Published in final edited form as:
Cancer Res. 2010 December 1; 70(23): 9875–9885. doi:10.1158/0008-5472.CAN-10-1100.

Blockade of the Ubiquitin Protease UBP43 Destabilizes the Transcription Factor PML/RARα and Inhibits Growth of Acute Promyelocytic Leukemia

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Abstract

New treatments for acute promyelocytic leukemia (APL) are needed. APL cell treatment with all-*trans*-retinoic acid (RA) degrades the chimeric, dominant negative-acting transcription factor PML/RARα, which is generated by chromosomal translocation. The E1-like ubiquitin-activating enzyme UBE1L associates with interferon stimulated gene ISG15 that binds and represses PML/RARα protein. Ubiquitin protease UBP43/USP18 removes ISG15 from conjugated proteins. In this study, we explored how RA regulates UBP43 expression and the effects of UBP43 on PML/RARα stability and APL growth, apoptosis and differentiation. RA treatment induced UBE1L, ISG15 and UBP43 expression in RA-sensitive but not RA-resistant APL cells. Similar *in vivo* findings were obtained in a transgenic mouse model of transplantable APL and in the RA response of leukemic cells harvested directly from APL patients. UBP43 knockdown repressed PML/RARα protein levels and inhibited RA-sensitive or RA-resistant APL cell growth by destabilizing the PML domain of PML/RARα. This inhibitory effect promoted apoptosis but did not affect the differentiation response in these APL cells. In contrast, elevation of UBP43 expression stabilized PML/RARα protein and inhibited apoptosis. Taken together, our findings define UBP43 as a novel candidate drug target for APL treatment.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest exist.

Keywords

UBP43/USP18; UBE1L; ISG15; acute promyelocytic leukemia; and PML/RARα

Introduction

Acute promyelocytic leukemia (APL) is a distinct subset of myeloid leukemia (FAB, M3) (1). The t(15;17) rearrangement present in APL fuses the promyelocytic leukemia gene (PML) on chromosome 15 with the retinoic acid receptor α (RARα) on chromosome 17 (2,3). PML/RARα is a dominant-negative translocation product etiologic for APL (2,3). Engineered PML/RARα transgenic mice spontaneously develop leukemia, as reviewed (4,5). All-*trans*-retinoic acid (RA) successfully treated APL patients by triggering leukemic cell differentiation (6). One retinoid mechanism activated is the induced proteasomal degradation of the RARα domain of PML/RARα that inhibits its dominant-negative effects, as previously reviewed (4,5).

Another mechanism activated by RA-treatment independently affects stability of the PML domain of PML/RARα protein. This engaged the E1-like ubiquitin-activating enzyme (UBE1L), which associates with the interferon-stimulated gene 15 (ISG15) (4,7–9). ISG15 conjugation of modified proteins is removed by the ubiquitin protease 43 (UBP43/USP18) (4,10,11).

Ubiquitination can regulate diverse biological processes, including proteasomal degradation of proteins. ISG15, like ubiquitin, can complex with proteins and the functional consequences of this are under active study (10,11). ISG15 conjugation is executed by an enzymatic cascade that includes UBE1L, an E2-conjugating enzyme (UbcH8), and E3 ligases (12). ISG15 conjugation is specifically removed by UBP43, a member of the ubiquitin specific protease (USP) family (13,14). Upon type I interferon (IFN) or lipopolysaccharide (LPS) treatment, ISG15, UBP43, and UBE1L are each rapidly up-regulated (13,15–17).

Regulation of ISG15ylation by UBP43 is associated with diverse biological processes (18,19). For example, UBP43 over-expression can inhibit cytokine-induced terminal differentiation of monocytic leukemia cells (20). UBP43-deficient cells were reported as hypersensitive to IFN treatment (13,21). Studies in engineered mice revealed that UBP43 is important for homeostasis of ISG15-conjugated proteins, which exert specific biologic effects (22,23).

RA-mediated differentiation of APL cells is accompanied by PML/RARα proteolysis through caspase- and proteasome-dependent degradation pathways, as previously summarized (4,5,24). Loss of PML/RARα expression and induction of apoptosis in APL cells followed arsenic treatment (25) or transfection of ribozymes that targeted PML/RARα for repression (26). RA-treatment augmented expression of UBE1L, a retinoid target that repressed PML/RARα protein (8,9). This also increased ISG15 expression in APL cells (7–9). These proteins repressed PML/RARα expression through a mechanism distinct from that of the ubiquitin-proteasome pathway (7–9,27,28).

This study explored whether RA regulated UBP43 expression. The kinetics of augmented UBP43 expression in retinoid sensitive versus resistant APL cells were related to UBE1L and ISG15 expression profiles. Anti-UBP43 antisera were derived for UBP43 immunoblot and enzymatic activity studies in APL cells. The consequences of gain or loss of UBP43 expression on PML/RARα stability as well as on APL growth, apoptosis and differentiation

were each examined. The UBE1L-ISG15-UBP43 pathway was studied before and after RA-treatment of a transgenic APL model and of leukemic cells harvested directly from APL patients. These findings define the ubiquitin protease UBP43 as a tractable pharmacologic target for treatment of APL.

Materials and Methods

Cell Culture

RA-sensitive NB4-S1 and RA-resistant NB4-R1 APL cell lines (7) were cultured in Advanced RPMI 1640 (Invitrogen, Carlsbad, CA) media with 2% fetal bovine serum (FBS, Gemini, Calabasas, CA), 4mM L-glutamine (Invitrogen), 100units/ml penicillin (Invitrogen), and 100μg/ml streptomycin (Invitrogen) (8,9). BEAS-2B immortalized human bronchial epithelial (HBE) cells were cultured in serum-free LHC-9 media (Biofluids, Akron, OH) (7,8). COS-7 cells were cultured in Advanced DMEM (Invitrogen) media with 10% FBS, 4mM L-glutamine, 100units/ml penicillin, and 100μg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Real-time RT-PCR Assays

Total cellular RNA was isolated using Trizol Reagent (Invitrogen) and cDNA synthesis was performed using the High-Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA) and a Peltier Thermal Cycler (MJ Research, Waltham, MA). Real-time RT-PCR assays were performed with iTaq Fast SYBR Green Supermix with an ROX Kit (Bio-Rad, Hercules, CA) and the 7500 Fast Real-time PCR System (Applied Biosystems). Three independent replicate experiments were performed. Primers were: human UBP43 forward primer: 5’-GAGGCTGGACGCTTGCAT-3’ and reverse primer: 5’-AGCACGACTTCACTTCCAGGAA-3’; human UBE1L forward primer: 5’-TGGGCACCTTGTGTCATAAGC-3’ and reverse primer: 5’-CTCAGAGTGAGAATGCCAGGG-3’; human ISG15 forward primer: 5’-TGTCGGTGTCAGAGCTGAAG-3’ and reverse primer: 5’-GCCCTTGTTATTCCTCACCA-3’; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer: 5’-ACGTGTCAGTCAGTGGTGGACCT-3’ and reverse primer: 5’-GTCCACCACCCTGTTGCTG-3’. The following primers detected murine species: UBP43 forward primer: 5’-TTGGGCTCCTGAGGAAACC-3’, and reverse primer: 5’-CGATGTTGTGTAAACCAACCAGA-3’; UBE1L forward primer: 5’-CTACGAGCGACTCCATATACCT-3’ and reverse primer: 5’-TACACACAGGGTAGGGAGCAT-3’; ISG15 forward primer: 5’-AACTGCAGCGAGCCTCTGA-3’ and reverse primer: 5’-CACCTTCTTCTTAAGCGTGTCTACAG-3’; GAPDH forward primer: 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse primer: 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Expression Plasmids and Transient Transfection

The pcDNA4-UBP43, pSG5-UBE1L, His_6-tagged-pcDNA3-ISG15, pCMV-hemagglutinin (HA)-PML/RARα, pCMV-HA-PML, and pCMV-HA-RARα expression vectors were previously described (7–9). The full-length coding region of human UBP43 was cloned into the pRetroX-IRES-ZsGreen1 vector (Clontech, Mountain View, CA) at BamH1 and Not1 restriction endonuclease sites to engineer the pRetroX-IRES-ZsGreen1-UBP43 retrovirus. The insertless retrovirus served as a control. The enhanced green fluorescent protein (EGFP) expression plasmid (EGFP-N2, Clontech), shRNA-UBP43 retroviruses (Open Biosystems, Huntsville, AL) and an insertless control vector (Open Biosystems) were purchased.

Independent transient transfection of logarithmically growing BEAS-2B and COS-7 cells was accomplished using Effectene Transfection Reagent (Qiagen, Valencia, CA) and optimized methods (29). EGFP expressing plasmids were used to assess transfection efficiencies. Transfections were conducted in triplicate and each experiment was independently replicated three times.

Independent transient transfection of NB4-S1 and NB4-R1 APL cells with small interfering RNAs (siRNAs) was accomplished using the Nucleofector Technology (Lonza, Walkersville, MD) (9). The siRNAs targeting UBP43 or a RISC-free control siRNA were synthesized (Dharmacon, Lafayette, CO). Different siRNAs targeted UBP43 and were: UBP43 siRNA 1 (5'-CTGCATATCTTCTGGTTTA-3') and UBP43 siRNA 2 (5'-GGAAGAAGACAGCAACATG-3'). Transfection efficiency was monitored by co-transfecting the siGLO Green Transfection Indicator (Dharmacon).

Generation of Stable UBP43 Expressing APL Transfectants

The pRetroX-IRES-ZsGreen1-UBP43 retroviral vector and an insertless control retrovirus were independently transfected into the RetroPack PT67 Packaging Cell Line (Clontech) using FuGENE 6 (Roche, Indianapolis, IN). Viral supernatants from transfectants were used to transduce NB4-R1 or NB4-S1 cells with 4μg/ml polybrene (Sigma, Milwaukee, WI). GFP positive cells were harvested 48 hours later using a FACStar Plus cytometer (Becton Dickinson, San Jose, CA). This was repeated a week later to enrich for transductants, as before (7). Doubly selected cells were studied. Three independent experiments were conducted with each study performed in triplicate.

The short hairpin RNA (shRNA) retrovirus selected for knock-down of UBP43 was isolated from PT67 cells and used to transduce independently NB4-S1 and NB4-R1 cells. Stable transductants were selected after 14 days of puromycin (2μg/ml, Sigma) treatment. Several candidate shRNAs were transduced into NB4 cells and the one that most prominently knocked-down UBP43 was chosen for study.

Generation of Anti-UBP43 Antisera

Two rabbit polyclonal antibodies were derived (Covance, Denver, PA) against human UBP43 protein using one peptide nearer to the amino terminus (FDVDSKPLKTLEDALHC, anti-UBP43-1) than the other (CGKKTRGKQVLKLTHLPQ, anti-UBP43-2). Antibody specificities were confirmed by immunoblot analyses of COS-7 transfected cells with the pcDNA4-UBP43 construct versus controls. Pre-immune antisera were used as additional controls.

Immunoblot Analyses

APL and other cells were lysed with ice-cold radioimmunoprecipitation (RIPA) buffer using optimized methods (8,9,30). Lysates were size-fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assays before transfer to nitrocellulose membranes (Whatman, Piscataway, NJ). Primary antibodies for immunoblot analyses were a rabbit polyclonal antibody that recognized PML-RARα (Abcam Inc, Cambridge, MA), a murine monoclonal antibody that recognized HA-tagged proteins (Babco, Richmond, CA) and a goat polyclonal antibody that recognized actin (Santa Cruz, Santa Cruz, CA). Anti-mouse and anti-rabbit antisera were purchased (Amersham, Piscataway, NJ) as was an anti-goat (Santa Cruz) antiserum. These were used as respective secondary antibodies. Quantifications of signals were scored as before (7–9). To assess PML/RARα protein stability after UBP43 transfection, cells were treated with or without cycloheximide (CHX, 40μg/ml, Sigma) for indicated time periods.

Measurement of UBP43 Activity

UBP43 enzymatic activity in APL cells was assayed using established methods (31). Briefly, NB4-S1 cells were lysed in RIPA buffer and protein supernatants were incubated with or without HA-ISG15-vinylmethyl ester (HA-ISG15-VME, Boston Biochem Inc, Cambridge, MA) at 37°C for 60 min before incubation with protein G agarose beads (Pierce, Rockford, IL) and an anti-HA antibody (Santa Cruz) for at least 2 hours at 4°C to precipitate complexes. The HA-ISG15-VME probe forms an irreversible covalent bond with active deubiquitinases. Immunoblot analyses were independently performed using an anti-HA (Rockland, Gilbertsville, PA) or an anti-UBP43 antibody (anti-UBP43-1 or anti-UBP43-2).

Proliferation and Apoptosis Assays

The day before proliferation assays were conducted, the desired cells (1×10^5 cells/ml) were plated onto individual 6-well tissue culture plates. Three independent wells were seeded in each experiment with triplicate independent replicate experiments performed. Proliferation was measured using the CellTiter-Glo Assay Kit (Promega, Madison, WI) and established methods (29). Apoptosis was measured by Annexin V:FITC positivity by flow cytometry using the Annexin V Assay Kit (AbD Serotec, Raleigh, NC). Apoptosis within engineered APL cell lines was assayed with the Caspase-Glo 3/7 Assay Kit (Promega).

Expression Studies in Transgenic APL Mice

Murine transgenic APL studies used previously optimized methods (32). The experiments were performed after review and approval by Dartmouth's Institutional Animal Care and Use Committee (IACUC). Briefly, four female FVB mice (seven-weeks-old) were tail vein injected with 2×10^5 transgenic APL donor cells for each time point. Two of these mice were treated with RA and two were treated with dimethyl sulfoxide (DMSO) as a vehicle control. Twenty days after these injections, RA (2mg) was intraperitoneally (ip) injected into each of two mice and the same DMSO volume was administered to each control mouse. Clinical APL was evident 20 days after donor APL cell injections (32). RA-treated and control mice were independently sacrificed at indicated time points using IACUC-approved procedures. APL cells were harvested from spleens, as in prior work (32). Total RNA was isolated as before (7–9) for real-time RT-PCR assays. Findings from all mice treated in each respective arm were pooled for analyses.

UBP43 Expression in Human APL Cells

Fresh APL cells were harvested as part of an Institutional Review Board (IRB) approved protocol using previously optimized methods (32). APL cells were cultured in the presence of RA or vehicle (DMSO). RNA was harvested 24 hours after RA (1μM) or vehicle treatments and differentiation response was scored by the percentage of nitrotetrazolium blue (NBT) stained cells after 5 days of RA (100nm) or vehicle treatments. Real-time RT-PCR assays for UBE1L, ISG15, UBP43 and GAPDH were each performed after 24 hours of RA-treatment and results were compared to vehicle controls.

Statistical Analyses

Two-tailed T-tests were used. Results appear as means ± standard deviation (SD). Statistical significance is noted in the text and figures as well as with these symbols: $P < 0.01$ (*) and $P < 0.005$ (**).

Results

UBP43 Expression in APL Cells

UBE1L, a retinoid target (7,8), along with its ubiquitin-like partner ISG15, destabilized PML/RARα (9) and cyclin D1 (33) via ISG15 conjugation to these respective proteins. That the ISG15 deconjugase UBP43 antagonized UBE1L-mediated repression of these proteins (9,33) implicated UBP43 as a pharmacologic target. This study determined RA-treatment effects on mRNA expression profiles of UBE1L, ISG15, and UBP43 in RA-sensitive NB4-S1 and in RA-resistant NB4-R1 APL cells using real-time RT-PCR assays (Fig. 1A). UBE1L and ISG15 mRNA expression profiles were augmented by RA-treatment beginning at 12 hours and these increased further in NB4-S1 cells at later time points (Fig. 1A). The maximal induction observed after RA-treatment was at 72 hours for UBE1L, at 96 hours for ISG15, and at 48 hours for UBP43.

In contrast, UBE1L, ISG15 and UBP43 inductions were markedly blunted despite RA-treatment of NB4-R1 APL cells. UBP43 mRNA expression (relative to vehicle controls) was augmented by RA-treatment of NB4-S1 cells after observed increases in UBE1L and ISG15 expression. This delayed UBP43 induction was consistent with an indirect RA-treatment effect on UBP43 expression in APL cells. This was supported by bioinformatic analysis of the UBP43 promoter. This did not reveal the presence of retinoic acid response elements in the UBP43 promoter and UBP43 reporter activity in transfected BEAS-2B cells was not appreciably affected by RA-treatment (data not shown).

UBP43 Protein Expression in APL Cells

Different rabbit polyclonal antibodies recognizing UBP43 protein were derived (see Materials and Methods) to examine RA-treatment effects on UBP43 protein expression in APL cells. To confirm specificity of the two anti-UBP43 antibodies, COS-7 cells that did not basally express detectable UBP43 protein were transfected with a human UBP43 or an insertless control vector. As expected, COS-7 UBP43 transfectants expressed UBP43 protein identified by either anti-UBP43-1 or anti-UBP43-2 antibodies, but cells transfected with an insertless vector did not express this protein (Fig. 1B and Fig. 1C). In each case, pre-immune serum was used as a negative control (Fig. 1B and Fig. 1C).

UBP43 is reported as the protease specific for ISG15 (13,34). To confirm that the expected complex between ISG15 and UBP43 formed in APL cells, NB4-S1 cell lysates were immunoprecipitated with an anti-HA antibody, before immunoblotting independently with an anti-HA or the anti-UBP43-1 antibody. UBP43 conjugated with ISG15 in these APL cells, as confirmed by an anti-HA or the anti-UBP43-1 antibody (Fig. 2A, two left panels). HA-tagged ISG15 is identified (hatched arrow, left panel) along with other possibly non-specific species in Fig. 2A (left and middle panels). UBP43 is expressed and active in these cells. Fig. 2A (right panel) shows that UBP43 can remove ISG15 from PML/RARα protein.

RA-regulation of UBP43 protein was next examined. Induction of UBP43 protein followed RA-treatment of RA-sensitive NB4-S1 cells, but not of RA-resistant NB4-R1 cells (Figs. 2B and 2C). Quantifications of signals appear below these immunoblots. RA-treatment augmented UBE1L and ISG15 protein expression and destabilized PML/RARα protein (8,9). UBP43 mRNA induction followed that of UBE1L and ISG15 mRNAs, raising the possibility that UBP43 directly affected PML/RARα protein stability. Experiments were conducted to examine this.

UBP43 Affected PML/RARα Stability

UBE1L repressed PML/RARα protein by targeting the PML, but not the RARα domain of PML/RARα (9). UBP43 antagonized this UBE1L effect (9). UBP43 effects on stabilities of different PML/RARα domains were uncovered by transient co-transfection experiments using constructs (9) expressing full-length PML/RARα, or respective PML or RARα domains of PML/RARα. Immunoblot analyses revealed that UBP43 co-transfection in BEAS-2B cells enhanced expression of both full-length PML/RARα (Fig. 3A, construct 1) and the PML domain of PML/RARα (Fig. 3A, construct 2), but no effects were observed on the expressed RARα domain of PML/RARα (Fig. 3A, construct 3). Quantification for each respective signal is presented in the corresponding right panels of Fig. 3A. Similar transfection efficiencies were achieved in each arm of the experiments as confirmed by EGFP expression vector co-transfection and immunoblot experiments, as displayed in this figure. Engineered UBP43 over-expression did not appreciably affect the proportion of EGFP-expressing transfected cells (data not shown and Fig. 3A).

To study further UBP43 effects on PML-RARα protein stability, UBP43 was co-transfected with an HA-tagged PML-RARα expression vector in BEAS-2B cells in the presence and absence of the protein synthesis inhibitor CHX. UBP43 transfection increased PML-RARα protein stability, despite CHX treatment (Fig.3B).

UBP43 and Apoptosis

Prior work found that UBE1L triggered PML/RARα degradation and apoptosis in APL cells (7–9). Whether UBP43 affected apoptosis in APL cells by targeting PML-RARα protein was studied. To ascertain effects of UBP43 on PML/RARα expression, two different siRNAs targeting UBP43 and a RISC-free control siRNA were independently transfected into NB4-S1 cells (Fig. 3C, left panel). Knock-down of UBP43 by each of these UBP43-targeting siRNAs significantly decreased UBP43 and PML/RARα immunoblot expression versus controls in APL cells. Actin expression was unaffected (Fig. 3C). Compared with RISC-free siRNA controls, knock-down of UBP43 by each siRNA targeting UBP43 significantly augmented apoptosis, as confirmed by annexin V and propidium iodide (PI) staining and fluorescence activated cell sorting (FACS) analysis (Fig. 3D). Compared to RISC-free control cells, UBP43 knock-down in NB4-S1 cells augmented apoptosis at day 1 and this increased over the 3 days of this study. A decline in PML/RARα protein accompanied this (Fig. 3C).

To confirm and extend these transient UBP43 knock-down findings, stable retroviral shRNA-mediated repression of UBP43 was achieved. The consequences of this on PML/RARα expression and on apoptosis were studied in NB4 transductants. Stable knock-down of UBP43 was engineered by retroviral transductions and puromycin selection of the desired shRNA independently expressed in NB4-R1 and NB4-S1 cells. Fig. 4A established that shRNA-mediated UBP43 knock-down reduced endogenous PML/RARα expression in both transduced APL cell lines (relative to insertless vector controls). Stable UBP43 knock-down in NB4-R1 and NB4-S1 cells significantly increased apoptosis in both cells (Fig. 4B). RA-treatment of UBP43 knock-down NB4-S1 cells did significantly promote apoptosis, but did not affect differentiation response (supplemental Fig. 1). Fig. 4B showed that compared with these respective control transfectants, UBP43 knock-down in these APL cells produced significant (**, $P < 0.005$) apoptosis.

UBP43 over-expression was independently achieved in NB4-S1 and NB4-R1 cells, as shown in Fig. 5. Unlike UBP43 knock-down, retroviral-mediated UBP43 over-expression augmented PML/RARα expression in both APL cell lines (Fig. 5A) and reduced apoptosis (Fig. 5B) relative to insertless vector controls.

UBP43 Regulated APL Cell Growth

Since UBP43 knock-down promoted apoptosis in APL cells by targeting PML/RARα for repression, effects of UBP43 knock-down on growth were studied in NB4 cells with engineered loss or gain of UBP43 expression. CellTiter-Glo assays confirmed that UBP43 knock-down conferred a marked repression of NB4-S1 and NB4-R1 cell growth (Fig. 4C). In contrast, engineered UBP43 over-expression significantly (**, $P < 0.005$) promoted growth, as compared to insertless vector controls for both APL cell lines (Fig. 5C).

RA-Treatment of APL Mice

To determine whether RA induced UBP43 expression *in vivo*, a murine transgenic transplantable APL model was studied (35). Clinical evidence of APL occurred in recipients 20 days after transgenic APL cell injections into FVB mice. Mice were then sacrificed and RNA was isolated from harvested spleens (see Materials and Methods). UBE1L, ISG15 and UBP43 mRNA expression profiles were each significantly augmented after RA-treatment versus vehicle control-treated APL mice (Fig. 6A). These findings extended results from cultured APL cells to the setting of APL in mice.

RA-Treatment of APL Cells from Patients

Whether RA increased UBE1L, ISG15 and UBP43 mRNA expression profiles in cultures of leukemic cells was studied in APL cells harvested directly from patients. APL cells from two different RA-responsive cases augmented UBE1L, ISG15 and UBP43 mRNA expression after 24 hours of RA-treatment relative to controls (Fig. 6B). No significant change in UBE1L expression was observed, but when the ISG15 and UBP43 results after RA-treatment were pooled, significant changes were observed ($P = 0.0024$, $N = 3$). RA-treatment also caused differentiation to occur in RA-sensitive cases, as shown by increased NBT positive APL cells (Fig. 6B). Cells from a representative RA-resistant APL case with an inactivating PML/RARα ligand-binding domain mutation similar to that in NB4-R1 cells (36,37) were examined. Deregulated UBE1L, ISG15 and UBP43 expression and minimal NBT augmentation were observed, despite RA-treatment of these APL cells (Fig. 6B).

Discussion

This study builds on prior work that revealed retinoid treatment of APL cells augmented UBE1L and ISG15 expression and ISG15ylation (8,9) by showing that the deconjugase, UBP43, is also regulated by RA-treatment of APL cells. RA-induction of UBP43 mRNA did not increase until 48 hours (Fig. 1A). That retinoid effects on UBP43 were indirect was consistent with bioinformatic analysis and UBP43 reporter assay results. RA-mediated induction of UBP43 occurred in RA-sensitive, but not in RA-resistant APL cells (Figs. 1 and 2). RA-treatment also augmented UBE1L and ISG15 mRNA expression in RA-sensitive, but not in RA-resistant APL cells. Increased UBE1L and ISG15 expression accompanied RA-treatment, but occurred earlier than did UBP43 induction. This indicated a negative regulatory loop likely exists to limit ISG15ylation (8) via induction of UBP43. Assays displayed in Fig. 2A showed that UBP43 was expressed and functionally active in APL cells.

PML/RARα expression is diagnostic for APL (2,3). Leukemic cell growth and differentiation are linked to PML/RARα expression (4,5). Loss of PML/RARα markedly affected APL cell growth. Prior work revealed that ribozyme-mediated repression of PML/RARα was anti-leukemogenic at least partly by triggering apoptosis of RA-sensitive and resistant APL cells (26,37). RA and arsenic trioxide also individually triggered PML/RARα degradation and conferred differentiation or apoptosis, respectively, as reviewed (38). RA-treatment caused PML/RARα degradation through caspase- and proteasome-dependent

mechanisms (9,28,39). RA engaged caspase-3, which targeted PML/RARα for repression (39). Likewise, UBE1L was induced by RA-treatment and this repressed PML/RARα expression as well as promoted apoptosis (7).

IFN treatment augmented UBE1L, ISG15 and UBP43 expression (13,15–17). Retinoids can cross-talk with UBE1L and ISG15 protein expression (7–9,33). This was previously implicated in regulating PML/RARα stability and apoptosis (7,9). That UBP43 is an anti-neoplastic target was suggested by previous retinoid work (7,9).

The present study extended that work by showing UBP43 regulated PML/RARα protein stability. Prior findings indicated that PML/RARα can undergo ISG15ylation and target the PML domain for repression, but in contrast RA affected PML/RARα stability through its RARα domain (9). These findings reveal that two distinct degradation pathways are activated by RA-treatment. In the current study, transfection experiments found that UBP43 also has domain-specific PML/RARα effects that targeted for repression the PML but not RARα domain, in accord with prior work that studied UBE1L effects in APL cells (9). Future work should determine which residue(s) in the PML domain of PML/RARα undergo ISG15 modification.

This study directly linked UBP43 to PML/RARα stabilization by showing that increased PML/RARα protein stability followed UBP43 transfection, despite CHX treatment (Fig. 3B). As expected, knock-down of UBP43 destabilized PML/RARα protein and promoted apoptosis in APL cells (Figs. 4 and 5). Yet, differentiation was not apparently affected as was the case following ribozyme-dependent or arsenic-mediated PML/RARα degradation (25,26,37). In contrast, UBP43 over-expression stabilized PML/RARα protein and promoted APL cell growth (Figs. 4 and 5), indicating that PML/RARα exerts anti-apoptotic effects in APL.

Prior work directly implicated PML in regulation of apoptosis and proliferation (40). PML effects on cell growth might be from its ability to promote apoptosis through intrinsic or extrinsic pathways (41). PML affects apoptosis through different mechanisms, either by serving as a transcriptional modification and activation platform for the p53 tumor suppressor, or by regulating expression and localization of apoptotic regulators, as reviewed (40–42). Prior work found that arsenic treatment increased PML assembly into PML-nuclear bodies, important for PML regulation of apoptosis. This assembly depends on post-translational modification of PML with SUMO-1 (40–42). SUMO is related to ISG15, but SUMOylation engages distinct effects, as reviewed (40–42).

The hypothesis that UBP43 is an anti-neoplastic target is consistent with recent work reported with the deubiquitinase USP9X, which promoted cancer cell survival by regulating stability of a pro-survival BCL2 family member (43). Analogous to that study, it was found here that knock-down of another deconjugase, UBP43, destabilized PML/RARα protein and triggered apoptosis while gain of UBP43 expression opposed these effects. Notably, UBP43 was found to be active in APL cells (Fig. 2A). Findings from mice and patients with APL revealed that UBP43 is expressed in APL cells and regulated by RA-treatment (Fig. 6). Given this, pharmacological inhibition of UBP43 would likely affect not only APL biology, but also APL therapy.

In summary, this study identified UBP43 as an anti-neoplastic target in APL. UBP43 directly affected PML/RARα stability and regulated APL cell growth. Knock-down of UBP43 repressed PML/RARα and this in turn reduced APL cell growth by promoting apoptosis. Pharmacologic targeting of the UBE1L-ISG15-UBP43 pathway, especially through inhibition of UBP43, is an appealing therapeutic approach to consider in APL and other malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Sutisak Kitareewan for his helpful consultation. This work was supported by National Institutes of Health (NIH) and National Cancer Institute (NCI) grants RO1-CA087546 (E.D.), RO1-CA111422 (E.D.), RO1-CA062275 (E.D.), R01-CA56771 (R.G.), by a Samuel Waxman Cancer Research Foundation Award (E.D.), by a Prouty Grant from the Norris Cotton Cancer Center (E.D.), and by an American Cancer Society Institutional grant (S.J.F.). E.D. is an American Cancer Society Clinical Research Professor supported by a generous gift from the F. M. Kirby Foundation.

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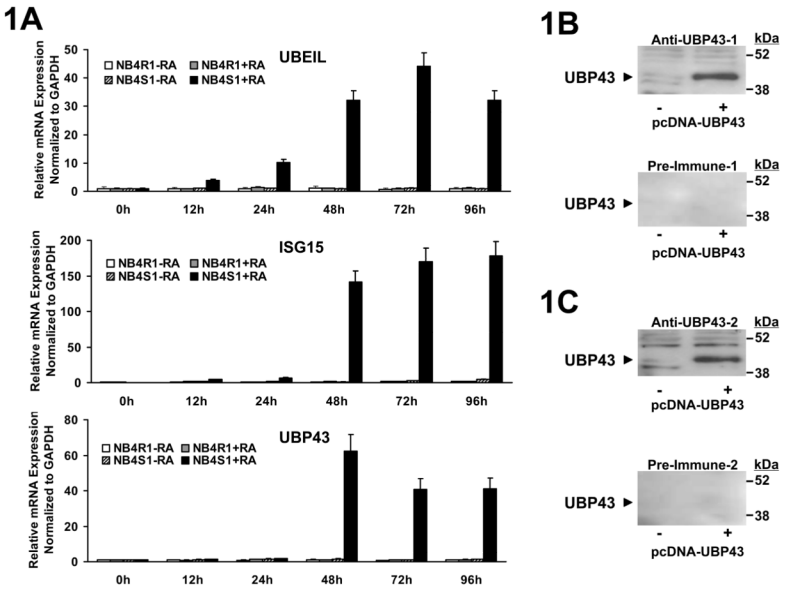

Fig. 1.

All-*trans*-retinoic acid (RA) regulation of UBE1L, ISG15, and UBP43 mRNA expression in RA-sensitive versus RA-resistant acute promyelocytic leukemia (APL) cells. (A) RA-sensitive NB4-S1 cells and RA-resistant NB4-R1 APL cells were each cultured with RA (1μM) or with vehicle for the indicated times in hours (h). UBE1L, ISG15, UBP43 and GAPDH mRNAs were each assessed by real-time RT-PCR assays. Similar results were obtained from three independent experiments. A representative result is shown. RA-treatment induced UBE1L and ISG15 before UBP43 expression in NB4-S1 cells, but these species were not induced in NB4-R1 APL cells. (B and C) Immunogenic peptides generated the indicated anti-UBP43 polyclonal antibodies (see Materials and Methods). UBP43 protein was detected in COS-7 cells transfected with pcDNA4-UBP43 (+), but not with an insertless control pcDNA4 vector (-). The 43-kDa UBP43 protein was detected using these respective antibodies. This protein was not detected when pre-immune antisera for each antibody was probed to each respective filter. Molecular weight size markers are displayed.

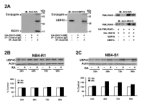

Fig. 2.

UBP43 is expressed and active in APL cells. (A) ISG15-UBP43 conjugation was detected in NB4-S1 APL cells. Immunoprecipitation (IP) with an anti-HA antibody followed by immunoblotting (IB) with a second anti-HA antibody (left panel) or an anti-UBP43 antibody (middle panel) revealed ISG15-UBP43 complex formation in NB4-S1 APL cells pre-incubated with HA-ISG15-vinylmethyl ester (HA-ISG15-VME). Closed arrows depict positions of UBP43 conjugated with ISG15, the open arrow indicates unconjugated UBP43 protein, and the hatched arrow shows the position of HA-tagged ISG15. The absence of HA-tagged ISG15 detection in the first lane of the left panel of Fig. 2A is due to no HA-tagged ISG15 addition to this lysate. Other species detected in the left and middle panels likely represent non-specific signals. The right panel indicates that UBP43 can remove ISG15 from PML/RARα protein following anti-HA immunoprecipitation of HA-tagged PML/RARα protein followed by anti-HA or anti-ISG15 immunoblotting (IB) to detect PML/RARα. Molecular weight size markers are displayed. (B and C) Independent immunoblot expression for UBP43 in NB4-R1 and in NB4-S1 APL cells treated with RA (1μM) or vehicle (DMSO) for the indicated hours (h). No appreciable change in UBP43 expression occurred after RA-treatment of NB4-R1 APL cells, as shown in panel B. In contrast, UBP43 was induced after 48 hours of RA-treatment in NB4-S1 APL cells, as displayed in panel C. Quantifications of respective signals are presented.

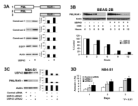

Fig. 3.

Effects of UBP43 transfection on PML/RARα protein stability. (A) Three different constructs, designated 1 through 3, assessed PML/RARα protein domain stabilities after engineered UBP43 over-expression. The EGFP expression vector served as a transfection control. Actin expression confirmed similar amounts of loaded proteins. Quantifications of these indicated immunoblot signals appear in the right panels. Full-length PML/RARα and the PML, but not RARα domains of PML/RARα were stabilized by UBP43. (B) Effects of UBP43 on transfected HA-tagged PML/RARα in BEAS-2B cells. Immunoblot expression for PML/RARα and actin is shown in the presence (+) or absence (−) of cycloheximide (CHX; 40μg/mL) treatment. UBP43 stabilized exogenous PML/RARα protein despite CHX-treatment, as detected by the anti-HA antibody. Quantification of signals is provided. (C and D) UBP43 knock-down in APL cells. (C) Transient transfection of two independent UBP43 targeting siRNAs versus an inactive control siRNA (see Materials and Methods) reduced UBP43 and decreased PML/RARα immunoblot expression in NB4-S1 cells, as in panel C. (D) UBP43 knock-down by each siRNA targeting UBP43 significantly (*, $P < 0.01$) induced apoptosis versus inactive control siRNA in panel D.

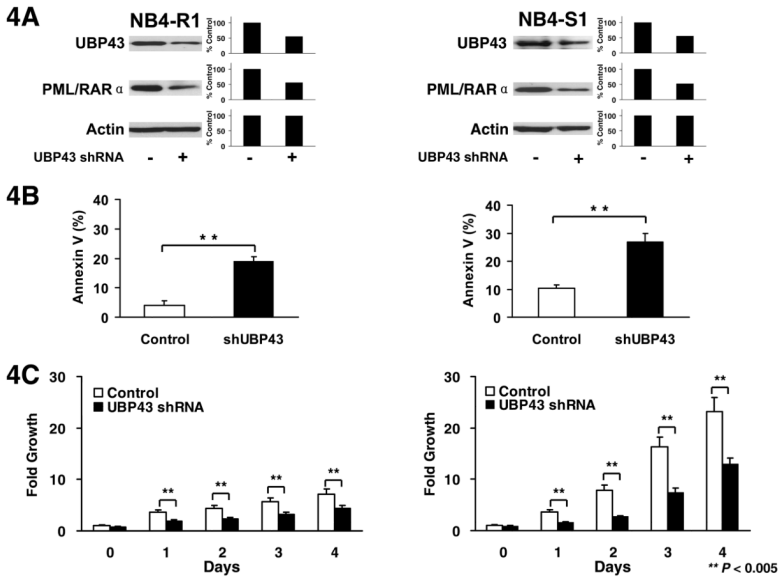

Fig. 4.

(A) Stable UBP43 knock-down by retroviral transduction of UBP43 shRNA in APL cells. UBP43 shRNA transduction (+) destabilized endogenous PML/RARα protein expression, as compared to an insertless retrovirus vector (−) independently in NB4-R1 (left panel) and NB4-S1 APL cells (right panel). Actin expression confirmed similar protein loading. (B) Retroviral-mediated knock-down of UBP43 triggered significant apoptosis in NB4-R1 (left panel) and NB4-S1 (right panel)APL cells. (C) Independent retroviral-mediated knock-down of UBP43 in NB4-R1 and NB4-S1 APL cells significantly inhibited proliferation as compared to an insertless retroviral vector. Significant changes are depicted (**, $P < 0.005$).

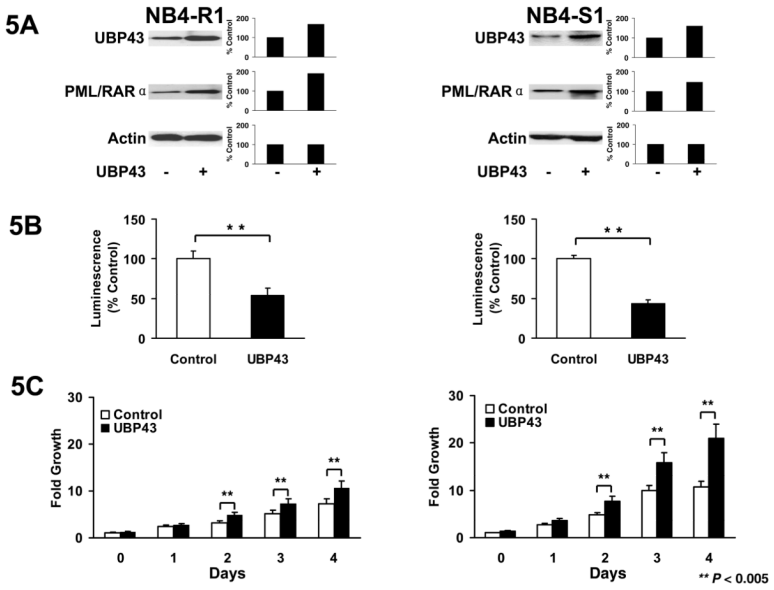

Fig. 5.

Gain of UBP43 expression in APL cells. (A) Independent UBP43 immunoblot analyses were performed in the indicated APL cell lines engineered after UBP43 (+) or an insertless (−) retroviral transduction. Increased UBP43 expression augmented PML/RARα expression in NB4-R1 (left panel) and NB4-S1 APL cells (right panel). Actin expression confirmed similar protein loading. (B) UBP43 retroviral transduction inhibited apoptosis in NB4-R1 and NB4-S1 cells versus insertless control transduction, as measured by these caspase 3/7 assays. (C) Engineered gain of UBP43 expression augmented proliferation of NB4-R1 and NB4-S1 APL cells versus insertless vector controls. Significant changes are depicted (**, P < 0.005).

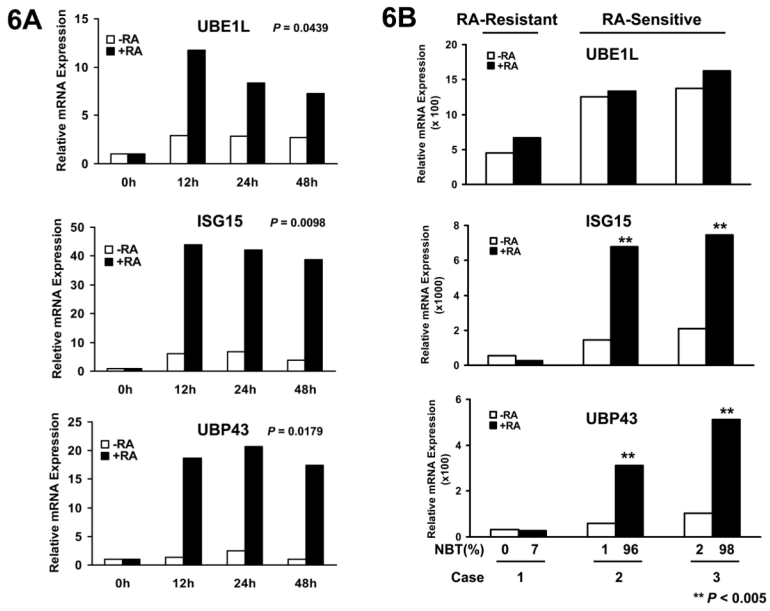

Fig. 6.

RA-treatment of a murine transgenic transplantable APL model and of APL cells harvested directly from patients. (A) UBE1L, ISG15 and UBP43 mRNA expression profiles were each studied in RA-treated versus vehicle control-treated APL mice (see Materials and Methods). After RA-treatment for the indicated hours (h), UBE1L, ISG15 and UBP43 mRNAs were each increased in the isolated spleens. (B) RA-treatment induced UBE1L, ISG15 and UBP43 mRNA expression in cultured human APL cells from two representative RA-responsive cases, but not appreciably in a representative RA-resistant APL case. Expression profiles were studied after RA-treatment or no treatment and differentiation response was assessed by nitrotetrazolium blue (NBT) staining as described in the Materials and Methods. NBT positive cells appeared after RA-treatment of RA-responsive, but minimally in RA-resistant APL cells from these cases. Significant changes are indicated (**, $P < 0.005$).