Human TFDP3, a Novel DP Protein, Inhibits DNA Binding and Transactivation by E2F*

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The two known DP proteins, TFDP1 and -2, bind E2Fs to form heterodimers essential for high affinity DNA binding and efficient transcriptional activation/repression. Here we report the identification of a new member of the DP family, human TFDP3. Despite the high degree of sequence similarity, TFDP3 is apparently distinct from TFDP1 in function. Although TFDP3 retained the capacity to bind to E2F proteins, the resulting heterodimers failed to interact with the E2F consensus sequence. In contrast to the stimulatory effect of TFDP1, TFDP3 inhibited E2F-mediated transcriptional activation. Consistent with this observation, we found that ectopic expression of TFDP3 impaired cell cycle progression from G1 to S phase instead of facilitating such a transition as TFDP1 does. Sequence substitution analysis indicated that the DNA binding domain of TFDP3 was primarily responsible for the lack of DNA binding ability of E2F-TFDP3 heterodimers and the inhibition of E2F-mediated transcriptional activation. Fine mapping further revealed four amino acids in this region, which were critical for the functional conversion from activation by TFDP1 to suppression by TFDP3. In conclusion, these studies identify a new DP protein and a novel mechanism whereby E2F function is regulated.

The E2F transcription factors are involved in the regulation of a wide variety of fundamental life processes ranging from cell cycling and growth to apoptosis and cell differentiation and development. Typically, E2F activity is mediated by a group of heterodimers composed of an E2F protein and a DP protein. The E2F family includes eight known members, which can be divided into four distinct subfamilies based upon their structural features, their transcriptional properties, and the molecules with which they interact. The first subfamily consists of E2F1 to -3. They are periodically expressed during the cell cycle, interact exclusively with the retinoblastoma (Rb)7 tumor suppressor protein and are required for S-phase entry in the cell cycle (1–3). An additional role specific for E2F1 is the induction of apoptosis (4–6). The second subfamily is composed of E2F4 and -5, whose function is mainly regulated by p130 and p107 (7–11). These two E2F proteins are expressed at nearly constant levels through the cell cycle and are generally considered to be critical for cell cycle exit and differentiation (12, 13). The third subfamily contains a single member, E2F6. It lacks the typical transactivation/pocket protein (Rb, p130, and p107) binding domain but retains the dimerization domain for DP proteins (14–17). E2F7 and E2F8 belong to the fourth subfamily. They possess two distinct DNA binding domains only, which are organized to mimic an E2F-DP heterodimer (18–22). E2F6 to -8 primarily act as transcription repressors and are capable of blocking E2F-mediated transcriptional activation of a subset of E2F targets. Thus, the multiple E2F proteins constitute a complex regulatory network with diversified functions.

The DP family contains two well characterized members, TFDP1 and -2. These two proteins share high homology in the DNA binding/heterodimerization domain but diverge from each other in the C terminus (23–26). Due to the lack of a transactivation domain, DP proteins themselves have no transcriptional activity. Instead, they exert a regulatory function by dimerizing with E2F proteins. In fact, the heterodimerization of E2F-DP is essential for both high affinity DNA binding and efficient transcriptional regulation by E2Fs (24, 27–29). As heterodimers, the E2F-DP complexes bind to the consensus E2F DNA recognition site TTT(C/G)GCGC(C/G) identified in a large number of cellular promoters. This could lead to either activation or repression of the target genes, depending on the specific E2F members involved. E2F1 to -3, for example, usually lead to the activation of genes critical for DNA synthesis and cell cycle progression. E2F4 and -5, on the other hand, recruit Rb and related proteins to E2F-regulated promoters and actively repress gene expression (30, 31).

In consideration of the pivotal role of E2F in cell cycle control, it has been speculated that deregulated E2F activity con-

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7 The abbreviations used are: Rb, retinoblastoma; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; GFP, green fluorescent protein.
ttributes to tumor development. As cofactors for E2Fs, DP proteins have been reported to cause transformation of cells in conjunction with activated ras, indicating a proto-oncogenic potential (32). Studies by Bargou et al. (33), however, suggest that DPs may also act as a tumor suppressor. In their hands, cell transformation was achieved using a dominant negative TFDP1 mutant (33). The underlying mechanism for these apparently opposing effects is not well understood.

In the search for tumor-associated antigens expressed in hepatocellular carcinoma, we identified a protein sharing high homology with human TFDP1 (34). In the present study, functional characterization of this novel member of the DP family (TFDP3) was pursued. First, its interaction with E2F proteins was analyzed using GST pull-down and co-immunoprecipitation assays. The putative E2F-TFDP3 complex was then examined for the capacity to bind to the E2F consensus sequence and to regulate E2F-dependent transcription. Subsequently, a series of substitution constructs were created to reveal the structural basis for the functional differences between TFDP3 and TFDP1. Finally, the influence of TFDP3 on E2F-driven cell cycle progression and cell growth was explored by cotransfection of E2F3 with TFDP3. As a result, our studies have identified a new negative regulator of the E2F transcription factors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—COS-7, HeLa, HEK-293, and the human liver cell line LO2 were maintained in Dulbecco’s modified Eagle’s medium with 10% (v/v) newborn calf serum. Lipofectamine 2000 reagent (Invitrogen) was used for the delivery of plasmids into cells.

**Plasmid Constructs**—To generate the expression constructs for TFDP3, TFDP1, and the various TFDP3/TFDP1 substitution mutants as indicated in Fig. 7, full-length coding sequences were amplified by PCR. Upon sequence verification, the PCR products were inserted into the HindIII-BamHI sites of pCDNA3-FLAG, pCMV-HA-E2F1, pCMV-HA-E2F2, pCMV-HA-E2F3, and pGL3 TATA basic 6 × E2F luciferase constructs have been described previously (18, 35). pCDNA3-HA-E2F4 and pCDNA3-HA-E2F5 were provided by Dr. H. B. Shu (Peking University, China). pGEX-4T2 was used to express glutathione S-transferase (GST) fusion proteins of E2F1 to -6 for the in vitro binding assay. pEGFP-N1 (Clontech) was used to express E2F proteins fused to the enhanced green fluorescent protein (EGFP) for subcellular localization of E2F.

**In Vitro Binding Assay**—GST and E2F fusion proteins were prepared by following standard procedures. 35S-labeled TFDP3 protein was produced by in vitro transcription (T7 polymerase) and translation in the presence of radioactive [35S]methionine using TNT® Quick Coupled Transcription/Translation system (Promega) according to the manufacturer’s protocol in a final volume of 50 μl/assay. For the in vitro binding reaction, appropriately purified GST or GST-E2F fusion proteins bound to glutathione-agarose beads were added to in vitro translated TFDP3 in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mg/ml lysozyme, 0.5 mM phenylmethylsulfonyl fluoride, 50 mg/ml leupeptin, 50 mg/ml protease inhibitor, 50 mg/ml aprotinin, and 50 mM dithiothreitol. After incubation for 2.5 h at 4 °C, the beads were collected and washed four times in lysis buffer to remove unbound protein. The protein bound to the beads was then released and fractionated on a 12.5% polyacrylamide gel. TFDP3 was detected by exposing the dried gel to an X-ray film.

**Co-immunoprecipitation and Western Blot Analysis**—To analyze the interaction of TFDP3/TFDP1 with E2F in vivo, HeLa cells were co-transfected with E2F and TFDP3/TFDP1. The transfected cells were washed twice in phosphate-buffered saline and resuspended in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride. The expression levels were monitored by Western blot of the cell lysate with antibodies specific for the hemagglutinin (HA) tag (for E2Fs) or the FLAG tag (for DPs). For immunoprecipitation, the lysate was incubated with anti-FLAG or anti-HA antibodies at a final concentration of 2 μg/ml with 25 μl of protein A-agarose (Roche Applied Science) for at least 2 h at 4 °C. The precipitates were separated on polyacrylamide gels and blotted onto nylon membranes. These blots were then probed anti-HA (for samples immunoprecipitated with anti-FLAG) or anti-FLAG antibodies (for samples immunoprecipitated with anti-HA).

**Immunofluorescence Staining**—COS-7 cells in 24-well plates were transfected with E2F and TFDP3/TFDP1. After 24 h, cells were fixed for 20 min at -20 °C with methanol (precooled at -70 °C) and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. After blocking, the fixed cells were incubated with 1 μg/ml anti-FLAG antibody for 1 h at room temperature, thoroughly washed, and stained with TRITC-conjugated goat anti-mouse IgG antibody (diluted to 1:100 with 1% bovine serum albumin in phosphate-buffered saline) for 1 h at room temperature. E2Fs were traced by EGFP, and the nuclei were counterstained with 10 μg/ml Hoechst 33342 (Sigma).

**Electrophoretic Mobility Shift Assay**—An electrophoretic mobility shift assay was performed as previously described (36). 6 × E2F-Luciferase Reporter Assay—LO2 cells were transfected with E2F and TFDP3/TFDP1 as indicated in the figure legends, together with a 6 × E2F luciferase reporter gene. To normalize the transfection efficiency, 140 ng of pRL-SV40 Renilla luciferase reporter plasmid was added to each transfection as an internal control. The luciferase activities were determined using a dual specific luciferase assay kit (Promega).

**Fluorescence-activated Cell Sorting Analysis**—An asynchronous population of HEK-293 cells in log phase was transfected with the indicated plasmids together with Us9-GFP (37). After 48 h, cells were harvested and stained with propidium iodide. Green fluorescent protein (GFP)-positive cells were gated for analysis of DNA content according to standard protocols.

**Colony Formation Assay**—HEK-293 cells were transfected with the indicated plasmids, along with pEGFP as a marker. 24 h after transfection, GFP-positive cells were isolated by fluorescence-activated cell sorting and added into culture medium containing 0.4% (w/v) agar and 10% (v/v) newborn calf serum. Cells were then poured onto a 6-well plate with a bottom layer of 0.8% agar at 200 cells/well. These plates were maintained at 37 °C under 5% CO2 for 2 weeks. Colonies containing more than 50 cells were counted.
Statistical Analysis—Statistical evaluation for colony formation was performed by Student’s t test. *p* < 0.05 was accepted as statistically significant.

RESULTS

Characteristics of TFDP3—Human TFDP3 (also called HCA 661 with NCBI nucleotide accession number CAI42694) was initially isolated as a novel cancer-testis antigen in our screening for tumor-associated antigens (34, 38). Located on chromosome X, TFDP3 contains a single exon encoding a 405-amino acid protein. As shown in Fig. 1, TFDP3 shares a high degree of sequence homology with TFDP1 (75.2% amino acid identity). In addition, TFDP3 resembles TFDP1 in overall structure, which includes a heterodimerization domain, a DNA binding
domain, and a typical RRXYD E2F DNA recognition motif in the DNA binding domain. Moreover, similar to TFDP1 and -2, TFDP3 possesses a C terminus composed of multiple acidic amino acids (15 of the last 20 amino acids are acidic). These features strongly suggest that TFDP3 is a third member of the DP family.

We subsequently performed a thorough search of the NCBI genomic data base for a human TFDP3 homolog in other species. In rhesus, three TFDP-like genes, represented by XR013178, XM001112439, and XP001097146, respectively, were identified. Although XM00112439 encodes TFDP2, XR013178 and XP001097146 had been previously named TFDP1 despite the apparent sequence variations. More careful analysis revealed that XR013178 encodes a protein almost identical to human TFDP1, whereas the protein dictated by XP001097146 is more closely related to human TFDP3 than TFDP1 (86% versus 76% identity). Therefore, we believe that XP001097146 is actually the rhesus homolog of human TFDP3. Another potential TFDP3-coding sequence, although incomplete, was found in chimpanzee. Mouse and rat, on the other hand, seem to have no TFDP3-like gene, suggesting that TFDP3 might arise later in evolution. Phylogenetic analysis indicated that TFDP3 segregates from TFDP1 and TFDP2, forming a distinct subfamily (Fig. 1C). Alignment of the DNA binding domain of various DP proteins revealed that some of the well conserved amino acids in TFDP1 and -2 are no longer retained in TFDP3 (Fig. 1D). The significance of such substitutions will be further discussed below.

Interaction of TFDP3 with E2F1 to -6—As a member of the DP family, TFDP3 would be expected to directly interact with the E2F proteins. To test for such a potential, in vitro translated, 35S-labeled TFDP3 was incubated with in vitro translated, 35S-labeled TFDP3. The bound TFDP3 proteins were visualized by exposure to x-ray film following SDS-gel electrophoresis. TFDP3 (lane 1) and GST protein (lane 2) served as positive and negative controls, respectively. B, co-immunoprecipitation for interaction in vivo. HeLa cells were co-transfected with TFDP3-FLAG and E2F-HA constructs, and the cell lysate was analyzed. Top, cell lysate was immunoprecipitated with anti-FLAG, and the blot was probed with anti-HA; middle and bottom, cell lysate was analyzed for E2F and TFDP3 expression using anti-HA and anti-FLAG, respectively. The arrowheads indicate the mobility of the specific E2F species and the TFDP3 protein. C, competitive E2F binding by TFDP3 and TFDP1. Cells were transfected with 3 μg of E2F3, 3 μg of TFDP1, and an increasing amount of TFDP3 at 3, 6, or 12 μg. Empty vectors were used to compensate for a total of 18 μg of plasmid DNA per transfection. Top, cell lysate was immunoprecipitated with anti-HA, and the blot was probed with anti-FLAG; middle and bottom, cell lysate was analyzed for TFDP3/TFDP1 and E2F3 expression using anti-FLAG and anti-HA, respectively. The arrowheads indicate the mobility of the TFDP3, TFDP1, and E2F3 proteins.
mids of TFDP3 and E2Fs in pairs, and the expression levels of these proteins were monitored by immunoblotting the lysates with antibodies against the tags (HA for E2Fs and FLAG for TFDP3) (Fig. 2B, middle and bottom). TFDP3 was immunoprecipitated from the cell lysate with anti-FLAG antibodies, and the precipitates were then probed with anti-HA antibody. E2Fs were observed to co-precipitate with TFDP3 (Fig. 2B, top), indicating that TFDP3 and E2Fs can interact in vivo.

To further determine the relative affinity of TFDP3 versus TFDP1 for E2Fs, cells were co-transfected with HA-tagged E2F3 and FLAG-tagged TFDP1 and -3. Upon immunoprecipitation of E2F3 with anti-HA antibodies, the precipitates were examined for the presence of TFDP1 and -3, which were distinguished by size. We found that TFDP3 effectively competed with TFDP1 for E2F in a dose-dependent manner (Fig. 2C, top). Taken together, these data show that TFDP3 can bind with a high affinity to each member of the E2F family, most likely through the heterodimerization domain to which TFDP1 has been shown to bind.

Subcellular Localization of TFDP3 and Its Translocation Induced by E2F—To reveal the subcellular distribution of TFDP3, COS-7 cells were transfected with FLAG-tagged DP and/or EGFP-tagged E2F expression constructs. Localization of E2F and DP proteins was followed by autofluorescence and immunofluorescence, respectively. When applied alone, both TFDP3 and -1 were localized in the cytoplasm (Fig. 3A) (similar results were obtained in HeLa cells; data not shown). Also found in the cytoplasm are E2F4 (Fig. 3A) and E2F5 (data not shown), whereas E2F3 (Fig. 3A), E2F1 and -2 (data not shown) were restricted to the nucleus.

We subsequently investigated how the localization of TFDP3 was affected by interaction with E2F proteins. Similar to TFDP1, TFDP3 translocated to the nucleus of transfected cells in the presence of E2F3 (Fig. 3B) and E2F1 or -2 (data not shown). In contrast, both TFDP3 and -1 remained in the cytoplasm after co-transfection with E2F4 (Fig. 3B) or E2F5 (data not shown). TFDP3 therefore appears to be directed to the same cellular compartments as TFDP1 whether expressed alone or in combination with E2Fs.

DNA Binding Defect of the E2F-TFDP3 Complex—Having confirmed the direct interaction between TFDP3 and E2F, we next examined the lysate of TFDP3-transfected cells for the presence of binding activity to the consensus E2F DNA recognition sequence by electrophoretic mobility shift assay. A basal level of binding activity was detected in HeLa cells, which was not significantly altered following transfection with TFDP3 or any of the individual E2F members tested (Fig. 4, compare lanes 2, 3, 6, and 9 with lane 1). However, consistent with previous studies (23, 26–28), co-transfection of E2F with TFDP1 resulted in a marked increase in DNA binding activity (Fig. 4, compare lane 5 with lane 3, lane 8 with lane 6, and lane 11 with lane 9). On the other hand, co-transfection of TFDP3 caused no change (Fig. 4, compare lane 4 with lane 3, lane 7 with lane 6, and lane 10 with lane 9) despite similar expression levels of exogenous proteins in the cell lysate (data not shown). Therefore, although TFDP3 interacted with E2Fs as efficiently as TFDP1, the resulting E2F-TFDP3 complexes were defective in DNA binding.

Inhibition of Transcriptional Activity of E2F by TFDP3—The DNA binding defect of the E2F-TFDP3 complex prompted us...
to explore the impact of TFDP3 on E2F-mediated transcriptional activation. Different combinations of the E2F (E2F1 to -5) and DP (TFDP1 and -3) expression vectors were introduced into L02 cells together with a reporter plasmid containing six E2F DNA binding sites. As shown in Fig. 5A, E2F alone generated a remarkable increase in luciferase activity over the empty vector control (10–30-fold, depending on the specific E2F members tested), and the enzymatic activity was further increased by co-transfection with TFDP1. In sharp contrast to the synergistic effect of TFDP1, TFDP3 demonstrated an inhibitory effect on E2F-mediated transcriptional activation of the reporter gene, as suggested by the much reduced luciferase activity in cells co-transfected with E2F and TFDP3.

Since E2F4 and -5 were found to be predominantly restricted to the cytoplasm and TFDP1 and -3 had no significant impact on their subcellular localization (Fig. 3), the transactivation of the reporter by E2F4 and -5 and the modifying effect of TFDP1 and -3 on their activities is somehow surprising. Nevertheless, similar effects have been reported in previous studies with E2F4 and TFDP1 (10). One may assume that nondetectable amounts of E2F4 and TFDP1 are in the nucleus, which are sufficient to activate transcription.

The mechanism by which TFDP3 repressed E2F-dependent transcription remains to be determined. As one possibility, TFDP3 may compete with endogenous TFDP1 to form inactive complexes with E2F proteins. To test this hypothesis, we compared the luciferase activities in L02 cells transfected with E2F and TFDP1, together with increasing amounts of TFDP3. The transcriptional activity of all combinations of E2F-TFDP1 was significantly inhibited in the presence of TFDP3. Moreover, there was clearly a dose-dependent response. At a 4-fold excess, TFDP3 completely abolished the enhancing effect of TFDP1 on E2F activity (Fig. 5B). These results support the notion that TFDP3 functions as a competitive inhibitor.

Up to this point, we had shown that TFDP3 could counteract the transcriptional activation induced by E2F transfection. Is endogenous E2F activity also affected by TFDP3? Indeed, we found that the base-line level of E2F activity in L02 cells was inhibited by TFDP3 in a dose-dependent manner, which is the reverse of the dose-dependent stimulatory effect of TFDP1 (Fig. 5C). Similar results were obtained using a luciferase reporter containing the E2F-responsive element from the cyclin A2 promoter, a natural target of E2F (data not shown), ruling out the possibility of an artifact somehow associated with the synthetic E2F promoter construct.

TFDP3 Induces G1 Arrest and Cell Growth Inhibition—The biochemical studies described above established TFDP3 as a negative regulator of the E2F activity. Given the complexity of the E2F family of transcription factors, it was next of interest to determine how TFDP3-mediated inhibition affected the behavior of intact cells. To this end, we first evaluated the effect of TFDP3 on cell cycle progression. Cells were transfected with E2F3, along with TFDP3 or TFDP1. The transfected population was then analyzed to determine the percentage of cells in G1, S, and G2/M phases. As shown in Fig. 6A, transfection with E2F3 resulted in a significant decrease in the percentage of cells in G1 and a concomitant increase in the percentage of S phase cells. Moreover, such changes were further enhanced by co-transfection with TFDP3. In contrast, the E2F3-mediated effect was largely abolished by co-transfection with TFDP3. Next, we sought to determine the influence of TFDP3 on colony formation of transfected cells. As demonstrated in Fig. 6B, TFDP3 significantly reduced the colony-forming capacity of HEK-293 cells. More intriguing, similar inhibition was also observed even in the presence of exogenous TFDP1, suggesting that TFDP3 plays a dominant role. Taken together, these data are consistent with a model in which TFDP3 can act as a negative regulator of E2F function.

The Molecular Basis of TFDP3-mediated Inhibition of E2F Activity—To dissect the molecular basis of the functional difference between TFDP3 and TFDP1, we created a panel of constructs with sequence substitutions between TFDP3 and TFDP1 (Fig. 7). Following introduction of these constructs into
L02 cells, the expression of mutant proteins was confirmed by Western blotting with anti-FLAG antibody (Fig. 8A). Thereafter, we estimated the effect of the mutants on the transcriptional activities of a representative E2F member, E2F4.

First, we tested a series of constructs with the substitution of individual functional domains to grossly map the region that dictates the stimulatory or inhibitory function. Activities similar to those of the parent proteins were maintained in the constructs generated from replacement of the heterodimerization domain (TFDP3SH and TFDP1SH) or the C-terminal half (1D3H/3D1H). On the other hand, each of the constructs with the DNA binding domain substituted, including TFDP3SD/TFDP1SD and TFDP3SDH/TFDP1SDH, demonstrated a function opposite to that of the wild-type molecule. TFDP3 harboring a DNA binding domain derived from TFDP1 acquired the capacity to stimulate E2F activity, whereas TFDP1 with a TFDP3 DNA binding domain showed inhibitive activity (Fig. 8B). Therefore, it is the DNA binding domain that distinguishes TFDP3 from TFDP1 in function.

Next, we sought to determine the critical amino acids that confer the inhibitory effect of TFDP3 within the DNA binding domain. Based on the analysis of sequence differences between TFDP3 and TFDP1, we performed a single amino acid substitution (TFDP3164 and TFDP1169) in the RRXYD DNA recognition motif and the replacement of a 13-amino acid region (TFDP3109–121 and TFDP1114–126) proposed to be involved in the heterodimerization contacts and DNA backbone contacts (39, 40). Surprisingly, these substitutions had no effect on the function of either TFDP3 or TFDP1 (Fig. 8B). More substitution mutants were then made, covering virtually all of the remaining regions showing amino acid differences within the DNA binding domain, including TFDP3130–145, TFDP3148–161, and TFDP3179–190. Although the last two substitutions failed to affect the inhibitory function of TFDP3, TFDP3130–145 substitution led to the loss of inhibition (Fig. 8C). Among the 16 amino acid residues in this region, TFDP3 and TFDP1 show differences in seven. To evaluate the contribution of each of these residues, we created seven single-amino acid substitution mutants (TFDP3130, TFDP3131, TFDP3134, TFDP3135, TFDP3140, TFDP3142, and TFDP3145). It was of interest that none of these were significantly different from wild-type TFDP3 in function (Fig. 8C). Therefore, it is likely that the abolition of TFDP3-mediated inhibition will require simultaneous substitutions of multiple amino acids in this region.

Although the TFDP3130–145 mutant no longer inhibited E2F activity, this substitution was not sufficient to convert TFDP3 into a stimulatory molecule like TFDP1, suggesting
that other residues outside this region may be required for the optimal function of TFDP3 or TFDP1. We therefore carried out a series of combination substitutions involving aa 109–121 or 130–145 in the α2 helix. Substitution of these residues in TFDP3 with those at the corresponding positions of TFDP1, Thr → Lys at 121, Cys → Tyr at 130, Glu → Asn at 131, and Lys → Glu at 140, were sufficient to convert TFDP3 into a stimulatory molecule like TFDP1.

DISCUSSION

In this study, we have characterized a novel human protein, TFDP3. This new member of the DP family shares a high degree of sequence homology with TFDP1 and -2. Moreover, it has demonstrated certain functional properties common to DP proteins. Specifically, TFDP3 interacts with E2F1 to -6 in vitro and in vivo and co-localizes with E2F1 to -3 in the nucleus and with E2F4 and -5 in the cytoplasm in transfected cells. Nevertheless, TFDP3 appears to exert an influence different from that of TFDP1 on E2F activities. Although dimerization with TFDP1 dramatically increases the DNA binding capacity of E2F, the E2F-TFDP3 complex fails to bind to the consensus E2F DNA recognition sequence. More intriguingly, the transcriptional activation driven by either endogenous or exogenous E2F is substantially suppressed by overexpression of TFDP3, which is opposite to the enhancing effect of TFDP1. Hence, our studies have identified a new and functionally distinctive member of the DP family.

The original member of the DP family, TFDP1, was first isolated in 1993 (23). Two years later, the second member of this family, TFDP2 (also called DP3 in mice), was identified (25, 26). TFDP1 and TFDP2 both function to enhance the DNA binding and the transcriptional activities of E2F. By targeting the DNA binding domain, Wu et al. (39) generated a series of TFDP1 mutants that were dominant negative in function. The new DP family member identified in this study, TFDP3, resembles these mutants in many ways. In particular, these molecules all retained the capacity to interact with E2F, but the resulting complexes failed to bind to DNA, and their ectopic expression caused the suppression of E2F activities and a G1 arrest in cell cycle progression. With a view to these latter features, we propose a model in which TFDP3 acts as an endogenous negative regulator for E2F, where TFDP3 competes with the activating DP family members for E2F binding, leading to the formation of transcriptionally inactive heterodimers and hence the inhibition of cellular E2F activities.

To summarize these findings, we showed that the distinctive functions of TFDP3 and TFDP1 are largely determined by their DNA binding domains. More specifically, this function involves four key amino acid residues, including one at position 121 in the α1 helix and three others at positions 130, 131, and 140 in the α2 helix. Substitution of these residues in TFDP3 with those at the corresponding positions of TFDP1, Thr → Lys at 121, Cys → Tyr at 130, Glu → Asn at 131, and Lys → Glu at 140, were sufficient to convert TFDP3 into a stimulatory molecule like TFDP1.

**Functional Analysis of Human TFDP3 Protein**
FIGURE 7. Schematic illustration of TFDP3 and TFDP1 mutants. The transcriptional property of the mutants is indicated to the right. +, transcriptional activation at the level of wild-type TFDP1; −, transcriptional inhibition similar to wild-type TFDP3; +/−, a transcriptional activity close to E2F4 alone. A, mutants derived from gross substitution of individual domains. UNT, unknown functional N terminus; DBD, DNA binding domain; HD, heterodimerization domain; UCT, unknown functional C terminus. B, mutants derived from substitution of specific regions or residues in the DNA binding domains. The regions or residues that originated from TFDP1 are shaded in gray.
lack of the C-terminal heterodimerization domain. It therefore seems clear that DP activities are fine tuned by multiple mechanisms.

In the analysis for the molecular basis of the functional divergence between TFDP3 and TFDP1, we found that it is the DNA binding domain that confers the capacity for inhibition by...
TFDP3 or stimulation by TFDP1, since an interchange of the DNA binding domains in these two molecules led to complete reversal in function. Fine mapping revealed that the inhibitory effect of TFDP3 involves four key amino acid residues, including Thr¹²¹, Cys¹³⁰, Gln¹³¹, and Lys¹⁴⁰. Substitution of these residues with those at the corresponding positions of TFDP1 (Lys¹²⁶, Tyr¹³⁵, Asn¹³⁶, and Glu¹⁴⁵) was sufficient to render TFDP3 stimulatory to E2F activity. Notably, these residues are well conserved in all known DP proteins in a wide variety of species except for a substitution of Glu¹⁴⁵ by a similar residue Asp in DPL-1 of Caenorhabditis elegans and AtDPa of Arabidopsis thaliana. On the other hand, three of the four substitutions in human TFDP3 (Thr¹²¹, Cys¹³⁰, and Lys¹⁴⁰) are also identified in the putative TFDP3 protein of rhesus (Fig. 1D). The phylogenetic conservation of these residues further highlights their importance in specification of stimulatory versus inhibitory function of DP proteins.

To understand how these four residues may contribute to the unique function of TFDP3, we performed computer-aided modeling of the tertiary structures of the DNA binding domains of DP proteins, making use of Swiss-Pdbviewer and SWISS-MODEL (42) with TFDP2 (Protein Data Bank entry 1CF7) (40) as a template. The DNA binding domain of TFDP3 was predicted to assume a winged helix structure composed of three α-helices and three β-sheets, which is typical of DP proteins (Fig. 9).

In TFDP1 (Lys¹²⁶ and Glu¹⁴⁵) or TFDP2 (Lys⁸¹ and Glu¹⁰⁰), residues Lys and Glu, which correspond in position to Thr¹²¹ and Lys¹⁴⁰ in TFDP3, are spatially close to each other with a distance of 2.81 or 2.82 Å, allowing the formation of a salt bridge that links the α1 and α2 helices. This type of interaction, however, is not favored in TFDP3 because of the relatively long distance between Thr¹²¹ and Lys¹⁴⁰ (6.23 Å). Single amino acid substitution of Thr¹²¹ with Lys (as in the TFDP3¹²¹ mutant) or Lys¹⁴⁰ with Glu (as in TFDP3¹⁴⁰) reduces the distance to 4.07 and 5.41 Å, respectively. Distance in this range is still nonpermissive for the formation of a salt bridge. On the other hand, simultaneous substitution of both residues, Thr¹²¹ with Lys and Lys¹⁴⁰ with Glu, reduces the distance to 2.81 Å, and an effective interaction is thus restored (Fig. 9B).

Tyr of TFDP1 (Tyr¹³⁵) and TFDP2 (Tyr⁹⁰) is positioned to interact with another Tyr of TFDP1 (Tyr¹⁷⁰) and TFDP2 (Tyr¹²⁵), which is in direct contact with DNA. The π stacking created by these two aromatic residues allows an optional occupancy of space and creates a strong hydrophobic core in the structure (Fig. 9C). In TFDP3, the first Tyr is replaced by Cys¹³⁰, leading to the disruption of the favored configuration.
Far defy any easy classification of E2Fs, particularly the activating members, into the conventional “oncogenes” or “tumor suppressors.” For example, on the one hand, forced expression of E2F1 in epidermal or hepatocellular cells induces spontaneous skin or liver tumors (47, 48), implying an oncogenic potential. On the other hand, mice deficient in E2F1 also develop a broad spectrum of tumors (49), supporting a tumor-suppressive function. These bimodal activities may stem from the fact that E2Fs can affect both cell proliferation and cell death (50). Under normal circumstances, the coupling of these two events ensures a fail-safe mechanism for apoptosis to occur in the event of any irreversible damage in the cell cycle. In case of E2F deregulation, such a mechanism could result in either uncontrolled cell proliferation or accumulation of cell mutants, either of which may lead to the development of tumors.

TFDP3 was initially identified as a gene highly expressed in hepatocellular carcinomas but not in normal liver tissues (34). This restricted pattern suggests a potential role in tumorogenesis. Surprisingly, the present study showed that overexpression of TFDP3 in HEK293 cells counteracted the enhancing effect of E2F3 on G1-S transition and colony formation in soft agar. A putative explanation for this paradox is that the induction of TFDP3 in tumor tissues is a response to hostile microenvironments, such as hypoxia and nutrition deprivation, and the consequent slowdown in growth actually favors tumor development in the long run. In a preliminary experiment, we observed the induction of TFDP3 transcription in several nonexpressing cell lines following serum starvation (data not shown). Efforts are being undertaken to identify the functional relevance of this induction. Alternatively, the “forced” expression of TFDP3 does not necessarily fully recapitulate the function of endogenous proteins within a specific cellular context. Previous studies have demonstrated that the known DP proteins can display both tumor-promoting and -suppressing activities, and the specific action is highly context-dependent (32, 33). In line with this thinking, one may speculate that TFDP3 preferentially interacts with the repressor E2Fs in tumor cells. By displacing/preventing them from binding to target promoters, TFDP3 could block E2F-mediated repression, thereby promoting tumorigenesis. Therefore, it would be interesting to determine if TFDP3 binds to specific E2F members in normal and tumor cells.

In conclusion, we have identified a new and functionally distinct member of the DP family. In contrast to the enhancing effect of known DP proteins, TFDP3 suppresses E2F-mediated transcriptional activation and cell cycle progression. This functional conversion seems to result from amino acid substitutions at a few critical positions. Further clarification of its biological functions may influence our current perspective of how E2F activities are orchestrated in normal cells and how they are disturbed in such pathological conditions as cancer.

Addendum—While this manuscript was in preparation, Milton et al. (51) published their studies on TFDP3, which they called DP4. Results from both their study and ours demonstrated a distinct inhibitory function of TFDP3 on the E2F activities. However, our study defined in detail the structural basis for the functional divergence between TFDP3 and TFDP1. Moreover, we tested this new
protein against almost the entire family of E2F proteins. As to the nomenclature, we adopted the name TFDP3 recommended by the HUGO Gene Nomenclature Committee, since it maintains the consistent order of this family of proteins.

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