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Original Articles

ATXN3 promotes breast cancer metastasis by deubiquitinating KLF4

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ABSTRACT

Krüppel-like factor 4 (KLF4) is an important transcription factor implicated in a variety of essential cellular processes. Aberrant KLF4 expression is closely related to tumourigenesis and tumour progression. The rapid turnover of the KLF4 protein indicates an important role for the posttranslational modifications (PTMs) of KLF4. To date, E3 ligases mediating KLF4 ubiquitination have been widely reported, yet the deubiquitinating mechanism of KLF4 remains largely unknown. We screened a library of 65 deubiquitinating enzymes and identified ATXN3 as a deubiquitinating enzyme of KLF4. Subsequent immunoprecipitation assays confirmed that ATXN3 bound to KLF4, mediating the deubiquitination and stabilization of KLF4 protein levels. Furthermore, we demonstrated that ATXN3 promoted breast cancer cell metastasis via KLF4 *in vitro* and *in vivo*. Finally, the protein expression analysis of human breast cancer specimens demonstrated that ATXN3 significantly correlated with KLF4. High ATXN3/KLF4 expression was associated with a poor prognosis in breast cancer patients. Collectively, we identified ATXN3 as a novel deubiquitinating enzyme of KLF4, providing a new explanation for breast cancer metastasis, and proposed ATXN3 as a potential target for breast cancer metastasis treatment.

1. Introduction

Breast cancer is the most common malignant tumour and the leading cause of cancer-related death in women worldwide [1]. Early stage breast cancer is considered a potentially curable disease. Once the cancer begins to metastasize, the survival rate decreases significantly [2]. However, the precise mechanism that underlies breast cancer metastasis remains largely unknown. Therefore, it is urgent to elucidate the mechanisms underlying breast cancer metastasis and identify novel therapeutic targets for metastatic patients.

Krüppel-like factor 4 (KLF4) is a cell fate determination transcription factor that plays a critical role in numerous cellular processes, including cell cycle progression, apoptosis, genome stability, migration, metabolism and reprogramming [3–5]. The aberrant expression of KLF4 has been reported in many types of cancer [6]. Accumulating evidence has shown that KLF4 plays various roles in different malignancies [4]. It is reported to be a tumour suppressor in oesophageal, gastrointestinal, colon, lung and bladder cancers, while it acts as an oncogene in skin, breast and pancreatic cancers [7]. Yu et al. reported that KLF4 promotes cell migration and invasion by modulating the Notch signalling pathway in breast cancer [8]. Our previous study suggested that KLF4 promotes 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced breast cancer cell migration through the transcriptional regulation of S100A14 [9].

The KLF4 level changes rapidly in response to various environmental factors [10,11], indicating that the regulation of KLF4 is a swiftly modulated, environmentally relevant process. KLF4 is regulated at both the transcriptional and posttranscriptional levels. Recent studies have revealed the mechanism that underlies KLF4 modulation via posttranslational modifications (PTMs) [12–14]. The ubiquitin-proteasome system (UPS) is a dynamic homeostatic process. As proteins are ubiquitinated by E3 ligases to undergo degradation, they also tend to maintain their stability through deubiquitination, which is mediated by deubiquitinating enzymes. To date, several E3 ligases have been identified to mediate the ubiquitination of KLF4 in a context-relevant manner, including Cdh1/APC, βTrCP, FBXO32, Mule, and pVHL

Abbreviations: KLF4, Krüppel-like factor 4; PTM, posttranslational modification; UPS, ubiquitin-proteasome system; DUB, deubiquitinating enzyme; CHX, cycloheximide

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[15–19]. Cdh1/APC facilitates KLF4 ubiquitination upon TGF-β stimulation [15]. β-TrCP1 or βTrCP2 regulate KLF4 ubiquitination and degradation in response to KLF4 phosphorylation caused by ERK1/2 [16]. Mule mediates KLF4 K48-linked ubiquitination and promotes T cell proliferation and cell cycle progression [18]. The tumour suppressor pVHL is also reported to mediate KLF4 ubiquitination and degradation to inhibit breast cancer cell proliferation [19]. Our previous studies showed that FBXO32 mediates KLF4 ubiquitination and degradation under the active p38/MAPK phosphorylation cascade [17]. However, the mechanism underlying KLF4 deubiquitination remains to be elucidated.

ATXN3 (also known as Ataxin-3, ATX3, AT3, or MJD) belongs to the MJD family, which is one of the five main cysteine deubiquitinating enzyme families. Numerous substrates of ATXN3 have been reported, including p53 [20], Chk1 [21], CHIP [22], histone H2B [23], BECN1 [24], Parkin [25], and HDAC [26], suggesting that ATXN3 is a ubiquitous deubiquitinating enzyme regulating the deubiquitination and subsequent stabilization of various proteins [27].

In this study, we screened a deubiquitinating enzyme library and identified ATXN3 as a novel deubiquitinating enzyme for KLF4 in breast cancer. We verified that ATXN3 stabilizes KLF4 through deubiquitination in breast cancer and promotes cancer metastasis. Furthermore, ATXN3 correlates with KLF4 in breast cancer tissues and is higher in metastatic nodes than in *in situ* breast carcinoma. In addition, we found that high expression of both ATXN3 and KLF4 is associated with a poor prognosis in breast cancer patients.

2. Materials and methods

2.1. Cell lines and cell culture

HEK293T cells and the breast cancer cell lines MCF-7, MDA-MB-231, and 4T1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). U2OS cells were purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China).

HEK293T and 4T1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) with 10% foetal bovine serum (FBS; HyClone, South Logan, UT, USA) under 37 °C and 5% CO_2 culture conditions. MCF-7 cells were maintained in DMEM containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and 10% FBS (HyClone) under 37 °C and 5% CO_2 culture conditions. MDA-MB-231 cells were maintained in Leibovitz's L-15 medium (Gibco) with 10% FBS (HyClone) under 37 °C and 0% CO_2 culture conditions. U2OS cells were maintained in McCoy's 5A medium (Gibco) with 10% FBS (HyClone) under 37 °C and 5% CO_2 culture conditions.

2.2. Plasmids and transfection

The deubiquitinating enzyme library was purchased from Addgene (Watertown, MA, USA). The full-length and deletion mutant constructs of ATXN3 were generated by PCR amplification of the full-length sequence of human ATXN3 and subcloning into pIRES-Flag-HA and pEGFP-C2 vectors, which were preserved in our laboratory. The ATXN3 wild-type and catalytic mutant (C14A) constructs were purchased from Addgene. The KLF4 ubiquitination-deficient mutant construct was generated using a site-directed mutagenesis kit (Stratagene, San Diego, CA, USA). Sequences of the primers and small hairpin RNAs (shRNAs) used for vector construction or mutagenesis in this study are listed in the Supplementary Table. All constructs were verified by DNA sequencing. For transfection, cells were seeded until reaching 70% confluency and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.3. RNA inference

The small interfering RNA (siRNA) specifically targeting KLF4 was purchased from Dharmacon (L-005089-00; Lafayette, CO, USA). The siRNA specifically targeting ATXN3 was purchased from Sigma (St. Louis, MO, USA). Lipofectamine 2000 (Invitrogen) was used for transfection. The transfection procedure was performed according to the protocol for Lipofectamine 2000 (Invitrogen). Cell lysates were collected after 48 h of transfection and denatured for immunoblot assays. The siRNA sequences are listed in the Supplementary Table.

2.4. Western blot analysis

Cells were harvested and lysed in NP-40 lysis buffer containing 1 × protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was determined using BCA Reagent (Thermo Scientific, Rockford, IL, USA). Immunoblot analysis was performed using the primary antibodies anti-KLF4 (sc-20691, Santa-Cruz, Dallas, TX, USA; 1:1000), anti-KLF4 (ab72543, Abcam, Cambridge, MA, USA; 1:1000), anti-ATXN3 (MAB5360, Millipore, Burlington, MA, USA; 1:2500), anti-GAPDH (RLM3029, Ruiying Biological, Beijing, China; 1:1000), anti-β-actin (20536-1-AP, Proteintech, Rosemont, IL, USA; 1:20000), anti-HA (RLM3003, Ruiying Biological; 1:1000), anti-Flag-tag (20543-1-AP, Proteintech; 1:5000), and anti-GFP (ab1218, Abcam; 1:5000) and an HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody. For Western blot analysis, signals were detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). The data were quantified using NIH ImageJ software (NIH, Bethesda, MD, USA).

2.5. Immunoprecipitation assay

HEK293T cells were transfected with mammalian expression plasmids to assess exogenous protein overexpression. Cells were harvested 48 h after transfection and lysed with TNE buffer (50 mM Tris-HCl, pH = 7.4; 150 mM NaCl; 1 mM EDTA; and 0.1% Triton X-100) containing 1 × protease inhibitor cocktail (Roche). Whole cell lysates were incubated with anti-Flag M2 affinity gel (Sigma) on a rotator at 4 °C overnight. Beads were washed 5 times with phosphate-buffered saline (PBS) and then boiled for 10 min in 2 × SDS-PAGE loading buffer. The immunoprecipitated proteins were detected by Western blot analysis.

2.6. Cell migration and invasion assays

MCF7, MDA-MB-231, and 4T1 cells were mixed with serum-free medium at a density of 3×10^5 cells/ml, 5×10^6 cells/ml, and 3×10^6 cells/ml, respectively. A 100 µl volume cell suspension was seeded into the upper compartment of a transwell insert with an 8 µm pore polycarbonate membrane precoated with or without Matrigel. The lower compartment was filled with 600 µl of complete culture medium. The procedure was conducted as instructed by the manufacturer (Corning Incorporated, New York, NY, USA). The transwell inserts were fixed and stained with a crystal violet solution 24 h after cell seeding. Cells on the bottom side of the pore membrane were counted across four random microscopic fields and averaged. The experiments were repeated three times, with two replicates per independent experiment. The error values were calculated as the standard deviation (SD) for a representative experiment.

2.7. In vivo metastasis assay

4T1 cells were suspended in 1 × PBS at a density of 5×10^5 cells/ml. A 100 µl volume cell suspension was injected subcutaneously into the mammary pad of each BALB/c mouse (Charles River, Beijing, China). Each group contained at least 7 mice. The mice were sacrificed 35 d after tumour cell injection. The lungs were harvested, fixed in 4%

polyoxymethylene, and stained with picric acid to count the metastatic nodules. Approval for the animal experiments was obtained from the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences Cancer Hospital.

2.8. Immunohistochemistry

The human breast cancer and paired adjacent tissue samples used in the tissue array were obtained from the Cancer Hospital Chinese Academy of Medical Sciences (Beijing, China). The study was approved by the ethics committee of the hospital. All patients provided informed consent. The tissue microarray containing human breast *in situ* carcinoma and paired metastatic lymph nodule specimens was purchased from Alenabio (BR10010e, Xian, China). The immunohistochemical staining procedure was conducted according to the immunoperoxidase method. The concentrations of antibodies used for incubation were diluted according to the antibody datasheets. The slides were scanned and analysed by an Aperio scanning system (Aperio, San Diego, CA, USA). The specimens were quantified based on the staining intensity and percentage of positive cells. Negative staining was defined as 0, weak positive staining was defined as 1, positive staining was defined as 2, and strong positive staining was defined as 3.

2.9. Kaplan-Meier analysis

This analysis was performed with the online Kaplan-Meier Plotter tool for breast cancer [28]. Patients were divided according to the median value. Probe 221841_s_at was used for KLF4, and probe 217321_x_at was used for ATXN3. Post-progression survival was calculated and tested by the log-rank test; p values < 0.05 were considered significant.

2.10. Statistical analysis

The statistical analysis in this study was conducted using GraphPad Prism 7.0 (San Diego, CA, USA) and IBM SPSS(Armonk, NY, USA). The differences in protein levels between the two groups, the numbers of migrated or invasive cells in the transwell assay, and the numbers of pulmonary metastatic nodules in the mouse metastasis assay were analysed using an unpaired Student's t-test. The correlation between ATXN3 and KLF4 expression in the tumour samples was calculated by Pearson's correlation coefficients. In the breast cancer tissue array, the differences in ATXN3 expression between *in situ* and met were assessed by a paired *t*-test (two-tailed); p values < 0.05 were considered significant.

3. Results

3.1. Identification of ATXN3 as a potential deubiquitinating enzyme of KLF4

We used a library of deubiquitinating enzymes to screen for a deubiquitinating enzyme that directly targets KLF4. The library contains 65 deubiquitinating enzymes that are members of five deubiquitinating enzyme families. To search for deubiquitinating enzymes that regulates KLF4 proteolysis, we packed this library into a lentivirus system and generated a total of 65 U2OS stable cell lines expressing each of the 65 deubiquitinating enzymes. The screening strategy aimed to focus on the candidate deubiquitinating enzymes that increased the KLF4 protein level (Fig. 1A). The basal KLF4 level was analysed in the 65 established U2OS stable cell lines overexpressing deubiquitinating enzymes. An obvious increase in endogenous KLF4 protein levels was observed in 13 of the 65 stable cell lines compared with those in the control cells (Fig. 1B, Fig. S1). We chose the following 4 deubiquitinating enzyme candidates based on a literature search: ATXN3, USP48, USP14, and USP50. Then, a coimmunoprecipitation assay was performed to test the interaction between the 4 candidates and endogenous KLF4. The Western blot assay results indicated that only ATXN3 interacted with endogenous KLF4 (Fig. 1C). We then performed exogenous and endogenous coimmunoprecipitation assays to confirm the binding between ATXN3 and KLF4 (Fig. 1D and E). An immunofluorescence assay showed that KLF4 and ATXN3 at least partially co-localized in MCF-7 cells (Fig. 1F). Thus, ATXN3 was identified as a potential deubiquitinating enzyme of KLF4.

3.2. Mapping of the binding region between ATXN3 and KLF4

As a zinc finger transcription factor, KLF4 has three zinc-finger domains in its C-terminus, mediating the interaction with a high GT DNA sequence to regulate downstream target genes. Within its N-terminus, KLF4 has a transactivation domain and a repressive domain, which regulates the transcriptional activity of KLF4 by modulating KLF4 binding efficiency to DNA [29]. ATXN3 belongs to the MJD deubiquitinating enzyme family. The family has a Josephin domain on the N-terminus, facilitating its binding with a ubiquitin chain or substrates [30]. The enzyme also has three ubiquitin-binding motifs (UIMs) on the C-terminus that coordinate its interaction with E3 ligases and fulfil its ubiquitin binding and cutting functions [31]. To identify the regions of KLF4 that interact with ATXN3, we engineered a series of Flag-tagged truncated mutants of KLF4 (Fig. 2A) and then co-transfected them with GFP-tagged ATXN3. The coimmunoprecipitation assay showed that the KLF4 truncated mutant with amino acids 200–300 interacted with ATXN3 (Fig. 2B). A fine mapping assay within amino acids 200–300 on KLF4 indicated that the region containing amino acids 220–240 is critical for KLF4 binding with ATXN3 (Fig. 2C and D). We then constructed a series of Flag-tagged truncated mutants of ATXN3 to determine the binding regions of ATXN3 (Fig. 2E). The results showed that the truncated mutant with amino acids 1–205 on ATXN3 facilitated its binding to KLF4 (Fig. 2F). Taken together, these results show that the regions containing amino acids 220–240 of KLF4 and amino acids 1–205 of ATXN3 facilitated their interaction with each other.

3.3. ATXN3 stabilizes KLF4 by mediating its deubiquitination

To validate the regulation of KLF4 expression and the deubiquitination of ATXN3 in breast cancer cells, we transfected siRNAs targeting ATXN3 into MCF-7 cells. The ablation of ATXN3 decreased the protein level of KLF4 (Fig. 3A). In contrast, ATXN3 overexpression increased KLF4 protein levels in MDA-MB-231 cells (Fig. 3B). Consistent with these observations, ATXN3 depletion accelerated KLF4 degradation (Fig. 3C), while ATXN3 upregulation prolonged the half-life of KLF4 (Fig. 3D). To verify the function of ATXN3 in deubiquitinating KLF4, we overexpressed ATXN3 in HEK293T cells and conducted a ubiquitination assay with KLF4. We observed a remarkable reduction in covalent ubiquitin binding to KLF4 after ATXN3 upregulation (Fig. 3E), suggesting that ATXN3 represses KLF4 degradation through a ubiquitin-proteasome pathway. Consistent results were observed in MDA-MB-231 cells (Fig. S2). Cysteine 14 was previously reported to be a catalytic site of ATXN3 [32]. We mutated cysteine 14 to alanine to generate a catalytically dead mutant. Then, we transfected the C14A mutant and wild-type plasmids into cells. The catalytically dead ATXN3 mutant abrogated KLF4 upregulation and enhanced the KLF4 ubiquitination level compared with wild-type ATXN3 (Fig. 3B and F), indicating that the functions of ATXN3 are dependent on its deubiquitinating catalytic activity. We also generated an amino acids 220–240 deletion mutant of KLF4 (KLF4 Δ220–240). Immunoprecipitation assays demonstrated that the deletion of amino acids 220–240 on KLF4 blocked its binding with ATXN3 and increased the KLF4 ubiquitination level (Fig. 3G). Collectively, these results suggest that ATXN3 functions as a deubiquitinating enzyme to stabilize KLF4 by inhibiting ubiquitin chain formation.

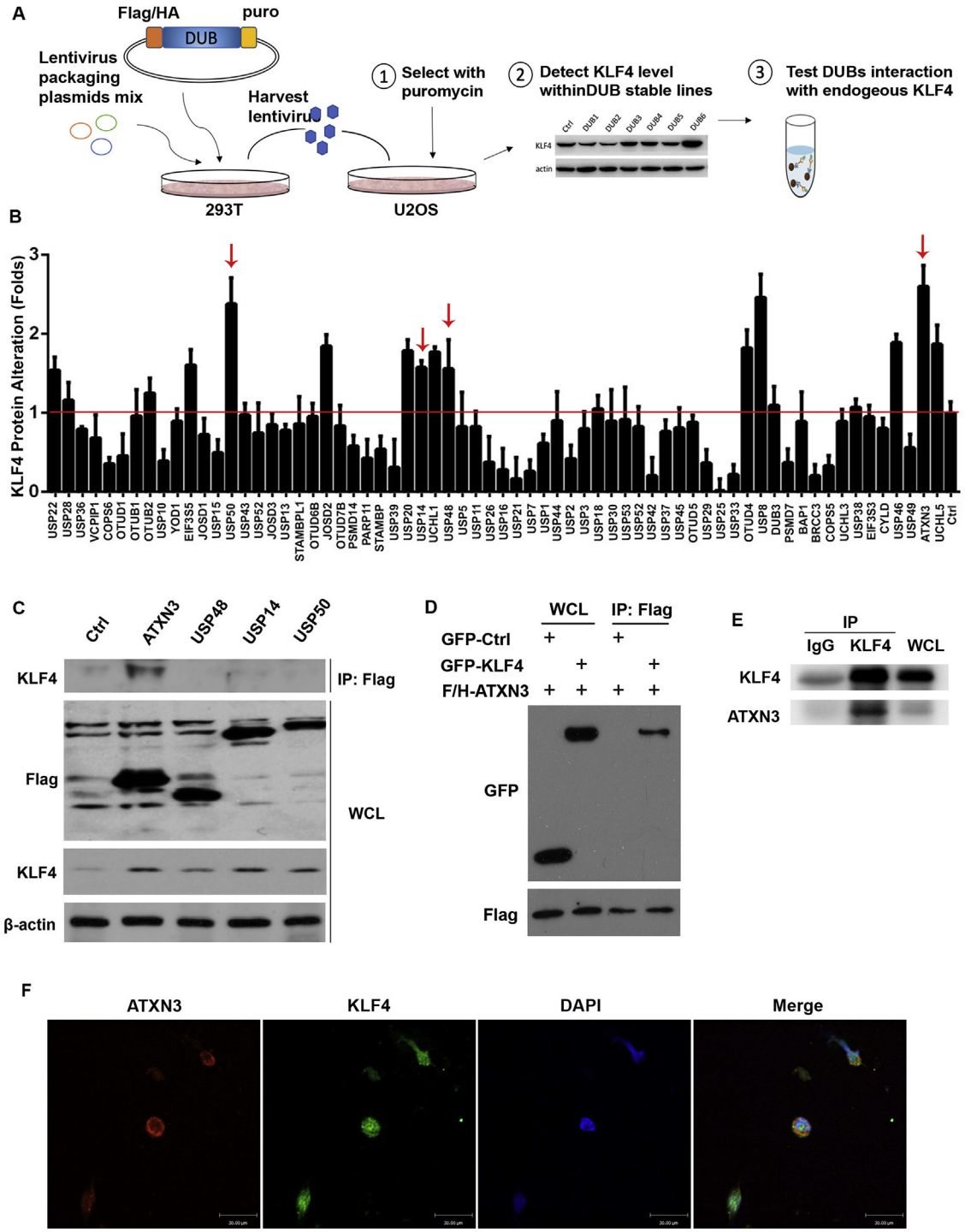

Fig. 1. Identification of ATXN3 as a potential deubiquitinating enzyme of KLF4. A. Diagram of the screening strategy. B. Relative KLF4 expression levels in U2OS cells stably expressing deubiquitinating enzymes. C. Coimmunoprecipitation assay showing that ATXN3 and KLF4 physically interact with each other in U2OS cells. D. An exogenous immunoprecipitation assay indicated binding between ATXN3 and KLF4 in HEK293T cells. E. An endogenous immunoprecipitation assay indicated binding between ATXN3 and KLF4 in MCF-7 cells. F. An immunofluorescence assay demonstrated that ATXN3 and KLF4 at least partially colocalized in MCF-7 cells.

3.4. ATXN3 promotes breast cancer metastasis by stabilizing KLF4

We next explored the roles of ATXN3 via modulating KLF4 in breast cancer metastasis. Using two parental breast cancer cell lines, MDA-MB-231 and 4T1, we established cell lines with stable ATXN3 overexpression or deletion and performed transwell assays *in vitro* to evaluate the potential function of ATXN3 in tumour migration and invasion. Knocking down ATXN3 significantly decreased cancer cell migration and invasion (Fig. 4A and B). Furthermore, we overexpressed KLF4 in ATXN3 knockdown cells and performed a rescue experiment to verify whether the functions of ATXN3 in cell migration and invasion require KLF4. Increased KLF4 expression partially recovered the effect of ATXN3 knockdown (Fig. 4C and D), indicating that ATXN3 promotes breast cancer cell migration and invasion, at least partially, through the regulation of KLF4. We next detected EMT markers in ATXN3 knockdown and ATXN3 overexpression/KLF4 knockdown breast cancer cells (Fig. 4E). The results suggested that ATXN3 regulated KLF4 to promote epithelial marker expression and inhibit mesenchymal markers

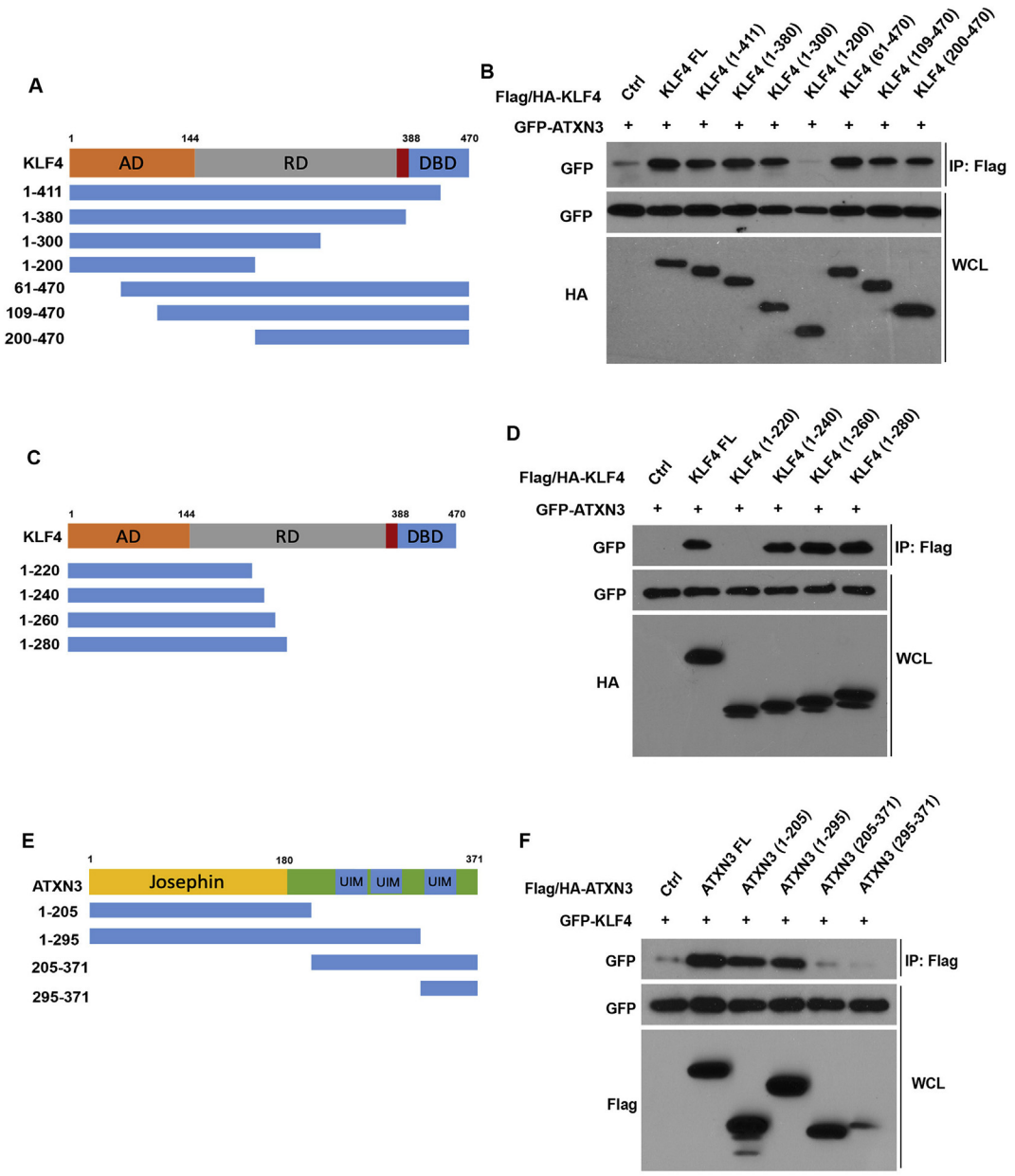

Fig. 2. Mapping of the ATXN3 and KLF4 binding regions. A. A schematic diagram of human KLF4 domains and the strategy for constructing a series of KLF4 deletion mutants. B. Identification of the region on KLF4 that mediates its interaction with ATXN3 (amino acids 200–300). C. Fine mapping of the KLF4 region containing amino acids 200–300. D. Identification of the region on KLF4 that interacts with ATXN3 (amino acids 220–240). E. A schematic diagram of the structure of human ATXN3 and the strategy for constructing a series of ATXN3 deletion mutants. F. Identification of the region on ATXN3 that facilitates its binding with KLF4 (amino acids 1–205).

expression, providing the explanation that the ATXN3-KLF4 axis mediates breast cancer metastasis via EMT regulation. We further used an *in situ* injection mouse model for *in vivo* metastasis evaluation. The ectopic overexpression of ATXN3 dramatically increased the number of lung metastatic nodules, whereas KLF4 depletion decreased the number of ATXN3-induced lung metastatic nodules, demonstrating that ATXN3 functions via KLF4 in breast cancer metastasis *in vivo* (Fig. 4F and G).

3.5. ATXN3 expression positively correlates with KLF4, and high ATXN3/KLF4 expression predicts a poor prognosis in breast cancer patients

We performed an immunohistochemistry analysis of a breast cancer tissue array to explore the relationship between ATXN3 and KLF4 expression at the protein level. ATXN3 and KLF4 were coexpressed and positively correlated in breast cancer tissue (Fig. 5A and B). We then used a tissue array including *in situ* breast carcinoma and paired metastatic lymph nodes to perform an immunohistochemistry analysis and found that ATXN3 was higher in metastatic lymph nodes than in *in situ* carcinoma (Fig. 5C), validating a potential role for ATXN3 in breast cancer metastasis. Moreover, a Kaplan-Meier survival analysis showed that the high expression of both ATXN3 and KLF4 was associated with poor survival probabilities (Fig. 5D). In conclusion, ATXN3 correlates with KLF4 and predicts a poor prognosis in breast cancer patients.

4. Discussion

We previously identified that pVHL and FBXO32 function as E3 ligases of KLF4, mediating KLF4 ubiquitination and degradation [17,33]. However, the deubiquitinating process of KLF4 remains largely unknown. Here, we report the deubiquitinating enzyme of KLF4 for the

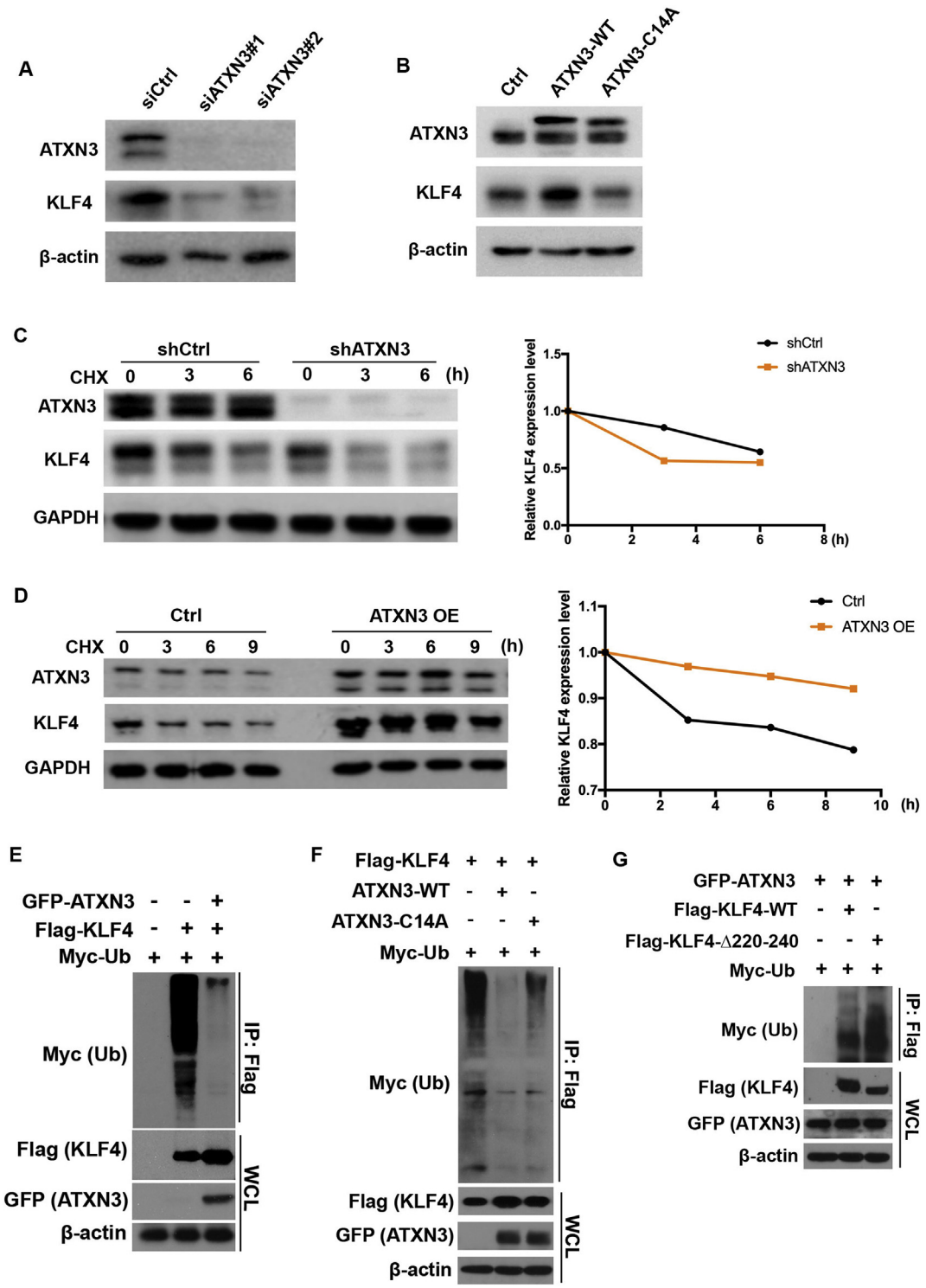

Fig. 3. ATXN3 stabilizes and deubiquitinates KLF4. A. ATXN3 knockdown in MCF-7 cells decreased KLF4 protein levels. B. Upregulated wild-type ATXN3 expression increased KLF4 protein levels in MDA-MB-231 cells, while the catalytically dead mutant of ATXN3 abrogated the KLF4 increase. C. ATXN3 depletion accelerated KLF4 degradation after CHX treatment in MCF-7 cells. D. ATXN3 overexpression attenuated KLF4 degradation after CHX treatment in MDA-MB-231 cells. E. ATXN3 accumulation led to a reduced level of KLF4 ubiquitination. F. Wild-type ATXN3 remarkably abolished ubiquitin binding on KLF4, whereas the C14A mutant restored KLF4 ubiquitination. G. ATXN3 failed to reduce the ubiquitination level of mutant KLF4 with amino acids 220–240 deleted.

first time. Our data show that ATXN3 interacts with KLF4 to antagonize KLF4 ubiquitination. Increased ATXN3 prolongs the half-life of KLF4 by inhibiting KLF4 proteasome-associated degradation. ATXN3 promotes breast cancer metastasis *in vitro* and *in vivo* in a KLF4-dependent manner.

In our experiment, we observed that KLF4 and ATXN3 interacted with each other through amino acids 220–240 on KLF4 and amino acids 1–205 on ATXN3. Kim et al. reported that amino acids 1–296 on KLF4 mediate protein degradation [34]. The 1–296 region contains the ATXN3-binding region of amino acids 220–240, which is consistent with our results. In addition, amino acids 1–205 on ATXN3 in the KLF4-binding region are restricted to the Josephin domain of ATXN3, which

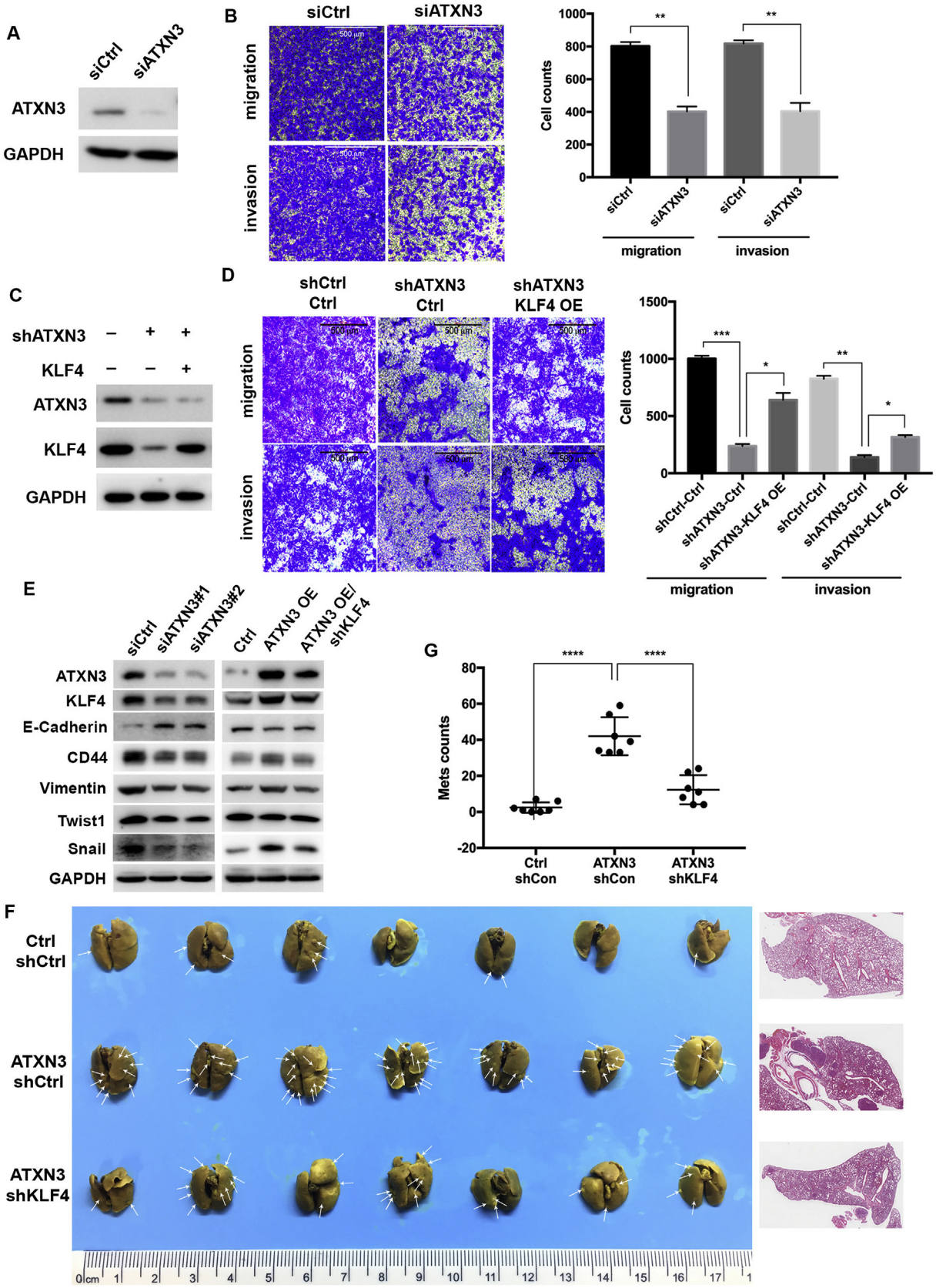

Fig. 4. ATXN3 promotes breast cancer metastasis by targeting KLF4. A. ATXN3 depletion efficiency in MDA-MB-231 cells. B. ATXN3 depletion reduces the proportions of migrated and invaded MDA-MB-231 cells. The experiments were repeated three times, and the results from only one experiment are shown. $^{**}p < 0.01$, mean ± SD. C. ATXN3 depletion and KLF4 overexpression efficiencies in 4T1 cells. D. Upregulated KLF4 expression reversed ATXN3 depletion-induced metastasis inhibition. The experiments were repeated three times, and the results from one experiment are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, mean ± SD. E. EMT markers detection in MDA-MB-231 cells. F. Lung metastasis nodules after the injection of 4T1 cells into mouse mammary glands for 5 weeks are shown. KLF4 knockdown reduced the number of ATXN3-accumulated metastatic nodules in the lungs. G. The number of nodules in F was calculated. The data are presented as the mean ± SD, $^{****}p < 0.0001$.

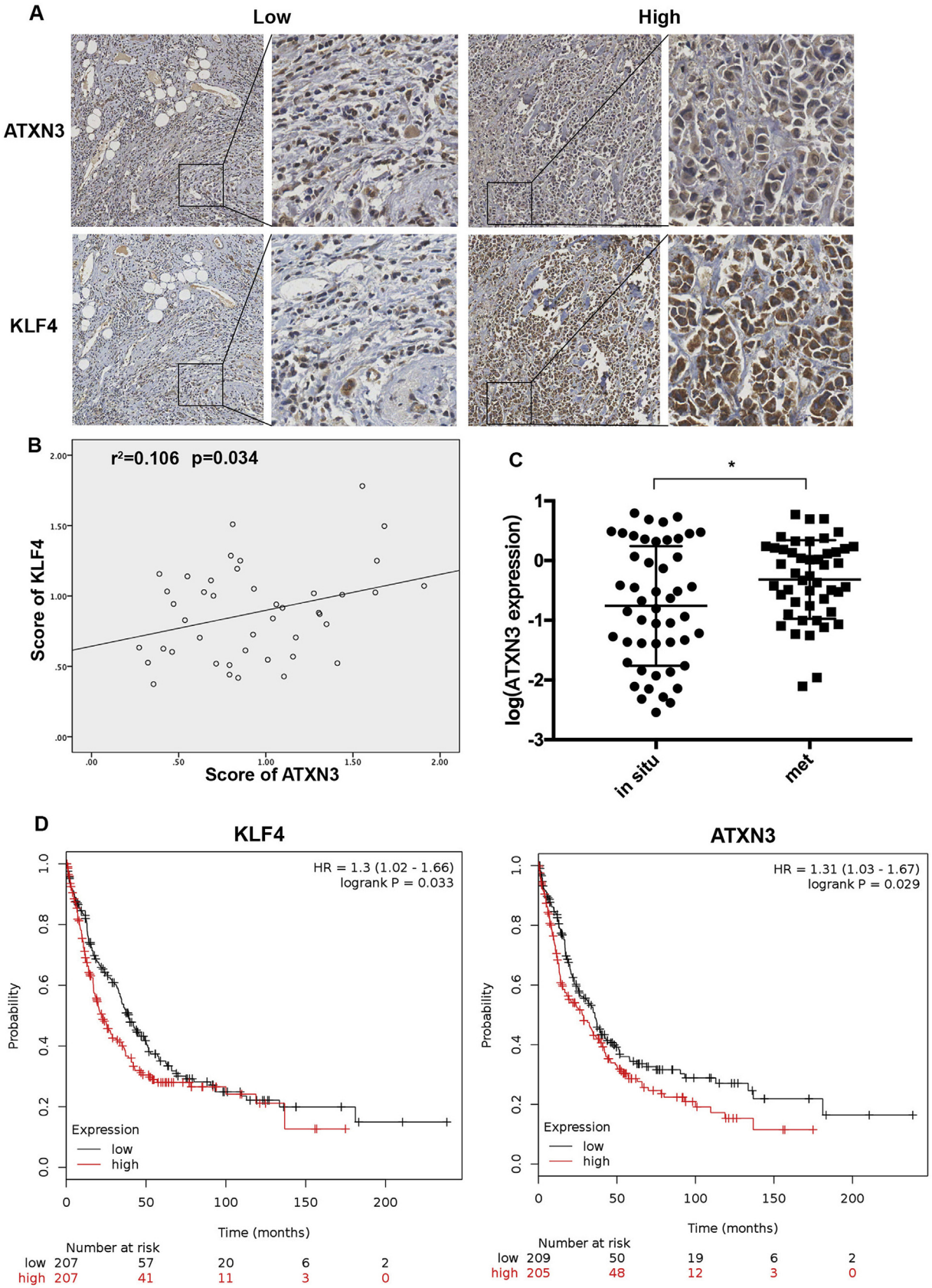

Fig. 5. ATXN3 and KLF4 correlate with each other, and aberrant ATXN3 accumulation is associated with a poor prognosis in breast cancer patients. A & B. KLF4 correlated with ATXN3 in breast cancer samples. C. Accumulation of ATXN3 expression in metastatic lymph nodes (met) compared with that *in situ* was observed. *$p < 0.05$, mean ± SD. D. High ATXN3 and KLF4 expression correlates with a poor prognosis in breast cancer patients.

is also consistent with the function of the Josephin domain to mediate the binding of deubiquitinating enzymes to their substrates.

Previous studies have shown that KLF4 plays a bifunctional role in different types of cancer. It acts as either a tumour suppressor or an oncogene in a context-relevant manner [3,35]. Controversy even exists regarding whether KLF4 promotes or inhibits breast cancer metastasis [9,36]; thus, there is an urgent need for a better understanding of the regulatory mechanism of KLF4. In this study, we identified ATXN3 as a deubiquitinating enzyme that stabilizes KLF4 in breast cancer. As shown in the data presented in ProteomicsDB and the Genevestigator platform, ATXN3 is highly expressed in many tissues, including the pancreas, B lymphocytes, bladder, and testes, but not in normal breast tissue. In contrast, ATXN3 is relatively highly expressed in breast cancer. Similarly, KLF4 expression is significantly higher in breast cancer tissues than in adjacent normal tissues [8,37]. We speculate that ATXN3 upregulation increases KLF4 expression in breast carcinoma and subsequently leads to the accelerated breast cancer metastasis. Therefore, our study partially explains why KLF4 acts as an oncogene in breast cancer. However, the cause of the increase in ATXN3 is not yet clear, and the underlying mechanism by which KLF4 promotes breast cancer metastasis still needs further exploration.

In the metastasis rescue experiments, KLF4 knockdown or overexpression only partially restored the metastatic phenotype in breast cancer cells, indicating that ATXN3 probably influenced some other substrates in addition to KLF4. Because the cell mobility-associated proteins p53, HDAC, and Parkin, have also been reported to be substrates of ATXN3 [38–40], it is likely that the increased metastatic rate of breast cancer cells in response to ATXN3 upregulation was a combination effect, thus explaining why KLF4 could only partially recover ATXN3-induced metastatic rate changes.

In summary, our study revealed new information regarding KLF4 PTMs by demonstrating that ATXN3 is the deubiquitinating enzyme of KLF4 in breast cancer. We also elucidated that ATXN3 promotes breast cancer metastasis by deubiquitinating and stabilizing KLF4, revealing the role of the KLF4 deubiquitinating process in breast cancer metastasis and suggesting that ATXN3 is a potential target for breast cancer treatment.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.09.012.

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