Preclinical Development

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.

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 amplification (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, rexinoid) 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; 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/RARα in APL cells (11) and augmented cyclin D1 expression in HBE cells (10). These findings implicated UBP43 as directly regulating the stability of
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 UBP43-dependent mechanisms that regulated lung cancer growth and tumorigenesis. Previous work (10) was extended by reporting gain of UBP43 expression preferentially stabilized cyclin D1 protein. UBP43 knockdown destabilized cyclin D1, but not other examined G1 cyclins. This was confirmed by UBP43 transfection and treatment with cycloheximide (CHX), the protein synthesis inhibitor, which increased cyclin D1 protein stability versus controls. UBP43 transduction reduced apoptosis and increased cyclin D1 expression as well as growth of HBE and lung cancer cells. In contrast, UBP43 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 UBP43 expression. To explore directly whether UBP43 enzymatic activity mediated this, an enzymatically-inactive UBP43 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 UBP43 expression. To ascertain clinical relevance, UBP43 immunohistochemical expression profiles were studied using new assays and a normal-malignant human lung tissue array. UBP43 was significantly increased in the malignant versus normal lung tissues. A direct relationship was found between UBP43 and cyclin D1. Results were confirmed and extended using a normal-malignant tissue array. UBP43 was significantly increased in the malignant group, thus validated human or mouse UBP43 species (Open Biosystems). Empty vectors (Open Biosystems) were controls (11). Indicated UBP43 C residues were transversely to serine (S) residues (to render UBP43 enzymatically inactive) using the QuickChange Site-Directed Mutagenesis Kit (Strategene). DNA sequence analyses confirmed engineered species.

Transient transfection of logarithmically growing cells was accomplished using the Effectene Transfection Reagent (Qiagen; refs. 11 and 26). EGF-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 UBP43, cyclin D1, or a RISC-free control siRNA were synthesized (Dharmacon). Two siRNAs that independently targeted UBP43 were: human UBP43 siRNA1 (5'-CTGGATATCTTCGTTTA-3'), human UBP43 siRNA2 (5'-GGAGAAGACCGCCAGCATG-3'), murine UBP43 siRNA1 (5'-CGTTGGTGTTCCACGCAGA-3'), and murine UBP43 siRNA2 (5'-AGGAACCTCGAGGACGGAAA-3'). Two siRNAs that independently targeted human cyclin D1 were: cyclin D1 (5'-ACAACTTCTCCTCTCCTACTA-3', siRNA3) and (5'-GTTCGTGGCCTCTAAGATG-3', siRNA4). Transfection efficiencies were monitored by cotransfecting the siGLO Green Transfection Indicator (Dharmacon).

**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-UBP43, pSG5-UBE1L, pCMV-hemagglutinin (HA)-ISG5, 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-IREZ-ZsGreen1-UBP43 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 UBP43 species (Open Biosystems). Empty vectors (Open Biosystems) were controls (11). Indicated UBP43 C residues were transversely to serine (S) residues (to render UBP43 enzymatically inactive) using the QuickChange Site-Directed Mutagenesis Kit (Strategene). DNA sequence analyses confirmed engineered species.

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**Generation of stable UBP43 transfectants**

The pRetroX-IREZ-ZsGreen1-UBP43 retroviral vector or an empty retrovirus was transfected into the RetroPack PT67 Packaging Cell Line (Clontech) using Effective 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 FACSStar 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 UBP43 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 independently engineered with loss or gain of UBP43 expression. To ascertain clinical relevance, UBP43 immunohistochemical expression profiles were studied using new assays and a normal-malignant human lung tissue array. UBP43 was significantly increased in the malignant versus normal lung tissues. A direct relationship was found between UBP43 and cyclin D1. Results were confirmed and extended using a normal-malignant tissue array with diverse tumor types. Taken together, these findings implicate UBP43 as a tractable target for lung and other cancers.
examined. The 2 with the most efficient UBP43 knockdowns were studied.

Real-time PCR assays
For real-time reverse transcription (RT) assays, primers were: human UBP43 forward primer 5'-GAGGCTG-GAGCGTTGCAT-3' and reverse primer 5'-AGCAGC-ACCTCACCCTCCAGGA-3'; human cyclin D1 forward primer 5'-AATCCTGACCCGTCCCTC-3' and reverse primer 5'-CCACCTGACTTTGTTCAACCA-3'; human gly- ceraldehyde-3-phosphate dehydrogenase (GAPDH) for- ward primer 5'-ACCTTGGCAATTTGAGG-3' and reverse primer 5'-ACACATTGGGGTGGAGGAAC-3'. Assays were conducted using established methods (11).

Immunoblot analyses
Cells were lysed with ice-cold radioimmunoprecipita- tion buffer using sodium dodecyl sulphate-poly- acrylamide gel electrophoresis (SDS-PAGE) assays (10, 11, 26). Primary antibodies for immunoblot analyses were 2 independently developed polyclonal anti- bodies recognizing UBP43 (11), rabbit polyclonal anti- body against cyclin D1 (Santa Cruz), murine monoclo- nal 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 anti- bodies 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 μg/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, inde- pendent stable transductants with loss or with gain of UBP43 were each treated with or without RA (Sigma), IFN (PBL), or cisplatin (Sigma). Empty retroviruses expression were independently probed for exogenously expressed cyclin D1, cyclin D2, cyclin D3, or cyclin E proteins. Only cyclin D1 depended on this activity was explored. Active site cys- teine residues of UBP43 (C64 and C65) were transversed to murine residues of UBP43 (C64 and C65) were transversed to

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 × 10⁶ cells from each transfectant were indi- vidually 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 Reg- istry 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 specifici- ties 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 meth- ods (22).

Statistical analyses
Two-tailed T tests were used. Results were presented as means ± 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 inde- pendently 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 resis- tant to UBP43 effects on its stability (Fig. 1C).

UBP43 enzymatic activity is required for ISG15 decon- jugation (28). Whether cyclin D1 stabilization by UBP43 depended on this activity was explored. Active site cys- teine 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
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/RARα 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 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 right panel. 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 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. 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.
apoptosis marker at day 1. This marker increased over 3 days of study.

Forced cyclin D1 expression antagonized UBP43 knockdown effects on apoptosis in A549 (Fig. 2C) and ED-1 (data not shown) lung cancer cells. The respective increases or decreases in UBP43 and cyclin D1 expression profiles were confirmed (Supplementary Fig. S2A and B). As expected, engineered expression of wild type, but not enzymatically inactive UBP43 antagonized the effects of siRNA-mediated UBP43 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 UBP43 was achieved in lung epithelial or cancer cells. Effects of loss of UBP43 expression on cyclin D1 and apoptosis were studied in ED-1 and BEAS-2B transductants. Stable UBP43 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 UBP43 knockdown reduced endogenous cyclin D1 protein expression in transductants versus controls. UBP43 knockdown significantly increased apoptosis in both examined cell lines (Fig. 3B, left and Supplementary Fig. S4B, left).

Gain of UBP43 expression was independently achieved in ED-1 and BEAS-2B cells in Fig. 3A (right) and Supplementary Fig. S4A (right). Unlike UBP43 knockdown, retroviral-mediated UBP43 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.

UBP43 effects on growth of ED-1 and BEAS-2B cells were studied independently after loss and also after gain of UBP43 expression. CellTiter-Glo assays confirmed UBP43 knockdown markedly repressed growth of these cells (Fig. 3C and Supplementary Fig. S4C, left). In contrast, UBP43 overexpression significantly increased (P < 0.01) growth (vs. empty vectors) of these cells (Fig. 3C, right and Supplementary Fig. S4C, right).
Tumorigenicity

UBP43 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 UBP43 knockdown. Results were compared with empty vector transductants. Four weeks after injections, lung lesions were scored. ED-1 cells independently engineered with reduced UBP43 expression using different UBP43-repressing shRNAs produced significantly fewer (P < 0.01) lung cancers versus controls (Fig. 3D). Representative micrographs 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 3. Engineered UBP43 knockdown versus UBP43 overexpression in ED-1 lung cancer cells. A, stable UBP43 knockdown by lentiviral-mediated shRNA transduction or engineered UBP43 overexpression via retroviral-mediated transduction of UBP43 in ED-1 cells. UBP43 shRNA transduction (+) destabilized endogenous cyclin D1 protein expression as compared with a transfected empty vector (−; left). Retroviral transduction (+) of UBP43 increased UBP43 expression and augmented cyclin D1 expression in ED-1 cells (right). Actin expression confirmed similar protein loading in each lane. Quantification of signals is provided. B, lentiviral-mediated knockdown of UBP43 triggered significant apoptosis (left). UBP43 retroviral-mediated transduction inhibited apoptosis (right) in ED-1 cells versus the empty control vector transduction. C, lentiviral-mediated knockdown of UBP43 significantly inhibited proliferation (left) and engineered gain of UBP43 expression independently augmented proliferation (right) of ED-1 cells versus these empty control transduced cells. D, engineered UBP43 knockdown in ED-1 cells signifcantly reduced (P < 0.01) tumorigenicity of these transduced lung cancer cells. The indicated transfected UBP43 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 UBP43 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.
Antineoplastic drug responses

UBP43 is an IFN and retinoid-regulated species (10, 11, 13, 30, 31). This implied that regulating UBP43 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 UBP43 expression (see Supplementary Fig. S1). Individual treatment effects of RA, cisplatin, or IFN on growth and apoptosis assays were studied. UBP43 knockdown followed by RA, cisplatin, or IFN treatments increased growth-inhibitory and proapoptotic effects of each agent (Fig. 4). In contrast, forced UBP43 expression antagonized these ED-1L cellular effects (Fig. 5).

Differential UBP43 expression profiles were examined in normal versus malignant lung tissues. Two different polyclonal anti-UBP43 antibodies detected UBP43 in immunoblot assays (11). These antibodies were used in immunohistochemical assays. Both the anti-UBP43 antibody recognizing a domain nearer to the amino terminus (anti-UBP43-1) than the second anti-UBP43 antibody (anti-UBP43-2) detected UBP43 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 UBP43, cyclin D1, and cyclin E immunohistochemical expression. UBP43 levels from 75 NSCLC cases (see Venn diagram) versus normal lung are shown in Fig. 6C. UBP43 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 UBP43 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 UBP43 levels.

Discussion

This study advances previous work (10) by showing the ubiquitin protease UBP43 can regulate cyclin D1 stability. UBP43 is important in IFN and immune responses, leukemogenesis, among other biological effects (11, 12, 14,
UBP43 acts by removing ISG15 from conjugated proteins (12, 13). Our previous work implicated UBP43 in regulating stability of the oncogenic protein, PML/RARα (11). This study identified cyclin D1 as a growth regulatory protein whose stability was also affected by UBP43. UBE1L-ISG15-UBP43 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 UBP43 stabilized cyclin D1 (but not other D-type cyclins or cyclin E; Fig. 1A). This established UBP43 as a critical regulator of cyclin D1 protein stability. Cyclin D1 stabilization by UBP43 required UBP43 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 UBP43 repressed cyclin D1 expression. Specific cyclin D1 residues affected proteasomal degradation (26). This work showed cyclin D1 stabilization by UBP43 also depended on lysines within cyclin D1 (Fig. 1C).

Cyclin D1 can affect apoptosis, as reviewed (32). UBE1L is extended here by showing UBP43 regulates cyclin D1 and apoptosis, antineoplastic drug responses, and tumorigenicity. An inhibitor of UBP43 should exert antineoplastic effects. Its effects would likely cooperate with RA, cisplatin, and IFN. Loss of UBP43 expression augmented apoptosis (15). Loss of UBP43 expression caused a similar effect. UBP43 was shown here to regulate cyclin D1 expression, cell growth, apoptosis, and tumorigenicity. UBP43 knockdown also inhibited lung cancer formation in vivo (Fig. 3D). As expected, gain of UBP43 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 UBP43 can modulate responses to antineoplastic agents. UBP43 was found in this study to regulate response to RA, cisplatin, and IFN. Loss of UBP43 expression increased growth inhibitory and proapoptotic effects of these agents, but gain of UBP43 expression antagonized these activities (Figs. 4 and 5). These findings have therapeutic implications. An inhibitor of UBP43 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). UBP43 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 UBP43 and cyclin D1, but not cyclin E (Fig. 6C). Yet, UBP43 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 UBP43 regulates cyclin D1.
expression as well as apoptosis, proliferation, and tumorigenicity of cancer cells.

Interactions between UBE1L-ISG15-UBP43 pathway members may prove important. Intriguingly, these species and their E2 (UbcH8) and E3 (Herc5a) undergo ISG15ylation (35), indicating an autoregulatory UBP43 loop might exist. Therapeutic implications are highlighted by finding that UBP43 is overexpressed in diverse human cancers (Fig. 6).

In summary, this study uncovered UBP43 as an antineoplastic target. Enzymatically active UBP43 directly affected cyclin D1 stability. UBP43 knockdown repressed cyclin D1 expression, promoted apoptosis, and cooperated with effects of antineoplastic agents. Loss of UBP43 also repressed cyclin D1 expression and reduced tumorigenicity in a murine syngeneic lung cancer model. UBP43 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 UBP43 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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Figure 6. UBP43 expression profiles in paired normal-malignant tissue arrays. A, immunohistochemical assays for UBP43 expression levels were conducted using 2 different anti-UBP43 antibodies. Blocking peptides for each respective antibody were used. UBP43 protein expression was detected in the normal human lung. These peptides blocked UBP43 staining by each respective antibody. B, UBP43 immunohistochemical expression was enhanced in the malignant versus normal human lung tissues. C, differential expression profiles for UBP43, 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 UBP43 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 UBP43 expression (right). D, representative immunohistochemical expression of cyclin D1 and cyclin E in human lung cancers. E, differential UBP43 immunohistochemical expression profiles in a tissue array with diverse normal-malignant human tissues. Symbols refer to $^*$, $P < 0.05$ and $^{**}$, $P < 0.01$. 

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