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CD44s is a crucial ATG7 downstream regulator for stem-like property, invasion, and lung metastasis of human bladder cancer (BC) cells

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Abstract

Over half a million US residents are suffering with bladder cancer (BC), which costs a total \$4 billion in treatment annually. Although recent studies report that autophagy-related gene 7 (ATG7) is overexpressed in BCs, the regulatory effects of ATG7 on cancer stem-like phenotypes and invasion have not been explored yet. Current studies demonstrated that the deficiency of ATG7 by its shRNA dramatically reduced sphere formation and invasion in vitro, as well as lung metastasis in vivo in human invasive BC cells. Further studies indicated that the knockdown of ATG7 attenuated the expression of CD44 standard (CD44s), while ectopic introduction of CD44s, was capable of completely restoring sphere formation, invasion, and lung metastasis in T24T(shATG7) cells. Mechanistic studies revealed that ATG7 overexpression stabilized CD44s proteins accompanied with upregulating USP28 proteins. Upregulated USP28 was able to bind to CD44s and remove the ubiquitin group from CD44s' protein, resulting in the stabilization of CD44s protein. Moreover, ATG7 inhibition stabilized AUF1 protein and thereby reduced *tet1* mRNA stability and expression, which was able to demethylate *usp28* promoter, reduced USP28 expression, finally promoting CD44s degradation. In addition, CD44s was defined to inhibit degradation of RhoGDI β , which in turn promotes BC invasion. Our results demonstrate that

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Author contributions CH, HH, and JZ conceived and designed the studies. HY, YL, JL, XC, HL, CH, and JZ detected the cells' biological function, conducted the RT-PCR assays, carried out the Western Blot and luciferase reporter assays, and performed the statistical analysis. HY, ZT, JX, and XH carried out the animal studies. CH, JZ, and WD drafted the manuscript. All authors read and approved the final manuscript.

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CD44s is a key ATG7 downstream regulator of the sphere formation, invasion, and lung metastasis of BCs, providing significant insight into understanding the BC invasions, metastasis, and stem-like properties.

Introduction

Through asymmetric cell division, stem cells renew themselves to produce differentiated tissue or organ-specific cells [1]. Cancer stem cells (also known as tumor-propagating cells or tumor-initiating cells) have characteristics of self-renewal capability, tumorigenic capacity and pluripotency, which are responsible for the heterogeneity in some tumors [2, 3]. Muscle-invasive bladder cancer (MIBC) and nonmuscle-invasive bladder cancer (NMIBC) are two major clinicopathological phenotypes of BC [4–6]. MIBCs can be grouped into two subtypes: basal and luminal. Pathological characteristics of luminal BC are papillary and of stromal infiltration [7]. In contrast to luminal BCs, basal MIBCs are mainly associated with sarcomatoid and squamous features with extremely aggressive behaviors while expressing a few biomarkers, such as BC stem cell (CSC) biomarker CD44 and others (p63, KRT5, KRT14, and EGFR) [4–6, 8]. Growing evidence indicates that basal MIBC contains a small population of CSCs, which is thought to be associated with BC invasion and metastasis [8]. The ATG (autophagy-related) proteins participate in the biogenesis of autophagosomes both in normal conditions and to a higher degree in responses to stress [9]. Due to its complexity, autophagy has been regarded as a double-edged sword that either promotes or suppresses human cancers, which depends on the cancer stage, the upstream regulators, and the downstream effectors of autophagy [10–12]. ATG7 is a critical protein for intracellular autophagic responses [13]. Our most recent studies demonstrated that N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced mouse MIBCs are basal MIBCs with ATG7 overexpression [14]. Furthermore, the inhibition of ATG7 abolishes the abnormal growth behavior of human BC cells through the ETS2/miR-196b/FOXO1/p27 pathway both in vitro and in vivo [14].

CD44, has multiple isoforms and is one of the major cancer stem cell markers for various epithelial tumors [15]. Cancer stem cell markers (CD24, CD44, CD47, and CD133) are responsible for cancer-specific survival of the human BC patients, which are differentially expressed in urothelial cells [16]. Although it has been reported that knockdown of ATG7 or BECN1 leads to alters of the CD44⁺/CD24^{low/-} population by regulating secretion of CD24 and IL-6 in breast cancer cells [17], the regulatory effects of ATG7 on stem-like sphere formation and their association with BC invasion and metastasis have never been explored. In this study, we demonstrated that ATG7 knockdown-specific promoted CD44s protein degradation, which consequently impacted on sphere formation, invasion, and lung metastasis of human BC cells.

The deubiquitylating enzymes (DUBs) have been reported to modulate the ubiquitination process by counteracting the activities of the E3 ligases, which are also implicated in human cancer [18]. During genotoxic stress, the USP family in DUBs plays an important role in regulation of cell cycle, DNA-damage response, and physiological homeostasis of ubiquitination process [19]. In the N-terminal region, USP28 contains ubiquitin-interacting

motifs and ubiquitin-associated domain [20]. Disrupting USP28 destabilizes LSD1 protein, which decreases breast cancer stem cell-like characteristics in vitro and suppresses tumorigenicity in vivo [21]. High expression level of USP28 has been regarded as an independent predictor of survival for human BC [22]. However, the molecular mechanisms underlying USP28 upregulation and its role in human BC are far from understood. Here, we discovered that USP28 could directly bind to CD44s and promoted the deubiquitination of CD44s, in turn increasing this protein stability.

TET1 regulates DNA methylation and gene expression through oxidizing methylated cytosine into 5-hydroxymethylcytosine (5hmC) [23]. Full length TET1 (TET1FL) contains a CXXC domain which could bind to unmethylated CpG islands (CGIs) and thereby protecting CGIs from aberrant methylation [24]. It has been reported that TET1 demethylates the vimentin promoter and finally promotes cisplatin-resistance in ovarian cancer [25]. TET1 demethylates PTEN promoter and finally inhibits gastric cancer growth and metastasis [26]. However, to the best of our knowledge, nothing is known regarding the implication of TET1 in BC development. In this study, we uncovered that overexpressed ATG7 promoted AUF1 protein degradation, and in turn stabilized *tet1* mRNA, further led to USP28 upregulation through its promoter hypomethylation, and consequently resulting in CD44s protein accumulation, sphere formation, invasion/metastasis of human BC cells.

Results

ATG7 expression was required for sphere formation of human high invasive BC T24T cells

Our previous studies have demonstrated that ATG7 is overexpressed in human BC tissues and mouse MIBC tissues, and plays a positive role in the promotion of BC cell growth both in vitro and in vivo by inhibiting *p27* transcription in human highly invasive BC cells [14]. To test whether ATG7 is associated with the acquisition of CSC-like properties, two sets of different shRNAs (#1 and #2) specific targeting ATG7 were stably transfected into highly invasive human BC cell lines, and their ATG7 knockdown transfectants were identified as shown in Fig. 1a–c. ATG7 knockdown in T24T cells dramatically inhibited sphere formation as compared to the nonsense transfectants (Fig. 1d and e). Moreover, we stably transfected the inducible ATG7 overexpression plasmid into T24T cells. Upon doxycycline (Dox) treatment, ATG7 protein expression was induced and the sphere formation was increased as compared to its vector control transfectants (Fig. 1f–h). Further, inhibition of autophagy by Bafilomycin A1 (Baf A1) significantly impaired sphere formation of T24T cells (Figs. S1A–S1C). These results revealed that ATG7 is associated with sphere formation. Accordingly, the expression levels of pluripotency-associated transcription factors, such as NANOG, SOX2, CD44, CD47, CD133, and OCT4 were evaluated, whereas only CD44 and CD133 were consistently inhibited in all three ATG7 knockdown high invasive BC cell lines (Fig. 1a–c). In addition, CD44 protein expression was elevated in the inducible ATG7 overexpression transfectants with Dox treatment (Fig. 1h). Those results suggest that ATG7 overexpression might be required for sphere formation by regulating expression of CD44 and CD133 in high invasive BC cells.

CD44s, but not CD133, was an ATG7 downstream effector for sphere formation promotion in human high invasive BC cells

Unlike the canonical CD44 standard isoform, CD44 variable isoforms (CD44v) expression are highly restricted and specific, which have been reported to be responsible for growth of various tumors [27]. To determine which of CD44 isoforms, including CD44s, CD44v3, CD44v6, and CD44v10, were involved in ATG7 regulation of sphere formation in human BC cells, the mRNA levels of those *cd44* isoforms were evaluated in T24T(Nonsense), T24T (shATG7), and CD44-positive control human glioblastoma stem-like D456 cells. Only *cd44s* mRNA was remarkably upregulated in T24T(shATG7) cells (Fig. 1i), and other mRNAs, including *cd44v3*, *cd44v6*, and *cd44v10*, were almost undetectable in both T24T(Nonsense) and T24T (shATG7) cells, whereas *cd44s*, *cd44v3*, *cd44v6*, and *cd44v10* mRNA expression were detectable in the positive control D456 cells (Fig. 1i). These results indicate that CD44s is a major isoform of CD44 that might play a role in ATG7 promotion of human BC cell sphere formation. To test the biological contribution of CD44s and CD133 in ATG7-regulating sphere formation, the constructs that express CD44s and CD133 were stably transfected into T24T(shATG7#1) cells, respectively, which were identified as shown in Fig. 1j and k. In comparison to T24T (shATG7#1) cells, ectopic expression of CD44s could completely restore the sphere formation, a stem-like property, that was inhibited due to ATG7 knockdown (Fig. 1l and m), while constitutively overexpression of CD133 did not show observed reversible effect on sphere formation under same experimental conditions (Fig. 1n and o). Given the concern on the possibility that cell aggregation maybe exist and interfere with the observation of the stem cell sphere formation when a large number of cells (>100–1000 per well) were seeded in a six-well plate [28], single cell-derived sphere formation assay was further performed by seeding 90 cells in a 96-well plate (Fig. S2A). The results showed that sphere formation rate in T24T(Nonsense) cells was about 37% (Fig. S2B), while ATG7 knockdown resulted in a dramatical inhibition of sphere formation to about 10% (Fig. S2B). In contrast, CD44s ectopic expression in T24T(shATG7#1) cells completely restored the sphere formation as compared to T24T(shATG7#1) cells (Fig. S2C). These results are consistent with the observation of sphere formation assay in six-well plates (Fig. 1e and m), demonstrating that CD44s, and not CD133, is critical for ATG7-mediated novel positive regulation of sphere formation in human high invasive BC cells.

Ectopic expression of CD44s in T24T(shATG7) cells reversed the reduction of BC cell invasion and lung metastatic ability due to ATG7-deficient via stabilization of RhoGDIβ in T24T cells

To evaluate the association of ATG7-regulated CD44s with BC invasion, the invasive ability of T24T(shATG7/CD44s) was compared with those in T24T(shATG7) and T24T (Nonsense) transfectants. The results indicated that CD44s overexpression in T24T(shATG7#1) cells restored the invasive ability in comparison to T24T(shATG7#1) cells, which showed a reduced invasive activity due to ATG7-deficient (Fig. 2a and b). This result provides a strong direct evidence showing that CD44s is not only a biomarker of cancer stem cell marker, but also acts as ATG7 downstream molecule responsible for BC invasion. To test whether ATG7-regulated CD44s was associated with the lung metastatic behavior of T24T cells, the lateral tail vein i.v. injection of the T24T(Nonsense), T24T(shATG7), and T24T(shATG7/CD44s) cells into nude mice was carried out and the

lung metastatic abilities were evaluated. As shown in Fig. 2c–e, ATG7 deficient in the transfectant of T24T (shATG7) attenuated the lung metastatic tumor formation (3.2 tumors/mouse, $n = 5$) in comparison to 17.4 tumor/mouse in the mice injected with T24T(Nonsense) control group, whereas overexpression of CD44s, T24T(shATG7/CD44s), restored the lung metastasis. Those results strongly reveal that CD44s was a critical ATG7 downstream mediator that is responsible for human BC cell invasion and lung metastasis.

Our most recent studies have found that RhoGDI β is an important player for upregulated XIAP promotion of BC invasion and lung metastasis by increasing matrix metalloproteinase-2 (MMP-2) protein expression [29, 30]. To investigate the mechanisms underlying CD44s promoting ATG7-mediated BC invasion and lung metastasis, potential downstream RhoGDI β expression was first examined. The results showed that ATG7 knockdown impaired RhoGDI β protein abundance, which could be completely reversed by CD44s restoration as compared to that in T24T(shATG7#1) cells (Fig. 2f), whereas there was no observable restoration of other potential candidates, including LC3-II or AUF1 in T24T(shATG7/CD44s) cells in comparison to T24T(shATG7#1) cells. Our results strongly reveal that CD44s is the critical downstream factor responsible for ATG7 upregulation of RhoGDI β protein through autophagy-independent axis.

To elucidate how CD44s increases RhoGDI β protein expression, the effect of ATG7 was assessed on *rhogdi β* mRNA abundance in T24T(shATG7#1), T24T(shATG7#1/CD44s), and T24T(Nonsense) cells. The results showed that *rhogdi β* mRNA was downregulated in ATG7-deficient cells, CD44s ectopic expression in T24T(shATG7#1) cells only showed a slight effect on *rhogdi β* mRNA level, which was not comparable to its effect on RhoGDI β protein level as compared to T24T(shATG7#1) cells (Fig. 2g), indicating that CD44s reversed RhoGDI β protein level through the mechanisms that are dependent on neither transcription nor mRNA stability. Therefore, we next tested whether CD44s regulated RhoGDI β protein stability. CD44s overexpression remarkably increased RhoGDI β protein stability in comparison to T24T(shATG7#1) cells (Fig. 2h). Collectively, our results demonstrate that CD44s promotes ATG7-mediated BC invasion and lung metastasis through preventing RhoGDI β protein from the degradation through autophagy-independent axis.

ATG7 promoted USP28 protein expression, which directly bound to and deubiquitinated CD44s protein and inhibited CD44s protein degradation

To investigate the mechanisms of ATG7 upregulating CD44s protein, *cd44s* mRNA levels were first examined. The results showed that *cd44s* mRNA was upregulated in ATG7-deficient transfectants (Fig. 3a), excluding the possible ATG7 promotion of *cd44s* mRNA expression. We next tested whether ATG7 regulated CD44s protein degradation. The result indicated that ATG7 knockdown remarkably promoted CD44s protein degradation (Fig. 3b), indicating that ATG7 expression is required for CD44s protein stabilization. Ubiquitin conjugation and deconjugation participates in modulating protein degradation with a powerful regulatory system. Ubiquitination levels are modulated by E1, E2, and E3 enzymes through a balance of the ubiquitination, while deubiquitination is regulated by deubiquitinating enzymes (DUBs) [31]. With ~85 members, the USP (ubiquitin-specific protease) family is the largest family of DUBs [32]. USP4 could bind to the catalytic domain

with its ubiquitin-like (UBL) domain and competes with ubiquitination enzyme binding [33]. USP8 could negatively regulate the lysosome-degraded proteins ubiquitination, including tropomyosin-related kinase A (TRKA) [34], the epidermal growth factor receptor (EGFR) [35] and AMPA receptor [36]. USP28 can bind to and deubiquitates several target proteins in DNA-damage pathway, resulting in stabilization of their targeted protein, including p53BP1 and Chk2 [37]. To determine the upstream regulators mediating CD44s protein stabilization by ATG7, USP4, USP8, USP28, ITCH, and XIAP were evaluated and compared with T24T(shATG7) vs. T24T (Nonsense) cells. As shown in Fig. 3c, while USP4, USP8, ITCH, and XIAP protein expressions were comparable between T24T(shATG7) and T24T (Nonsense) cells, USP28 was dramatically attenuated in T24T(shATG7) cells. Moreover, we found that USP28 protein expression was dramatically reduced in non-metastatic T24 cells in comparison with that in the metastatic derivative T24T cells (Fig. 3d). Thus, we anticipated that USP28 might be an ATG7-regulated DUBs that is associated with ATG7 upregulation of CD44s protein stability. To test this notion, we transfected Flag-USP28 expression construct into T24T (shATG7#1) cells and the stable transfectant, T24T (shATG7/Flag-USP28), was established (Fig. 3e). As expected, USP28 overexpression in T24T(shATG7#1) cells led to increased CD44s protein expression, as well as restoration of sphere formation (Fig. 3e–g). Consistently, the introduction of USP28 expression in T24T(shATG7#1) cells increased in CD44s protein stability (Fig. 3h). Our results demonstrate that USP28 might be a key DUB that links ATG7 to CD44s for ATG7 stabilization of CD44s, and in turn promoting sphere formation, and invasion and lung metastasis of human BC cells.

To determine whether USP28 acted as the deubiquitination enzyme stabilizing CD44s protein, Flag-USP28 plasmid was transfected into T24T(shATG7#1) cells. The immunoprecipitation assay was then performed to test the interaction of USP28 with CD44s protein, as well as the effect of USP28 on CD44s protein ubiquitination. As shown in Fig. 3i, CD44s was specifically presented in the immunocomplex pulled down of Flag-USP28 using the anti-Flag antibody, revealing that USP28 interacts with CD44s protein in the intact T24T cells. Consistently, the overexpression of Flag-USP28 abolished CD44s protein ubiquitination in the presence of exogenous wild type (WT) ubiquitin in the immunocomplex pulled down of CD44s by anti-CD44s antibody (Fig. 3j). Together, our results demonstrate that USP28 is a key in the deubiquitination of enzyme that is regulated by ATG7, and binds to CD44s protein and remove the ubiquitin group from CD44s protein, and in turn stabilizing CD44s protein and promoting sphere formation, invasion, and lung metastasis of human BC cells.

ATG7 promoted *usp28* transcription through inhibiting its promoter hypermethylation

To investigate the mechanisms of ATG7 upregulating USP28 protein, we first examined *usp28* mRNA levels in ATG7 knockdown transfectants. The results showed that *usp28* mRNA was markedly decreased (Fig. 4a). Upon treatment with Actinomycin D (Act D), *usp28* mRNA degradation rates in UMUC3(Nonsense) cells became much faster than in UMUC3(shATG7) cells (Fig. 4b), excluding the possibility that ATG7 stabilizes of *usp28* mRNA. Therefore, we next transfected human *usp28* promoter (from –1966 to –251)-driven luciferase reporters into T24T(Nonsense), T24T(shATG7#1), and T24T (shATG7#2) cells.

The results showed that knockdown of ATG7 decreased human *usp28* promoter-driven reporter transcription activity (Fig. 4c), revealing that ATG7 promotes *usp28* promoter transcription. Epigenetic hypermethylation in the CpG-rich promoter regions of cancer-related genes has been documented as an essential mechanism in human cancer development [38]. To investigate whether ATG7 knockdown-inhibited *usp28* transcription is due to its promoter hypermethylation, we firstly bioinformatically analyzed the potential CpG Island of human *usp28* promoter (Fig. 4d) using MethPrimer v1.1 beta software and then assessed the methylation of *usp28* promoter at the predicted methylated region from -432 to -309 [39]. Two independent predicted primer sets were used in the methylation-specific PCR (MS-PCR) system to amplify the methylated and unmethylated regions. As shown in Fig. 4e, knockdown of ATG7 upregulated the methylated DNA (M), produced a 113-bp band, accompanied with the unmethylated DNA (U) downregulation, and produced a 109-bp band. Our results clearly demonstrate that ATG7 knockdown inhibited *usp28* transcription through mediates hypermethylation of *usp28* promoter.

TET1-mediated *usp28* promoter demethylation contributed to ATG7 upregulation of CD44s and cancer stem-like properties in human BC cells

To further investigate the role of promoter hypermethylation in ATG7 knockdown-mediated USP28 downregulation, 5-aza-2'-deoxycytidine, the DNA methyltransferase (DNMT) inhibitor was used to inhibit genomic DNA methylation in human BC cells. The results showed that with 5-aza-2'-deoxycytidine treatment, the inhibition of *usp28* promoter activity was reversed, USP28 protein and its downstream CD44s protein abundance were also increased by ATG7 knockdown (Fig. 5a and b). These results strongly reveal that ATG7 knockdown-mediated *usp28* promoter hypermethylation leads to CD44s protein downregulation in human BC cells. DNMTs transfer a group of methyl from the universal methyl donor, S-adenosyl-L-methionine, to the 5-position of cytosine residues in DNA [40]. The Ten-eleven translocation (TET) enzymes (TET1, TET2, and TET3) exhibit the dioxygenase activity to convert 5-mC to 5-hmC [41]. To discover the DNMTs' or TETs' effect on the *usp28* promoter hypermethylation, we first evaluated the potential effect of ATG7 knockdown on *tets* and *dnmts* mRNA expression, including *tet1*, *tet2*, *tet3*, *tdg*, *dnmt1*, *dnmt3a*, and *dnmt3b*. The results demonstrated that ATG7 inhibition specifically decreased *dnmt3a*, *dnmt3b*, *tet1*, and *tet2* mRNA expression, but showed an effect on *dnmt1* or *tdg* (Fig. 5c). Moreover, ATG7 knockdown in T24T and UMUC3 cells showed consistently attenuation of protein expression of TET1, DNMT3A, and DNMT3B (Fig. 5d and e). Given functional of three enzymes in regulation of DNA methylation, only TET1 was functionally anticipated to be involved in ATG7 regulation of *usp28* promoter demethylation. To explore this possibility, we stably transfected HA-TET1 into T24T(shATG7#1) cells as shown in Fig. 5f. TET1 overexpression markedly promoted USP28 and CD44a protein expression, and rescued cancer stem-like properties of T24T(shATG7) cells (Fig. 5g and h). Consistently, TET1 overexpression also abolished ATG7 knockdown-caused *usp28* promoter hypermethylation (Fig. 5i). Collectively, our results strongly demonstrate that ATG7 overexpression-mediated TET1 upregulation contributes to *usp28* promoter demethylation, thereby increasing USP28 expression, consequently resulting in CD44s protein accumulation, and finally enhancing BC stem sphere formation, invasion, and lung metastatic activity.

ATG7 stabilized *tet1* mRNA through attenuating AUF1 protein expression

As above, in ATG7 knockdown transfectants, TET1 protein and mRNA were profoundly impaired (Fig. 5c–e). We firstly explored the possibility that ATG7 stabilizes *tet1* mRNA. Upon Act D treatment, *tet1* mRNA degradation rates became much faster in UMUC3(shATG7) cells than in UMUC3(Nonsense) cells (Fig. 6a), revealing that ATG7 expression plays an essential role in maintaining *tet1* mRNA stability. Our most recent studies have shown that ATG7 overexpression promotes autophagic removal of AUF1 protein, thereby stabilizing *rhogdiβ* mRNA in BC cells (Zhu J and Huang C, unpublished data). Thus, we knocked down AUF1 in UMUC3(shATG7#1) cells and T24T cells by using shRNAs-specific targeting human AUF1. As expected, AUF1 knockdown led to increased expression of TET1, USP28, and CD44s protein (Fig. 6b and c), as well as promotion of cancer stem-like cell sphere formation (Fig. 6d and e). RNA-IP assay was then performed to determine whether AUF1 is able to directly bind to *tet1* mRNA in HEK 293T cells with HA-AUF1 ectopically overexpressed. The results showed that AUF1 did bind to *tet1* mRNA (Fig. 6f). Collectively, our results demonstrate that ATG7 autophagic removal of AUF1 promotes *tet1* mRNA stabilization, which demethylates *usp28* promoter, and in turn resulting in accumulation of CD44s protein, finally promoting cancer stem-like properties, invasion and lung metastatic activity of human high invasive BC cells as shown in Fig. 6g.

Discussion

Autophagy promotes cytoplasm and cellular organelle degradation to possesses the maintenance of a proper cellular homeostasis, and therefore, is emerging as an attractive therapeutic target in human cancers, such as human BC [42]. Autophagy has been reported to play a critical role in the CSCs origin, maintenance, and systemic distribution [43–45]. Our recent studies show that ATG7 is overexpressed in BBN-induced basal MIBCs and plays an oncogenic role in BC tumorigenic growth in vitro and in vivo [14]. Knockdown of autophagy genes ATG5 or ATG7 has been reported to reduce human hematopoietic stem/progenitor cells frequencies both in vitro and in vivo [46]. Moreover, via Parkin-dependent mitochondrial clearance and oxidative stress modulation, autophagy activation supports EMT-mediated CD44^{High} cell generation, which is associated with poor prognosis in esophageal squamous cell carcinoma (ESCC) patients [47]. The current study identified that ATG7 overexpression was required for cancer stem-like properties in human BC cells through stabilizing CD44s protein. We observed that CD44s ectopic expression in T24T(shATG7) cells could completely restore the invasive ability in vitro and lung metastasis in vivo. Moreover, inhibition autophagy by Baf A1 reduced the sphere formation of BC, indicating that autophagy exerts the oncogenic role on cancer stem-like properties in human BC cells (Fig. S1B and S1C). However, the knockdown of BECN1 in T24T cells promoted CD44s protein expression, inhibited TET1, USP28, and RhoGDIβ expressions, but showed no effect on AUF1 expression and sphere formation in comparison to its scramble nonsense transfectants (Fig. S3A–S3C). It is noted that knockdown of BECN1, inhibited the conversion of LC3 from LC3-I to LC3-II and promoted the invasion ability of BC (Fig. S3A, S3D and S3E), indicating that BECN1-dependent autophagy mediated anti-cancer effect on BC invasion. It has been proposed that autophagy exhibits four functional forms in response to chemotherapeutic drugs in a cell type-specific and drug-specific

manner: cytotoxic, cytoprotective, cytostatic, and non-protective [48]. Due to the complexity, autophagy functions as a double-edged sword that either protect or suppress human bladder cancer, depending on the stage of cancer, the stimuli of autophagy, and the downstream effectors [10–12, 49, 50]. Our most recent studies have shown that autophagic responses mediated by Sestrin 2 (SESN2) and BECN1 mainly results in autophagic death of human bladder cancer cells [51, 52], whereas ATG7-mediated autophagic responses promote human BC growth [14]. Consequently, it is essential and highly significant to identify the functional types of autophagy and underlying mechanisms induced by different experimental conditions. Therefore, the further exploration of exact mechanisms underlying the diversity effects of autophagy on BC development are currently ongoing in our research group. Collectively, our results define a first link among ATG7 overexpression, stem-like properties, and the invasion/metastasis of human BC cells, which offers new insight into the therapeutic targeting of ATG7/CD44 for advanced human bladder cancers.

CD44 is a transmembrane glycoprotein widely expressed in pathological and physiological systems [53], and contributes to tumor metastasis and cancer stem cells [54–56]. According to recent studies observed in breast cancers, the cleaved intracellular domain of CD44 activates stem cell factors, such as NANOG, SOX2, and OCT4, and results in promoting tumorigenesis of breast cancer [57]. Moreover, CD44 and CD44v9 knockdown in human BC cells (HT1376 and U5637) increases E-cadherin and decreases N-cadherin, snail, and slug epithelial–mesenchymal transition marker protein expression and further inhibits human BC migration and invasion [15]. Mir-34a exerts the major metastasis and angiogenesis suppressive role by directly targeting CD44, would be helpful as a therapeutic approach to block human BC metastasis [58]. Our results from current studies showed that ATG7 upregulated the expression of CD44s protein and enhanced human bladder cancer cells stem-like properties. Our finding showing that ectopic expression of CD44s rescued the invasion and metastasis in ATG7 knockdown BC cells, providing a direct evidence demonstrating that CD44s is not only a biomarker for CSCs, but also acts as an ATG7 downstream molecule responsible for human BC cell invasion and metastasis.

RhoGDI β was found to be overexpressed in multiple human cancers. It has been reported that the patients with higher RhoGDI β expression have poor overall survival than those with low RhoGDI β expression relative to their adjacent normal colorectal cancer, gastric cancer, and pancreatic cancers [59–61]. Our recent studies also find that RhoGDI β overexpression is observed in over 90% (11 of 12) of human BC tissues when compared to that in their paired adjacent normal tissues, and in mouse muscle-invasive bladder carcinoma that are caused by exposure to BBN [29]. Our studies show that RhoGDI β promotes BC cell invasion and lung metastasis through elevating MMP2 expression [30]. Mechanistic studies reveal that RhoGDI β overexpression inhibits miR-200c expression, which reduces JNK2 protein translation inhibition, in turn promoting Sp1 expression and its targeting gene *mmp-2* promoter transcription, and finally promoting BC invasion. Herein, we found that ATG7 knockdown concurrently impaired invasion, lung metastasis, and expression of CD44s and RhoGDI β , while ectopic expression of CD44s rescued all of invasion, lung metastasis, and RhoGDI β expression in ATG7-deficient cells. These results provide strong direct evidence revealing that RhoGDI β is a CD44 downstream molecule for ATG7 promotion of BC invasion and metastasis.

The USP family plays an important role in signaling pathways and cellular processes [62]. In colon carcinoma, USP28 antagonizes the activity of the SCFFBW7 ubiquitin ligase complex to regulate the Myc stability and further promotes cell differentiation [63]. High expression of USP28 is also thought as a potential prognostic marker for human BC [22]. Nevertheless, the molecular mechanisms of USP28 protein upregulation and the biological function of USP28 on human BC stem-like properties has never been explored before. In this study, we found that ATG7 overexpression increased USP28 protein expression. Intriguingly, ectopic expression of Flag-USP28 in T24T(shATG7) cells promoted CD44s protein expression and human BC sphere formation, revealing that the increased USP28 expression positively correlated with CD44s upregulation. Further studies illustrated that USP28 could directly bind to CD44s and promoted the deubiquitination of CD44s, in turn increasing its protein stabilization. Therefore, the USP28/CD44s axis is critical in controlling the stem-like properties of human BCs.

DNA methylation, one of the epigenetic modifications, play crucial role in the regulation of gene expression, and alterations in this modification are involved in cancer initiation and progression [64]. Growing evidence showed that specific genes affecting cancer onset and progression are hypermethylated and transcriptionally silenced in many cancers [65, 66]. Herein, we found that specific knockdown ATG7 resulted in *usp28* promoter hypermethylation as comparison to scramble nonsense transfectants in human BC cells, and increased USP28 expression with 5-aza-2-deoxycytidine treatment, strongly indicating that the promoter hypermethylation was responsible for USP28 transcription downregulation due to ATG7 knockdown. TET proteins are α -ketoglutarate and iron(II)-dependent dioxygenases, and they hydroxylate 5-methylcytosine to 5-hydroxymethylcytosine and further to 5-formylcytosine and 5-carboxylcytosine [67], thereby playing a role in modulation of DNA demethylation. However, nothing is known about the related enzymes and the functional role in human bladder cancer progression. Our current elucidation shows that inhibition of ATG7 reduced TET1 expression, and that ectopic expression of HA-TET1 in T24T (shATG7#1) cells promoted *usp28* promoter demethylation and further enhanced its expression, accompanied by increasing CD44s expression and bladder cancer cell stem-like properties. These results demonstrate the contribution of TET1 in the regulation of *usp28* promoter demethylation, as well as its crucial role in human BC cancer stem-like properties, invasion and metastasis.

In summary, our studies have revealed a new TET1/USP28/CD44/RhoGDI β pathway that is responsible for the oncogenic role of ATG7 in invasion, metastasis, and stem-like properties in human BC cells. Specifically, ATG7 overexpression inhibits AUF1 protein expression, which stabilizes *tet1* mRNA to increase its protein expression. Moreover, upregulated TET1 directly demethylates USP28 promoter, thereby enhancing USP28 transcription and expression, which binds to CD44s protein, and remove the ubiquitin group from the ubiquitinated CD44s protein, resulting in stabilization of CD44s protein to mediate stem-like property of human BC cells. In addition, CD44s is defined to inhibit RhoGDI β degradation, in turn promoting human BC cell invasion and lung metastasis. Given the recent findings of the ATG7's crucial role in promoting BC tumorigenic growth, our new results provide the potential for developing an ATG7-based-specific therapeutic strategy for human BC patient treatment.

Materials and methods

Cell lines, plasmids, antibodies, and other reagents

These details are described in Supplementary Information.

Western Blot analysis

Western Blotting was performed in our previous publication [29].

Luciferase report assay

Luciferase reporter assays were performed as described previously [68].

Semiquantitative or quantitative RT-PCR

Semiquantitative or quantitative RT-PCR was performed to examine the mRNA expression level, as described previously [14, 69], and the primers were described in Supplementary table 1.

RNA-IP assay

RNA immunoprecipitation was performed as described previously [14]. The RNAs were extracted by TriZol reagent. Semiquantitative RT-PCR was performed to detect the presence of TET1 in the immune complex.

Immunoprecipitation

Immunoprecipitation was performed as described previously [29].

DNA extraction, bisulfite DNA modification, and methylation-specific PCR

According to the manufacturer's guidelines, DNeasy Blood and Tissue Kit (Qiagen, Gaithersburg, MD, USA) was used to extract Genomic DNA from the indicated cells. Sodium bisulfite modification of DNA and subsequent purification was performed according to the manufacturer's guidelines for sodium bisulfite conversion of unmethylated cytosine in DNA using EpiTect Bisulfite kit (Qiagen). Bisulfite-treated genomic DNA was subjected to an optimized methylation-specific PCR, which was performed as previously described [70].

Cell migration, invasion, and lung metastatic assay

These details are described in Supplementary Information.

Sphere formation assay

The indicated cells were cultured in a six-well ultra-low attachment culture plate for 5 or 7 days, and the images were taken under an Olympus DP71 microscopy (Olympus America Inc. Center Valley, PA, USA), and the number of spheroids formed cells in each image was counted by the software "Image J"_ENREF_30 [30] for at least six random images. For single cell-derived sphere formation assay, 90 of the indicated cells were seeded into each of 96-well plate. After culture for 2 weeks, total spheroid colonies in each 96-well plate was marked and photographed. The results were presented as the percentage of the spheroid colonies.

Statistical analysis

Statistical analysis was performed using Prism 5.0 Software (GraphPad software, San Diego, CA). Student's *t*-test was employed to determine the significance of differences between various groups. All data are presented as the means of triplicate assays $\pm$ SD. The differences were considered significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. Ying QL, Wray J, Nichols J, Battle-Morera L, Doble B, Woodgett J, et al. The ground state of embryonic stem cell self-renewal. *Nature*. 2008;453:519–23. [PubMed: 18497825]
2. Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell*. 2012;21:283–96. [PubMed: 22439924]
3. Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell*. 2012;10:717–28. [PubMed: 22704512]
4. Blaveri E, Simko JP, Korkola JE, Brewer JL, Baehner F, Mehta K, et al. Bladder cancer outcome and subtype classification by gene expression. *Clin Cancer Res*. 2005;11:4044–55. [PubMed: 15930339]
5. Sjö Dahl G, Lauss M, Lövgren K, Chebil G, Gudjonsson S, Veerla S, et al. A molecular taxonomy for urothelial carcinoma. *Clin Cancer Res*. 2012;18:3377–86. [PubMed: 22553347]
6. Damrauer JS, Hoadley KA, Chism DD, Fan C, Tiganelli CJ, Wobker SE, et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. *Proc Natl Acad Sci USA*. 2014;111:3110–5. [PubMed: 24520177]
7. Warrick JI, Walter V, Yamashita H, Chung E, Shuman L, Amponsa VO, et al. FOXA1, GATA3 and PPAR γ cooperate to drive luminal subtype in bladder cancer: a molecular analysis of established human cell lines. *Sci Rep*. 2016;6:38531. [PubMed: 27924948]
8. Chan KS, Volkmer J-P, Weissman I. Cancer stem cells in bladder cancer: a revisited and evolving concept. *Curr Opin Urol*. 2010;20:393–7. [PubMed: 20657288]
9. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol*. 2013;15:713–20. [PubMed: 23817233]
10. Dickstein RJ, Nitti G, Dinney CP, Davies BR, Kamat AM, McConkey DJ. Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther*. 2012;13:1325–38. [PubMed: 22895070]
11. Lin C, Tsai SC, Tseng MT, Peng SF, Kuo SC, Lin MW, et al. AKT serine/threonine protein kinase modulates baicalin-triggered autophagy in human bladder cancer T24 cells. *Int J Oncol*. 2013;42:993–1000. [PubMed: 23354080]
12. Mani J, Vallo S, Rakel S, Antonietti P, Gessler F, Blaheta R, et al. Chemoresistance is associated with increased cytoprotective autophagy and diminished apoptosis in bladder cancer cells treated with the BH3 mimetic (-)-Gossypol (AT-101). *BMC Cancer*. 2015;15:224. [PubMed: 25885284]
13. Mandelbaum J, Rollins N, Shah P, Bowman D, Lee JY, Tayber O et al. Identification of a lung cancer cell line deficient in atg7-dependent autophagy. *Autophagy*. 2015; [Epub ahead of print].

14. Zhu J, Li Y, Tian Z, Hua X, Gu J, Li J, et al. ATG7 over-expression Is crucial for tumorigenic growth of bladder cancer in vitro and in vivo by targeting the ETS2/miRNA196b/FOXO1/p27 axis. *Molecular therapy. Nucleic Acids*. 2017;7:299–313. [PubMed: 28624205]
15. Kobayashi K, Matsumoto H, Matsuyama H, Fujii N, Inoue R, Yamamoto Y, et al. Clinical significance of CD44 variant 9 expression as a prognostic indicator in bladder cancer. *Oncol Rep*. 2016;36:2852–60. [PubMed: 27599396]
16. Hofner T, Macher-Goeppinger S, Klein C, Schillert A, Eisen C, Wagner S, et al. Expression and prognostic significance of cancer stem cell markers CD24 and CD44 in urothelial bladder cancer xenografts and patients undergoing radical cystectomy. *Urol Oncol*. 2014;32:678–86. [PubMed: 24631171]
17. Maycotte P, Jones KL, Goodall ML, Thorburn J, Thorburn A. Autophagy supports breast cancer stem cell maintenance by regulating IL6 secretion. *Mol Cancer Res : MCR*. 2015;13:651–8. [PubMed: 25573951]
18. Wang X, Liu Z, Zhang L, Yang Z, Chen X, Luo J, et al. Targeting deubiquitinase USP28 for cancer therapy. *Cell Death Dis*. 2018;9:186. [PubMed: 29415985]
19. Peth A, Besche HC, Goldberg AL. Ubiquitinated proteins activate the proteasome by binding to Usp14/Ubp6, which causes 20S gate opening. *Mol Cell*. 2009;36:794–804. [PubMed: 20005843]
20. Komander D, Clague MJ, Urbe S. Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol*. 2009;10:550–63. [PubMed: 19626045]
21. Wu Y, Wang Y, Yang XH, Kang T, Zhao Y, Wang C, et al. The deubiquitinase USP28 stabilizes LSD1 and confers stem-cell-like traits to breast cancer cells. *Cell Rep*. 2013;5:224–36. [PubMed: 24075993]
22. Guo G, Xu Y, Gong M, Cao Y, An R. USP28 is a potential prognostic marker for bladder cancer. *Tumour Biol: J Int Soc Oncodev Biol Med*. 2014;35:4017–22.
23. Good CR, Madzo J, Patel B, Maegawa S, Engel N, Jelinek J, et al. A novel isoform of TET1 that lacks a CXXC domain is over-expressed in cancer. *Nucleic Acids Res*. 2017;45:8269–8281. [PubMed: 28531272]
24. Yin X, Xu Y. Structure and function of TET enzymes. *Adv Exp Med Biol*. 2016;945:275–302. [PubMed: 27826843]
25. Han X, Zhou Y, You Y, Lu J, Wang L, Hou H, et al. TET1 promotes cisplatin-resistance via demethylating the vimentin promoter in ovarian cancer. *Cell Biol Int*. 2017;41:405–14. [PubMed: 28150354]
26. Pei YF, Tao R, Li JF, Su LP, Yu BQ, Wu XY, et al. TET1 inhibits gastric cancer growth and metastasis by PTEN demethylation and re-expression. *Oncotarget*. 2016;7:31322–35. [PubMed: 27121319]
27. Zavros Y Initiation and maintenance of gastric cancer: a focus on CD44 variant isoforms and cancer stem cells. *Cell Mol Gastroenterol Hepatol*. 2017;4:55–63. [PubMed: 28560289]
28. Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell*. 2011;8:486–98. [PubMed: 21549325]
29. Zhu J, Li Y, Chen C, Ma J, Sun W, Tian Z, et al. NF-kappaB p65 overexpression promotes bladder cancer cell migration via FBW7-mediated degradation of RhoGDIalpha protein. *Neoplasia*. 2017;19:672–83. [PubMed: 28772241]
30. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671–5. [PubMed: 22930834]
31. Faesen AC, Luna-Vargas MP, Sixma TK. The role of UBL domains in ubiquitin-specific proteases. *Biochem Soc Trans*. 2012;40:539–45. [PubMed: 22616864]
32. Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, et al. A genomic and functional inventory of deubiquitinating enzymes. *Cell*. 2005;123:773–86. [PubMed: 16325574]
33. Zhou F, Xie F, Jin K, Zhang Z, Clerici M, Gao R, et al. USP4 inhibits SMAD4 monoubiquitination and promotes activin and BMP signaling. *EMBO J*. 2017;36:1623–39. [PubMed: 28468752]
34. Ceriani M, Amigoni L, D'Aloia A, Berruti G, Martegani E. The deubiquitinating enzyme UBPY/USP8 interacts with TrkA and inhibits neuronal differentiation in PC12 cells. *Exp Cell Res*. 2015;333:49–59. [PubMed: 25662281]

35. Mizuno E, Iura T, Mukai A, Yoshimori T, Kitamura N, Komada M. Regulation of epidermal growth factor receptor downregulation by UBPY-mediated deubiquitination at endosomes. *Mol Biol Cell*. 2005;16:5163–74. [PubMed: 16120644]
36. Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, et al. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. *J Neurosci*. 2014;34:16637–49. [PubMed: 25505317]
37. Zhang D, Zaugg K, Mak TW, Elledge SJ. A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response. *Cell*. 2006;126:529–42. [PubMed: 16901786]
38. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002;3:415–28. [PubMed: 12042769]
39. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics*. 2002;18:1427–31. [PubMed: 12424112]
40. Jin B, Robertson KD. DNA methyltransferases, DNA damage repair, and cancer. *Adv Exp Med Biol*. 2013;754:3–29. [PubMed: 22956494]
41. Murata A, Baba Y, Ishimoto T, Miyake K, Kosumi K, Harada K, et al. TET family proteins and 5-hydroxymethylcytosine in esophageal squamous cell carcinoma. *Oncotarget*. 2015;6:23372–82. [PubMed: 26093090]
42. Chandrasekar T, Evans CP. Autophagy and urothelial carcinoma of the bladder: a review. *Investig Clin Urol*. 2016;57(Suppl. 1):S89–97.
43. Gong C, Song E, Codogno P, Mehrpour M. The roles of BECN1 and autophagy in cancer are context dependent. *Autophagy*. 2012;8:1853–5. [PubMed: 22960473]
44. Keith B, Simon MC. Hypoxia-inducible factors. *stem Cells, Cancer Cell*. 2007;129:465–72.
45. Quail DF, Taylor MJ, Postovit LM. Microenvironmental regulation of cancer stem cell phenotypes. *Curr Stem Cell Res Ther*. 2012;7:197–216. [PubMed: 22329582]
46. Gomez-Puerto MC, Folkerts H, Wierenga AT, Schepers K, Schuringa JJ, Coffier PJ, et al. Autophagy proteins ATG5 and ATG7 are essential for the maintenance of human CD34(+) hematopoietic stem-progenitor cells. *Stem Cells*. 2016;34:1651–63. [PubMed: 26930546]
47. Whelan KA, Chandramouleeswaran PM, Tanaka K, Natsuizaka M, Guha M, Srinivasan S, et al. Autophagy supports generation of cells with high CD44 expression via modulation of oxidative stress and Parkin-mediated mitochondrial clearance. *Oncogene*. 2017;36:4843–58. [PubMed: 28414310]
48. Gewirtz DA. The four faces of autophagy: implications for cancer therapy. *Cancer Res*. 2014;74:647–51. [PubMed: 24459182]
49. Lin YC, Lin JF, Wen SI, Yang SC, Tsai TF, Chen HE. et al. Inhibition of high basal level of autophagy induces apoptosis in human bladder cancer cells. *J Urol*. 2016;195:1126–35. [PubMed: 26519656]
50. Ying L, Huang Y, Chen H, Wang Y, Xia L, Chen Y, et al. Downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer. *Mol Biosyst*. 2013;9:407–11. [PubMed: 23295831]
51. Liang Y, Zhu J, Huang H, Xiang D, Li Y, Zhang D, et al. SESN2/sestrin 2 induction-mediated autophagy and inhibitory effect of isorhapontigenin (ISO) on human bladder cancers. *Autophagy*. 2016;12:1229–39. [PubMed: 27171279]
52. Hua X, Xu J, Deng X, Xu J, Li J, Zhu DQ, et al. New compound ChlA-F induces autophagy-dependent anti-cancer effect via upregulating Sestrin-2 in human bladder cancer. *Cancer Lett*. 2018;436:38–51. [PubMed: 30118841]
53. Chetty C, Vanamala SK, Gondi CS, Dinh DH, Gujrati M, Rao JS. MMP-9 induces CD44 cleavage and CD44 mediated cell migration in glioblastoma xenograft cells. *Cell Signal*. 2012;24:549–59. [PubMed: 22024282]
54. Nishino M, Ozaki M, Hegab AE, Hamamoto J, Kagawa S, Arai D, et al. Variant CD44 expression is enriching for a cell population with cancer stem cell-like characteristics in human lung adenocarcinoma. *J Cancer*. 2017;8:1774–85. [PubMed: 28819374]
55. Bartakova A, Michalova K, Presl J, Vlasak P, Kostun J, Bouda J. et al. CD44 as a cancer stem cell marker and its prognostic value in patients with ovarian carcinoma. *J Obstet Gynaecol*. 2017;38:1–5. [PubMed: 28934892]

56. Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, et al. CD44+/CD24– breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res.* 2006;8:R59. [PubMed: 17062128]
57. Cho Y, Lee HW, Kang HG, Kim HY, Kim SJ, Chun KH. Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. *Oncotarget.* 2015;6:8709–21. [PubMed: 25909162]
58. Yu G, Yao W, Xiao W, Li H, Xu H, Lang B. MicroRNA-34a functions as an anti-metastatic microRNA and suppresses angiogenesis in bladder cancer by directly targeting CD44. *J Exp Clin Cancer Res.* 2014;33:779. [PubMed: 25551284]
59. Li X, Wang J, Zhang X, Zeng Y, Liang L, Ding Y. Overexpression of RhoGDI2 correlates with tumor progression and poor prognosis in colorectal carcinoma. *Ann Surg Oncol.* 2012;19:145–53. [PubMed: 21861235]
60. Fang Y, Yi J, Lizhi L, Qiucheng C. Rho GDP dissociation inhibitor beta promotes cell proliferation and invasion by modulating the AKT pathway in hepatocellular carcinoma. *DNA Cell Biol.* 2014;33:781–6. [PubMed: 25180836]
61. Yi B, Zhang Y, Zhu D, Zhang L, Song S, He S, et al. Overexpression of RhoGDI2 correlates with the progression and prognosis of pancreatic carcinoma. *Oncol Rep.* 2015;33:1201–6. [PubMed: 25573518]
62. Lee BH, Lee MJ, Park S, Oh DC, Elsasser S, Chen PC, et al. Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature.* 2010;467:179–84. [PubMed: 20829789]
63. Popov N, Wanzel M, Madiredjo M, Zhang D, Beijersbergen R, Bernards R, et al. The ubiquitin-specific protease USP28 is required for MYC stability. *Nat Cell Biol.* 2007;9:765–74. [PubMed: 17558397]
64. Ciesielski P, Jozwiak P, Krzeslak A. [TET proteins and epigenetic modifications in cancers]. *Post Hig Med Dosw.* 2015;69:1371–83.
65. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer.* 2004;4:707–17. [PubMed: 15343277]
66. Baylin SB. DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol.* 2005;2(Suppl. 1):S4–11. [PubMed: 16341240]
67. Chen Q, Yin D, Zhang Y, Yu L, Li XD, Zhou ZJ, et al. MicroRNA-29a induces loss of 5-hydroxymethylcytosine and promotes metastasis of hepatocellular carcinoma through a TET-SOCS1-MMP9 signaling axis. *Cell Death Dis.* 2017;8:e2906. [PubMed: 28661477]
68. Huang H, Zhu J, Li Y, Zhang L, Gu J, Xie Q, et al. Upregulation of SQSTM1/p62 contributes to nickel-induced malignant transformation of human bronchial epithelial cells. *Autophagy.* 2016;12:1687–703. [PubMed: 27467530]
69. Zhu J, Zhang J, Huang H, Li J, Yu Y, Jin H, et al. Crucial role of c-Jun phosphorylation at Ser63/73 mediated by PHLPP protein degradation in the chelation of cell transformation. *Cancer Prev Res.* 2014;7:1270–81.
70. Zhou C, Huang C, Wang J, Huang H, Li J, Xie Q, et al. LncRNA MEG3 downregulation mediated by DNMT3b contributes to nickel malignant transformation of human bronchial epithelial cells via modulating PHLPP1 transcription and HIF-1 α translation. *Oncogene.* 2017;36:3878–89. [PubMed: 28263966]

Fig. 1.

CD44s, but not CD133, acted as an ATG7 downstream effector being responsible for ATG7-mediated sphere formation in human high invasive BC cells. **a-c** ATG7 knockdown constructs (#1 and #2) were stably transfected into T24T, T24, UMUC3 cells, respectively. The knockdown efficiency of ATG7 protein and its effects on other protein expression were assessed by Western Blot. **d** and **e** T24T(shATG7#1), T24T(shATG7#2), and T24T(Nonsense) cells were subjected to determination of sphere formation abilities according to the manufacturer's instruction, the number of spheroid formed cells was counted as described in section of "Materials and Methods". The symbol (*) indicates a significant inhibition ($p < 0.05$). **f** and **g** Sphere formation abilities of T24T(Vector), and T24T(pTRE-ATG7) cells were determined with 10 µg/ml Dox treatment using the same

method as described above. The asterisk (*) indicates a significant increase in comparison to vector control transfectant ($*p < 0.05$). **h** The inducible ATG7 overexpression plasmid was stably transfected into T24T cells, whole cell extracts from T24T(Vector), and T24T(pTRE-ATG7) cells treated with or without 10 $\mu\text{g/ml}$ Dox for 12 h were subjected to Western Blot. **i** Whole cell extracts from D456, T24T(shATG7#1), T24T(shATG7#2), and T24T(Nonsense) cells were subjected to Real-time PCR for determination of variant isoforms of *cd44* mRNA. **j** and **k** Western Blot was used to detect the indicated proteins in T24T (Nonsense), T24T(shATG7#1), T24T(shATG7#1/CD44s), T24T (shATG7#1/Vector), and T24TshATG7#1/GFP-CD133) cells. **l-o** Sphere formation abilities of T24T(Nonsense), T24T(shATG7#1), T24T(shATG7#1/CD44s), T24T(shATG7#1/Vector), and T24T (shATG7#1/GFP-CD133) cells were determined using the same method as described above. The asterisk (*) indicates a significant decrease in comparison to scramble nonsense transfectant ($*p < 0.05$), while the symbol (**) indicates a significant increase in comparison to T24T(shATG7#1) cells ($**p < 0.05$)

Fig. 2.

Introduction of CD44s into T24T(shATG7) cells restored cell invasion and lung metastatic ability via stabilization of RhoGDI β . **a** and **b** Invasion abilities of T24T(shATG7#1), T24T(shATG7#1/CD44s), and T24T(Nonsense) cells were determined using BD Bio-coatTM matrigelTM invasion chamber. The migration ability was determined using the empty insert membrane without the matrigel, while the invasion ability was evaluated using the same system except that the matrigel was applied. **b** The invasion ability was normalized to the insert control according to the manufacturer's instruction. The asterisk (*) indicates a significant inhibition in comparison to T24T (Nonsense) cells (* p < 0.05), while the symbol (**) indicates a significant increase in comparison to T24T(shATG7#1) (** p < 0.05). **c-e** T24T(Nonsense), T24T(shATG7#1), and T24T(shATG7#1/CD44s) cells were intravenously inoculated into nude mice, respectively. The representative images of the lungs and lung surface metastatic foci were photographed and counted after fixation in a neutral-buffered formalin/Bouin's fixative solution. The results were presented as mean $\pm$ SD from different mice and the asterisk (*) indicated a significant decrease in comparison to T24T(Nonsense), while the symbol (**) indicates a significant increase in comparison to T24T(shATG7#1) (p < 0.05). **f** The whole cell protein extracts obtained from T24T(Nonsense), T24T(shATG7#1), and T24T(shATG7#1/CD44s) were subjected to Western Blot for determination of protein expression as indicated. **g** Real-time PCR was applied to compare the mRNA levels of *rhogdi β* among T24T(Nonsense), T24T(shATG7#1), and T24T (shATG7#1/CD44s) cells, and *gapdh* was used as internal control. The asterisk (*) indicated a significant decrease in comparison to T24T (Nonsense). **h** T24T(shATG7#1) and T24T(shATG7#1/CD44s) cells were pretreated with or without MG132 in the presence or

absence of CHX for 12 h. The cell extracts were then subjected to Western Blot to determine protein degradation for time points as indicated

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Fig. 3.

ATG7 inhibited CD44s protein degradation via promoting protein expression of USP28, which directly bound to and deubiquitinated CD44s protein. **a** Real-time PCR was applied to compare the mRNA levels of variant isoforms of *cd44s* between T24T(Nonsense) and T24T(shATG7) cells, and *gapdh* was used as internal control. **b** T24T(Nonsense) and T24T(shATG7#1) cells were pretreated with or without MG132 for 12 h, and the cells were then subjected to Western Blot to determine protein degradation in the presence of CHX for time points as indicated. **c-e** The cell extracts from T24, T24T and their stable transfectants

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were subjected to Western Blot for determination of protein expression as indicated. **f** and **g** T24T(Nonsense), T24T (shATG7#1), and T24T(shATG7#1/Flag-USP28) cells were subjected to determination of sphere formation abilities using the same method as described above. The asterisk (*) indicates a significant decrease in comparison to scramble nonsense transfectant, while the symbol (**) indicates a significant increase in comparison to T24T(shATG7#1) cells (* $p < 0.05$). **h** T24T(shATG7#1/Vector) and T24T(shATG7#1/Flag-USP28) cells were pretreated with or without MG132 for 12 h, and the cells were subjected to Western Blot to determine protein degradation in the presence of CHX for time points as indicated. **i** Co-immunoprecipitation was performed with anti-Flag antibody-conjugated agarose beads in the indicated cells. Immunoprecipitates (IP) were then subjected to immunoblotting for the detection of CD44s. **j** 293T cells were transfected with constructs of CD44s-pwz1-blast in combination with Ubiquitin-WT, together with pcDNA3.1 or Flag-USP28 constructs as indicated. Forty-eight hours after transfection, cells were lysed and co-immunoprecipitated with anti-CD44s antibody-conjugated agarose beads, and then immunoblotted with anti-HA, anti-Flag, and anti-CD44s antibodies for detection of CD44s ubiquitination

Fig. 4.

ATG7-mediated *usp28* transcription by promoting its promoter demethylation in human BC cells. **a** Real-time PCR was applied to compare the mRNA levels of *usp28* among T24T(shATG7#1), T24T (shATG7#2), and T24T(Nonsense) cells, and *gapdh* was used as internal control. **b** UMUC3(shATG7#1), UMUC3(shATG7#2), and UMUC3(Nonsense) cells were seeded into six-well plates. After synchronization, the cells were used to determine *usp28* mRNA stability in the presence of Actinomycin D (Act D) by using Real-time PCR. *gapdh* was used as internal control. **c** Human *usp28* promoter-driven luciferase reporter was employed to evaluate *usp28* promoter transcriptional activity in the indicated cells. The results were normalized by internal TK activity. Bars represent mean $\pm$ SD from three independent experiments. Student's *t*-test was used to determine the *p*-value, **p* < 0.05. **d** MethPrimer v1.1 beta software was used to bioinformatically analyze the potential CpG Island of human *usp28* promoter, and the blue shaded region indicates the potential CpG Island of human *usp28* promoter (from -432 to -309). **e** The methylation status of the

usp28 promoter in the methylation region were determined using the MS-PCR assay in the indicated cells. A primer set was used to evaluate the methylated (M) and unmethylated (U) copies of *usp28* promoter in the methylation region. Methylated control was used as the positive control (P), whereas unmethylated control was used as the negative control (N). A 113-bp PCR product represents the methylated state and a 109-bp PCR product stands for the unmethylated allele

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Fig. 5.

TET1-mediated *usp28* promoter demethylation contributed to ATG7 upregulation of CD44s and cancer stem-like properties of human BC cells. **a** The indicated cells were treated with 5-aza-2'-deoxycytidine (5 μM) for 72 h. The relative levels of human *usp28* promoter-driven luciferase activity was evaluated in the indicated cells. The results were normalized by internal TK activity. Bars represent mean ± SD from three independent experiments. Student's *t*-test was utilized to determine the *p*-value, **p* < 0.05. The symbol (**) indicates a significant increase in comparison to T24T(shATG7#1) cells with medium control (***p* < 0.05). **b** The indicated cells were treated with 5-aza-2'-deoxycytidine (5 μM) for 72 h. Cell lysates extracted from the cells were then subjected to Western Blot to determine USP28 and CD44s protein expression, β-Actin was used as a protein loading control. **c** Real-time PCR was applied to compare the mRNA levels of *dnmt1*, *dnmt3a*, *dnmt3b*, *tet1*, *tet2*, and *tdg* among T24T(Nonsense), T24T(shATG7#1), and T24T(shATG7#2) cells, *gapdh* was used as internal control. **d** and **e** Cell lysates extracted from the indicated cells were subjected to Western Blot to determine DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, and TDG protein expression, β-Actin was used as a protein loading control. **f** HA-TET1 expression constructs were stably transfected into T24T (shATG7#1) cells, and the stable transfectants were identified by Western Blotting. **g** and **h** T24T(Nonsense), T24T(shATG7#1), and T24T(shATG7#1/HA-TET1) cells were subjected to determination of sphere formation

abilities using the same method as described above. The asterisk (*) indicates a significant decrease in comparison to scramble nonsense transfectant, while the symbol (**) indicates a significant increase in comparison to T24T(shATG7#1) cells (* $p < 0.005$). **I** The methylation status of the *usp28* promoter in the methylation region was determined using the MS-PCR assay in the indicated cells

Fig. 6.

ATG7 stabilized *tet1* mRNA through attenuating AUF1 protein expression in human BC cells. **a** UMUC3(shATG7#1), UMUC3 (shATG7#2), and UMUC3(Nonsense) cells were seeded into six-well plates. After synchronization, the cells were used to determine *tet1* mRNA stability in the presence of Act D by using Real-time PCR. *gapdh* was used as an internal control. **b** and **c** Cell lysates extracted from the indicated cells were subjected to Western Blot to determine AUF1, TET1, USP28, and CD44s protein expression, GAPDH was used as a protein loading control. **d** and **e** The indicated cells were subjected to determination of sphere formation abilities using the same method as described above. The asterisk (*) indicates a significant increase in comparison to scramble nonsense transfectant (* $p < 0.05$). **f** HA-AUF1 construct was transfected into 293T cells and HA-AUF1 protein was pulled down with anti-HA beads. The mRNAs bound to AUF1 protein were used to carry out RT-PCR for determination of presence of *tet1* mRNA in the immunocomplex. **g** The proposed mechanisms underlying ATG7 promotion of human sphere formation and invasion of human high invasive BC cells: ATG7 overexpression inhibits AUF1 expression, which in turn resulting in *tet1* mRNA stabilization, and demethylating *usp28* promoter, and thereby increasing USP28 expression, further leading to accumulation of CD44s protein, finally promotion of cancer stem-like properties and invasion of human BC cells