

Evidence for the Ubiquitin Protease UBP43 as an Antineoplastic Target

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Abstract

New pharmacologic targets are needed for lung cancer. One candidate pathway to target is composed of the E1-like ubiquitin-activating enzyme (UBE1L) that associates with interferon-stimulated gene 15 (ISG15), which complexes with and destabilizes cyclin D1. Ubiquitin protease 43 (UBP43/USP18) removes ISG15 from conjugated proteins. This study reports that gain of UBP43 stabilized cyclin D1, but not other D-type cyclins or cyclin E. This depended on UBP43 enzymatic activity; an enzymatically inactive UBP43 did not affect cyclin D1 stability. As expected, small interfering RNAs that reduced UBP43 expression also decreased cyclin D1 levels and increased apoptosis in a panel of lung cancer cell lines. Forced cyclin D1 expression rescued UBP43 apoptotic effects, which highlighted the importance of cyclin D1 in conferring this. Short hairpin RNA-mediated reduction of UBP43 significantly increased apoptosis and reduced murine lung cancer growth *in vitro* and *in vivo* after transplantation of these cells into syngeneic mice. These cells also exhibited increased response to all-*trans*-retinoic acid, interferon, or cisplatin treatments. Notably, gain of UBP43 expression antagonized these effects. Normal-malignant human lung tissue arrays were examined independently for UBP43, cyclin D1, and cyclin E immunohistochemical expression. UBP43 was significantly ($P < 0.01$) increased in the malignant versus normal lung. A direct relationship was found between UBP43 and cyclin D1 (but not cyclin E) expression. Differential UBP43 expression was independently detected in a normal-malignant tissue array with diverse human cancers. Taken together, these findings uncovered UBP43 as a previously unrecognized antineoplastic target. *Mol Cancer Ther*; 11(9); 1968–77. ©2012 AACR.

Introduction

Lung cancer is the leading cause of cancer mortality for men and women in the United States (1). The 5-year survival rate for lung cancer patients is only 16% (1). Innovative ways to combat lung cancer are needed.

Carcinogenesis is a multistep process. Frequent genetic changes arise in human lung carcinogenesis and some drive this process (2). Previous work highlighted cyclin D1 as a chemopreventive or therapeutic target, as reviewed (3, 4). Increased cyclin D1 expression occurs early during lung carcinogenesis (5, 6) through gene amplifi-

cation (7) or allele-specific expression imbalance (6), among other mechanisms. These and other findings implicated cyclin D1 as an antineoplastic target in the lung.

Lung carcinogenesis is inhibited by induced cyclin D1 proteasomal degradation. This occurred after treatment with retinoid X receptor (RXR, retinoid) or retinoic acid receptor (RAR, retinoid) agonists (2, 8, 9). Cyclin D1 protein is also repressed by activating a pathway constituted by these retinoid regulated species: ubiquitin activating enzyme E1-like (UBE1L), interferon-stimulated gene 15 (ISG15) and ubiquitin protease UBP43 (UBP43/USP18; refs. 10 and 11). This study examined whether UBP43 was an antineoplastic target.

UBE1L associates with ISG15, the ubiquitin-like protein family member; ISG15 conjugation is specifically removed by UBP43, the ubiquitin-specific protease (USP) family member (12, 13). Deregulation of the UBE1L-ISG15-UBP43 pathway occurs in diverse tumor types (14–18). All-*trans*-retinoic acid (RA)-treated human bronchial epithelial (HBE) and acute promyelocytic leukemia (APL) cells increased UBE1L, ISG15, and UBP43 expression (11, 15, 16, and data not shown). Gain of UBP43 expression stabilized PML/RAR α in APL cells (11) and augmented cyclin D1 expression in HBE cells (10). These findings implicated UBP43 as directly regulating the stability of

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cyclin D1 and other growth-regulatory proteins. Evidence for cyclin D1 as a chemopreventive target also came from mouse models and clinical studies (3, 5, 8, 9, 19–23).

This study uncovered UB43-dependent mechanisms that regulated lung cancer growth and tumorigenesis. Previous work (10) was extended by reporting gain of UB43 expression preferentially stabilized cyclin D1 protein. UB43 knockdown destabilized cyclin D1, but not other examined G1 cyclin proteins. This was confirmed by UB43 transfection and treatment with cycloheximide (CHX), the protein synthesis inhibitor, which increased cyclin D1 protein stability versus controls. UB43 transduction reduced apoptosis and increased cyclin D1 expression as well as growth of HBE and lung cancer cells. In contrast, UB43 knockdown decreased cyclin D1 expression and inhibited growth and increased apoptosis in murine and human lung cancer cells. This significantly decreased lung cancer formation in FVB mice transplanted with syngeneic lung cancer cells that were engineered with reduced UB43 expression. To explore directly whether UB43 enzymatic activity mediated this, an enzymatically-inactive UB43 species was engineered and used in these experiments.

To elucidate therapeutic implications, growth, and apoptosis assays were carried out after individual treatments with RA, interferon (IFN), or cisplatin in lung cancer cells independently engineered with loss or gain of UB43 expression. To ascertain clinical relevance, UB43 immunohistochemical expression profiles were studied using new assays and a normal-malignant human lung tissue array. UB43 was significantly increased in the malignant versus normal lung tissues. A direct relationship was found between UB43 and cyclin D1. Results were confirmed and extended using a normal-malignant tissue array with diverse tumor types. Taken together, these findings implicate UB43 as a tractable target for lung and other cancers.

Materials and Methods

Cell culture

The ED-1 murine lung cancer cell line was cultured in RPMI 1640 (Invitrogen) medium with 10% FBS (Gemini), 4 mmol/L L-glutamine (Invitrogen), 100 units/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen; ref. 24). ED-1L cells were isolated as a single-cell subclone of ED-1 cells. H23, HOP62, and A549 lung cancer cell lines were cultured as before (25). BEAS-2B immortalized HBE cells were cultured in serum-free LHC-9 medium (Biofluids; refs. 10 and 11). PT67 and 293T cells were cultured in DMEM (Invitrogen) medium with 10% FBS (Gemini), 4 mmol/L L-glutamine (Invitrogen), 100 units/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified incubator with 5% CO₂. Cell lines were obtained from and authenticated (using genotypic and phenotypic assays) by American Type Culture Collection,

except for ED-1 cells that were previously authenticated (24).

Expression plasmids and transient transfection

The pcDNA4-UB43, pSG5-UBE1L, pCMV-hemagglutinin (HA)-ISG15, pCMV-HA-cyclin D1, lysine-less pCMV-HA-cyclin D1 33–269 (mut-cyclin D1), pCMV-HA-cyclin D2, pCMV-HA-cyclin D3, pCMV-HA-cyclin E, enhanced green fluorescent protein (EGFP) expression plasmid, and pRetroX-IRES-ZsGreen1-UB43 retroviruses were previously described (10, 11, 26). Flag-tagged-UbcH8 was purchased (Addgene). Short hairpin RNA (shRNA) retroviruses and lentiviruses were used to knockdown human or mouse UB43 species (Open Biosystems). Empty vectors (Open Biosystems) were controls (11). Indicated UB43 cysteine (C) residues were transversed to serine (S) residues (to render UB43 enzymatically inactive) using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). DNA sequence analyses confirmed engineered species.

Transient transfection of logarithmically growing cells was accomplished using the Effectene Transfection Reagent (Qiagen; refs. 11 and 26). EGFP-expressing plasmids assessed transfection efficiencies. Transfections were in triplicate. Each experiment was independently replicated 3 times.

Transient transfection of cells with the desired small interfering RNAs (siRNA) was accomplished using Lipofectamine 2000 (Invitrogen) reagent. Different siRNAs that targeted UB43, cyclin D1, or a RISC-free control siRNA were synthesized (Dharmacon). Two siRNAs that independently targeted UB43 were: human UB43 siRNA1 (5'-CTGCATATCTTCTGGTTTA-3'), human UB43 siRNA2 (5'-GGAAGAAGACAGCAACATG-3'), murine UB43 siRNA1 (5'-CGTTGTTTGTCCAGCACGA-3'), and murine UB43 siRNA2 (5'-AGGAACTC-GAGGACGGAAA-3'). Two siRNAs that independently targeted human cyclin D1 were: cyclin D1 (5'-ACAACTTCTGTCTACTA-3', siRNA3) and (5'-GTTTCGTG-GCCTCTAAGATG-3', siRNA4). Transfection efficiencies were monitored by cotransfecting the siGLO Green Transfection Indicator (Dharmacon).

Generation of stable UB43 transfectants

The pRetroX-IRES-ZsGreen1-UB43 retroviral vector or an empty retrovirus was transfected into the RetroPack PT67 Packaging Cell Line (Clontech) using Effectene Transfection Reagent (Qiagen). Viral supernatants transduced cells with polybrene (4 µg/mL; Sigma). Green fluorescent protein positive cells were harvested 48 hours later using a FACStar Plus cytometer (Becton Dickinson). This was repeated a week later. Three independent experiments were performed, each in triplicate.

The shRNA retrovirus (for human cells) and lentivirus (for murine cells) for UB43 knockdowns were generated in PT67 cells or 293T cells, respectively. Stable selection was achieved with puromycin (2 µg/mL; Sigma) treatment. Five candidate shRNAs were individually

examined. The 2 with the most efficient UBP43 knock-downs were studied.

Real-time PCR assays

For real-time reverse transcription (RT) assays, primers were: human UBP43 forward primer 5'-GAGGCTG-GACGCTTGCAT-3' and reverse primer 5'-AGCACG-ACCTCACTTCCAGGAA-3'; human cyclin D1 forward primer 5'-AACTACCTGGACCGCTTCCT-3' and reverse primer 5'-CCACTTGAGCTTGTTACCA-3'; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5-ACCTTTGGCATTGTGGAGG-3' and reverse primer 5'-ACACATTGGGGGTAGGAACA-3'. Assays were conducted using established methods (11).

Immunoblot analyses

Cells were lysed with ice-cold radioimmunoprecipitation buffer using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assays (10, 11, 26). Primary antibodies for immunoblot analyses were 2 independently developed polyclonal antibodies recognizing UBP43 (11), rabbit polyclonal antibody against cyclin D1 (Santa Cruz), murine monoclonal antibody against HA-tagged proteins (Covance), murine monoclonal antibody for Flag-tagged proteins (OriGene), and a goat polyclonal antibody for actin (Santa Cruz). Secondary antimouse and antirabbit antibodies were purchased (Amersham) as was an anti-goat (Santa Cruz) antibody. Quantifications of signals were as before (11). To assess cyclin D1 protein stability after UBP43 transfection, cells were treated with or without CHX (40 μ g/mL; Sigma).

Proliferation and apoptosis assays

Growth was measured using the CellTiter-Glo Assay Kit (Promega; ref. 19). Triplicate replicate experiments were carried out. To assess for drug responses, independent stable transductants with loss or with gain of UBP43 expression were each treated with or without RA (Sigma), IFN (PBL), or cisplatin (Sigma). Empty retroviruses served as controls. Apoptosis was measured by Annexin V:FITC positivity and flow cytometry using the Annexin V Assay Kit (AbD Serotec). Cell lines were assayed with the Caspase-Glo 3/7 Assay Kit (Promega).

In vivo tumorigenicity and statistical assays

Lentiviral-mediated UBP43 knockdown was achieved in ED-1 cells. Cells were harvested in phosphate buffered saline (PBS) supplemented with 10% mouse serum (Invitrogen); 8×10^5 cells from each transfectant were individually injected into tail veins of respective 8-week-old female FVB syngeneic mice. Ten mice were used in each experimental arm with replicate experiments conducted. Mice were sacrificed 4 weeks after injections after an Institutional Animal Care and Use Committee-approved protocol. Tissues were formalin-fixed, processed, and hematoxylin and eosin stained (27). Scoring of lung tumors was as in previous work (25).

Normal-malignant tissue arrays

Resected non-small cell lung carcinomas (NSCLC) with case-matched adjacent histopathologically normal lung tissues were examined using a normal-malignant lung tissue array from the New Hampshire State Cancer Registry and the Dartmouth-Hitchcock Tumor Registry (25). Studies were reviewed and approved by Dartmouth's Institutional Review Board for human subjects.

Normal and malignant lung tissues were each formalin-fixed and processed as before (27). Tissues were independently examined for cyclin D1 and cyclin E immunohistochemical expression profiles (10, 27). Results were compared with those obtained with different polyclonal anti-UBP43 antibodies used to detect UBP43 immunohistochemically (11). Antibody specificities were confirmed using blocking peptides.

To extend analyses to other human cancers, a normal-malignant tissue array with lung and other cancers was purchased (Life Span Biosciences) and probed for UBP43 immunohistochemical expression. UBP43 levels were scored by the reference pathologist (V. Memoli), who quantified cytoplasmic staining using established methods (22).

Statistical analyses

Two-tailed *T* tests were used. Results were presented as means \pm SD. Statistical significance was noted with these symbols: **P* < 0.05 and ***P* < 0.01.

Results

In vitro studies

Previous work implicated UBP43 as an antineoplastic target that opposed UBE1L effects on cyclin D1 (10, 16). To determine whether UBP43 affected stability of other G1 cyclins, HBE cells were transfected with UBP43 and independently probed for exogenously expressed cyclin D1, cyclin D2, cyclin D3, or cyclin E proteins. Only cyclin D1 was appreciably stabilized by gain of UBP43 expression (Fig. 1A). UBP43 transfection also increased endogenous cyclin D1 protein, but not cyclin D1 mRNA expression (Supplementary Fig. S1 and data not shown). To establish whether UBP43 affected cyclin D1 protein stability, UBP43 was cotransfected with HA-tagged cyclin D1 into BEAS-2B cells treated +/– CHX. UBP43 increased exogenous cyclin D1 protein stability +/– CHX treatments (Fig. 1B). Specific lysines in cyclin D1 affected its stability (26). Because lysines are targets of ISG15 conjugation (10), it was expected that lysine-less cyclin D1 would be resistant to UBP43 effects on its stability (Fig. 1C).

UBP43 enzymatic activity is required for ISG15 deconjugation (28). Whether cyclin D1 stabilization by UBP43 depended on this activity was explored. Active site cysteine residues of UBP43 (C64 and C65) were transversed to serines to render UBP43 inactive (designated UBP43-S64, S65 or Mut-UBP43). Figure 1D shows transfection of ED-1 lung cancer cells with this mutant UBP43 species did not appreciably affect cyclin D1 stability versus cells

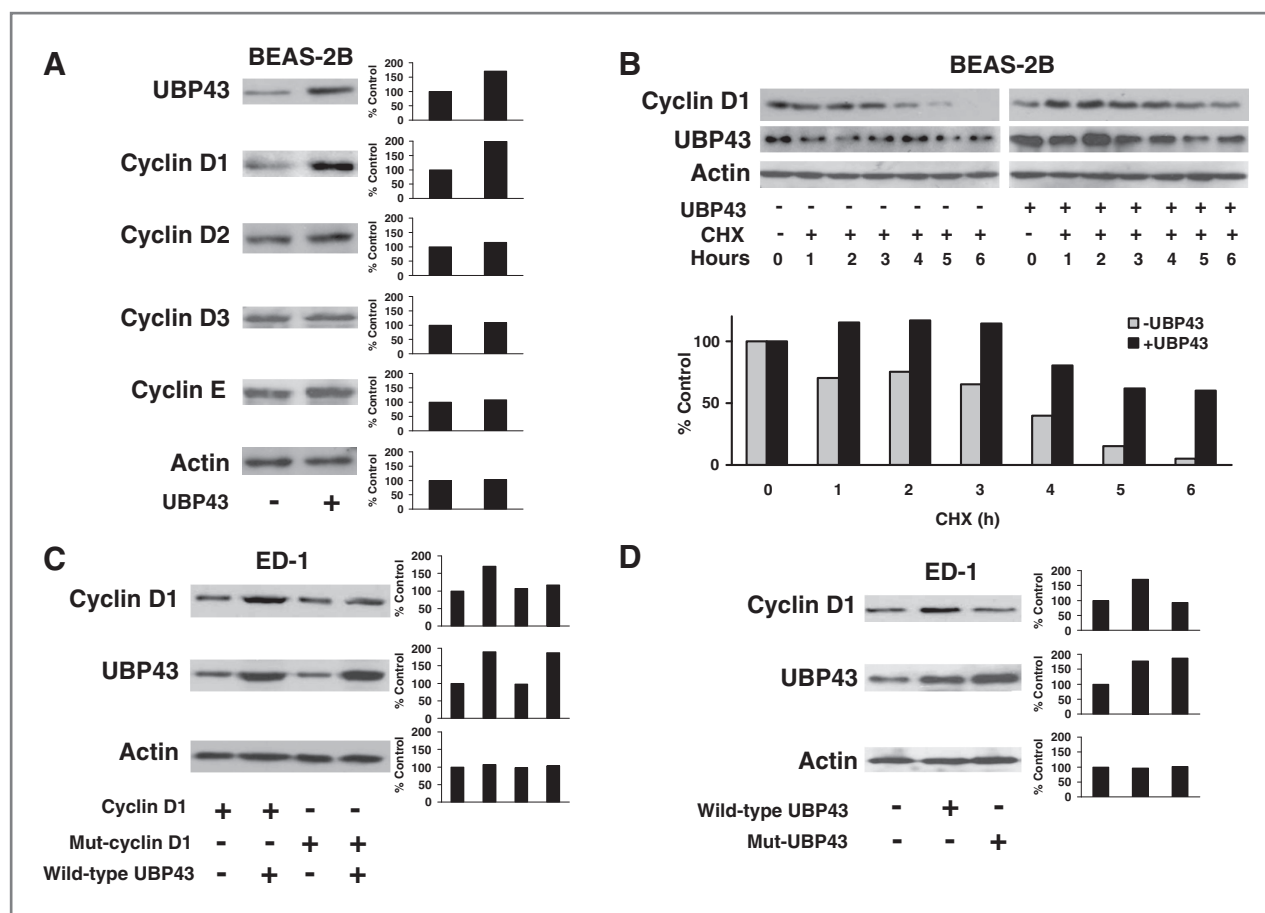


Figure 1. Effects of UBP43 on cyclin D1 protein stability. **A**, effects of UBP43 on individual transiently-transfected D-type and cyclin E species. UBP43 transfection (+) or empty vector transfection (-) was accomplished in BEAS-2B HBE cells with immunoblot analyses subsequently performed. Actin expression served as a loading control. Quantification of each respective signal is displayed in the right panel. The percent change in expression of each of these cyclins relative to respective actin expression is presented. **B**, effects of UBP43 cotransfection on transfected HA-tagged cyclin D1 immunoblot expression in the presence (+) or absence (-) of cycloheximide (CHX) treatment of BEAS-2B cells. UBP43 stabilized (vs. actin control) exogenous cyclin D1 protein, which was detected by an anti-HA antibody. Exogenous cyclin D1 expression was enhanced despite CHX treatment. Quantification of signals is provided in the panel below this immunoblot. **C**, consequences of UBP43 transfection on wild-type cyclin D1 and independently on lysine-less cyclin D1 (Mut-cyclin D1) species. These respective species were independently transfected into ED-1 lung cancer cells in the presence (+) or absence (-) of cotransfected UBP43. Compared with effects on wild-type cyclin D1, UBP43 did not appreciably augment lysine-less cyclin D1 expression. Quantification of signals is provided in right panels. **D**, effects of cyclin D1 protein stability on wild-type (wild-type UBP43) or an enzymatically inactive UBP43 species (Mut-UBP43) after their respective cotransfections. Wild-type and mutant UBP43 species were independently cotransfected into ED-1 cells along with HA-tagged-cyclin D1 species. Compared with wild-type UBP43, this mutant UBP43 species did not stabilize cyclin D1 expression. Quantification of these respective signals is shown in the right panel.

transfected with wild-type UBP43 that increased cyclin D1 expression.

UBP43 is the protease specific for ISG15 (13, 29). Complexes between ISG15 and UBP43 were detected in APL cells (11). Whether catalytically inactive UBP43-S64.S65 affected ISG15 conjugation or cyclin D1 stability was studied in lung cancer cells individually transfected without exogenous UBP43, with wild-type UBP43, or with this inactive UBP43 species. As expected, expression of the enzymatically inactive UBP43 in A549 cells did not affect overall ISG15 conjugation while overexpression of wild-type UBP43 markedly reduced this conjugation (Fig. 2A).

Gain of UBP43 expression increased cyclin D1 and PML/RAR α expression (10, 11). Whether UBP43

repression affected apoptosis in lung cancer cells via changes in cyclin D1 levels was studied. Two different siRNAs that targeted UBP43 and an inactive control siRNA were each independently transfected into murine ED-1 lung cancer and BEAS-2B HBE cells as well as A549, H23, and HOP62 human lung cancer cell lines (Fig. 2B). Knockdown of UBP43 by each of these UBP43-targeting siRNAs substantially decreased UBP43 and cyclin D1 immunoblot expression versus control cells (Fig. 2B). Actin expression was unaffected. Compared with controls, siRNA-mediated knockdown of UBP43 significantly augmented apoptosis (Fig. 2B, bottom). Supplementary Fig. S1A revealed UBP43 knockdown (vs. controls) in ED-1 cells augmented an

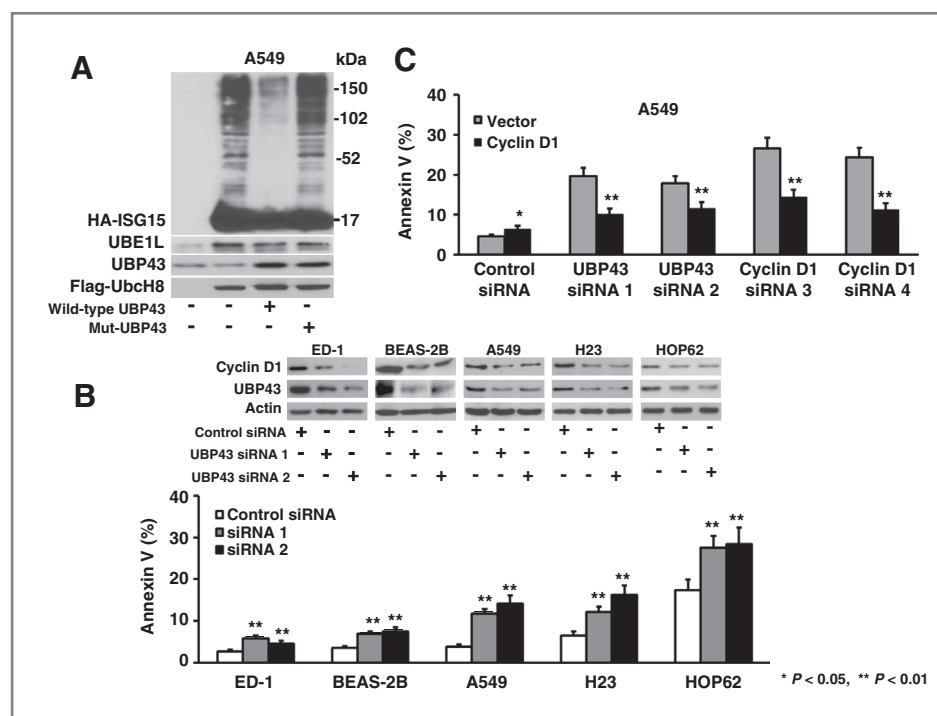


Figure 2. UBPA3 effects on ISG15-conjugation and cellular apoptosis. **A**, A549 lung cancer cells were transiently transfected with vector-control or plasmids containing HA-ISG15, UBE1L, or Flag-tagged-UbcH8 species in the absence or the presence of wild-type or the described mutant UBPA3 species. The level of overall ISG15 conjugation was assessed with an anti-HA antibody that recognized the HA-tagged ISG15. As compared with wild-type UBPA3 transfection, transfected mutant UBPA3 (Mut-UBPA3) species did not reduce ISG15 conjugates, consistent with its enzymatic inactivity. **B**, UBPA3 knockdown in lung cancer and HBE cells caused apoptosis. Independent transient transfection of 2 independent UBPA3-targeting siRNAs versus a control siRNA for 24 hours reduced UBPA3 and cyclin D1 immunoblot expression in ED-1, BEAS-2B, A549, H23, and HOP62 cells (top). UBPA3 knockdown by each siRNA-targeting UBPA3 significantly induced an apoptosis marker versus transfected control siRNA (bottom). **C**, cyclin D1 involvement in UBPA3-dependent apoptosis in A549 lung cancer cells. Assays confirmed that siRNA-mediated knockdown of UBPA3 led to an induced apoptosis marker (gray bars) versus control siRNA. Transient transfection of independent cyclin D1-targeting siRNAs increased apoptosis versus a control siRNA. Cotransfection of cyclin D1 with respective siRNA-targeting UBPA3 or cyclin D1 species significantly reduced apoptosis (black bars). The expected changes in expressed UBPA3 and cyclin D1 mRNAs as well as findings from rescue experiments are shown in Supplementary Fig. S2. Standard deviation bars are shown. *, $P < 0.05$ and **, $P < 0.01$.

apoptosis marker at day 1. This marker increased over 3 days of study.

Forced cyclin D1 expression antagonized UBPA3 knockdown effects on apoptosis in A549 (Fig. 2C) and ED-1 (data not shown) lung cancer cells. The respective increases or decreases in UBPA3 and cyclin D1 expression profiles were confirmed (Supplementary Fig. S2A and B). As expected, engineered expression of wild type, but not enzymatically inactive UBPA3 antagonized the effects of siRNA-mediated UBPA3 knockdown on cyclin D1 expression (Supplementary Fig. S2C). Representative FACS analyses are displayed in Supplementary Fig. S3.

To confirm and extend these findings, stable shRNA-mediated repression of UBPA3 was achieved in lung epithelial or cancer cells. Effects of loss of UBPA3 expression on cyclin D1 and apoptosis were studied in ED-1 and BEAS-2B transductants. Stable UBPA3 knockdown was engineered by lentiviral (for ED-1) or retroviral (for BEAS-2B) transductions followed by puromycin selection of desired shRNAs in these cells. Figure 3A and Supplementary Fig. S4A (left) showed shRNA-mediated UBPA3

knockdown reduced endogenous cyclin D1 protein expression in transductants versus controls. UBPA3 knockdown significantly increased apoptosis in both examined cell lines (Fig. 3B, left and Supplementary Fig. S4B, left).

Gain of UBPA3 expression was independently achieved in ED-1 and BEAS-2B cells in Fig. 3A (right) and Supplementary Fig. S4A (right). Unlike UBPA3 knockdown, retroviral-mediated UBPA3 expression augmented cyclin D1 in both of these cell lines (Fig. 3A, right and Supplementary Fig. S4A, right) and significantly reduced apoptosis (Fig. 3B, right and Supplementary Fig. S4B, right) versus empty vector controls.

UBPA3 effects on growth of ED-1 and BEAS-2B cells were studied independently after loss and also after gain of UBPA3 expression. CellTiter-Glo assays confirmed UBPA3 knockdown markedly repressed growth of these cells (Fig. 3C and Supplementary Fig. S4C, left). In contrast, UBPA3 overexpression significantly increased ($P < 0.01$) growth (vs. empty vectors) of these cells (Fig. 3C, right and Supplementary Fig. S4C, right).

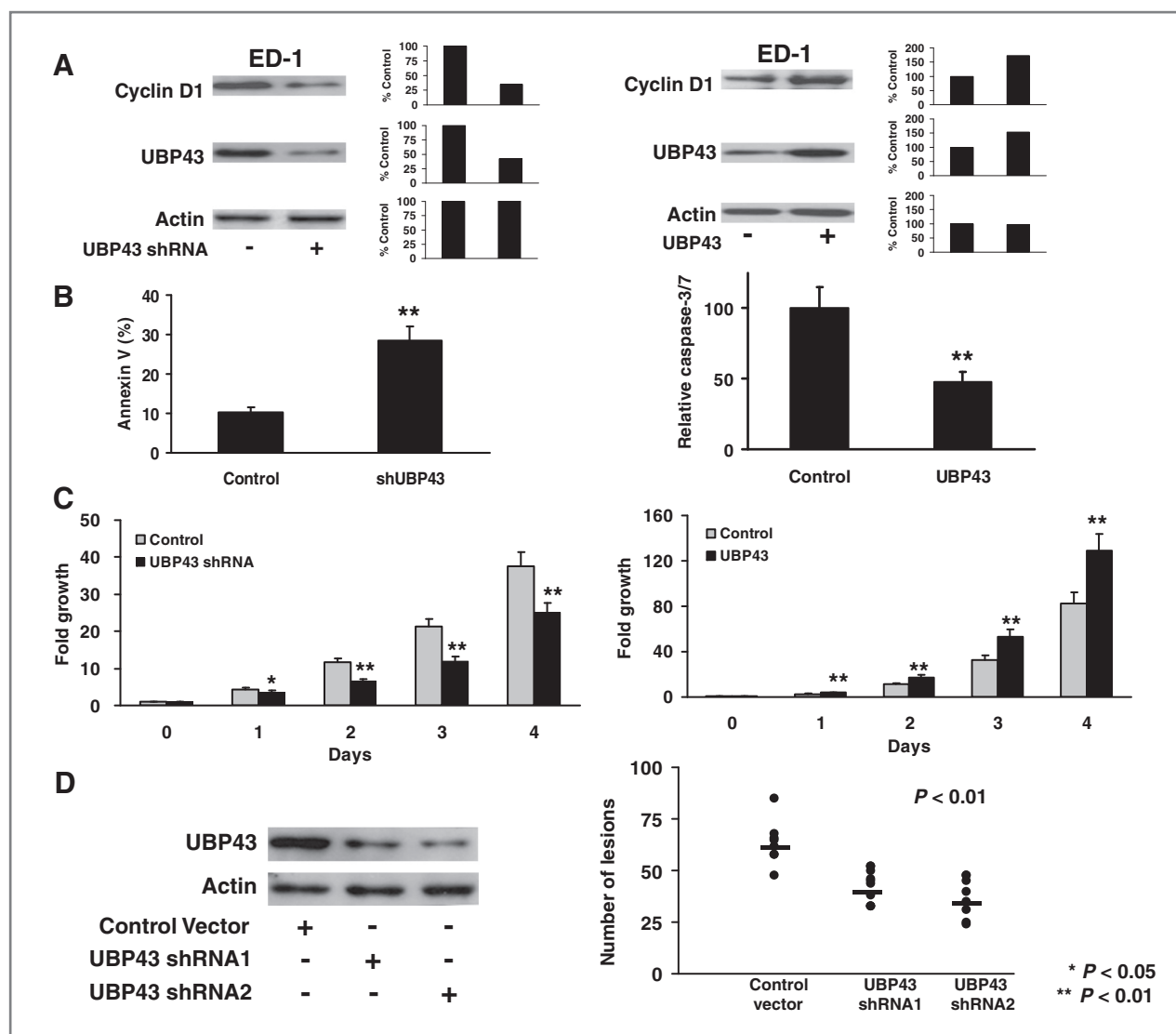


Figure 3. Engineered UB43 knockdown versus UB43 overexpression in ED-1 lung cancer cells. **A**, stable UB43 knockdown by lentiviral-mediated shRNA transduction or engineered UB43 overexpression via retroviral-mediated transduction of UB43 in ED-1 cells. UB43 shRNA transduction (+) destabilized endogenous cyclin D1 protein expression as compared with a transfected empty vector (-; left). Retroviral transduction (+) of UB43 increased UB43 expression and augmented cyclin D1 expression in ED-1 cells (right). Actin expression confirmed similar protein loading in each line. Quantification of signals is provided. **B**, lentiviral-mediated knockdown of UB43 triggered significant apoptosis (left). UB43 retroviral-mediated transduction inhibited apoptosis (right) in ED-1 cells versus the empty control vector transduction. **C**, lentiviral-mediated knockdown of UB43 significantly inhibited proliferation (left) and engineered gain of UB43 expression independently augmented proliferation (right) of ED-1 cells versus these empty control transduced cells. **D**, engineered UB43 knockdown in ED-1 cells significantly reduced ($P < 0.01$) tumorigenicity of these transduced lung cancer cells. The indicated transfected UB43 shRNA knockdowns of ED-1 cells repressed *in vivo* lung tumorigenicity after tail vein injections into FVB mice. The horizontal bars represented the respective median numbers of lung cancers for UB43 shRNA-mediated transduction of ED-1 cells relative to mice injected with control vector transduced ED-1 cells. Each circle in this figure represents an individual mouse. Standard deviation bars are shown. *, $P < 0.05$ and **, $P < 0.01$.

Tumorigenicity

UB43 knockdown effects on tumorigenicity were next studied. FVB mice were injected via their tail veins with syngeneic ED-1 cells (24) engineered with different lentiviral-mediated shRNAs to achieve stable UB43 knockdown. Results were compared with empty vector transductants. Four weeks after injections, lung lesions were scored. ED-1 cells independently engineered with

reduced UB43 expression using different UB43-repressing shRNAs produced significantly fewer ($P < 0.01$) lung cancers versus controls (Fig. 3D). Representative photomicrographs of lung tumors that formed after tail-vein injections of these respective engineered cells are presented in Supplementary Fig. S5A. *In vitro* growth of these transductants (vs. parental ED-1 cells) is displayed in Supplementary Fig. S5B.

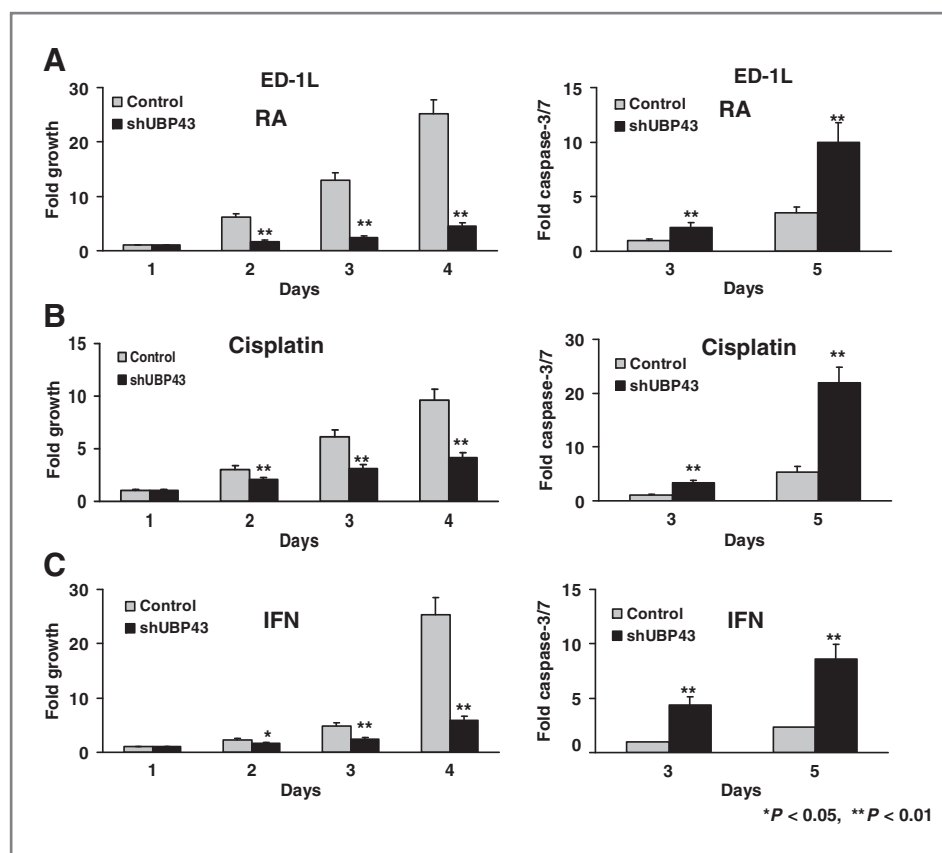


Figure 4. Growth and apoptotic effects of drug treatments in lung cancer cells engineered with UBPA3 knockdown (designated as shUBPA3) versus control vector (Control). A, treatment with RA (1 $\mu\text{mol/L}$) inhibited cell growth (left) and triggered a significant increase in apoptosis (right) in these ED-1L cells having lentiviral-mediated UBPA3 knockdown. B, cisplatin (2.5 $\mu\text{mol/L}$) treatments of the same UBPA3-mediated knockdown cells inhibited proliferation (left) and promoted apoptosis (right). C, IFN (1,000 U) treatments decreased cell growth and increased apoptosis in the same UBPA3 knockdown cells. Standard deviation bars are shown. *, $P < 0.05$ and **, $P < 0.01$.

Antineoplastic drug responses

UBPA3 is an IFN and retinoid-regulated species (10, 11, 13, 30, 31). This implied that regulating UBPA3 levels would affect response to these and other antineoplastic agents. A single-cell subclone of ED-1 cells (ED-1L) was engineered with gain and also independently with loss of UBPA3 expression (see Supplementary Fig. S1). Individual treatment effects of RA, cisplatin, or IFN on growth and apoptosis assays were studied. UBPA3 knockdown followed by RA, cisplatin, or IFN treatments increased growth-inhibitory and proapoptotic effects of each agent (Fig. 4). In contrast, forced UBPA3 expression antagonized these ED-1L cellular effects (Fig. 5).

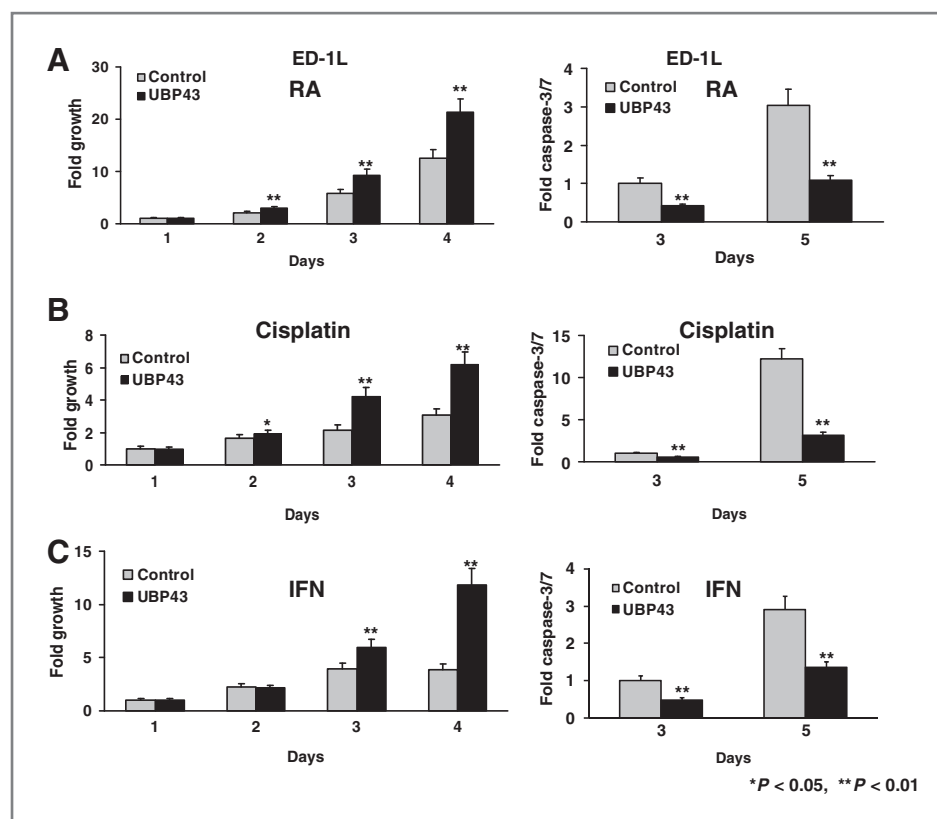
Differential UBPA3 expression profiles were examined in normal versus malignant lung tissues. Two different polyclonal anti-UBPA3 antibodies detected UBPA3 in immunoblot assays (11). These antibodies were used in immunohistochemical assays. Both the anti-UBPA3 antibody recognizing a domain nearer to the amino terminus (anti-UBPA3-1) than the second anti-UBPA3 antibody (anti-UBPA3-2) detected UBPA3 immunohistochemical expression in the normal lung with specificity confirmed using blocking peptides (Fig 6A). A representative lung cancer and a representative adjacent normal lung tissue are displayed in Fig. 6B. To extend this analysis, a paired normal-malignant human lung tissue array from the New Hampshire and Dartmouth lung cancer registry (25) was

examined individually for UBPA3, cyclin D1, and cyclin E immunohistochemical expression. UBPA3 levels from 75 NSCLC cases (see Venn diagram) versus normal lung are shown in Fig. 6C. UBPA3 expression was significantly increased in the malignant versus adjacent normal lung tissues ($P = 0.04$ adenocarcinoma and $P = 0.02$ for squamous cell carcinoma). Significant associations were found between UBPA3 and cyclin D1, but not for cyclin E (Fig. 6C). There was not a significant difference in survival in lung cancer cases with high versus low UBPA3 levels. Representative individual immunohistochemical assays for cyclin D1 and cyclin E are shown in lung cancers (Fig. 6D). To determine whether UBPA3 was differentially expressed in other cancers, a normal-malignant tissue array was examined from many cancer subtypes. Immunohistochemical findings in Fig. 6E and Supplementary Fig. S6 revealed increased UBPA3 expression in diverse cancers versus corresponding normal tissues. Reduced or absent UBPA3 expression was found in several kidney cancers or normal as well as malignant prostate tissues.

Discussion

This study advances previous work (10) by showing the ubiquitin protease UBPA3 can regulate cyclin D1 stability. UBPA3 is important in IFN and immune responses, leukemogenesis, among other biological effects (11, 12, 14,

Figure 5. Growth and apoptotic effects of drug treatments in lung cancer cells engineered with UB43 overexpression (designated UB43) versus control vector (Control). A, treatment with RA (1 $\mu\text{mol/L}$) reduced growth inhibition (left) and significantly decreased apoptosis (right) in engineered ED-1L cells having UB43 overexpression. B, cisplatin (2.5 $\mu\text{mol/L}$) treatments inhibited growth suppression (left) and apoptosis (right) in ED-1L cells overexpressing UB43 relative to controls. C, IFN (1,000 U) treatments increased cell growth and decreased apoptosis in UB43 overexpressing ED-1L cells versus controls. Standard deviation bars are shown. *, $P < 0.05$ and **, $P < 0.01$.



17, 18). UB43 acts by removing ISG15 from conjugated proteins (12, 13). Our previous work implicated UB43 in regulating stability of the oncogenic protein, PML/RAR α (11). This study identified cyclin D1 as a growth regulatory protein whose stability was also affected by UB43. UBE1L-ISG15-UB43 pathway effects on protein stability, apoptosis, antineoplastic drug responses, and tumorigenicity are summarized in Supplementary Fig. S7.

Aberrant cyclin expression occurs in lung carcinogenesis (5–7). This study found that UB43 stabilized cyclin D1 (but not other D-type cyclins or cyclin E; Fig. 1A). This established UB43 as a critical regulator of cyclin D1 protein stability. Cyclin D1 stabilization by UB43 required UB43 enzymatic activity (Figs. 1D and 2B and Supplementary Fig. S2C), but not *de novo* protein synthesis (Fig. 1). Cyclin D1 destabilization was engaged in diverse cells (Fig. 2B). Reduction of UB43 repressed cyclin D1 expression. Specific cyclin D1 residues affected proteasomal degradation (26). This work showed cyclin D1 stabilization by UB43 also depended on lysines within cyclin D1 (Fig. 1C).

Cyclin D1 can affect apoptosis, as reviewed (32). UBE1L also regulated cyclin D1 stability and apoptosis (10, 15). Results presented here build on these findings by showing UB43 knockdown decreased cyclin D1 expression and significantly increased apoptosis in lung cancer cells (Fig. 2B). Forced cyclin D1 expression antagonized proapoptotic effects of UB43 knockdown (Fig. 2C), indicating cyclin D1 played a key role in this process.

Increased UBE1L expression augmented apoptosis (15). Loss of UB43 expression caused a similar effect. UB43 was shown here to regulate cyclin D1 expression, cell growth, apoptosis, and tumorigenicity. UB43 knockdown also inhibited lung cancer formation *in vivo* (Fig. 3D). As expected, gain of UB43 expression antagonized these growth and apoptotic effects.

Combination therapy is a tenet of cancer therapy (2, 33). A desired regimen is one where a target such as UB43 can modulate responses to antineoplastic agents. UB43 was found in this study to regulate response to RA, cisplatin, and IFN. Loss of UB43 expression increased growth inhibitory and proapoptotic effects of these agents, but gain of UB43 expression antagonized these activities (Figs. 4 and 5). These findings have therapeutic implications. An inhibitor of UB43 should exert antineoplastic effects. Its effects would likely cooperate with RA, cisplatin, IFN, and other agents.

Translational research was extended by studies of paired normal and malignant tissue arrays (Fig. 6). UB43 immunohistochemical expression was significantly increased in the malignant versus normal lung. The same paired normal-malignant lung tissue array was probed for cyclin D1 and cyclin E expression. A significant association was found between UB43 and cyclin D1, but not cyclin E (Fig. 6C). Yet, UB43 levels alone did not predict prognosis in lung cancer (Fig. 6D). Previous work implicating UBE1L as a tumor suppressor in the lung (34) is extended here by showing UB43 regulates cyclin D1

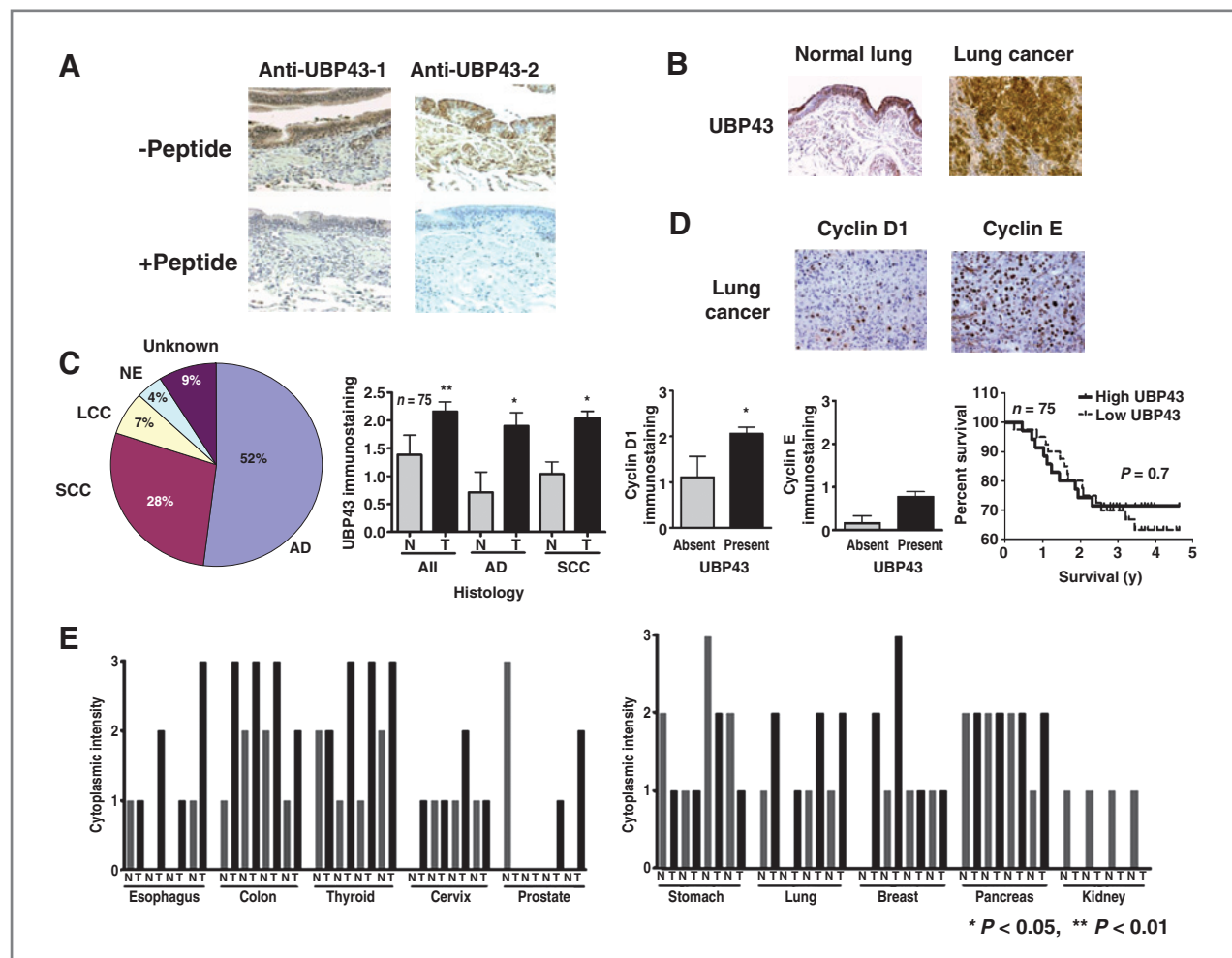


Figure 6. UBPA3 expression profiles in paired normal-malignant tissue arrays. A, immunohistochemical assays for UBPA3 expression levels were conducted using 2 different anti-UBPA3 antibodies. Blocking peptides for each respective antibody were used. UBPA3 protein expression was detected in the normal human lung. These peptides blocked UBPA3 staining by each respective antibody. B, UBPA3 immunohistochemical expression was enhanced in the malignant versus normal human lung tissues. C, differential expression profiles for UBPA3, cyclin D1, and cyclin E in a paired normal-malignant lung tissue array. Different histopathologic lung cancers are shown (All, NSCLCs; AD, adenocarcinoma; and SCC, squamous cell carcinoma). Significant associations were found between UBPA3 and cyclin D1, but not with cyclin E. The Venn diagram displayed lung cancer histopathologies (NE, neuroendocrine; LCC, large cell carcinoma; AD, adenocarcinoma; and SCC, squamous cell carcinoma). There was no significant survival difference in lung cancer cases with high versus low UBPA3 expression (right). D, representative immunohistochemical expression of cyclin D1 and cyclin E in human lung cancers. E, differential UBPA3 immunohistochemical expression profiles in a tissue array with diverse normal-malignant human tissues. Symbols refer to *, $P < 0.05$ and **, $P < 0.01$.

expression as well as apoptosis, proliferation, and tumorigenicity of cancer cells.

Interactions between UBE1L-ISG15-UBPA3 pathway members may prove important. Intriguingly, these species and their E2 (UbcH8) and E3 (Herc5a) undergo ISG15ylation (35), indicating an autoregulatory UBPA3 loop might exist. Therapeutic implications are highlighted by finding that UBPA3 is overexpressed in diverse human cancers (Fig. 6).

In summary, this study uncovered UBPA3 as an anti-neoplastic target. Enzymatically active UBPA3 directly affected cyclin D1 stability. UBPA3 knockdown repressed cyclin D1 expression, promoted apoptosis, and cooperated with effects of antineoplastic agents. Loss of UBPA3

also repressed cyclin D1 expression and reduced tumorigenicity in a murine syngeneic lung cancer model. UBPA3 expression was augmented in human lung cancers (and other cancers) relative to the corresponding normal tissues. A therapeutic window likely exists in lung and other cancers for an inhibitor of UBPA3 to exert antitumorigenic effects. Future work should explore this possibility given the need for improved treatments for lung cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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