



## Research Paper

## Regulation of XIAP Turnover Reveals a Role for USP11 in Promotion of Tumorigenesis



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## ABSTRACT

The emerging regulatory role of deubiquitinases (DUBs) has been implicated in various fundamental processes and pathogenesis. To determine the pivotal role that DUBs play in mediating tumorigenesis, we have performed a non-biased screen of 67 human DUBs based on a mammary cell transformation assay. This led to the identification of USP11 as a critical determinant of mammary tumor initiation and progression. Using an approach of protein complex purification coupled with mass spectrometry, we further identified XIAP to be a target for USP11. We demonstrated that, while depletion of XIAP attenuates cell transformation, elevated USP11 significantly promotes the tumor colony formation through stabilization of XIAP. Molecular modeling coupled with mutagenesis analyses further revealed that Leu207 on the BIR2 domain of XIAP facilitates its interaction with USP11. Stabilization of XIAP due to its deubiquitylation by USP11 leads to the inhibition of cell anoikis and apoptosis, which in turn promotes tumorigenesis. Finally, immunohistochemical staining revealed that aberrant accumulation of USP11 correlates with elevated levels of XIAP in breast cancer tissues. We therefore propose that aberrant USP11, via stabilization of XIAP, promotes tumor initiation and progression.

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## 1. Introduction

The ubiquitin–proteasome system (UPS) serves a critical function by orchestrating protein–protein interactions, protein stability, protein subcellular localization, and activation/deactivation of functional proteins among other cellular activities (Skaar et al., 2014; Lipkowitz and Weissman, 2011). Malfunction of the UPS leads to various human diseases including tumor development, from initiation to invasion (Lipkowitz and Weissman, 2011; Popovic et al., 2014). While ubiquitin protein E3 ligase targets functional proteins for ubiquitylation followed by degradation, deubiquitinases (DUBs) catalyze the removal of the ubiquitin chain attached to the substrate protein, thereby preventing the degradation, or promoting the stabilization of the targeted protein (Christianson and Ye, 2014; Clague et al., 2013). The activity of UPS has been tightly linked to tumorigenesis through its impact on a variety of cellular processes, including modulation of oncogenic or tumor

suppressing signaling, cell cycle control, genome stability, apoptosis, immune surveillance, and angiogenesis (Clague et al., 2013; Lipkowitz and Weissman, 2011; Skaar et al., 2014; Hanahan and Weinberg, 2011). While the role of a large number of ubiquitin–protein ligases in regulating carcinogenesis has been extensively studied, the importance of various DUBs in tumor initiation and invasion has not drawn the same amount of attention in the field until recently, especially in breast cancer (Clague et al., 2013). To systematically determine the pivotal role that DUBs play in initiating mammary tumorigenesis, we screened a non-biased library of DUBs using a mammary gland cell malignant transformation assay. This endeavor led to the identification of ubiquitin-specific peptidase 11 (USP11) as a critical DUB that promotes mammary tumor initiation and progression. Using a TAP-purification coupled with mass spectrometry, we further identified X-linked inhibitor of apoptosis protein (XIAP) to be a target for USP11.

The role of USP11 has been reported recently in modulating a number of signaling cascades such as DNA damage response, TGF- $\beta$ , NF- $\kappa$ B and Notch signaling pathways (Ramakrishna et al., 2011; Schoenfeld et al., 2004; Wu et al., 2014; Sun et al., 2010; Al-Salihi et al., 2012). Several substrates have been linked to USP11, including BRCA2, I $\kappa$ B $\alpha$  and promyelocytic leukemia protein (PML) (Ramakrishna et al., 2011; Schoenfeld et al., 2004; Wu et al., 2014; Sun et al., 2010; Al-Salihi et

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al., 2012). Regulation of BRCA2 by USP11 in response to DNA damage signal leads to an enhanced survivability for cancer cells (Schoenfeld et al., 2004). USP11 could counteract ubiquitylation of I $\kappa$ B thereby suppressing NF $\kappa$ B activation (Sun et al., 2010). The role of USP11 in modulating TGF- $\beta$  signaling is through deubiquitylation of the type I TGF- $\beta$  receptor ALK5 (Al-Salihhi et al., 2012). In addition, USP11 could regulate PML-mediated brain tumor pathogenesis through stabilization of PML via antagonizing RNF4-facilitated PML ubiquitylation (Wu et al., 2014). Recent pathological studies further revealed the correlation of abnormal accumulation of USP11 with poor prognosis of breast cancer (Bayraktar et al., 2013). However, how exactly USP11 involves in breast cancer development and what is its deregulation of downstream substrate contributes to mammary tumorigenesis remains unknown. Thus, the identification of USP11 to be a potent player in promoting transformation of mammary gland epithelial cells and further elucidation of the mechanism by which USP11 regulates XIAP in promoting breast tumor initiation could advance our knowledge of molecular basis of cancer formation and provide novel target for anti-cancer therapy.

X-linked inhibitor of apoptosis protein (XIAP) is a member of the inhibitor of apoptosis (IAP) family. XIAP has been initially identified for its role in blocking apoptosis upon inhibition of the activation of caspase-9, -3 and -7 through the XIAP-mediated protein degradation (Deveraux and Reed, 1999). Previous studies demonstrated the role of XIAP in regulating various cellular processes, including TGF- $\beta$  signaling, NF- $\kappa$ B pathway, autophagy and inflammatory responses (Birkey Reffey et al., 2001; Gyrd-Hansen et al., 2008; Huang et al., 2013; Krieg et al., 2009). Mutations of XIAP in human have been linked to X-linked lymphoproliferative syndrome type 2 (XLP-2), a rare primary immunodeficiency (Rigaud et al., 2006). Aberrant stabilization of XIAP is thought to be one of the reasons for chemoresistance (Merlo and Ceconi, 2013; Schimmer et al., 2006). Recent studies revealed that the growth factor-induced phosphorylation of XIAP by AKT leads to the stabilization of XIAP through inhibiting its auto-ubiquitylation, which, in turn, leads to the inhibition of autophagy and tumorigenesis (Huang et al., 2013). Moreover, abnormal accumulated XIAP protein was observed in various types of human cancers such as leukemia, clear-cell renal carcinoma, prostate cancer, hepatocellular carcinoma and breast cancer, suggesting its oncogenic role in tumorigenesis (Schimmer et al., 2006; Xu et al., 2014; Berezovskaya et al., 2005; Mehrotra et al., 2010; Shi et al., 2008). Our identification of deubiquitylation of XIAP by USP11 adds up a novel layer for XIAP regulation. Characterization of relevance of USP11-XIAP axis in breast cancer initiation, progression and sensitization of breast cancer cell to chemodrugs could provide new strategy for anti-cancer therapy.

## 2. Materials and Methods

### 2.1. Cell Lines and Cell Culture

MCF10A, MDA-MB-231, MDA-MB-468, MCF-7, T47D, SKBR3 and BT474 cells were obtained from the American Type Culture Collection (Manassas, VA). The viral packaging line Phoenix-A cells were the gift from Edward V. Prochownik (University of Pittsburgh). All cells were maintained in DMEM supplemented with 5% or 10% FBS, 1  $\times$  antibiotic/antimycotic solution (100 units/ml streptomycin and 100 units/ml penicillin) (all from Invitrogen). All cells were cultured at 37  $^{\circ}$ C in a humidified atmosphere containing 5% CO $_2$ .

### 2.2. Purification of USP11 Complex and Mass Spectrometry

MCF10A cells stably expression FLAG/HA-tagged USP11 were washed twice with PBS and lysed with NP-40 buffer (1% NP40, 10% glycerol, 25 mM Tris-HCl [pH 7.9] and protease inhibitor cocktails). USP11-interacting proteins were purified by immunoprecipitation and washing four times with TBST buffer (137 mM NaCl, 20 mM Tris-HCl [pH 7.6], 0.1% Tween-20). The complex was eluted with 3 $\times$ FLAG peptide in TBS

buffer. The elute was then separated on SDS-PAGE followed by Coomassie blue staining. The interest bands were cut out for mass spectrometry analysis (Gamper et al., 2012; Zhang et al., 2010).

### 2.3. Plasmids and Constructs

The 67 retrovirus based FLAG-HA tagged deubiquitinases and Myc-tagged XIAP plasmids were ordered from Addgene. The USP11 and XIAP full-length and deletion mutant constructs were generated by PCR amplification of the full-length or partial coding sequence subsequent subcloning into mammalian expression vectors with FLAG or HA tag. The full-length USP11 subsequent subcloning into pCDNA6.2 with V5 tag. The full-length USP11 subsequent subcloning into pEGFP-C1 with GFP tag. The full-length XIAP subsequent subcloning into pDsRed2-C1 with DsRed2 tag. XIAP dot mutation plasmids were generated by replacement as C203A, K206A, L207P and H467A by site-directed mutagenesis.

### 2.4. RNA Inference

RNA Inference-siRNAs (Sigma) specifically targeted to USP11, XIAP or luciferase were synthesized and transfected into cells using Lipofectamine 2000. Cells were collected at 48 h post-transfection for immunoblotting assay. The synthesized siRNA sequences are as following: Luciferase, 5'-CGUACGCGAAUACUUCGA-3'; USP11, 5'-ACCGAUUCU AUUGGCCUAGUA-3'; XIAP, 5'-AAUAGUGCCACGCAGUCUACA-3' and 5'-GCCGGCUGUCCUGGCGCGAAA-3'; SIAH1, 5'-CAGGAAACAGUUGCA UGUAGUACA-3' and 5'-GCUGAUAGGAACACGCAAGCA-3'; ARTS, 5'-GGGUACAACUUGCCUAU-3' and 5'-GAUCAUGCAAACUGUGGAGAU-3'; USP9X, 5'-GAUGUGGGUCGUACAGCUAGUAUU-3' and 5'-CAACUUGCUGCUAGGUUCCUUAU-3'.

### 2.5. Quantitative Real Time PCR Arrays and Analysis

Total RNAs were isolated from MDA-MB-231 using the Trizol (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Invitrogen). Real-time PCR was performed on a StepOnePlus Real-Time PCR System (ABI) in duplicates with Fast SYBR Green Master Mix (Applied Biosystems). The cycle threshold (CT) defines the number of PCR cycles required for the fluorescent signal to cross the threshold. Primers are USP11 forward 5'-TGGAAAGCGAGGATTATGTGC-3', reverse 5'-ATGACCTTGCGTTCAATGGGT-3'; XIAP forward 5'-AGTGGTAGTCTGTTCAGCATCA-3', reverse 5'-CCGCACGGTATCTCTTCA-3'; GAPDH 5'-TGTTGCCATCAATGACCCCTT-3'; reverse 5'-CTCCACGACTACTCAGCG-3'.

### 2.6. Antibodies and Chemicals

Specific antibodies against USP11 (A301-613A) and USP9X (A301-351A) were purchased from Bethyl Laboratories (Montgomery, TX). Antibodies against  $\beta$ -actin, and FLAG were from Sigma-Aldrich. XIAP (E-2), HA (F-7) and Myc tag (9E10) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Active caspase-3 and PARP1 antibody were from Cell signaling (Beverly, MA). The immunohistochemistry antibody XIAP (Cat: PRS3331) and USP11 (Cat: SAB1300069) were purchase from Sigma. The cisplatin (S1166), MG132 (S2619), XIAP inhibitor Embelin and USP11 inhibitor Mitoxantrone (S2485) were from SelleckChem (Houston, TX).

### 2.7. Packaging of Virus and Infection

Packaging of Retrovirus was performed in Phoenix-AMPHO cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (Zhou et al., 2013). Briefly, 1 day before transfection, the culture dish was replaced with fresh medium without antibiotics. Culture dish was replaced with IMDM without any antibiotics when the

cultured cells reach to 90% confluence. 20  $\mu$ l of Lipofectamine 2000 was mixed in 0.5 ml of OPTI-MEM at 25 °C for 5 min. Then, 8  $\mu$ g of retrovirus DNA was mixed in another 0.5 ml of OPTI-MEM. The DNA and Lipofectamine™ 2000 mixture was combined at 25 °C for 20 min. The transfection mixture was poured onto the culture dish. The viral supernatant was harvested with a 10-ml syringe and needle. The viral supernatant was filtered with a 0.45  $\mu$ m filter and further mixed virus with fresh DMEM complete medium (4:1 ratio). The viral mixture was added to a culture plate for infection. The stable cell lines were generated by culturing the cells in the medium containing antibiotic puromycin (1  $\mu$ g ml<sup>-1</sup>).

## 2.8. Immunoblotting and Immunoprecipitation Assay

Cells were harvested and lysed in RIPA lysis buffer (50 mM NaCl, 50 mM Tris pH 7.4, 0.5% DOC, 0.1% SDS, 1% NP-40, 1 mM EGTA and protease inhibitor cocktail). The protein concentration was determined using Bio-Rad Protein Assay Reagent (Bio-Rad). Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA), and probed with the appropriate antibodies overnight at 4 °C. Membranes were washed three times with PBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoblots were visualized with enhanced chemiluminescence detection reagents (Millipore, Billerica, MA). Semi-quantification of data was performed using software NIH Image J.

For immunoprecipitation assay, cell pellets collected at designated time points were lysed in buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 mM NaF, and protease inhibitor cocktail) on ice for 30 min. Then, 27-gauge one-half-inch syringes were used to shred the DNA. The supernatants were collected after centrifugation at 12,000  $\times$ g for 30 min. Equal amount of protein lysates at designated time points were aliquoted, and equal amount of primary antibody was added to the above lysates. After rotation at 4 °C overnight, equal amounts of immobilized protein A/G beads (Pierce, Rockford, IL) were added to the tubes. After rotation again at 4 °C for 4 h, the beads were collected by centrifugation at 2500  $\times$ g for 3 min. Electrophoresis loading buffer was added to the beads after washing with IP wash buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 $\times$  protein inhibitor mixture) five times. After denaturing at 95 °C for 5 min, the supernatants were subject to Western blot. For ubiquitylation assay, cell pellets were lysed in 2% SDS and 5 mM dithiothreitol and diluted into 1% Nonidet P-40 buffer. The final concentrations in the lysate used for M2 beads immunoprecipitation were 0.2% SDS, 0.5 mM dithiothreitol, 1% Nonidet P-40, 50 mM Tris, pH 8, 150 mM NaCl, 10 mM MgCl<sub>2</sub> and protease inhibitor cocktail.

## 2.9. Soft Agar Colony Formation Assays

The tumorigenicity of USP11 and XIAP was measured by soft agar colony formation assays in duplicate in three independent experiments (Zhou et al., 2013; Hu et al., 2015). Briefly, 1-ml underlayers of 0.6% agar medium were prepared in 35-mm dishes by combining equal volumes of 1.2% noble agar and 2 $\times$  DMEM with 40% fetal bovine serum (Difco). The cells were trypsinized, centrifuged, and resuspended, and 1  $\times$  10<sup>4</sup> MCF10A cells were plated in 0.3% agar medium. 1-ml top layers of 0.6% agar medium were prepared and add. The surface was kept wet by addition of a small amount of growth medium. After 3 weeks, dishes were stained with 0.005% crystal violet and colonies were photographed and counted.

## 2.10. Clonogenic Assay

Cell were plated for 24 h, then culture medium was replaced with either complete medium (for non treated controls) or complete medium containing cisplatin for 1 h. Cells were then washed once in PBS and

replaced with fresh medium. After an additional 7 to 10 days of culture, cells were fixed with an acetic acid/methanol (1:3) solution and stained with a dilute crystal violet (0.33%, w/v) solution, and surviving colonies consisting of 50 or more cells were counted (Zhou et al., 2013).

## 2.11. MCF10A Acini Culture

The 3D matrigel culture was operated as previously reported (Debnath et al., 2003). In brief, Add 200  $\mu$ l Matrigel to 24 well plates, then place plate in cell culture incubator and allow 20 min for the matrigel to solidify. Trypsinized cells and resuspended MCF10A cells in 5000 cells/200  $\mu$ l medium containing 2.5% Matrigel and 5 ng/ml EGF. MCF10A were added onto solidified matrigel and further culture for 2 weeks.

## 2.12. Tissue Specimens

65 breast invasive ductal carcinoma and 48 adjacent normal tissue specimens were analyzed. None of the patients had received radiotherapy or chemotherapy before surgery. Clinical specimens were obtained at the time of surgery. The specimens were immediately fixed in 4% polyformaldehyde and completely embedded in paraffin (He et al., 2014). Clinical characteristics of the patients are summarized in supplemental Table 1.

## 2.13. Annexin V/Propidium Iodide (PI) Assay

Apoptotic cell death was measured by flow cytometric analysis after staining using FITC-conjugated Annexin V/PI Kit (BD PharMingen) following the manufacturer's instructions. In the population of Annexin V-positive cells, PI-negative or PI-positive cells were considered to be early apoptotic or late apoptotic (Hu et al., 2015).

## 2.14. In Vivo Tumorigenesis Assay

In orthotopic model, 5  $\times$  10<sup>6</sup> of MDA-MB-231 cells were injected into the mammary fat pads of 8-week-old female Crl:Nu-Foxn1(nu) nude mice; 1  $\times$  10<sup>7</sup> of MCF10A cells were injected into the mammary fat pads of 6-week-old female C.B17-SCID mice. Tumor size was measured weekly. The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) from University of Pittsburgh (Hu et al., 2015).

## 2.15. Modeling USP11 and XIAP Structure

A homology model of the catalytic domain of USP11 was built using Swiss-Modeller web server (Biasini et al., 2014). The template for the model was the catalytic domain of USP4 (pdb: 2Y6E) (Clerici et al., 2014), which has 64.5% sequence identity with the catalytic domain of USP11. Typically, a sequence identity of 60% or more between template (2Y6E) and target (USP11) is considered to yield a highly successful homology model (Xiang, 2006). This homology model of USP11 and the crystal structure of XIAP-BIR2 domain (pdb 4WVS) was used to simulate the USP11-XIAP interaction.

## 2.16. Simulating USP11-XIAP Interactions

The interaction between USP11 and XIAP BIR2 domain was simulated using ClusPro3 (Comeau et al., 2004). This software uses a fully automated algorithm to model protein-protein interactions by performing docking simulations. The algorithm evaluates millions of putative complexes and first selects those with favorable surface complementarities. The resulting complexes are filtered based on good electrostatic interactions and desolvation energies for further clustering. The models of the complexes are then rank-ordered and outputted according to their clustering properties. The model shown in this work is ranked 9th in this

ranking and was selected because of the proximity of the catalytic triad on USP11 and the region 200–210 on the XIAP, which are shown to be interacting experimentally.

### 2.17. Ethics Statement

This study protocol had been reviewed and approved by the ethical committees of Chinese Academy of Medical Sciences Cancer Hospital. All participants gave written informed consent.

### 2.18. Tissue Microarray and Immunohistochemistry

Tissue microarrays (10 mm tissue cores for each tissue) were constructed. Immunohistochemistry staining was carried out following standard streptavidin-biotin-peroxidase complex method. Briefly, section was deparaffinized, and nonspecific bindings were blocked with 10% normal goat serum for 30 min. Section was then incubated with antibody overnight at 4 °C. For negative controls, the primary antibody was replaced by non-immune serum. After immunostaining, the sections were scanned, and imaged by a single investigator who was not informed of the clinical characteristics. The value of the integral intensity was measured by Aperio's ImageScope software (Vista, CA) (He et al., 2014).

### 2.19. Statistical Analysis

Statistical analysis was performed using two-tailed, two independent or paired sample *t*-tests, one-way ANOVA tests depending on the number of groups with SPSS17.0 (SPSS, Chicago, IL) (He et al., 2014; Hu et al., 2015).

## 3. Results

### 3.1. Identification of USP11 as a Potent Promoter of Cell Transformation into Cancer Cells

Using transformation of mammary gland epithelial cells as a model system, we have systematically evaluated the role of 67 DUBs in transforming mammary gland epithelial cells into cancer cells (Sowa et al., 2009). To this end, 67 DUB genes carried by retroviral vector were utilized to establish stably expressed cell lines based on mammary gland epithelial cell MCF10A. Each of the 67 stable cell lines was then subjected to soft agar colony formation assay (Zhou et al., 2013; Hu et al., 2015). As shown in Fig. 1A, several DUBs were observed to promote colony formation with USP11 being the most potent DUB whose elevation dramatically promoted the transformation of mammary gland epithelial cells into cancer cells. The oncogenic role of USP11 in mammary tumor initiation was further validated by 3D matrigel based acini overgrowth assays (Hu et al., 2015; Debnath et al., 2003). As shown in Fig. 1B & C, while elevated expression of wild-type USP11 led to transformation of MCF10A and increased acini overgrowth, the expression of mutant USP11 without catalytic activity (Cys318 is replaced by Ala) failed to promote colony formation as well as acini overgrowth. In addition, results from the immune-staining coupled confocal microscopy indicate that inhibition of cell anoikis leads to the loss of basement attachment through the inhibition of caspase-3, which could be the mechanism behind the role of USP11 in promoting mammary tumorigenesis (Fig. 1D). Altogether, these results suggest that USP11 is a potent oncogenic player of mammary tumor transformation and its alteration in program cell death could be a pivotal event for tumor formation.

### 3.2. Increased Levels of USP11 Correlates with Breast Cancer Prognosis

To examine the possible clinical relevance of USP11 in breast cancer development, we measured the protein expression levels of

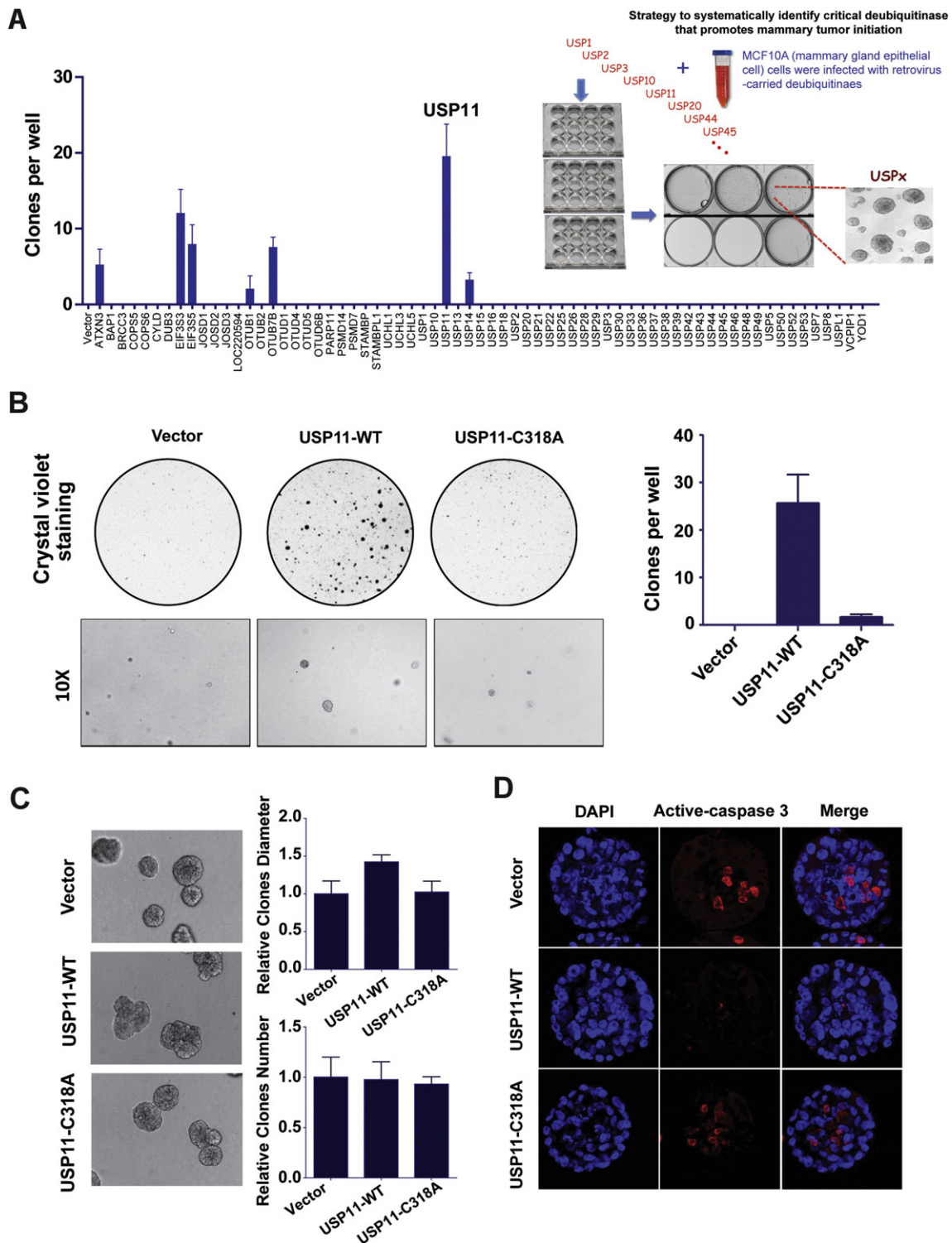
USP11 in various types of breast cancer cells as well as human breast tumor tissues in comparison to adjacent normal tissues by immunohistochemistry (IHC) (Hu et al., 2015). As shown in Fig. 2A, while USP11 expression is relatively low in mammary epithelial cell MCF10A and moderate in Her2 positive breast cancer cell SKBR3, a significant accumulation of USP11 is observed in triple negative and ER positive type of breast cancer cell lines, including MDA-MB-231, MDA-MB-468, MCF7 and T47D (Zhou et al., 2013; Hu et al., 2012). Furthermore, tissue arrays of 65 breast invasive ductal carcinoma and 48 adjacent normal tissue specimens were examined by IHC with anti-USP11 and visualized by DAB staining (He et al., 2015). As shown in Fig. 2B–C, the USP11 protein levels are significantly higher in breast tumor tissues than those in the adjacent normal tissues. The USP11 protein levels are also higher in well-moderate differentiation (W-M) and lymph node positive (LN+) breast cancer tissue than those in poor differentiation (P) and Lymph node negative (LN-) breast cancer tissue (Fig. 2D–F). In addition, the Kaplan-Meier survival assay for USP11 indicates that patients with overexpression of USP11 correlate with short DMFS time compared to patients with low USP11 in either overall breast cancer patients or ER positive patients (Fig. 2G & H). Thus, USP11 levels are higher in tissues from breast cancers associated with poor prognosis.

### 3.3. Identification of XIAP as a Putative Substrate for USP11

To search for the USP11 substrate(s) that facilitate the USP11-mediated mammary tumorigenesis, we used MCF10A cells expressing tagged USP11 and isolated USP11-substrate complexes by tandem immunoprecipitation (Gamper et al., 2012; Zhang et al., 2010; Sowa et al., 2009). These assays led to the identification of several USP11-binding partners, including XIAP, by mass spectrometry (Fig. 3A & B). The interaction of XIAP with USP11 was confirmed by blotting complexes having endogenous USP11 precipitated with XIAP specific antibodies (Fig. 3C) or complexes of overexpressed tagged USP11 with antibodies against Myc tag (Fig. 3D) and probing for XIAP. Finally, the co-localization of XIAP and USP11 in the cytosol was demonstrated by confocal microscopy (Fig. 3E & F).

### 3.4. USP11 is a DUB that Prevents XIAP from Ubiquitin-dependent Degradation

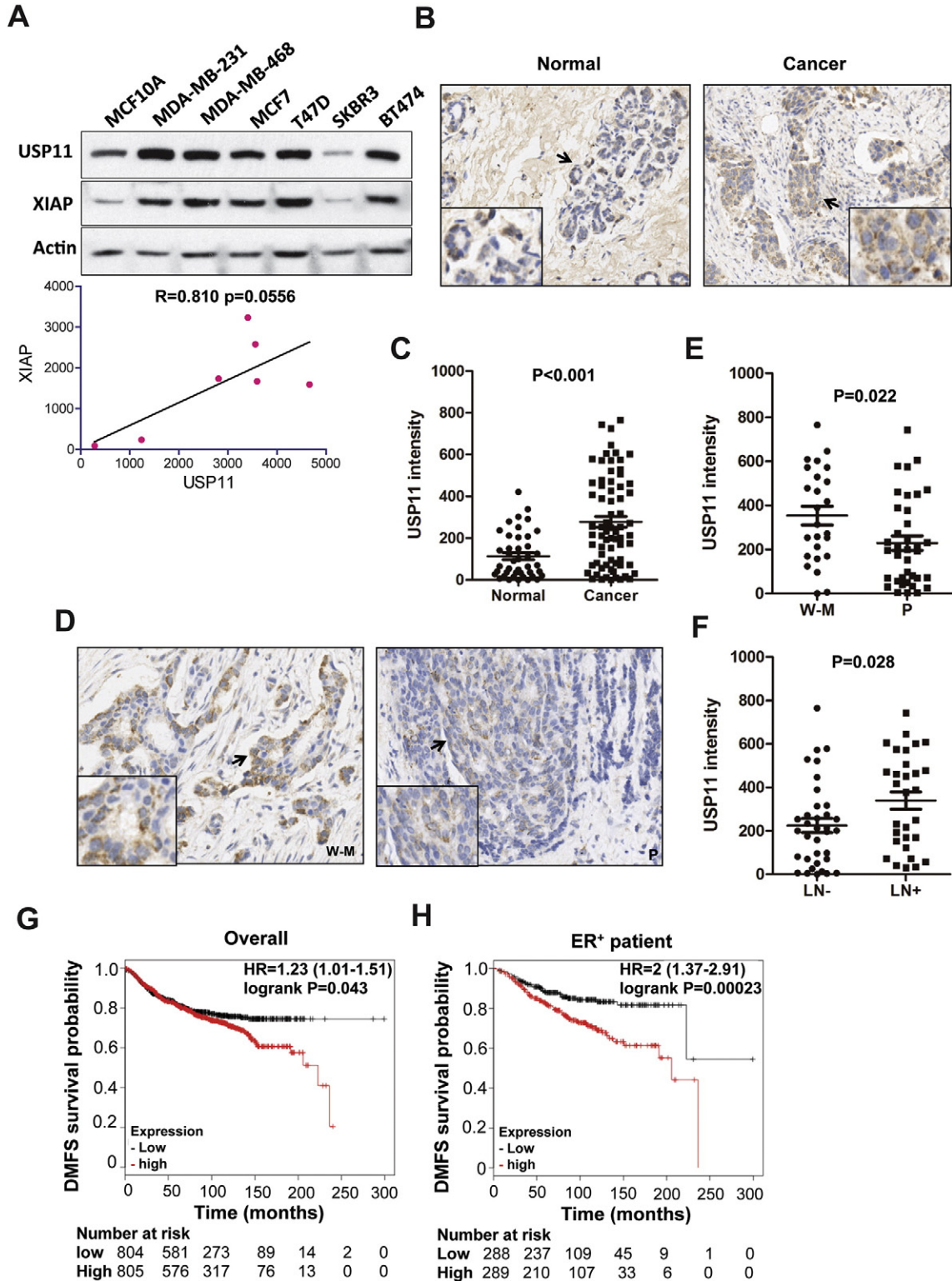
To characterize whether XIAP is a physiologically relevant USP11-substrate that mediates tumorigenesis, we examined the role of USP11 in regulating the abundance of XIAP, its turnover rate, and its ubiquitylation, along with the pathological correlation, if any, between USP11 and XIAP in human breast cancer specimens (Gamper et al., 2012; Hu et al., 2012; Zhou et al., 2013). We observed that the XIAP expression levels were tightly regulated by USP11, and that elevated expression of USP11 led to significant upregulation of both endogenous and exogenous XIAP, while as the mRNA level of XIAP has no change (Fig. 4A & B, Supplemental Figs. 1 & 2A). We further observed that while depletion of USP11 resulted in accelerated turnover rate for XIAP, elevation of USP11 led to a longer half-life for XIAP in mammary gland epithelial cells as well as breast cancer cells (Fig. 4C & D). Moreover, inhibition of USP11 by Mitoxantrone (It was originally developed as type II DNA topoisomerase inhibitor. Its inhibiting role against USP11 was recently identified by using a high throughput screening of USP11 enzyme activity (IC<sub>50</sub> = 3.15 μM)) resulted in a significant drop in XIAP levels (Fig. 4E) (Burkhart et al., 2013). The drop of XIAP protein levels in response to Mitoxantrone are caused due to accelerated turnover of XIAP protein but not mRNA level (Supplemental Fig. 2B–D). In addition, we measured the effect of the C318A mutant USP11 on XIAP ubiquitylation (Hu et al., 2015). As shown in Fig. 4F, the expression of wild-type USP11 but not USP11-C318A mutant results in significantly decreased XIAP



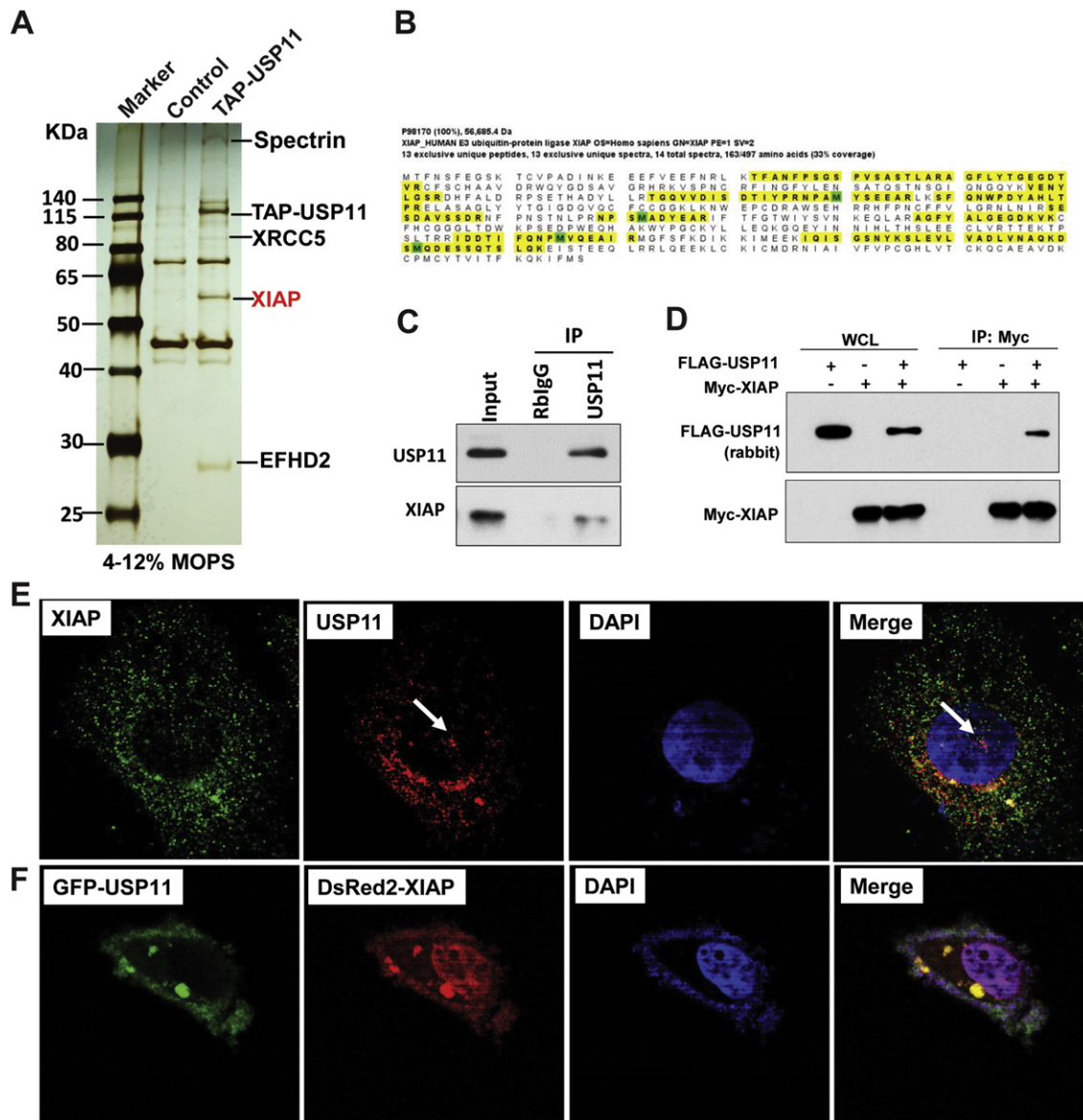
**Fig. 1.** Identification of USP11 as a potent determinant that promotes transformation of mammary gland epithelial cells. (A) 67 deubiquitinase genes carried by retroviral vector were utilized to establish stably expression cell line based on mammary gland epithelial cell MCF10A, respectively. The individual 67 stable cell lines were subjected to soft agar colony formation assay, respectively. The capacity of each deubiquitinase in promoting the transformation of mammary gland epithelial cell was systematically evaluated. Several deubiquitinases were observed to promote colony formation. USP11 turns to be the most potent one that promotes the transformation of mammary gland epithelial cells. (B) Confirmation of USP11 in transformation of mammary gland epithelial cells. Wild-type USP11 and USP11 deubiquitinase catalytic death mutant (cysteine 318 is replaced by alanine) were stably delivered into MCF10 cells. While USP11-WT dramatically drove MCF10A cell for colony formation, USP11-C318A failed to transform MCF10A cells. (C) Acini overgrowth analysis for USP11. Both USP11-WT and USP11-C318A were subjected to an acini overgrowth assay. The count of colony size and number were shown in right panel. USP11-WT but not USP11-C318A mutant in promoting MCF10A acini overgrowth was observed. (D) Staining of active caspase 3 and DAPI in MCF10A acini culture. USP11-WT but not USP11-C318A mutant inhibits the activation of caspase 3 in central cell population of acini culture.

ubiquitin-conjugates, suggesting that USP11 is the DUB that protects XIAP from turnover degradation and XIAP is a putative substrate that facilitates USP11-mediated oncogenic effect. This notion is further

supported by the significant pathological correlation between USP11 and XIAP in human breast tumor tissues in comparison to the adjacent normal tissues (Fig. 4G & H and Supplemental Fig. 3).



**Fig. 2.** Abnormal USP11 expression correlates with breast cancer prognosis. (A) Expression of USP11 and XIAP in mammary gland epithelial cell and various types of breast cancer cell lines. While USP11 expression is relatively lower in mammary epithelial cell and moderately in Her2 positive breast cancer cells, significant accumulation of USP11 is observed in triple negative and ER positive type of breast cancer cell lines, including MDA-MB-231, MDA-MB-468, MCF7 and T47D. The down panel showed the statistical analysis of western blotting staining of USP11 expression positively correlated with XIAP expression in breast cancer cell line ( $R = 0.810$ ,  $p = 0.0556$ ). (B) Tissue arrays of 65 breast invasive ductal carcinoma and 48 adjacent normal tissue specimens were subjected to immunohistochemistry with anti-USP11 and visualized by DAB staining. Representative normal and cancer tissue staining are shown. (C) Summary of B. (D) Representative staining of well-moderate (W-M) ( $n = 26$ ) and poor (P) ( $n = 37$ ) differentiation were shown. (E) Summary of D. (F) Summary of USP11 expression in lymph node negative (LN-) ( $n = 35$ ) and positive (LN+) ( $n = 30$ ) breast cancer tissue. (G and H) Kaplan-Meier survival assay of USP11 in overall breast cancer G and ER positive breast cancer H. Patients with USP11 overexpression shows short distant metastasis-free survival (DMSF) time in comparison with patients with low USP11 in either overall breast cancer patients or ER positive patient.



**Fig. 3.** Identification of XIAP as a putative substrate for USP11. (A) Engineering of TAP-USP11 stable expression clone and purification of USP11 protein complex. Proteins that interacted with USP11 were purified from MCF10A cells expressing FLAG and HA-tagged USP11 or MCF10A (control). Several proteins including XIAP as well as spectrin, XRCC5 and EFHD2 were found in the USP11 protein complex. (B) The sequences of mass spectrometry analysis for identification of XIAP (P9817) to be an interacting partner of USP11. The identified peptides were labeled in yellow. (C) Interaction between endogenous USP11 and XIAP was validated by co-immunoprecipitation. (D) Interaction between ectopically expressed FLAG-USP11 and Myc-XIAP was demonstrated using immunoprecipitation. (E) Validation of the interaction between endogenous USP11 and XIAP by immunostaining and confocal microscopy in breast cancer cell. Nuclei were stained using DAPI. USP11 and XIAP were observed to be colocalized in cytosol. The white arrow indicated the presence of nuclear USP11 staining. (F) GFP-USP11 and Dsred2-XIAP were cotransfected in MDA-MB-231 cells. Colocalization of GFP-USP11 and Dsred2-XIAP were observed.

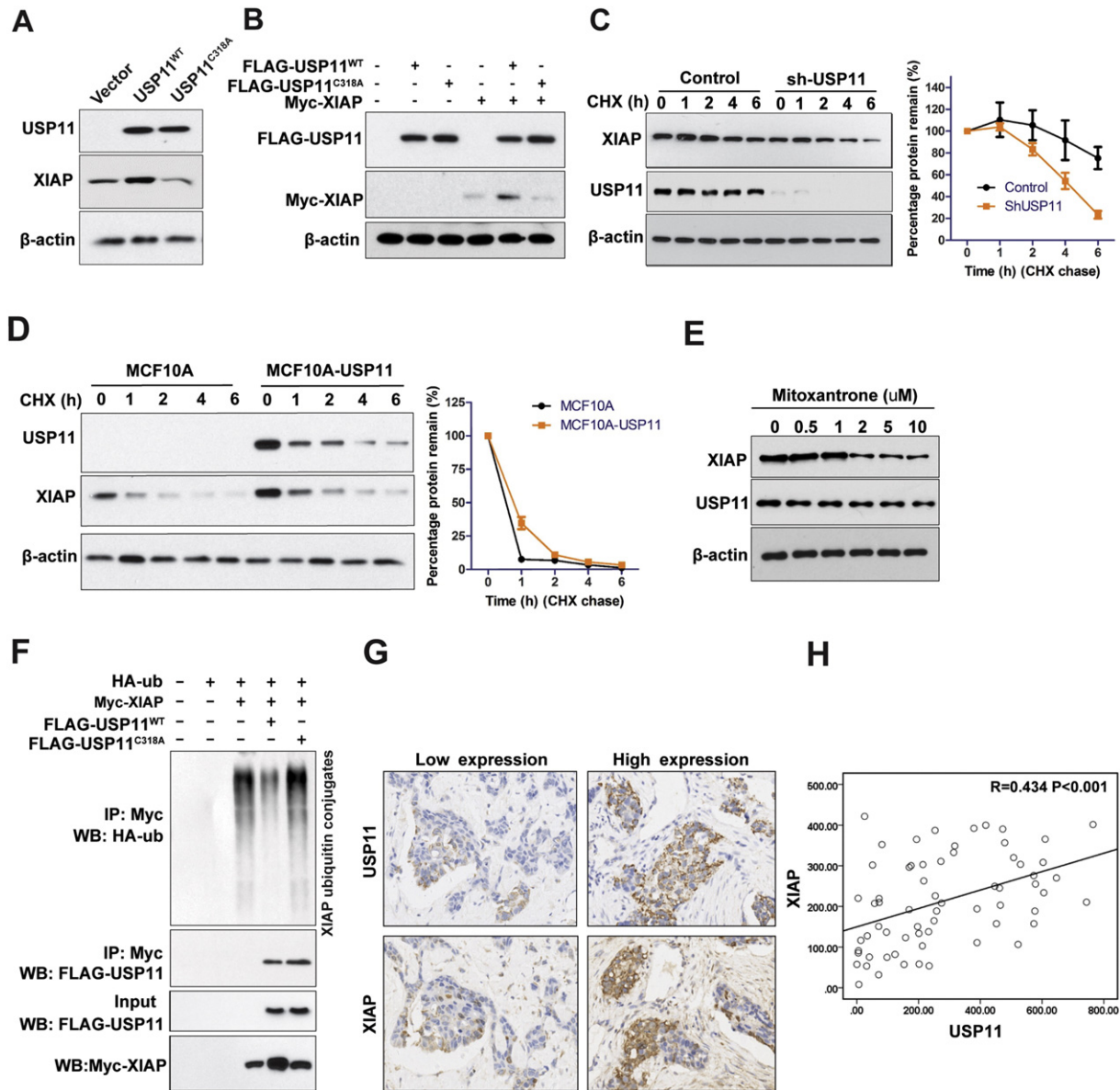
### 3.5. Identification of Molecular Motifs on USP11 and XIAP that Mediate Deubiquitylation of XIAP by USP11

Towards gaining a mechanistic understanding of the recognition of XIAP by USP11, we have next identified the molecular motifs on USP11 as well as on XIAP that facilitate their interaction (Hu et al., 2015). To date, a series of deletion mutants for both USP11 and XIAP were engineered as illustrated in Fig. 5A and C. Cotransfection of expression vectors coupled with co-immunoprecipitation helped us make a first assessment of the binding region between USP11 and XIAP (Zhang et al., 2010). As shown in Fig. 5B, the mapping result indicates that the amino acid stretch 503–963 on the carboxyl-terminus of USP11 is critical in facilitating its interaction with XIAP. As for XIAP, the first mapping narrowed down the range of amino acids that lie in its USP11-binding region to

residues 93–230 (Fig. 5D). A second round of mapping further narrowed down the range of USP11-binding residues to 10 amino acids (200–210) on the BIR2 domain of XIAP (Fig. 5E).

### 3.6. Identification of XIAP Leu207 as a Critical Residue in the Deubiquitylation of XIAP by USP11

The above analysis helped us narrow down the interfacial residues of the XIAP-USP11 complex to 503–963 (on USP11) and 200–210 (on XIAP). To further characterize and visualize the mechanism of interaction between USP11 and XIAP, we performed molecular docking simulations, using the computational methods described earlier (Hu et al., 2015). Our results indicate that Phe228, Phe229, Cys203 and Leu207 on XIAP are engaged in tight interactions with the USP11 catalytic



**Fig. 4.** USP11 as a deubiquitinase stabilizes XIAP from ubiquitin-dependent degradation. (A) While elevated expression of USP11-WT led to increased XIAP protein levels, expression of deubiquitinase catalytic death mutant USP11 failed to stabilize XIAP in MCF10A cells. (B) Expression of USP11-WT but not USP11-C318A stabilizes Myc tagged XIAP expression levels in HEK293 cells. (C) Depletion of USP11 leads to decreased turnover half-life of XIAP in MDA-MB-231 cells. The density of XIAP band was quantified, normalized to the internal control  $\beta$ -actin, which are presented in right panel. (D) Elevated expression of USP11 results in prolonged XIAP protein turnover half-life in MCF10A. The density of XIAP band was quantified, normalized to the internal control  $\beta$ -actin, which are presented in right panel. (E) Blockade of USP11 by pharmacological inhibitor Mitoxantrone destabilizes XIAP protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with Mitoxantrone at indicated concentration for 24 h. (F) Expression of USP11-WT but not USP11-C318A results in decreased XIAP ubiquitin-conjugates in HEK293 cell. HEK293T cells were transfected with the indicated plasmids for 48 h and then collected for immunoprecipitation assay with the FLAG tag antibody followed by the immunoblotting analysis with the HA and Myc tag antibody. (G and H) Elevated expression of USP11 and XIAP are significantly correlated in 65 cases human breast cancer tissue specimen. G Represents paired immunohistochemistry staining of USP11 and XIAP. H Shows statistical analysis of immunohistochemistry staining and indicates USP11 expression is positively correlated with XIAP expression in breast cancer ( $R = 0.434, p < 0.001$ ).

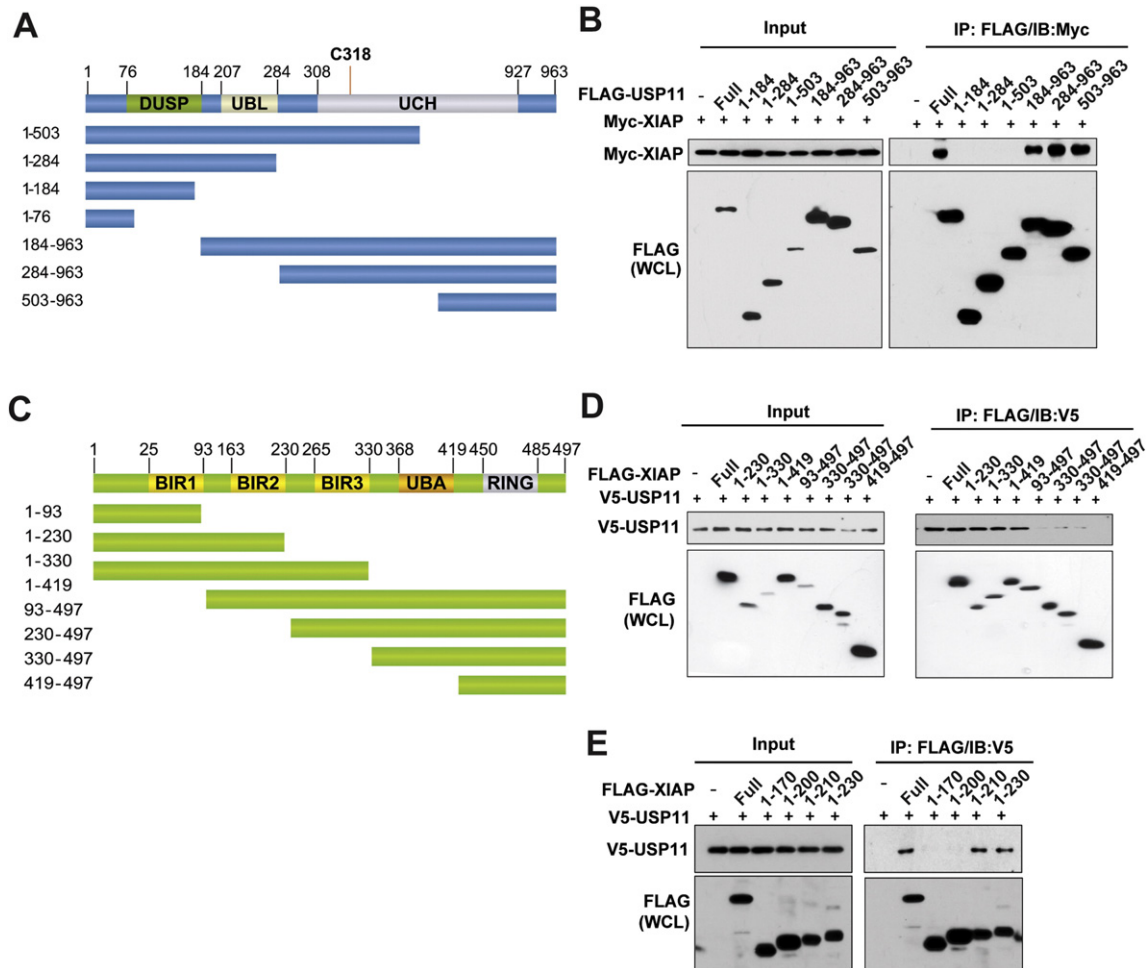
residues Cys318, His388 and Asp406 (Fig. 6A). In particular, we note the cation- $\pi$  interactions between Phe229 and His388. The precise positioning of F228 is enabled by the tight interactions of Leu297 with H220 and H223 and the disulfide bridge between C203 and C227 (Fig. 6B and Supplemental Fig. 4).

To validate the impact of Cys203 and Leu207 as well as Lys206 on XIAP in mediating the interaction of XIAP with USP11, we performed a multiple sequence alignment at that particular region (residues 200–210), using the aligned the amino acid stretch from 200 to 210 of human XIAP with mouse, rat and xenopus XIAP sequences as well as the human CIAP1 and CIAP2. As indicated in Fig. 6C, Cys203 and Leu207 (cysteine 203 and leucine 207 as well as Lys206) are conserved residues among these species. We thus then engineered point XIAP

mutant constructs for C203A, L207P and K206A, and further test the impact of these point mutations on XIAP turnover regulation. As shown in Fig. 6D–F, replacing Leu207 with proline, but not mutation of Cys203 or Lys206 to alanine, significantly destabilizes XIAP, presumably, due to the perturbation of the interfacial interaction between USP11 and XIAP and reduced deubiquitylation capacity of USP11. These results further suggested that Leu207 was the critical residue on XIAP in mediating the binding between USP11 and XIAP. Loss of interaction due to mutation of leucine 207 in the mutant L207P could prevent protection from degradation of XIAP afforded that is otherwise provided by USP11.

Given that the stability of XIAP is tightly regulated by USP11, we asked what the ubiquitin-protein ligase that governs XIAP ubiquitylation and





**Fig. 5.** Mapping of the binding interface between USP11 and XIAP. (A) Schematic diagram of human USP11 domains and strategy for engineering of a series of USP11 deletion mutants. (B) Identification of amino acid stretch 503–963 on the carboxyl-terminus of USP11 involved in mediating its interaction with XIAP. (C) Schematic diagram of human XIAP domains and strategy for engineering of a series of XIAP deletion mutants. (D) Identification of molecular region on XIAP, amino acid stretch 93–230, facilitating the interaction between XIAP and USP11. (E) Fine mapping of amino acid stretch 200–210 on the BIR2 domain of XIAP in mediating its interaction with USP11.

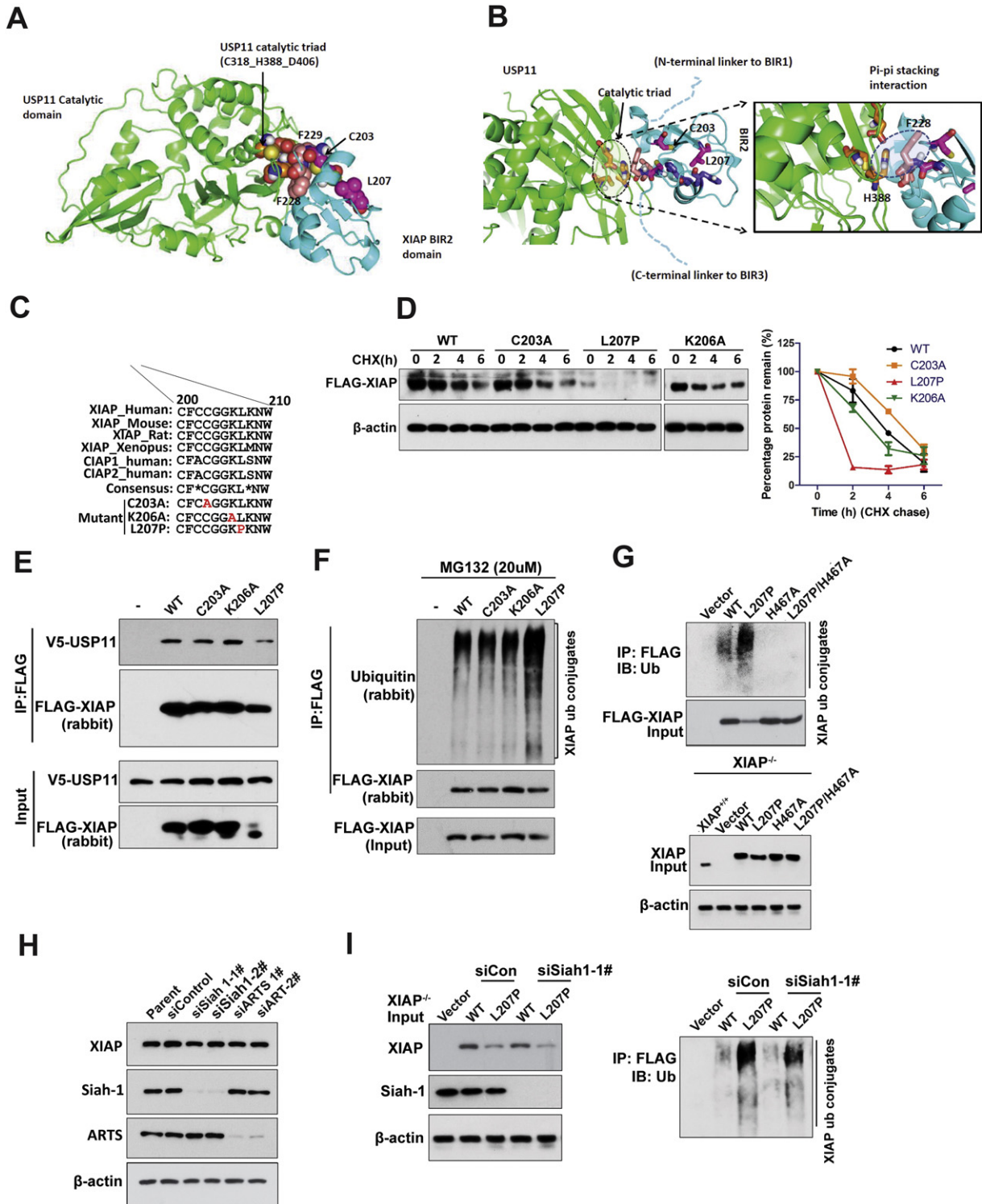
degradation is. It has been previously reported that while E3 ligase Siah1 targets XIAP for ubiquitylation, XIAP could also undergo self-ubiquitylation through its RING domain on the carboxyl-terminus (Garrison et al., 2011; Yang et al., 2000). To confirm the identity of the ubiquitin E3 ligase involved in mammary tumorigenesis, we have examined the effects of dissecting the RING domain using crispr depletion of XIAP, or knockdown of Siah1 (Ran et al., 2013). As shown in Fig. 6G–I and Supplemental Fig. 5, XIAP with mutation at the RING domain (H467A) fails to be ubiquitylated after XIAP crispr deletion but no effect was seen for Siah1 knockdown, suggesting that XIAP ubiquitylation is facilitated by its RING domain.

### 3.7. Stabilization of XIAP by USP11 Inhibits Anoikis and Promotes Tumor Transformation and Drug Resistance

Results from the above studies demonstrated that USP11 was a potent oncogenic factor that promotes mammary tumorigenesis by affecting cellular apoptotic response. XIAP is the substrate for USP11, which mediates the USP11-dependent tumorigenesis. Given that XIAP is an inhibitor of apoptosis, we next asked what is the physiological consequence of stabilizing XIAP by USP11 on tumorigenesis (Galban and Duckett, 2010; Merlo and Cecconi, 2013). To date, we have assessed the impact of USP11 in orchestrating apoptosis through regulating XIAP (Liu et al., 2006). We also tested the effect of alteration of XIAP by USP11 on MCF10A transformation

(Zhou et al., 2013). Moreover, we examined the effect of inhibition of USP11 or XIAP by pharmacological inhibitors on the sensitization of breast cancer cells to chemotherapy. Finally, we determined the impact of USP11 in mammary tumorigenesis using human breast cancer xenograft mouse model.

As shown in Fig. 7A & B, we observed that elevated expression of wild-type USP11, but not DUB-deficient mutant USP11, significantly inhibits activation of caspase-3 and caspase -9, cleavage of PARP1 and anoikis in mammary gland epithelial cells. In addition, expression of wild-type XIAP leads to the inhibition of anoikis, we observed that disruption of interaction between XIAP and USP11 by expression of XIAP L207P mutant (interaction deficient) significantly attenuates the inhibition of anoikis by XIAP (Supplemental Fig. 6). We further observed that depletion of XIAP attenuates the USP11-mediated colony formation in MCF10A cells Fig. 7C & D. Moreover, we compared the protein stability of XIAP-WT and XIAP-L207P and evaluated their capacity on promoting MCF10A colony formation. As shown in Fig. 7E, the stability of ectopically expressed XIAP-WT is higher than that of the interaction-deficient XIAP mutant. We also observed that the loss of interaction with USP11 (due to L207P mutation) significantly reduced the role of XIAP in MCF10A colony formation. Results from Fig. 7F indicate that depletion of USP11 leads to enhancement of apoptosis. In addition, preventing the effect of USP11 by Mitoxantrone or inhibition of XIAP by Embelin significantly sensitized triple negative breast cancer cells to the chemo-



**Fig. 6.** Identification of Leucine 207 as the critical residue that facilitates the interaction between USP11 and XIAP. (A) Molecular docking analysis of the BIR2 domain of XIAP and the USP11 catalytic core. USP11 is shown in green and XIAP in cyan. The amino acid C203 and L207 on XIAP are colored magenta. The USP11 catalytic triad (C318, H388, D406) are colored orange. (B) The cation-pi stacking interaction of BIR2 domain on XIAP with the USP11 catalytic triad, indicating that C203 and L207 are critical for the cation-pi interaction. The blow up shows in detail the cation-pi interaction between H388 and F228 on BIR2 domain of XIAP and USP11, respectively. (C) Alignment assay of the amino stretch 200–210 of human XIAP with mouse, rat and xenopus XIAP and human CIAP1, CIAP2. Three point-mutant as indicated (C203A, K206A and L207P) were constructed. (D) Pulse-chase analysis of the protein stability of three XIAP mutants, indicating that L207 on XIAP but not C203 or K206 is critical in facilitating interaction between XIAP and USP11. (E) Replacement of leucine by proline residue on XIAP leads to reduced interaction between USP11 and XIAP, which further results in destabilization of XIAP. (F) Replacement of L207 by proline residue on XIAP leads to destabilization of XIAP due to loss of protection from USP11. (G) XIAP is a self-ubiquitin protein ligase that catalyzes its own ubiquitylation. Wild-type XIAP and its mutants (L207P, H467A and L207/H467A) were transfected into XIAP<sup>-/-</sup> cells, respectively. XIAP protein complex was then pulled-down with M2-beads following by immunoblotting with antibody against ubiquitin. Abrogation of RING domain function on XIAP by the replacement of histidine 467 with alanine results in the failure of XIAP ubiquitylation. (H) Depletion of either Siah-1 or ARTS has no effect on XIAP expression in MDA-MB-231 cells. (I) No effect of Siah-1 depletion on XIAP ubiquitylation is observed in an ubiquitylation assay.

therapeutic agent cisplatin, doxorubicin as well as TNF $\alpha$  (Fig. 7G&H and Supplemental Figs. 7–9) (Burkhart et al., 2013; Nikolovska-Coleska et al., 2004). Finally, the result from the human breast cancer xenograft model shows that elevation of USP11-WT but not USP11-C318A significantly promotes mammary gland epithelial cell transformation and breast tumor progression (Fig. 7I–K and Supplemental Fig. 10). Altogether, our results suggest that the stabilization of XIAP by USP11 inhibits anoikis, promotes the development of cancer in mammary gland epithelial cells, and increases drug resistance in breast cancer cells.

#### 4. Discussion

Previous studies have implicated USP11 in tumorigenesis, although the underlying mechanism remained unclear. The work presented here establishes the inhibitor of apoptosis XIAP as a target of the deubiquitinase USP11 in human cells and xenograft mouse model, and provides concrete evidence for the coordinated-regulation of XIAP by its autoubiquitylation and USP11, to favor its deubiquitylation or stabilization, thus causing/assisting USP11-mediated tumor transformation. Accumulation of XIAP due to abrogated elevation of USP11 promotes tumor formation as well as progression because of its inhibition of anoikis in mammary gland epithelial cells and apoptosis in cancer cells, which enhances the escape of cells from regulated death. XIAP is directly bound to USP11 as shown here by both experimental and computational examinations. Our experiments in which USP11 was either overexpressed or depleted have documented the role of USP11 in XIAP stabilization and in determining the transformation of human mammary gland epithelial cells into cancer cells. These studies indicate that the DUB USP11 acts as a mediator of tumorigenesis by counteracting XIAP degradation and thus preventing the apoptotic response of the cells (Fig. 8).

DUB is a counteracting component of the ubiquitin-proteasomal system that together with ubiquitin-protein ligase regulates protein stability as well as subcellular localization of protein (Clague et al., 2013; Engel et al., 2016). DUB regulates protein function through several mechanisms, including direct binding to substrate and removing ubiquitin-chain from its target, modulating ubiquitin conjugating enzyme E2, counteracting ubiquitin E3 ligase and assisting proteasomal function (Clague et al., 2013; Engel et al., 2016). Several DUBs have been recently reported to be critical in tumorigenesis-related cellular processes (Ramakrishna et al., 2011; Wu et al., 2013; Popov et al., 2007; Schwickart et al., 2010). USP1 and USP28 were reported in regulating FNACD2 mono-ubiquitylation and PCNA ubiquitylation in order to maintain genome stability (Huang and D'Andrea, 2006; Nijman et al., 2005). USP28 regulates DNA damage response through stabilizing Chk2 and 53BP1 (Zhang et al., 2006). The role of several DUBs such as USP7, USP9x, USP28 and CYLD have been linked to programmed cell death and tumor formation (Ramakrishna et al., 2011; Wu et al., 2013; Popov et al., 2007; Schwickart et al., 2010). In addition, preclinical studies of several small molecule inhibitors for DUBs such as UCHL5 and USP14 shed a light on anti-cancer treatment suggesting that DUBs could be novel targets for future's cancer therapy. Our non-biased search for DUB in promoting mammary gland epithelial cell transformation unveils the potent oncogenic role of USP11 in driving mammary

tumor formation, which reveals a new layer of molecular basis of mammary tumor initiation and progression. Our observation that depletion of USP11 or inhibition of USP11 by a non-specific USP11 inhibitor Mitoxantrone significantly sensitized triple negative breast cancer cells to the chemo-drug cisplatin further supports USP11 as a potential target for anti-cancer treatment.

XIAP was previously demonstrated to be a fast-turnover protein (Galban and Duckett, 2010). Siah1 and XIAP itself is thought to be an ubiquitin-protein ligase that catalyzes XIAP for ubiquitylation following by degradation (Garrison et al., 2011; Galban and Duckett, 2010). Based on protein complex purification coupled with mass spectrometry, we have demonstrated that XIAP is a target of USP11, wherein USP11 directly binds to XIAP through Leu207 on XIAP and stabilizes it by deubiquitylation of XIAP. Very recently, it was reported that the XIAP protein levels could be also regulated by USP9x in small fraction of mitotic population (Engel et al., 2016) (Supplemental Fig. 1). Besides regulation of TGF- $\beta$  signaling, NF- $\kappa$ B pathway, autophagy and inflammatory responses formation (Ramakrishna et al., 2011; Wu et al., 2013; Popov et al., 2007; Schwickart et al., 2010), the most important function of XIAP is to inhibit caspase-9, caspase-3 and caspase-7 through protein degradation mechanism (Merlo and Cecconi, 2013). Using mammary gland epithelial cell based 3D matrigel culture (acini overgrowth assay) and breast cancer cell based apoptosis analyses, we explored the mechanism of the molecular cascade of USP11-XIAP in mammary tumor formation, in particular, the stabilization of XIAP by overriding USP11. During oncogenesis, abrogated accumulation of USP11 that targets XIAP for deubiquitylation protects it from protein degradation, thereby resulting in increased XIAP levels (Debnath et al., 2003). Our results show elevated XIAP directly leads to the inhibition of caspase 3, caspase 9, and decrease in PARP1 cleavage and Annexin V stained population that all together point towards inhibition of anoikis or cell apoptosis. While we do not know yet how USP11 is regulated in response to the upstream oncogenic signaling at this time, our validation analysis based on breast cancer mouse model and pathological analyses using human breast tumor specimen confirm the impact of USP11-XIAP axis in mammary tumor initiation and progression.

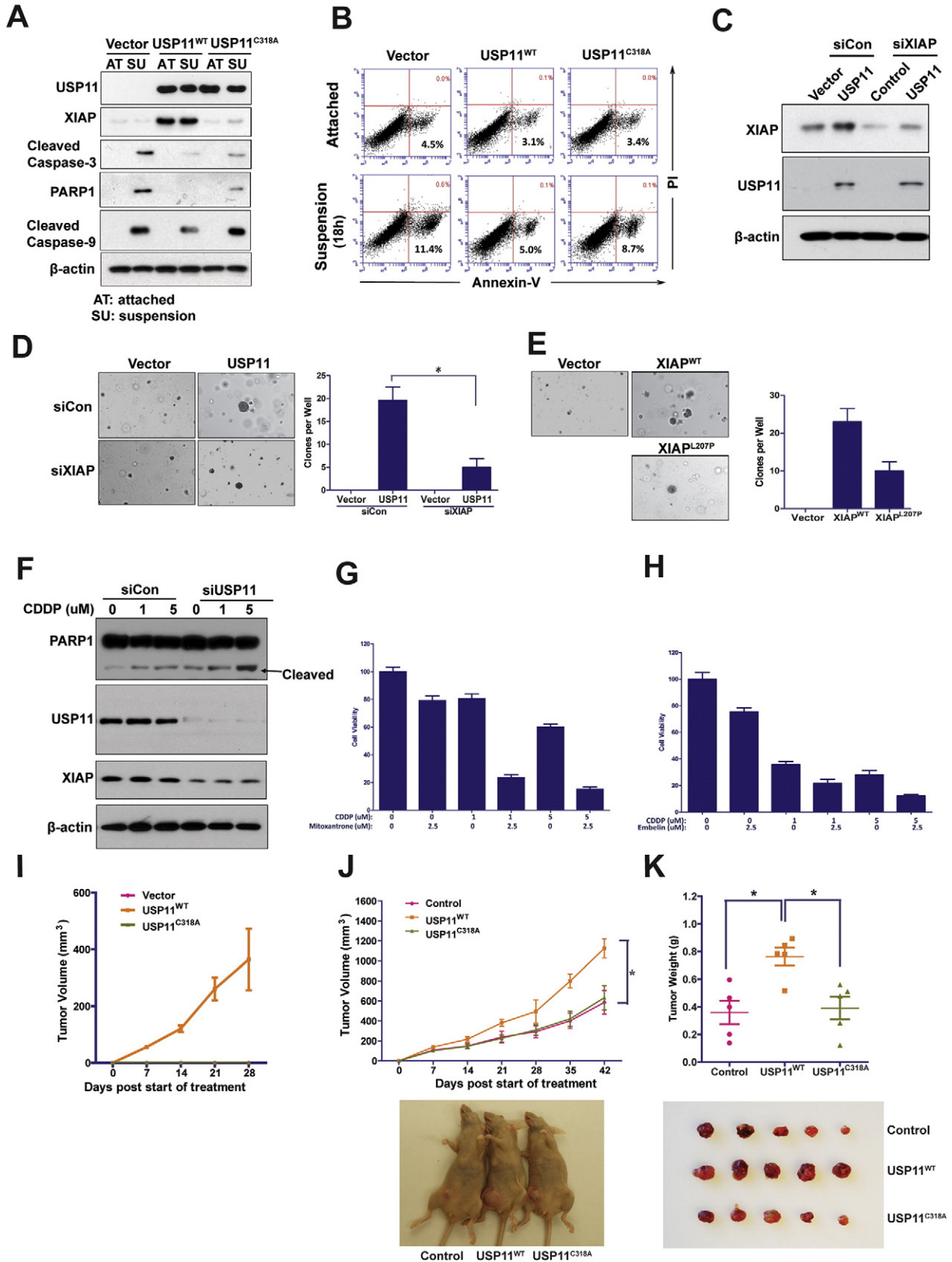
While we have identified the critical role of UPS11 in mammary tumorigenesis and further dissected the mechanism by which USP11 catalyzes XIAP for deubiquitylation, the mechanism that initiates the accumulation of USP11 in breast cancer tissue and its subsequent regulation during the tumor development still remains unknown. Results from our pilot studies suggest the USP11 is a fast-turnover protein with its stability regulated by the ubiquitin pathway. The USP11 protein levels are sensitive to growth factor signaling such as EGFR and IGFR signaling. We speculate that in the presence of oncogenic signaling, disruption of UPS that governs USP11 turnover could be a contributing factor towards USP11 accumulation. Thus, further efforts to elucidate the underlying mechanism of USP11 regulation by UPS in response to growth factor signaling will shed light on the role of USP11-XIAP interaction in regulating tumorigenesis. The present work isolates USP11 as a novel target for breast cancer treatment. Thus, strategy to intercept the USP11 function could be utilized for anti-breast cancer treatment. Indeed, depletion of USP11 or inhibition of USP11 by a non-specific inhibitor Mitoxantrone led to significantly sensitization of triple negative breast cancer cells to the chemo-therapeutic agent cisplatin further

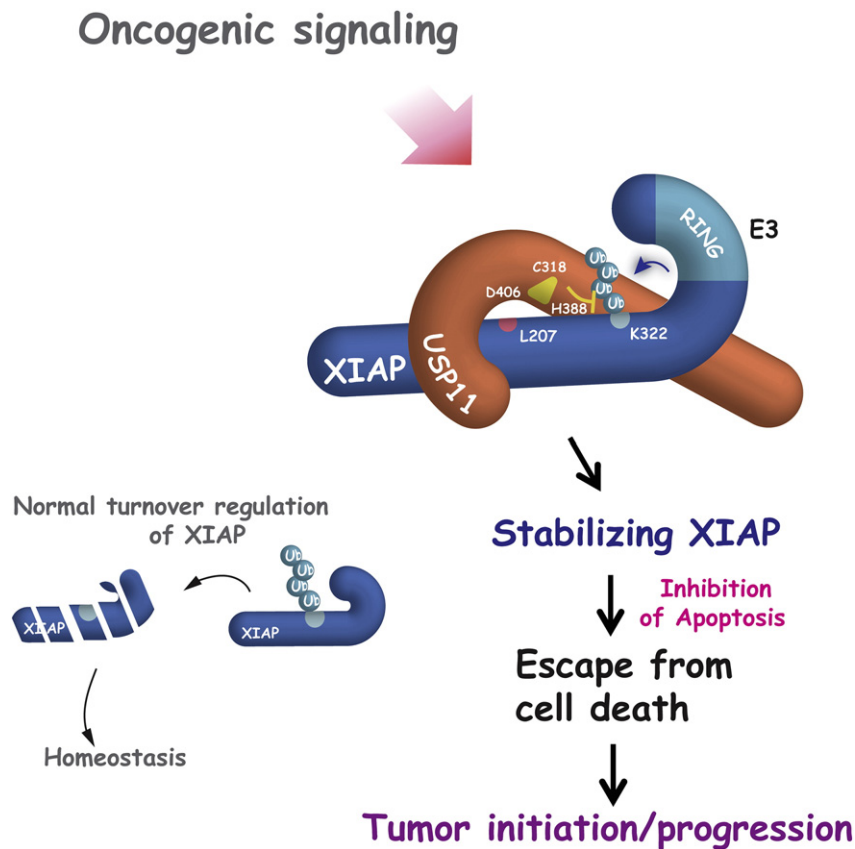
**Fig. 7.** Stabilization of XIAP by USP11 inhibits anoikis and promotes tumor transformation and drug resistance. (A, B) Elevated expression of USP11 inhibits anoikis in MCF10A. (A) Elevated expression of USP11-WT but not C318A significantly decreased activation of Caspase-3, -9 and cleavage of PARP1 in MCF10A cells. (B) The annexin-V-PI staining indicates the elevated USP11-WT but not USP11-C318A decreases anoikis in MCF10A cells. (C, D) Depletion of XIAP decreases USP11 dependent MCF10A transformation. (E) Elevated expression of XIAP-WT but not XIAP-L207P enhances MCF10A transformation. (F) Knockdown of USP11 in MDA-MB-231 cells promotes cell apoptosis in the presence of cisplatin treatment. PARP1 cleavage is marker for apoptosis. (G) Inhibition of USP11 by its inhibitor Mitoxantrone results in sensitizing MDA-MB-231 cells to cisplatin. (H) Inhibition of XIAP by its inhibitor Embelin leads to sensitizing MDA-MB-231 cells to cisplatin. (I, J, K) An in vivo mouse xenograft study was conducted to validate the oncogenic role of USP11 in breast tumorigenesis and tumor progression. (I)  $1 \times 10^7$  MCF10A cells with stable expression of USP11-WT or USP11-C318A were implanted into right mammary fat pad of SCID mice, the equal amount control MCF10A cells were implanted into left mammary fat pad of SCID mice. Tumor volume was measured once a week for 28 days after injection. Only the wild-type USP11 overexpression but not USP11 death mutant expression can drive MCF10A cells to form tumor after 28 days implanted. (J, K) MDA-MB-231 cells with stable expression of USP11-WT or USP11-C318A were implanted into mammary fat pad of nude mice. Tumor volume was measured once a week and mice were sacrificed 42 days after injection. (K) Lower panel, representative mice show tumor formation. Upper panel, tumor weight was measured. The results were presented as mean values  $\pm$  s.e.m. \* $p < 0.01$  using Student's *t*-test.

confirming the therapeutic value of targeting USP11. However, to date, no specific small molecule inhibitor for USP11 is available at this moment. Our molecular modeling work unveils a detailed mechanism by which XIAP is recognized and catalyzed by USP11. Such information in molecular level will allow development of specific pharmacological inhibitor of USP11 that could benefit to future anti-breast cancer therapy.

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**Fig. 8.** Schematic of a proposed model for the role of USP11 in stabilization of XIAP and mammary tumor transformation. USP11 is a potent determinant, whose abnormal accumulation could promote mammary tumor transformation and progression. XIAP is a self-ubiquitin protein ligase, whose protein stability is regulated by USP11. Under normal circumstance, constitutive activation of XIAP ubiquitylation and degradation self-catalysis is necessary for the maintenance of homeostasis. Stabilization of XIAP due to abnormal accumulation of USP11 could be a critical cause of mammary tumor transformation and progression.

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#### Conflicts of Interest

There are no conflicts of interest.

#### Author Contributions

Y.W. is the PI who managed the whole project. Y.W. and Z.Z. designed the experiments and wrote the manuscript. I.S. performed the structure modeling/simulation and wrote the related part of the manuscript with I.B. Z.L. contributes the deubiquitinase library, and Y.W. and Z.L. designed the non-biased screening. Z.Z. performed most experiments. A.L. performed experiment of immunohistochemistry and wrote the related part of the manuscript with Z.L. M.H. made some USP11 and XIAP constructs. Y.H. contributed to breast cancer model analysis. I.B. supervised the structure modeling/simulation and contributed to the discussion and revision.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2016.12.014>.

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