2N: cells in G_1, 4N: cells in G_2/M.

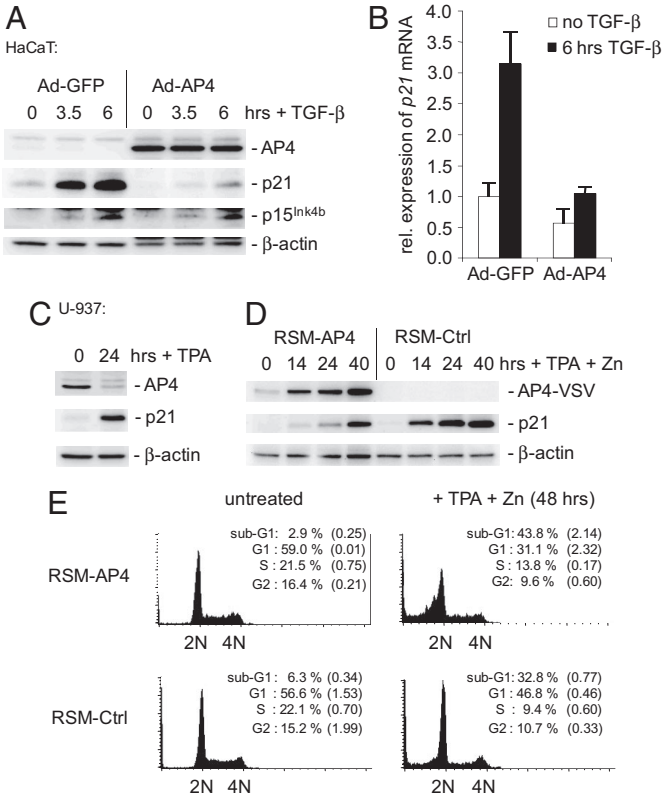

Fig. 5. AP4 antagonizes TGF-β and TPA mediated p21 induction. (*A*) HaCaT cells were infected with adenovirus encoding AP4 and eGFP (Ad-AP4) or eGFP alone (Ad-GFP). Twenty-four hours later, cells were treated with human, recombinant TGF-β (5 ng/ml) for the indicated periods. Expression of AP4-VSV, p21, $p15^{Ink4b}$ and β-actin was determined by immunoblotting. (*B*) Quantification of *p21* mRNA in HaCaT cells infected with adenoviruses encoding either AP4 and eGFP or eGFP alone. Twenty-four hours after infection, cells were treated with human recombinant TGF-β (5 ng/ml) for 6 h. mRNA expression of *p21* and β-actin was determined by qPCR analysis. The experiment was performed in duplicate. Error bars indicate standard errors. (*C*) U-937 cells were treated with 10 nM TPA (10 nM) for 24 h, and expression of AP4, p21, and, as a control for equal loading, β-actin was determined by immunoblotting. (*D*) U-937 RSM-AP4 or RSM-Ctrl cell pools were treated with 10 nM TPA and 100 μM zinc sulfate for the indicated periods. Expression of AP4-VSV, p21 and β-actin was determined by immunoblotting. (*E*) U-937 RSM-AP4 or RSM-Ctrl cells were treated with 10 nM TPA and 100 μM zinc sulfate for the indicated periods, and analyzed by flow cytometry. The experiment was repeated twice, and exemplary histograms representing 15,000 cells are shown. The percentage cell cycle distribution represent the average of two independent experiments. Standard errors are indicated. 2N: cells in G_1, 4N: cells in G_2/M.

5*E*). Moreover, ectopic AP4 increased the fraction of U-937 cells undergoing DNA synthesis after treatment with TPA (Fig. S8*B*). Taken together, these results indicate that ectopic AP4 interferes with the cell cycle arrest, which is part of the terminal differentiation program of myelomonoblasts.

Immunohistochemical analyses revealed that the expression of AP4 protein is restricted to the base of human colonic crypts, which is populated by nondifferentiated, proliferating stem and progenitor cells, as evidenced by the proliferation marker Ki67 and the absence of p21 expression (Fig. 6*A*). The expression pattern of AP4 was identical to the pattern described for c-MYC (20, 21). In the differentiated upper part of colonic crypts AP4 expression was not detectable, whereas p21 expression increased toward the top of the crypts, which contains terminally differentiated cells (21–23) (Fig. 6*A*). These results suggest that *AP4* may be involved in maintaining cells in a proliferative progenitor-like state. Primary colorectal carcinomas derived from 12 patients showed strong expression of AP4, which correlated with c-MYC and Ki67 protein expression in all cases analyzed (Fig. 6*B*, Fig. S9). Given the results described above, AP4 may represent an important mediator of c-MYC's oncogenic effects in colorectal carcinomas.

Discussion

In summary, our results establish AP4 as a c-MYC-inducible repressor of *p21*. In colorectal cancer c-MYC is generally deregulated because of mutations in the APC/β-catenin pathway (21, 24). Down-regulation of p21 consistently occurs during colorectal carcinogenesis (20, 25). Therefore, the AP4-mediated repression of *p21* may have an important role in colorectal carcinogenesis. Interestingly, repression of *p21* by c-MYC was also shown to play a critical role in anti-estrogen resistance during breast cancer therapy (26, 27).

The AP4 transcription factor forms a complex with geminin and the co-repressor SMRT that represses the human *PAHX-AP1* gene through recruitment of histone deacetylase 3 (HDAC3) (9). Other studies indicate that AP4 may block access of the TATA-box-binding protein (TBP) to the TATA box (8, 28). However, further studies are warranted to determine the molecular mechanism through which AP4 represses transcription.

Several previous studies addressed the molecular basis of repression of *p21* by c-MYC and multiple alternative mechanisms have been proposed (reviewed in ref. 29). One mode of repression of *p21* by c-MYC occurs via interference with the transcription factor Miz1 (30). Because the c-MYC transactivation domain, which is dispensable for binding of c-MYC to Miz1, is essential for repression of *p21*, other factors beside Miz1 have been proposed to participate in the c-MYC-mediated repression of *p21* (13). The c-MYC responsive region in the *p21* promoter has been mapped between −49 and +16 (13) and overlaps with two of the four AP4-binding sites characterized here. Here, these

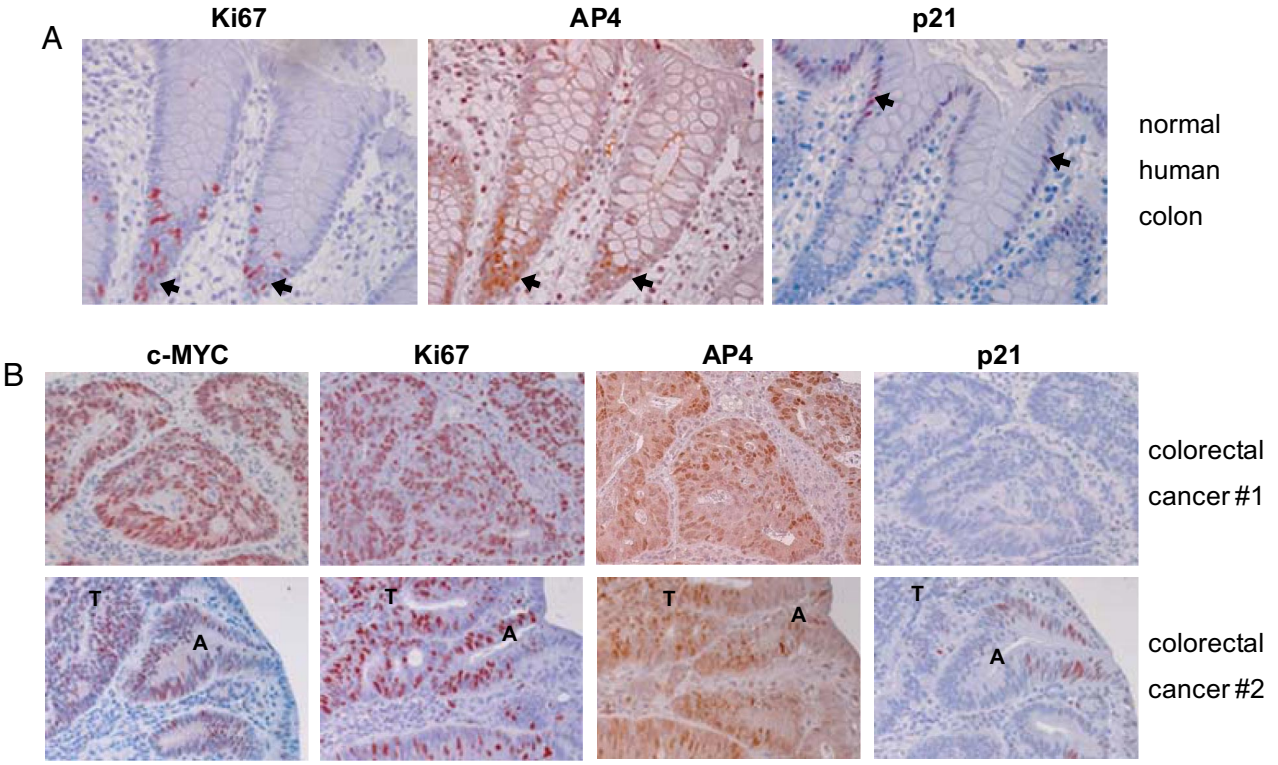

Fig. 6. AP4 expression in human colon and colorectal cancer. (*A*) Section of an endoscopic biopsy derived from a normal colonic region. Consecutive paraffin sections were stained with antibodies directed against Ki67, AP4 or p21 (arrows indicate the presence of positive cells). Magnification: ×200. (*B*) Sections of endoscopic biopsies derived from primary tumors of two patients are depicted. Consecutive paraffin sections were stained with antibodies directed against c-MYC, Ki67, AP4, or p21. Identical results were obtained with colorectal carcinoma biopsies from 10 additional patients (see Fig. S9). A, adenomatous polyp (dysplastic); T, tumor. Magnification: ×200.

two sites (A3+A4) were sufficient for *p21* repression by AP4, and their mutation rendered the *p21* promoter largely unresponsive to AP4. Mutations within putative Miz1-binding sites did not abolish *p21* repression by AP4, therefore suggesting a Miz1-independent mechanism. Other authors provided evidence that repression of *p21* by c-MYC may be mediated via interactions with Sp1/Sp3 and independently of the *Inr* sequence (31). Our results show that AP4 is not able to repress *p21* via Sp1/Sp3 transcription factor-binding sites.

TGF-β signaling provokes cell cycle arrest in the G_1-phase by inhibition of c-*MYC* and induction of the CDK inhibitors $p15^{Ink4b}$ and *p21* (reviewed in refs. 32 and 33). We could demonstrate that *AP4* interferes with TGF-β-mediated induction of *p21*. However, AP4-mediated repression of *p21* did not prevent the TGF-β-mediated cell cycle arrest (data not shown). Presumably, regulation of other genes by TGF-β, as $p15^{Ink4b}$ or *CDC25A* (32, 34) is sufficient to mediate cell cycle arrest in HaCaT keratinocytes. Other functions normally mediated by TGF-β-induced p21, such as terminal differentiation (35), may be affected in cells with deregulated *c-MYC* and *AP4* genes. Moreover, deregulation of AP4 may contribute to the morphogenetic changes observed during colon cancer formation by antagonizing the TGF-β pathway (36).

Down-regulation of c-MYC is required for terminal differentiation of many cell types (37). Elevated expression of c-MYC in the midgestational mouse is indicative of proliferation, whereas proliferative arrest and the onset of differentiation are accompanied by down-regulation of c-MYC (38). Decreased expression of c-MYC presumably explains the down-regulation of AP4 observed after TPA-mediated differentiation of myelomonoblasts and might also account for the decline of AP4 levels during the development of mouse brain observed by Kim *et al.* (9). Constitutive c-MYC expression blocks TPA-induced differentiation of U-937 cells by repressing p21 (13, 18). p21 itself plays an important role in monocytic differentiation and supports survival of differentiated cells by maintaining a stable cell cycle arrest (39, 40). In agreement with these findings, ectopic AP4 interfered with a stable G_1-arrest and increased the fraction of TPA-treated cells in S-phase. This effect of AP4 is likely due to repression of *p21* and the resulting increased CDK activity.

As p21 is a potent inhibitor of cyclin-dependent kinases; its repression by AP4 may contribute to the ability of c-MYC to activate CDKs (41–43). c-MYC-mediated repression of *p21* was shown to modulate the response to DNA damage by favoring the initiation of apoptosis vs. cell cycle arrest (44). In line with this observation, we found that expression of AP4 sensitizes cells toward DNA damaging agents, which are commonly used in cancer therapy. Further analyses of the processes regulated by AP4 may allow to selectively increase the sensitivity of AP4-expressing cancer cells to therapeutic agents in the future.

Materials and Methods

The analyses were performed as described previously (45–48). For details, see the *SI Text*. This contains paragraphs describing plasmids and siRNAs, cell lines, cell culture and reagents, generation of cell lines, Western blot analysis and antibodies, cell-based reporter assays, qPCR, ChIP assays, BrdU labeling for detection of DNA synthesis, DNA content analysis by FACS, proliferation assay, indirect immunofluorescence, tissue samples and immunohistochemistry, and generation of recombinant adenoviruses and infection of target cells. Furthermore, tables listing oligonucleotides used for site-directed mutagenesis (Table S1), qPCR (Table S2), or qChIP analyses (Table S3) are provided.

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