Human DMTF1 antagonizes DMTF1 regulation of the p14(ARF) tumor suppressor and promotes cellular proliferation

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Abstract: The human DMTF1 (DMP1) transcription factor, a DNA binding protein that interacts with cyclin D, is a positive regulator of the p14ARF (ARF) tumor suppressor. Our earlier studies have shown that three differentially spliced human DMP1 mRNAs, , and , arise from the human gene. We now show that DMP1 , and isoforms differentially regulate ARF expression and promote distinct cellular functions. In contrast to DMP1 , DMP1 and did not activate the ARF promoter, whereas only resulted in a dose-dependent inhibition of DMP1 -induced transactivation of the ARF promoter. Ectopic expression of DMP1 reduced endogenous ARF mRNA levels in human fibroblasts. The DMP1 - and -isoforms share domains necessary for the inhibitory function of the -isoform. That DMP1 may interact with DMP1 to antagonize its function was shown in DNA binding assays and in cells by the close proximity of DMP1 / in the nucleus. Cells stably expressing DMP1 , as well as shRNA targeting all DMP1 isoforms, disrupted cellular growth arrest induced by serum deprivation or in PMA-derived macrophages in the presence or absence of cellular p53. DMP1 mRNA levels in acute myeloid leukemia samples, as compared to granulocytes, were reduced. Treatment of acute promyelocytic leukemia patient samples with all-trans retinoic acid promoted differentiation to granulocytes and restored DMP1 transcripts to normal granulocyte levels. Our findings imply that DMP1 - and -ratios are tightly regulated in hematopoietic cells and DMP1 antagonizes DMP1 transcriptional regulation of ARF resulting in the alteration of cellular control with a gain in proliferation.

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Human DMTF1β antagonizes DMTF1α regulation of the p14^{ARF} tumor suppressor and promotes cellular proliferation☆

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ABSTRACT

The human DMTF1 (DMP1) transcription factor, a DNA binding protein that interacts with cyclin D, is a positive regulator of the p14ARF (ARF) tumor suppressor. Our earlier studies have shown that three differentially spliced human DMP1 mRNAs, α, β and γ, arise from the human gene. We now show that DMP1α, β and γ isoforms differentially regulate ARF expression and promote distinct cellular functions. In contrast to DMP1α, DMP1β and γ did not activate the ARF promoter, whereas only β resulted in a dose-dependent inhibition of DMP1α-induced transactivation of the ARF promoter. Ectopic expression of DMP1β reduced endogenous ARF mRNA levels in human fibroblasts. The DMP1β- and γ-isoforms share domains necessary for the inhibitory function of the β-isoform. That DMP1β may interact with DMP1α to antagonize its function was shown in DNA binding assays and in cells by the close proximity of DMP1α/β in the nucleus. Cells stably expressing DMP1β, as well as shRNA targeting all DMP1 isoforms, disrupted cellular growth arrest induced by serum deprivation or in PMA-derived macrophages in the presence or absence of cellular p53. DMP1 mRNA levels in acute myeloid leukemia samples, as compared to granulocytes, were reduced. Treatment of acute promyelocytic leukemia patient samples with all-trans retinoic acid promoted differentiation to granulocytes and restored DMP1 transcripts to normal granulocyte levels. Our findings imply that DMP1α- and β-ratios are tightly regulated in hematopoietic cells and DMP1β antagonizes DMP1α transcriptional regulation of ARF resulting in the alteration of cellular control with a gain in proliferation.

1. Introduction

The 761-amino acid cyclin D-binding myb-like protein (DMTF1; DMP1) was originally identified as a cyclin D binding protein with ETS-like transcription factor motifs [1]. Subsequently, it was found to be a positive transcriptional regulator of CD13/APN and the p14^{ARF} (ARF) tumor suppressor [2,3]. DMP1-mediated transcriptional activation of ARF gene increases ARF protein levels, which in turn binds MDM2 and blocks the cellular removal of p53 [4–6]. Furthermore, DMP1 can physically interact with p53 antagonizing the ubiquitination function of MDM2, thereby maintaining p53 levels [7]. Our earlier studies have shown that DMP1 is comprised of three differentially spliced human DMP1 mRNAs, DMP1α, β, and γ, which have unique expression patterns in normal, primary hematopoietic cells during differentiation [8]. The β and γ protein isoforms lack the DNA binding and C-terminal transactivation domains [8]. While DMP1α can function as a tumor suppressor and activate the CD13/APN and ARF promoters [2,3], DMP1β and γ have lost the capacity to function as CD13/APN transcriptional activators [8]. Recently, DMP1β was reported to be present in selected human breast cancer tissues, extending cell types expressing differentially spliced DMP1 [9].

DMP1 is emerging as pivotal protein possibly linking Ras/Raf/ERK growth promoting signals with the ARF/p53/p21 tumor suppressor network [10]. Studies in mDmp1-null and -heterozygous mice have implicated a critical and physiological role for mDmp1 in decreasing the incidence of leukemia and non-hematopoietic tumors [11]. Consistent with the role of mDmp1 in controlling oncogenic insults is the dramatically accelerated lymphomagenesis seen in mDmp1-null and -heterozygous mice in the presence of the oncogenic Eμ-Myc transgene [12]. Moreover, tumors can develop in mice without p53 mutations or ARF deletions, further emphasizing the regulatory importance of mDmp1 in p53 as well as non-p53 pathways [5,12]. In human non-small lung cell carcinomas, loss of heterozygosity of DMP1 was found

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in about 35% of the cancers evaluated (n = 51), which was mutually exclusive with mutations of p53 and/or Arf/ink4a [13]. These findings further underscore the importance of DMP1 in human oncogenic suppression.

hDMP1 is located on chromosome 7q21, a locus frequently altered in human acute myeloid leukemia (AML) and breast cancer [14–17]. Studies in Dmp1-null mice demonstrated a gain of proliferative function in hematopoietic stem and common myeloid progenitor cells, thus uncoupling cellular control of blood lineages that can give raise to leukemias [18]. That DMP1β has a function distinct from DMP1α in controlling cell progression was shown by its capacity to promote proliferation in macrophages, which are limited in proliferation after differentiation from monocytes [8]. The identification of increased DMP1β levels over α in human breast cancers and the promotion of mammary tumors in MMTV-DMP1β transgene mice, supports a disease progressive role for DMP1β, rather than the tumor suppressor function of DMP1 [9].

To interrogate the transcriptional and cellular roles of the β and γ DMP1 isoforms, we investigated their capacity to transactivate the ARF promoter, regulate endogenous ARF, and enhance proliferation. Our findings define a transcriptional pathway whereby the DMP1β isoform, but not γ, can dampen DMP1α-mediated ARF responses in a dominant negative fashion. Moreover, the DMP1β isoform supports a gain of cellular proliferation in the presence or absence of p53. Lastly, DMP1 message levels in primary acute myeloid leukemia samples, as compared to granulocytes, were significantly reduced. We propose that DMP1β antagonizes the function of DMP1α, thus altering cellular proliferative control.

2. Material and methods

2.1. Plasmids and lentiviral vectors

2.1.1. Human and mouse DMP1/EGFP cloning

The open reading frames of all three DMP1 isoforms were PCR amplified and TOPO cloned using the following primers: general forward primer 5′-G*AAGCTT*GCCGCCACCATGAGCACAGTGGAAGAGGATTC-3′, and the human α-specific 5′-G*CCGCGG*ATGACAGTTTACCAAATCTTCG AC-3′, human β-specific 5′-G*CCGCGG*TTCTTCATTCTTCTTCTTCCCATTTG AC-3′, human γ-specific 5′-GCCGCGGTTCTTCATTCTTCTTCTTCCCATTT GAC-3′ reverse primers, mouse β-specific 5′-GCCGCGGTTCTTCATTCT TCTTCTCCCTTCTGAC-3′ or γ-specific 5′-G*CCGCGG*TTCTTCATTCTTCTTC TTCCCATTTGAC-3′ reverse primers. The HindIII/SacII fragments were further subcloned into pEGFP-N1 resulting in DMP1 isoforms fused to enhanced green fluorescent protein (EGFP). A generic Kozak element was added for efficient transcription in mammalian cells.

2.1.2. Human DMP1 ΔMHR cloning

The sequence encoding amino acids 1 to 223 of DMP1α was PCR amplified using the forward primer described above and the 5′-G*CCGC GG*ATGGTTTCTGTCATCATACATGC-3′ reverse primer. The PCR product was TOPO cloned and the HindIII/SacII fragment was further subcloned into pcDNA3.1/V5/His. Cloning of the V5/His tagged DMP1α, β and γ expression vectors were described earlier [8].

2.1.3. Generation of the CGW lentiviral vector

The self-inactivating (SIN) CGW control vector was assembled by standard cloning techniques from the following elements: MND, myeloproliferative sarcoma virus LTR-negative control region deleted; cPPT-CTS, polypurine tract–central terminating sequence; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein; SAR, IFN-β-scaffold attachment region; and WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. The vector backbone was derived from CS-PRE, a derivative of HIV-CS (kind gift of H. Miyoshi, University of Tsukuba, Japan), which has unique *Bam*HI, *Sac*II, *Eco*RI, *Xba*I, *Hpa*I, and *Xho*I sites upstream of the WPRE. The MND LTR was cloned from the MND-HSPSV-EGFP plasmid (kind gift of D. Kohn, USC School of Medicine, Children's Hospital, Los Angeles, CA) and cloned into the *Bam*HI site. The 178-bp cPPT-CTS *Bam*H1/*Sma*1 fragment was amplified from HIV-1 molecular clone R8. The viral IRES and EGFP fragment were cloned from plasmid pIRES2-EGFP (Clontech, BD Biosciences). The 800 bp 3′ scaffold attachment region (SAR) element was cloned from plasmid pCL, by introducing *Xba*1/*Hpa*1 sites [8].

2.1.4. CGW-hDMP1β and pBABE-hDMP1β-puro cloning

The human DMP1β open reading frame was PCR amplified (primers: forward 5′-AAGCTTGCCGCCACCATGAGCACAGTGGAAGAGGATTC-3′ and reverse 5′-*CCGCGG*TTCTTCATTCTTCTTCTTCCCATTTGAC-3′), TOPO cloned and further subcloned into the EcoRI site of CGW (Suppl. Fig. 5A, top) or the HindIII/SacII sites of pBabe-puro [19].

2.1.5. CGW-shDMP$_3$ and shDMP$_5$ lentiviral vector cloning

The U6 promoter driven short hairpin (sh)RNA expression cassettes, shDMP$_3$ and shDMP$_5$, targeting the open reading frame or the 5′ untranslated region (Suppl. Fig. 5B, bottom), respectively, were PCR cloned using the pTZU6 + 1 hp plasmid (kindly provided by Dr. J.J. Rossi, Beckman Research Institute, City of Hope, Duarte, CA) as template and the following primers: forward 5′-*GGATCC*AAGGTCGGGCAGGAAGAGGG-3′ and reverse 5′-G*CTGCAG*AAAAAATAAATGGAAGCAGGGGATGTGTCTCTTGAACA CATCCCCTGCTTCCATTTACGGTGTTTCGTCCTTTCCAC-3′ for shDMP$_3$ and 5′-G*CTGCAG*AAAAAAGCACTTTGGAAGAACCAGGATTCTCTTGAAATCCTG GTTCTTCCAAAGTGCCGGTGTTTCGTCCTTTCCAC3′ for shDMP$_5$. Complementary shRNA sequences are underlined. The PCR fragment was TOPO cloned into pCR-XL-TOPO (Invitrogen) and the *BamHI*/*PstI* fragment was further subcloned 5′ of the MND promoter into the CGW vector.

2.1.6. pLKO.1 shDMP1

pLKO.1 lentiviral vectors expressing small hairpin (sh)RNAs targeting human DMTF1 (shDMTF1_375: NM_021145.1-375s1c1, shDMTF1_888: NM_021145.1-888s1c1, shDMTF1_2148: NM_021145.1-2148s1c1 and shDMTF1_2248: NM_021145.1-2248s1c1) or a non-targeting shRNA control (SHC002) were purchased from Sigma-Aldrich. Lentiviral production and transduction of the different cells was done as described [8].

2.2. Cell lines, primary cells and generation of stable transfectants

U937 and 293T cells were maintained in DMEM; MOLM-13, NB4, HT93 and HL60 cells in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin, and 2 mM glutamine, at 37 °C in 5% CO_2. Cells were serum-starved by incubation in 0.1% FBS containing medium after five washes with PBS. For the myeloid differentiation experiments, we used the human acute leukemia cell lines U937, MOLM-13 and HL60 or the human APL cell lines NB4 and HT93. Cells were seeded at a concentration of 2×10^5 cells/ml and treated with phorbol 12-myristate 13-acetate (PMA) as indicated or with 1 μM all-*trans* retinoic acid (ATRA). Cell culture reagents, media and differentiation-inducing agents were purchased from Sigma-Aldrich.

Peripheral granulocytes were isolated from buffy coats of healthy donors recruited at the Inselspital Bern, Switzerland, using Ficoll gradient density centrifugation (Lymphoprep, Axon Lab AG, Switzerland). Red cells in the bottom layer were treated with erythrocyte lysis buffer (Qiagen AG, Hombrechtikon, Switzerland) to yield a granulocytes fraction enriched (>95% purity). Fresh leukemic blast cells from AML patients at diagnosis obtained at the University Hospital Inselspital Bern were classified according to the French–American–British (FAB) classification and cytogenetic analysis. Informed consent was obtained from all patients. All leukemia samples had blast counts >90% after separation of mononuclear cells using a Ficoll gradient as described previously [8]. All protocols for the use of human sample uses were approved by the Cantonal Ethical Committee at the Inselspital and The Scripps Research Institute.

Normal human foreskin fibroblasts (BJ) and lung fibroblasts (IMR90) were maintained in 6-well plates and infected with

pBabe-puro and pBabe-puro-hDMP1β retroviral particles produced in LinX-A amphotropic packaging cells. After 48 h, cells were transferred to 100-mm dishes. Selection of stable cells was carried out in the presence of 1.0 μg/ml puromycin for one week. Lentiviral production and transduction of 293T, HeLa and U937 cells were done as described [8].

2.3. Transient transfections and reporter assays

293T cells were transiently transfected with pGL2 reporter plasmids containing an ARF promoter fragment (pGL2-ARFpro), an ARF promoter fragment with a mutated DMP1 consensus site (pGL2-ARFpro mutant) or a stretch of eight concatemerized DMP1 binding sites (pGL2-BS2) upstream of the *firefly luciferase* reporter gene (kindly provided by Dr. C.J. Sherr, St. Jude Children's Hospital, Memphis, TN) and the different human or mouse DMP1 isoform expression plasmids. Total DNA was kept constant with parent vector. Cells were lysed forty-eight hours after $CaPO_4$ transfection and were assayed for luciferase activity by a Dual-Luciferase™ Reporter System kit (Promega) [8]. Transfection efficiencies were normalized with an internal Renilla expressing plasmid (pRL-TK). Results, expressed as ratio of *Firefly* to *Renilla luciferase* (relative luciferase activity, RLA), are the means of three independent experiments measured in duplicate, and error bars represent standard deviations.

2.4. RNA extraction, human DMP1 variant specific RT-PCR and total human DMP1 qPCR assays

RNA extraction and cDNA synthesis were done as described [8]. The following primer and probe combinations were used to analyze the house keeping gene ABL, forward 5′-TGTGGCCAGTGGAGATAACACT-3′; reverse 5′-CCATTCCCCATTGTGATTATAGC-3′; probe 6FAM-5′-TAAG CATAACTAAAGGTGAAAAGCTCCGGGTCTTA-3′-TAMRA. For ARF, we used primers and a probe as described [20]. All samples were run under standard conditions on a 7900HT sequence detection system (Applied Biosystems).

To determine human DMP1 expression we used the following three assays: First, to quantify total hDMP1 gene expression the Taqman® Gene Expression Assays preloaded on low density arrays for hDMP1, HMBS and ABL1 were Hs00253517_m1, Hs00609297_m1 and Hs00245445_m1, respectively. Data were normalized to HMBS and ABL1 and analyzed as described [21]. Second, to specifically quantify hDMP1 α and β we used hairpin-unfolding-based RT-PCR [22]. The following primers were used: hDMP1α as described in Blancafort P et al. [23]; hDMP1β forward 5′-actgaacctgaccgtacaATTGAGAAGCTC AAGGAACAACTGT-3′, reverse 5′-CCATTTGACTGGTTGGAAGTTG-3′, ABL1 forward 5′-actgaacctgaccgtacaTGCCCTGCATTTTATCAAAGG-3′; reverse 5′-AAAGTCAGATGCTACTGGCCG-3′. The 5′ end of the target specific forward primer contains a tail sequence complementary to the 3′ end of the UniPrimer (see nts depicted in lower case) (EMD, Millipore). Expression was quantified according to the comparative threshold method using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{calibrator})$, and ΔCt is the Ct of the target gene subtracted from the Ct of the house keeping gene ABL1 [24,25]. Unfortunately, this hDMP1β assay, also highly specific, was not sensitive enough to reliably quantify endogenous expression of this splice variant. Thus, we only used this assay to confirm β-specific overexpression in transduced BJ and IMR90 cells. Third, we used a single primer pair encompassing the β/γ insertions to determine relative expression of the hDMP1 splice variants. This assay allows differentiation of the three isoforms based on size differences of the resulting PCR product and has been published earlier [8].

2.5. Band shift assays

Band shift assays were done as described [26]. Briefly, DMP1 isoform proteins were in vitro synthesized using rabbit reticulocyte lysates (TnT Quick Coupled Transcription/Translation System, Promega, USA). Annealed probes were radioactively labeled using 50 μCi of adenosine 5′-[γ32P]triphosphate at 6000 Ci/mmol (Amersham) and T4-Polynucleotide Kinase (Life Technology Invitrogen, USA). A probe containing the putative DMP1 binding site in the human ARF promoter (5′-GTCAGGTGACGGATGTAGCTAGG-3′) was used. Binding reactions were carried out in a total reaction volume of 15 μl containing 100 ng poly-(dIdC), 1 μl hot probe, 5 μl of programmed reticulocyte lysate, 200 ng of monoclonal anti-V5 antibody (Life Technology), and competing cold oligonucleotides where indicated, in binding buffer (10 mM TrisHCl (pH 8.0), 250 mM KCl, 500 μM EDTA, 0.1% Triton-X 100, 12.5% glycerol (v/v), 200 μM DTT) for 30 min at room temperature. Protein–DNA complexes were separated on 4% non-denaturing PAGE for 90 min at 1 mA/cm at 4 °C. Gels were dried and exposed to Kodak BioMax XAR Films at −80 °C. Band densities were determined using ImageQuant Software (GE Healthcare, USA).

2.6. Proximity ligation assay

To detect a possible interaction between DMP1α and β as well as between DMP1α/β and p53, we utilized the DuoLink in situ Proximity Ligation Assay (PLA) [27] according to the manufacturer's protocol (Sigma). Transient transfection of 293T cells was done using lipofectamine (Invitrogen), then, cells cultured on glass coverslips, were fixed with 4% PFA followed by 10 min incubation with 0.1% Triton X-100 solution in PBS and further blocked with PBS-containing 1% BSA and 0.1% Tween. Cells were immunolabeled with the following primary antibodies: V5-Probe (Santa Cruz; sc-81594) and EGFP (Santa Cruz; sc-8334) for 1 h at room temperature. Secondary antibodies with attached PLA probes specific to the primary antibodies were supplied by the manufacturer. We used confocal microscopy to detect protein-to-protein interaction at a 60× magnification. A fluorescence signal indicates that two proteins within cells are separated by <40 nm.

2.7. Confocal microscopy

293T and HeLa cells were grown on poly-L-lysine-coated glass coverslips and transiently transfected with expression plasmids containing DMP1β or γ C-terminally fused with EGFP. After 24 h, the cells were washed four times with HBSS. Cells were then fixed with 2% paraformaldehyde, washed once with PBS and treated with DNAse-free RNAse for 15 min at room temperature. After washing twice with PBS, the nuclei were stained with TO-PRO 3 (1:2000; Molecular Probes). The cells were examined using a laser confocal scanning microscope (BioRad). Images were processed using Adobe Photoshop (Adobe Systems).

2.8. Western blot analysis as well as cytoplasmic and nuclear fractionation

Whole cells were lysed in urea buffer consisting of 8 M urea and 0.5% Triton X-100 supplemented with protease inhibitor cocktail (Roche). 30 μg of total protein was analyzed by electrophoresis on a mini-protean TGX Stain-Free Precast gel (Bio-Rad). Antibodies used were anti-EGFP rabbit polyclonal antibody (Molecular Probes, Basel Switzerland) and goat anti-rabbit IRDye® 800CW (Li-Cor Biosciences). Blots were analyzed using the Odyssey infrared imaging system detection (Li-Cor Biosciences).

Cytoplasmic and nuclear fractionation was done by first lysing the cells in buffer A (HEPES 10 mM (pH 8.0), KCl 10 mM, $MgCl_2$ 1.5 mM, DTT 1 mM), centrifugation and recovering the supernatant. The cell pellet was re-suspended in buffer B (HEPES 20 mM (pH 8.0), $MgCl_2$ 1.5 mM, KCl 400 mM, NaCl 40 mM, EDTA 0.2 mM, DTT 1 mM, glycerol 25%) to lyse the nuclear envelope and nuclear proteins were extracted. Protein content was determined with a Bradford Assay (#500-0006; Bio-Rad) with BSA as a standard. 5 μg of cytoplasmic and nuclear protein was analyzed by electrophoresis on a mini protean TGX Precast gel (Bio-Rad). Primary antibodies used were anti-α-Tubulin (#3873; Cell

Signaling), anti-Histone H3 (#9715; Cell Signaling), and anti-EGFP (see above). Secondary antibodies used were anti-rabbit and anti-mouse IgG HRP-linked (NA93IV; GE Healthcare). Chemiluminescence signal was detected using an ECL-kit (170-5060; Bio-Rad).

2.9. Proliferation assays

Proliferative capacity was assessed by [^{3}H]thymidine or bromodeoxyuridine (BrdU) incorporation. 293T, HeLa and U937 transfectants were plated in 96-well plates at concentrations of 7.5×10^3 and 15×10^3/well and were incubated for serum conditions as indicated. [^{3}H]thymidine was added for the last five hours of cell treatment (1 μCi/well). Cells were harvested using a Mach III cell harvester (Tomtec) and γ-radiation was detected using a Wallac 1450 MicroBeta Liquid Scintillaton Counter (PerkinElmer). For the BrdU incorporation analysis, HL60 and MOLM-13 cells were cultured in 24-well plates and treated as indicated. BrdU incorporation was measured using the BrdU cell proliferation assay kit (Cell Signaling). Subsequent immunofluorescent staining of incorporated BrdU was followed by flow cytometric analysis using the BrdU Flow Kit (BD Biosciences, San Jose, CA, USA).

3. Results

3.1. Transcriptional activities of the human DMP1 isoforms

We have reported earlier on the splicing and resulting protein structures of the DMP1α, β and γ isoforms [8] and see Suppl. Fig. 1 for a DMP1 isoform diagram). Similar to our earlier findings utilizing the

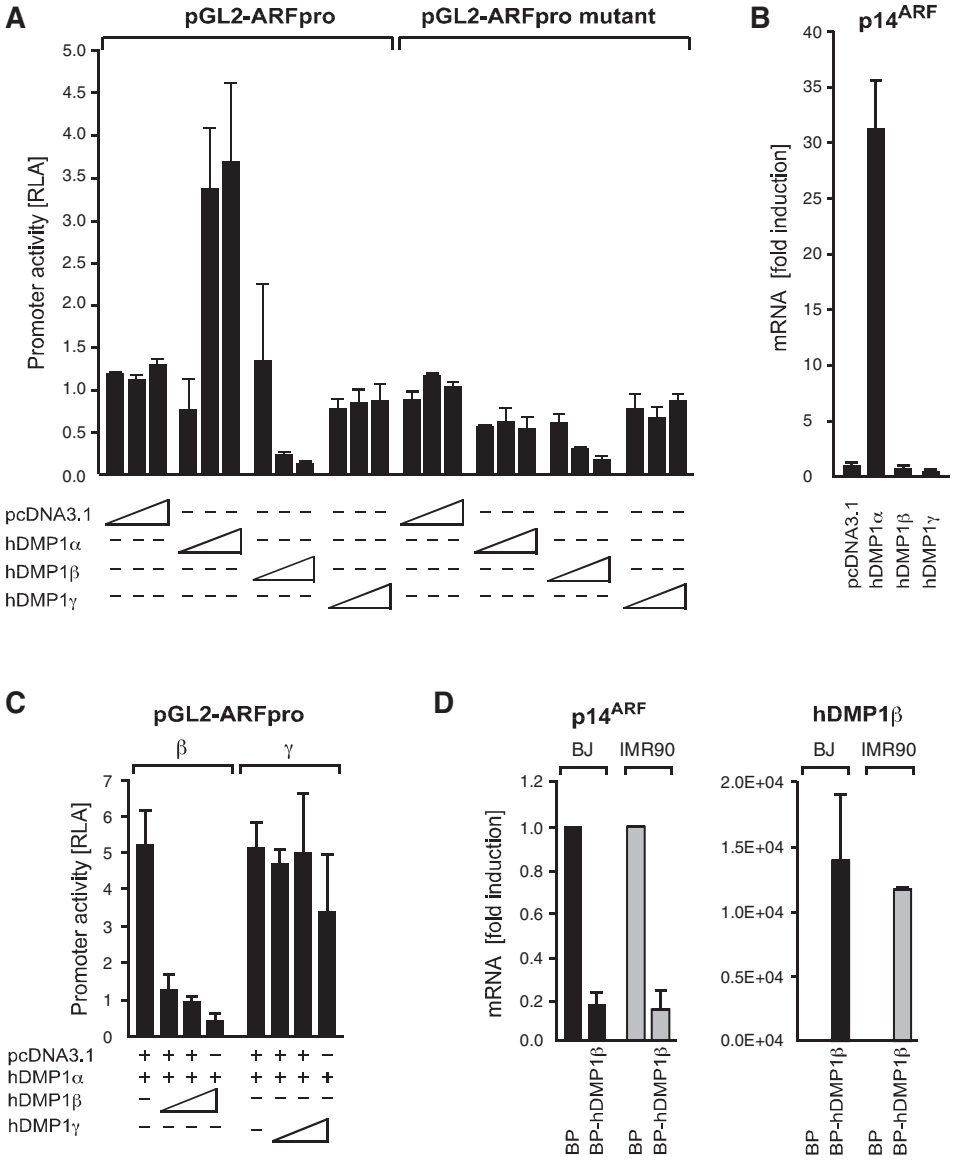

Fig. 1. Human DMP1β negatively regulates p14 ARF transactivation. A) Transcriptional response of the ARF promoter to the different DMP1 isoforms. 293T cells were transiently transfected with 4 μg of each reporter plasmid, 10 ng of the *Renilla luciferase* expression plasmid pRL-TK and with increasing amounts of the DMP1 isoform expression plasmids pcDNA3.1–DMP1α, β, γ or the control expression vector pcDNA3.1. The promoter activity is shown as relative luciferase activity (RLA). Results are the means ± S.D. of triplicate transfections. B) Primary human foreskin fibroblasts were transfected with expression vectors as in (1A). ARF transcripts were measured by qPCR. Specific gene expression was normalized to *ABL* expression. Results are given as fold expression of pcDNA3.1 transfected cells. The experiment was performed in duplicate. C) Co-expression of DMP1β and γ with DMP1α and its influence on ARF promoter activity. 293T cells were transiently transfected with 4 μg of each reporter plasmid, 10 ng of the *Renilla luciferase* expression plasmid pRL-TK, 1.5 μg pcDNA3.1–DMP1α and with increasing amounts (1.5, 3.0 and 6.0 μg) of the pcDNA3.1–hDMP1β or γ plasmids. Analysis was performed as described above. D) Retroviral expression of DMP1β in BJ or IMR90 primary human fibroblasts reduces endogenous ARF mRNA levels. Cells were transduced with empty (BP) and DMP1β expressing (BP-hDMP1β) pBabe-puro retroviral vectors. ARF and DMP1β mRNA levels of puromycin selected cell populations were measured by qPCR and normalized to *ABL* expression. Results are given as fold expression of mock-transduced cells. Mean and standard deviation (bars) is shown.

CD13/APN promoter [8], DMP1α, but not β or γ, activated the ARF promoter (Fig. 1A). In contrast to DMP1α or γ, increasing the amounts of exogenous DMP1β inhibited basal levels of ARF promoter activity. An ARF promoter reporter with a mutated DMP1 binding site, pGL2-ARFpro mutant, was used as negative control and showed no changes in activity when co-expressed with DMP1α, confirming the specificity of DMP1α transactivation. Studies utilizing plasmid expression of DMP1α, β and γ EGFP fusion proteins indicated that each isoform was present in cells after transient transfection (Suppl. Fig. 2A and B).

To assess whether endogenous cellular ARF can be regulated by the different DMP1 splice variants, we transiently expressed the splice variants in human primary BJ fibroblasts (Fig. 1B). We found a 30-fold induction of ARF mRNA levels in cells expressing DMP1α, confirming that elevated cellular levels of human DMP1α indeed caused increased endogenous ARF transcription. Similar to the findings in transient transfection assays with the ARF promoter, DMP1β and γ did not induce ARF transcription in primary fibroblasts. Given the findings shown in Fig. 1A and B, we next evaluated whether DMP1β and γ could inhibit DMP1α-induced activation of the ARF promoter. Cellular co-transfection of DMP1β with α resulted in a dose-dependent inhibition of DMP1α-induced transactivation of the ARF promoter (Fig. 1C, left). In contrast, DMP1γ did not significantly inhibit the ARF promoter activity induced by DMP1α (Fig. 1C, right), which is in agreement with our earlier published findings utilizing the CD13/APN promoter [8]. To determine if species conservation was apparent for DMP1, the mouse Dmp1β gene was scrutinized and an alternative splice acceptor site was identified (Suppl. Fig. 3A). Furthermore, the mouse β-isoform showed similar α inhibitory functions as the human isoform in DMP1 promoter transactivation reporter assays (Suppl. Fig. 3B). These results indicate that either the human or mouse DMP1β isoform antagonizes the transcriptional activity of the α-isoform. The species conservation of the alternative splice acceptor sites, protein, and function in human and mouse supports the potential cellular relevance of the β-isoform.

Next, we analyzed the effects of ectopically expressed DMP1β on endogenous ARF mRNA levels in human primary fibroblasts. Since the DMP1γ splice variant had minimal effect on antagonizing the transcriptional activation function of DMP1α (Fig. 1C, right panel), we focused our effort on DMP1β. Stably retroviral transduced human BJ and IMR90 fibroblast cell lines were generated using the pBabe-hDMP1β-puro (BP-hDMP1β), with the empty pBabe-puro (BP) retroviral vector as the control. After selection in puromycin we measured endogenous ARF levels by qPCR. We found an 80% downregulation of ARF in BJ and IMR90 cells expressing DMP1β as compared to the controls (Fig. 1D, left panel). We confirmed that the BP-hDMP1β transduced cells expressed 1.2 to 1.4×10^4 fold higher DMP1β levels than the BP control cells (Fig. 1D, right panel). Thus, continued ectopic expression of DMP1β in fibroblasts leads to damping of endogenous ARF mRNA levels.

3.2. Functional analysis of the truncated human DMP1 isoforms

The ARF promoter results imply that DMP1β antagonizes DMP1α activity. In contrast, DMP1γ does not affect transcriptional activity of DMP1α. The noted difference between the two short isoforms is the presence of 13 additional amino acids from intron 9 in DMP1γ as the result of an alternative splicing, which joins the N-terminal and C-terminal Myb-like homology domain remnants (Suppl. Fig. 1). We reasoned that the functional differences between DMP1β and γ might lie in this 13-amino acid sequence that is unique to the γ isoform (Suppl. Fig. 1). To better understand the role of the C-terminal Myb-like homology domain remnants (MHR), we compared the DMP1 MHR deletion mutant (ΔMHR), a mutant that lacks amino acids common to all three splice variants, to DMP1β and γ in a cell-based reporter assay, and measured the degree of inhibition of DMP1α transcriptional activity. Ectopic expression of DMP1α enhanced promoter reporter activity beyond the endogenous cellular activity, whereas co-ectopic expression of DMP1β and DMP1α resulted in high-level (80%) inhibition of DMP1α activity (Fig. 2A). The co-transfections of the ΔMHR mutant or DMP1γ with DMP1α and promoter reporter demonstrated similar low levels of inhibitory activity. These results indicate that the β-specific protein sequence is necessary for efficiently inhibiting α function, as the N-terminal transactivation and cyclin D-binding domain, and Myb-homology region common to all three DMP1 isoforms did not confer high levels of DMP1α inhibition.

Although DMP1γ differs from DMP1β by the addition of only 13 additional amino acids (Suppl. Fig. 1), it demonstrated distinct functional properties. Since nuclear localization is critical for DMP1 function, we wondered whether the differences in DMP1 isoform activity could be the result of cellular localization [28]. To investigate this possibility, we compared subcellular localization of DMP1β and γ isoforms by confocal microscopy. Since verified commercial antibodies are not available to differentiate the subcellular localization of endogenous DMP1β and γ from DMP1α, we transfected 293T and HeLa cells with different C-terminal EGFP-tagged versions of the DMP1 isoforms. Surprisingly, unlike the nuclear specific localization of DMP1α- [28] and β-EGFP fusion proteins, the DMP1γ signal was detected in the cytoplasm and nucleus, similar to the EGFP only control (Suppl. Fig. 4A). Therefore, DMP1γ appears to have an altered localization pattern relative to the other DMP1 isoforms. Next, we quantified DMP1–EGFP isoforms from cytoplasmic and nuclear compartments and determined that the γ protein isoform was less abundant than either the α- or β-isoforms, as evidenced by Western blot detection of the respective DMP1–EGFP fusion proteins (Suppl. Fig. 4B). Thus, DMP1γ cellular levels and, most importantly, nucleus localization are decreased relative to the DMP1α- or β-isoforms. These findings may provide a potential explanation for the lack of cellular DMP1γ activity observed during our DMP1 splice variant transfection and ectopic expression studies. Lastly, it should be pointed out that our earlier report [8], and a recent report by Maglic and colleagues [9], determined that in many cell types DMP1γ transcripts were not abundant, relative to DMP1α and β. Moreover, DMP1γ transcript levels remained constant during human hematopoietic cell differentiation [8].

Next, we asked whether the inhibitory activity of DMP1β is dependent on direct binding to the DMP1 site present in the ARF promoter. To this end we performed electrophoretic mobility shift assays (EMSAs) utilizing in vitro transcribed and translated V5-tagged DMP1α, β, or γ protein or the ΔMHR (Suppl. Fig. 1). Only the α, but not the β and γ isoforms or ΔMHR, was able to bind to a probe containing the DMP1 binding site of the ARF promoter that resulted in a gel shift (Fig. 2B). Moreover, the addition of a monoclonal antibody directed against the V5-tag led to a supershift of the DMP1α-probe complex, further confirming interaction specificity. In contrast, addition of a 300-fold molar excess of cold probe efficiently blunted the shift. Cell lysates from the control empty expression vector or the ΔMHR mutant failed to promote gel shifts, as would be expected. Lastly, co-incubation of DMP1α with increasing amounts of DMP1β decreased it's binding to the DNA probe to approximately 43% of DMP1α only (Fig. 2C). Our findings infer that DMP1α requires MHR-TAD for binding to DNA, the shorter isoforms do not bind the ARF promoter, and finally, DMP1β antagonizes DMP1α binding to DNA.

To determine if DMP1α/α and α/β were in close proximity in a cellular environment and co-localized to the nucleus, we utilized in situ proximity ligation methodology [27]. The proximity ligation method depends on the dual proximal binding by pairs of detection reagents to generate amplifiable DNA strands, which then can serve as markers for the detection of 2 proteins when they are <40 nm apart. We first determined that transient ectopic expression of DMP1α-EGFP and DMP1α-V5 were localized to the nucleus and DMP1α/α co-localized based on signal proximity (Fig. 2D, top row). Next, DMP1α-EGFP and DMP1β-V5 were evaluated in the same methodological fashion and found to generate a similar fluorescent signal pattern as seen with DMP1α/α (Fig. 2D, middle row). These results indicate that DMP1α/α and α/β co-localized within the nucleus and were within 40 nm of each other [27].

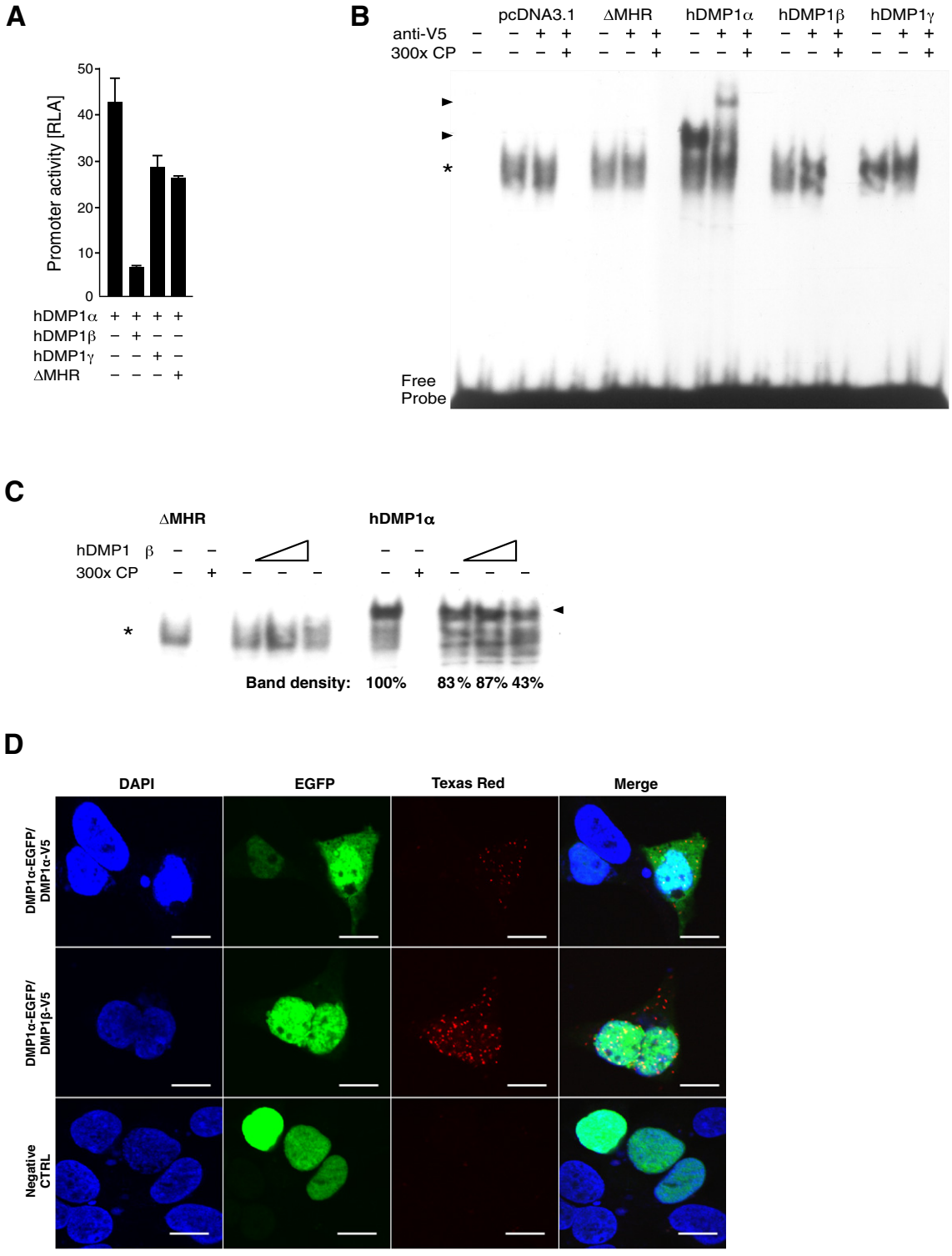

Fig. 2. Human DMP1β binds to the DMP1α isoform but not to the human ARF promoter. A) Role of the DMP1β specific amino acids in repressing DMP1α activity. 293T cells were transfected with 1.0 μg of the pGL2-BS2 DMP1 consensus site reporter, 20 ng of the *Renilla luciferase* expression plasmid pRL-TK, 1.5 μg pcDNA3.1–DMP1α expression plasmid together with 3.0 μg expression plasmids for either DMP1β, γ or the ΔMHR mutant as indicated. The ΔMHR mutant shows significantly less inhibition of DMP1α. Results are given as the mean ± S.D. of relative luciferase activity (RLA). B) Electrophoretic mobility shift assays (EMSAs) were performed by using ^{32}P-labeled ARF promoter oligonucleotides as probe with DMP1 splice variant proteins. Binding of the DMP1 variants (lower arrow) was subjected to competition with 300-fold molar excess of cold competitor (CP). ΔMHR mutant was used as negative control. Specificity of the binding was shown by preincubating the V5-tagged DMP1α protein and the probe with antiserum against V5, resulting in a band shift (upper arrow, supershift). *Nonspecific binding. C) Preincubation of DMP1α with β leads to a reduction in binding activity, approximately 43% of the full-length DMP1α protein. DMP1β protein was titrated against a constant amount of ΔMHR or DMP1α as indicated and subjected to EMSA. Densities of the bands were measured and the DMP1α only band density was arbitrarily set to 100%. D) Proximity Ligation Assay was performed on 293T cells transiently transfected with 1 μg of EGFP-tagged DMP1α and V5-tagged DMP1α or EGFP-tagged DMP1α and V5-tagged DMP1β. The Texas red fluorescence signal indicates that the two isoforms α and β were separated by <40 nm within the cells.

3.3. Human DMP1β provides a growth advantage in low serum conditions and during myeloid differentiation

Given our findings that DMP1β dampens DMP1α-induced transactivation of ARF, we directly tested whether either ectopic expression of the β-isoform or directly decreasing DMP1α levels altered growth patterns of 293T cells and the myelomonocytic cell line, U937, in tissue culture. To this end, stably transduced cell lines were generated utilizing lentiviral vectors expressing EGFP, as a reporter only control, the DMP1β–EGFP fusion protein, or two different short hairpin (sh) RNAs targeting the DMP1 open reading frame ($shDMP_3$) or the 5′ UTR ($shDMP_5$) (Suppl. Fig. 5A). Targeting only DMP1α is not possible given all splice variants share the α sequence (Suppl. Fig. 1). All cell lines utilized expressed the α-isoform and the lentiviral vector expression of the DMP1 shRNAs reduced DMP1 mRNA levels 60% and resulted in significant inhibition of a DMP1α specific luciferase reporter (data not shown).

The shRNA lentiviral vector stable expressing cell lines were enriched for EGFP expression by flow cytometry sorting and then tested in a proliferation assay ([^{3}H]-thymidine incorporation) in reduced (0.1%) serum levels. Low serum levels are known to reduce proliferation and survival in selected cell lines and the ectopic expression of certain oncogenes has been shown to reverse this condition. What was readily apparent when 293T and U937 cells expressing the β-isoform were cultured with low serum was a significant growth advantage as compared to the control lines expressing just EGFP (Fig. 3A). Similar results were obtained with HeLa cells expressing DMP1β (data not shown). Evaluation of 293T or U937 cells in low serum revealed that stably expressing $shDMP_3$ or $_5$ enhanced cell proliferation in a manner similar to cells ectopically expressing the DMP1β isoform (Fig. 3A). Increased cellular proliferation as determined by [^{3}H]-thymidine incorporation was further confirmed by cell counting (Fig. 3B). Thus, decreasing DMP1 levels or increasing DMP1β levels conferred similar cellular traits of increased proliferation in cultured cells maintained in low serum conditions.

During earlier studies on the role of DMP1α and β in CD13/APN regulation and myeloid differentiation, we noted that the U937 cell line treated with PMA underwent morphological differentiation, but maintained proliferation when DMP1β was ectopically expressed [8]. To further interrogate the role of DMP1 during monocyte to macrophage differentiation and gain of proliferative function in macrophages,

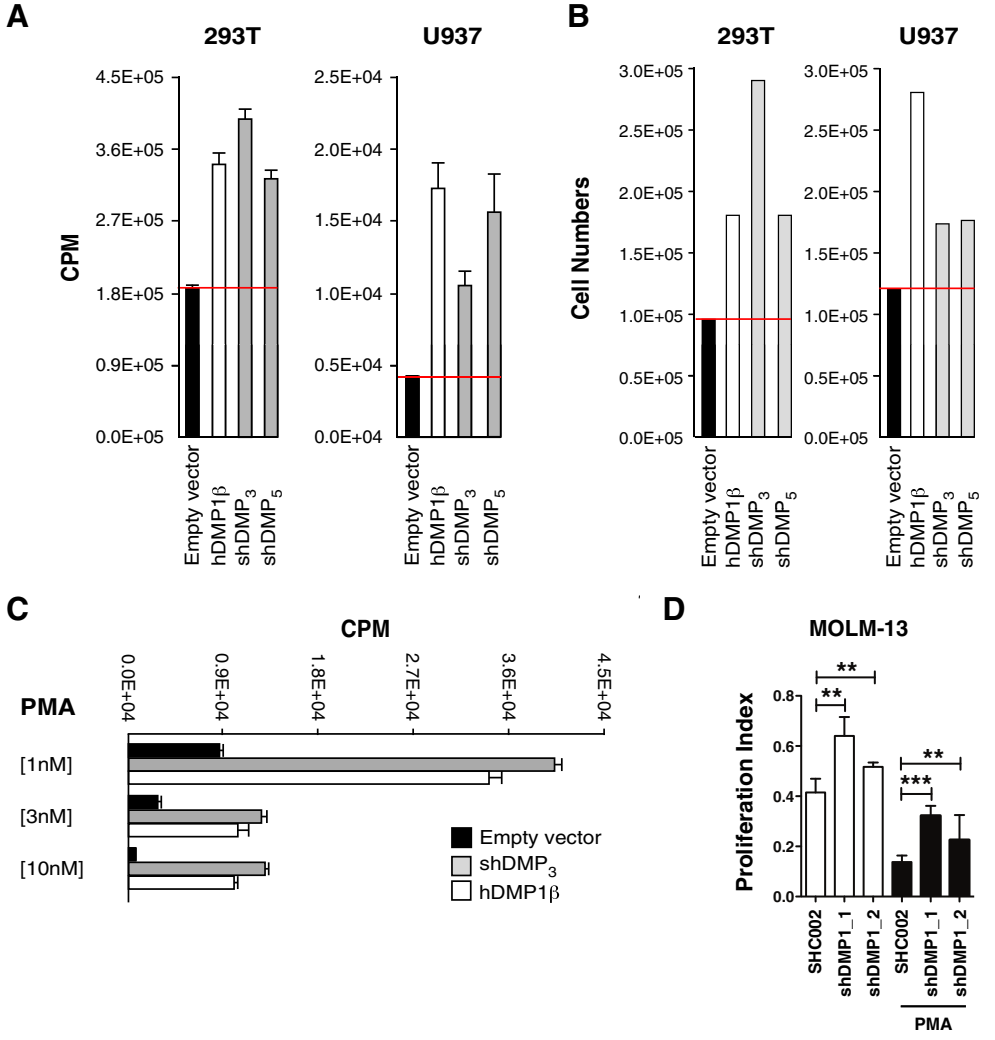

Fig. 3. Growth advantage of DMP1β or total DMP1 knockdown cells in low serum conditions and during myeloid differentiation. A) Proliferation of 293T and the myeloid U937 cells in low serum conditions, 0.1% fetal bovine serum, stably expressing DMP1β or $shDMP_{3/5}$ was measured by [^{3}H]thymidine incorporation in cells. Results are given as counts per minute (cpm) of β-radiation from cell cultures set up in triplicate. B) 293T and the myeloid U937 cells stably expressing DMP1β or $shDMP_{3/5}$ were cultured in low serum conditions, as in A above, and cell counts determined using a hemocytometer. C) Proliferation of U937 cells ectopically expressing DMP1β or $shDMP_3$ during myeloid differentiation. U937 DMP1β or $shDMP_3$ transfectants proliferation during phorbol 12-myristate 13-acetate (PMA) induced macrophage differentiation was measured as in (3A). D) Proliferation of MOLM-13 cells ectopically expressing shDMP1_1 or shDMP_2 short hairpin RNAs targeting the DMP1 mRNA or SHC002 control. Proliferation of DMP1 knockdown or control MOLM-13 cells treated with 10 ng/ml PMA was measured by BrdU incorporation and detected by absorbance at 450 nm. Absorbance is plotted as a relative proliferation index. Mean and error bars represent standard deviation, P is designated as *P, 0.05, **P, 0.01, ***P, and 0.001 from a parametric 2-tailed unpaired Student t test.

we differentiated U937 shDMP$_3$, DMP1β, and EGFP (empty vector) expressing cells with PMA (Fig. 3C). When EGFP expressing control cells were treated with increasing amounts of PMA (1, 3, 10 nM, and refer to Fig. 3A for proliferation without PMA-mediated differentiation), cells decreased proliferation (Fig. 3C), and underwent terminal differentiation (not shown). In contrast, U937 shDMP$_3$ or DMP1β expressing cells continued to proliferate despite terminal macrophage differentiation (Fig. 3C). Morphologically, PMA-treated U937 cells appeared similar whether cells were expressing shDMP$_3$, DMP1β, or EGFP (data not shown). Thus, loss of DMP1 or DMP1β ectopic expression provided for a gain of proliferation in U937 macrophages.

DMP1 regulates p53 through ARF or directly via physical interaction with p53 [2,7]. Furthermore, ARF has been reported to regulate tumor suppression independently of p53 (reviewed in [5]) and a recent report postulates that DMP1β may function independently of the ARF-p53 pathway [9]. To determine if loss of p53 alters macrophage proliferation when DMP1 was disrupted, we utilized the U937 cell line, which has a disruptive 46 amino acid deletion in the p53 gene [29]. As a control for the wild type p53 gene we utilized the myelomonocytic MOLM-13 cell line [30]. PMA treatment of a MOLM-13 cell line expressing scrambled shRNAs from integrated lentiviral vectors demonstrated loss of proliferation, which accompanies macrophage differentiation (Fig. 3D). However, in contrast, stable MOLM-13 cell line expressing lentiviral vector driven shDMP1_1 (combination of shDMTF1_375 and shDMTF1_2148) or shDMP1_2 (combination of shDMTF1_888 and shDMTF1_2248) demonstrated a significant gain of proliferative function upon PMA-induced macrophage differentiation (Fig. 3D). Assessment of DMP1 mRNA disruption in MOLM-13 cells by either shDMP1_1 or shDMP1_2 showed up to 50% transcript disruption in the 2 independent cell lines generated (Suppl. Fig. 5B). Lastly, the promyelocytic leukemia cell line HL-60, reported to not express p53 due to major gene deletions [31], also demonstrated a gain of proliferative in PMA-derived macrophages expressing the same DMP1 knockdown constructs (Suppl. Fig. 5C and D). Together, these findings indicate that disruption of DMP1 transcript expression promotes gain of proliferation in macrophages in the presence or absence of p53 and that ectopic expression of DMP1β induces a similar cellular effect.

3.4. Overall lower human DMP1 mRNA and distinct splice variant expression in primary acute myeloid leukemias (AML)

Molecular events leading to AML include a block of normal myeloid differentiation combined with gains of survival and proliferation. The potential role of DMP1 functioning as a tumor suppressor prompted us to investigate whether DMP1 mRNA expressions in primary acute myeloid leukemia (AML) samples, composed of different FAB subtypes, were altered. To this end, we assessed DMP1 transcript levels in 78 AML patient samples and, as myeloid cell controls, 5 peripheral blood granulocyte and three freshly isolated CD34$^+$ cell samples utilizing qPCR for total DMP1. DMP1 mRNA levels were given as differences in Ct-values compared to mRNA levels for the housekeeping genes HMBS and ABL1. DMP1 mRNA expression levels in AML patient samples were significantly lower than in granulocytes (Fig. 4A; Mann–Whitney U, $P < 0.01$), but similar to the CD34$^+$ cells.

Next, we assessed DMP1 mRNA splice variant patterns in 7 primary AML cell samples of different FAB subtypes (Fig. 4B). Since the human DMP1 qPCR methodology did not faithfully differentiate among the three splice variants, a semi-quantitative DMP1 splice variant specific RT-PCR assay was used, as described in Material and Methods Section 2.4 and [8]. The α, β, and γ plasmids were used as controls and provided equal band density, thus controlling for RT-PCR performance [24,25]. Band densities from each AML patient sample were measured and α/β ratios calculated. We found that 5/7 AML patient samples presented with enhanced expression of DMP1β transcripts relative to DMP1α (Fig. 4B), with an overall ratio of 0.9 ± 0.1 DMP1α to β transcripts in all 7 samples, similar to that reported from freshly isolated CD34$^+$ cells, but not lower than differentiating myeloid progenitors [8].

Since AML cells are blocked at early stages of myeloid development and express lower levels of DMP1 transcripts than granulocytes (Fig. 4A) and myeloid progenitors [8], we next asked whether the acute promyelocytic leukemia (APL — the M3 subtype of AML) cell lines, NB4 and HT93, and 2 primary patient samples were capable of regulating DMP1 expression during terminal granulocyte differentiation. To promote myeloid differentiation, all-*trans* retinoic acid-mediated (ATRA) treatment was utilized [32]. After ATRA-mediated differentiation of NB4 and HT93 cells to granulocytes, DMP1 mRNA levels increased 2.3-fold in NB4 cells and 3.5-fold in HT93 cells relative to the starting mRNA levels (Fig. 4C). This same pattern of increased DMP1 mRNA levels after ATRA treatment of APL cells was observed in two APL patients undergoing ATRA therapy (Fig. 4D). These findings demonstrate that DMP1 expression is decreased in AML cells, and DMP1 expression can be increased in APL cells when differentiated. The results imply that decreased levels of DMP1 transcripts, and enhanced DMP1β transcript levels (Fig. 4B) are coincident with a block in AML differentiation.

4. Discussion

Our original investigations of DMP1 expression in human hematopoietic cells uncovered 2 isoforms, β and γ, in addition to DMP1α [8]. The current study demonstrates that while the DMP1γ isoform is ineffective in modulating ARF activation or promoting discernable cellular phenotypes, the DMP1β isoform disrupts ARF activation mediated by DMP1α with promoter reporters and in fibroblasts. Of note, DMP1β also inhibited the basal activity of an ARF promoter reporter construct with a mutated DMP1 binding site. Since the DMP1α form did not activate the mutated ARF promoter, we speculate that the β-specific amino acids may lead to a gain-of-function that could impair additional ARF activators. Moreover, loss of DMP1 activity in cells, either through targeted message knockdown with shRNAs or by ectopic expression of DMP1β, effectively counteracts the cellular growth arrest triggered by either serum-deprivation or induction of myeloid differentiation, in the presence or absence of p53. In contrast to normal granulocytes, DMP1 transcripts in AML were decreased. In line with the identification of DMP1 isoforms and their potential cellular functions, the presence of mouse functional β-transcripts, together with the reported β-transcripts in ferrets, dogs, cats and sheep [33], a recent report of DMP1β transcripts and protein in breast cancer cells, and tumor promoting activity in MMTV–DMP1β transgenic mice [9], supports a physiological role for DMP1-isoforms in many animal species.

In order to understand mechanistically how DMP1β may regulate DMP1α, we investigated whether the DMP1β- and γ-isoforms compete directly with DMP1α for DNA binding site use. We showed that both DMP1β and γ isoforms, in contrast to DMP1α, do not bind DNA, which implies that these isoforms cannot compete directly with DMP1α for DNA binding. The lack of cellular activity of the DMP1γ isoform could be due to its low cellular levels and nuclear retention. It has been reported that the conserved lysine at position 319, missing in DMP1β and γ, appears to be necessary for DMP1–DNA interactions [3,34], consistent with our findings (Fig. 2B). How might then DMP1β disrupt the function of DMP1α? Our findings that co-incubation of DMP1α with β reduces DMP1α's binding to DNA suggests an alternative pathway that may interfere with the formation of α/α interaction and subsequent binding to the ARF promoter [35]. This phenomenon is not without precedent, as a number of examples exist for transcription factors functioning as homodimers and with functional disruption through heterodimer formation, such as the p73 tumor suppressor, STAT3 and the human glucocorticoid receptor [36–38]. Cellular co-localization of α/α and α/β in the nucleus, as visualized with in situ proximity ligation methodology, supports potential proximal α/α and α/β interactions. Although our findings would support heterodimer formation, which is a

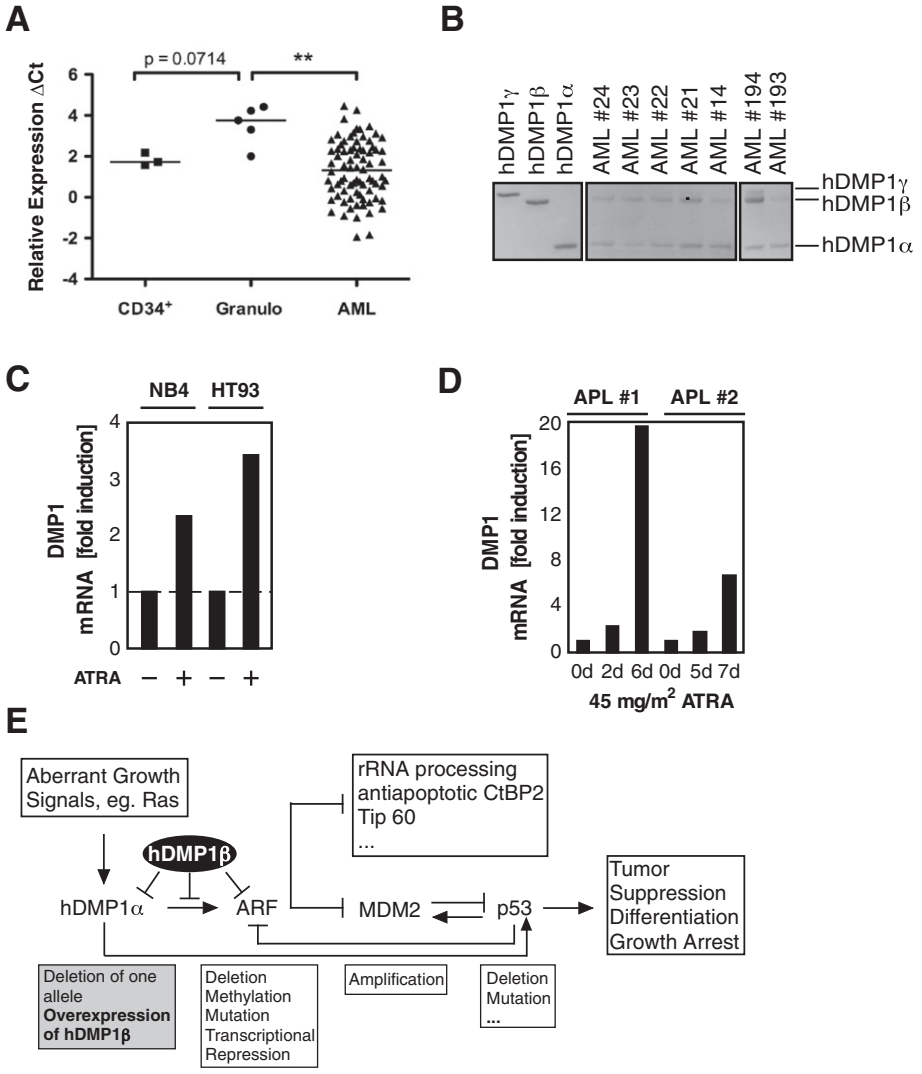

Fig. 4. Overall human DMP1 mRNA expression and splice variant analysis in primary acute myeloid leukemia (AML). A) Significantly lower expression of *DMP1* mRNA levels in AML blasts compared to granulocytes or $CD34^+$ cells from healthy donors. Values are the differences in Ct-values between DMP1 mRNA and the levels of the housekeeping genes HMBS and ABL1. **Mann–Whitney U, $p < 0.01$. B) DMP1β mRNA levels relative to DMP1α in primary leukemic patient samples. DMP1 splice variant cDNAs were used as size and efficiency controls for the PCR reaction. C) NB4 and HT93 acute promyelocytic leukemia cells were differentiated in vitro for 4 days using 1 μM *all-trans* retinoic acid (ATRA). mRNA levels are expressed as n-fold changes in regulation compared to untreated cells using HMBS mRNA expression as a reference gene. Average values from two experiments are shown. D) Two patients with newly diagnosed APL t(15;17) were treated with orally administered all-*trans* retinoic acid (ATRA) at a dosage of 45 mg/m^2 daily. DMP1 mRNA expression levels in blast cells were assessed by qPCR. Values were normalized to HMBS and day 0 as the experimental starting point. E) A model of the oncogenic DMP1β pathway. DMP1α is activated upon oncogenic signaling and can induce growth arrest via the ARF/MDM2/p53 pathway. In addition, DMP1α can induce p53-independent cell cycle stop via alternative targets of ARF such as CtBP2, Tip60 and rRNA processing or p53. DMP1β may promote proliferation by binding and inhibiting the anti-proliferative DMP1α or by DMP1α-independent pathways, possibly by an as of yet unknown function of the β-specific protein domain.

mechanism that is functionally appealing to explain the inhibitory mechanism of the β-isoform, we cannot exclude the possibility that the unique 35-amino acid protein domain of the β-isoform binds cellular cofactors that disrupt DMP1α-induced transcription of DMP1 regulated genes. In fact, the importance of this unique domain was striking in our experiments, where removing the 35-amino acid protein domain severely impaired the function of human DMP1β (Fig. 2A).

Despite the complexity of ARF regulation mediated by DMP1 isoforms, we propose a direct role for DMP1β in damping the cellular pathways controlling proliferation. We present evidence that ectopic expression of DMP1β, as well as significant downregulation of DMP1α together with other DMP1 splice variants, result in maintenance of proliferation of 293T, U937 and HeLa cell lines cultured in serum-deprived media. Moreover, we show that macrophage differentiation induced growth arrest of PMA-treated U937 cells was ineffective when the ratio of α/β isoform is inverted through ectopic expression of DMP1β or through disruption of DMP1 transcript levels. Additionally, the gain of proliferative function with disruption of DMP1 transcripts in PMA-induced macrophages was independent of mutated (HL-60 and U937 cells) or functional p53 (MOLM-13 cells). These findings imply that ectopic expression of DMP1β disrupts the delicate DMP1α/β balance, thus altering cellular proliferation control, possibly through ARF and independent of p53.

Our results connect cellular phenotypes resulting from the loss of DMP1 to that mediated by ectopic expression of DMP1β. The cell lines used in this study expressed wild type p53 or were p53-deficient either due to expression of viral oncogenes or p53 mutations. We propose that proliferation in DMP1 disrupted monocytic cells and macrophages occur via diminished ARF cellular function in a p53-independent fashion. Weber and colleagues have shown that DMP1α remains operational via ARF regulation in p53-deficient mouse cells, where mDmp1α induces growth arrest in p53-null mouse embryo fibroblasts, but not

in $p19^{ARF}$ or $p19^{ARF}$/p53 double-knockout mouse embryo fibroblasts [39]. Furthermore, both the activity of the ARF/p53 and the non-p53 pathways are impaired in Dmp1-null and heterozygous mice, suggesting a physiological role of mDmp1α in oncogenic as well as normal cellular control. p53-independent cell cycle and apoptosis control via ARF is most likely conducted by a wide range of ARF binding partners, such as B23/nucleophosmin, DP1 and Tip60 [40–42]. It has been proposed that DMP1β functions independently of the ARF-DMP1 axis [9], perhaps through direct interaction with p53. This mechanism is not without precedent as DMP1α has been shown to interact directly with p53 [7]. However, we cannot dismiss additional as of yet undiscovered target genes/pathways that may be important in DMP1β imparting cellular function. Nevertheless, our findings demonstrate that ectopic expression of DMP1β disrupts the delicate DMP1α/β balance, thus altering cellular proliferation control.

Molecular events leading to AML include both disruption/blocks at various stages of myeloid differentiation in combination with gains of survival and proliferation [32]. Recent studies have demonstrated that low levels of $p14^{ARF}$ are an independent predictor for poor survival in patients with AML [43]. Although it has been shown that AML1-ETO fusion protein expression represses activation of ARF [20,44,45], most AML samples demonstrating decreased levels of ARF were not AML1-ETO positive. We found that DMP1 transcripts are dampened in AML samples, with levels similar to freshly isolated $CD34^{+}$ cells, rather than granulocytes [8]. Furthermore, 5/7 of the AML patient samples demonstrated DMP1α/β transcript ratios below 1, indicating a greater abundance of DMP1β over DMP1α, and lower than the DMP1α/β transcript ratio of ≥1.5 reported for differentiating myeloid progenitors [8]. ATRA treatment of APL patient samples promoted differentiation to granulocytes and was coincident with an increase in DMP1 transcripts to levels similar to normal granulocytes. The increase in DMP1β transcript abundance in AML cells may reflect a myeloid stage specific condition associated with reduced tumor suppression and increased proliferation. Mallakin and colleagues have proposed that DMP1 is haplo-insufficient for tumor suppression in human lung and breast cancer [46–48]. In addition, the authors found that DMP1β over α transcript ratios were increased 54.8% in ER+/HER2- and 42.9% in triple negative primary breast tumors as compared to adjacent normal breast tissue [9]. A recent study by the same group has shown that increased DMP1β transcripts and proteins levels in breast cancer tumors correlated with increased relapse rate [9]. Future evaluations of DMP1 transcript variant levels and protein isoform amounts in additional types of human hematopoietic and solid cancers should prove insightful as to their involvement in specific cancer types.

We originally demonstrated that DMP1β is expressed during early stages of hematopoietic differentiation [8] and now show that DMP1β has the potential to blunt DMP1α regulation of ARF during myeloid cell differentiation. A recent publication by Kobayashi and Srour utilizing Dmp1-null and -hemizygous mice demonstrated enhanced proliferation in the stem and progenitor cell populations [18]. Both our earlier study and Kobayashi's and Srour's study underscore the importance of DMP1 in regulation of normal hematopoietic cellular proliferation and differentiation. Our current findings extend the cellular role of DMP1 isoform regulation by demonstrating that loss of DMP1 isoforms or increased expression of DMP1β result in a similar phenotype: Increased cell proliferation of myeloid cells. Our findings on AML, as well as those of Maglic and colleagues in breast cancer [9], point out a possible pathological role for the dysregulation of DMP1 isoforms. The ability of DMP1β to negatively regulate ARF places it in the ranks of other negative ARF regulators, such as Bmi-1, JunD, Tbx-2/3, Twist, and p53 [49–52]. It is tempting to speculate that a cellular balance of DMP1α/β fine-tunes ARF regulation during normal cellular differentiation and maintains growth/proliferation during differentiation by counterbalancing the potent effects of DMP1α, as shown in the diagram in Fig. 4E. Although limited human cancer samples have been evaluated for DMP1 splice variant expression to date, it is intriguing to propose that increased expression of DMP1β or loss of DMP1α will have similar functional consequences.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2015.07.009.

Transparency document

The Transparency document associated with this article can be found, in online version.

Abbreviations

FAB	French–American–British
PMA	phorbol 12-myristate 13-acetate;
h	human
m	mouse
nt	nucleotide
RLA	relative luciferase activity
shRNA	short hairpin RNA
VSV-G	vesicular stomatitis virus G protein
AML	acute myeloid leukemia. ARF, $p14^{ARF}$

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