Uev1A Promotes Breast Cancer Cell Migration and EMT by Up-Regulating CT45A Expression via the AKT Pathway

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Research article

Keywords: Uev1A, CT45A, AKT signaling pathway, NF-κB pathway, cell migration, EMT

Posted Date: December 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-122157/v1

Abstract

Introduction

UEV1A encodes a ubiquitin-E2 variant closely associated with tumorigenesis and metastasis, but its underlying mechanism in promoting tumorigenesis remain to be investigated.

Methods

In this study, we experimentally manipulated *UEV1A* and *CT45A* gene expression and monitored their effects on cancer-related gene expression, cell migration and the signal transduction cascade.

Results

It was found that *UEV1A* overexpression induces *CT45A* family gene expression in breast cancer cells. Indeed, ectopic expression of *UEV1A* is sufficient to induce CT45A and its downstream genes involved in tumorigenesis, epithelial-mesenchymal transition (EMT), stemness and metastasis, and promote cell migration and EMT. Consistently, depletion of CT45A abolishes the above effects, indicating that CT45A is a critical downstream effector of Uev1A. The Uev1A-induced cell migration and EMT is dependent on AKT signaling but independent of NF-κB signaling, indicating that CT45A acts downstream of the AKT pathway.

Conclusions

Based on previous reports and observations in this study, we propose that the Ubc13-Uev1A complex activates AKT through K63-linked polyubiquitination, which leads to enhanced *CT45A* expression, stimulated cell migration and EMT. Since similar effects were also observed in a colorectal cancer cell line, we propose that the Ubc13/Uev1A-AKT-CT45A axis promotes tumorigenesis and metastasis in a broad range of tissues.

Introduction

UEV1, also known as *CROC1* [1, 2] or *CIR1* [3], was identified as a mammalian homolog of yeast *MMS2* [4], as well as a potential proto-oncogene that is associated with tumorigenesis and metastasis [5-7]. Indeed, *UEV1* maps to a region (chromosome 20q13.2) where DNA amplification is frequently reported in breast cancers [8-11] and other tumors [12]. Ubiquitin (Ub)-conjugating enzyme variant (UEV, including Mms2 or Uev1 in mammalian cells) is a co-factor of Ubc13 [13] and absolutely required for Ubc13-mediated Lys63-linked polyubiquitin chain assembly [14-17]. To date, at least three *UEV1* splicing variants have been reported, among which Uev1A and Uev1C could promote K63-linked polyubiquitination by forming a complex with Ubc13 whereas Uev1B could not [18]. Uev1A differs from Uev1C in that it contains thirty additional amino acids at the N-terminus [18, 19].

Despite the fact that Uev1A and Mms2 are two major Uevs in mammalian cells and share a similar biochemical activity, they appear to function differently: Ubc13-Mms2 is required for DNA-damage response, whereas Ubc13-Uev1A is involved in NF-κB activation [18] and AKT activation [6]. Previous studies demonstrated that Uev1A-Ubc13 represses stress-induced apoptosis in HepG2 cells [20] and promotes breast and colon cancer metastasis through the NF-κB signaling pathway [19, 21]. Meanwhile, Uev1A-Ubc13 promotes breast cancer cell survival and chemoresistance through the AKT pathway [6]. Consistently, chemical inhibition of the Uev1A-Ubc13 interaction suppresses cells survival and proliferation of diffuse large B-cell lymphoma cells [22]. These results collectively indicate that Uev1A is involved in tumorigenesis and metastasis.

The PI3K/AKT signaling pathway is an essential node in mammalian cells and is closely associated with various biological functions including cell growth, survival, proliferation, migration, resistance to apopotosis, differentiation, metabolism and angiogenesis [23-26]. In addition, this pathway is frequently found to be abnormally activated and altered in many human malignancies, which induces chemoresistance and malignant transformation [27-30].

In this study we found that overexpression of *UEV1A* induced *CT45A* expression in breast cancer cells in an Ubc13-dependent manner, while depletion of Uev1 inhibited *CT45A* expression. This study revealed that ectopic expression of *CT45A* could upregulate expression of its downstream genes related to tumorigenic, EMT, stemness and metastasis, and promote breast cancer EMT and cell migration. A series of experimental results support a notion that *CT45A* is a critical downstream gene of the AKT signaling pathway but not the NF-κB signaling pathway. Since similar effects were also observed in a colorectal cancer cell line, the Uev1A-/Ubc13-AKT-CT45A axis in tumorigenesis may be a general phenomenon. Hence, this study suggests a potential therapeutic target in the treatment of breast and colorectal cancers.

Methods

Cell lines and culture

Human breast cancer cell lines MCF7 and MDA-MB-231, and human colon carcinoma cell line HCT116 were obtained from the American Type Culture Collection (ATCC, Manassan, VA, USA). The cells were cultured in Dubecco's modified Eagle medium (DMEM, HyClone), supplemented with 10% fetal bovine serum (FBS, HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C with 5% CO_2. MDA-MB-231-TR and MCF7 *UEV1A*-overexpressed stable cell lines were created as previously reported [6]. MDA-MB-231 and MCF7 *UEV1A*-knockdown stable cell lines were created by transfecting MDA-MB-231 and MCF7 cells with *UEV1A* shRNA lentiviral particles or negative control shRNA lentiviral particles-A (Santa Cruz Biotechnology, Inc), and selecting with 1 μg/ml puromycin dihydrochloride (Santa Cruz Biotechnology, Inc).

Plasmids and cell transfection

Human *UEV1A* and *CT45A* open reading frames (ORFs) were amplified as *Kpn*I-*Xho*I fragments and cloned into plasmid vector pcDNA4.0/TO/HA (+) as previously described [19]. The mutated Ubc13-binding site (F38E) in *UEV1A* was designed based on a previous study with Mms2-F13E [17]. The *CT45A* small interfering RNA (siRNA) was purchased from GenePharma (Shanghai, China). The sequence for *CT45A* siRNA is 5'-GGAGAGAAAAGGAUCAGAUUU-3'. The modified sequence for *UEV1A* small hairpin RNA (shRNA, sc-38606-v) and negative control shRNA (sc-108080) delivered by lentiviral particles were obtained from Santa Cruz Biotechnology, Inc. The lentiviral particle infection of MDA-MB-231 and MCF7 breast cancer cells was performed following instructions of the supplier.

RNA preparation and quantitative real-time RT-PCR (qRT-PCR)

Total RNAs were extracted from cultured MDA-MB-231, MCF7 breast cancer and HCT116 colorectal cancer cells using Trizol (Invitrogen, 15596018). First-strand cDNA was synthesized with 1 μg of total RNAs with TransScript® All-in-One First-Strand cDNA Synthesis SuperMix (TransGen, AT341-01) according to manufacturer's instructions. qRT-PCR analysis based on SYBR® Premix Ex Taq™ (Takara, RR420A) was performed on the BioRad real-time PCR machine. The specific primers are listed in Supplementary Table 1. The data analysis was performed using the $2^{-\Delta CT}$ comparative cycle threshold method [31] from three independent experiments.

Protein extraction and western blotting

Cells were grown to log phase and lysed with whole-cell extraction buffer (150 mM NaCl; 1% NP-40; 10% glycerol; 1 mM EDTA; 50 mM Tris; 1 mM PMSF) and protease inhibitor cocktail for mammalian cells (Roche). Proteins in cell extracts were separated by 8-12% SDS-PAGE gels and transferred to PVDF membrane. The membrane was blocked with 5% milk/BSA and incubated with specific primary antibodies followed by secondary antibodies. The following antibodies were used: anti-AKT (#4691, Cell Signaling Technology), anti-Phospho-Akt-Ser473 (#4060, Cell Signaling Technology), anti-Phospho-Akt-Tr308 (#13038, Cell Signaling Technology), anti-Tubulin (sc-166729, Santa Cruz), anti-HA (A-190-208A. BETHYL), anti-N-cadherin (#13116, Cell Signaling Technology), anti-E-cadherin (#3195S, Cell Signaling Technology), anti-Lamin B (sc-6216, Santa Cruz), anti-NF-κB p65 (sc-8008, Santa Cruz), goat anti-mouse IgG-horseradish peroxidase (HRP) (sc-2005, Santa Cruz) and goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz), donkey anti-goat IgG-HRP (sc-2033, Santa, Cruz).

Cell migration assay

In vitro cell migration ability was measured by a Transwell assay without Matrigel coating, using 8-μm-pore-size polycarbonate membrane filters in 24-well culture plates. After starving in FBS-free DMEM medium for 24 hours, 2×10^5 HCT116, 5×10^4 MDA-MB-231 and 2×10^5 MCF7 cells were seeded in the upper chamber, while the lower surface of the filter was coated with 10% FBS-DMEM as chemo-attractants. The cells were allowed to migrate for 24 hours and the cells migrated to the lower surface of the filter were counted in five random fields under a light-microscope at high magnification. These experiments were done at least in triplicate.

Statistical analysis

The statistical significance of differential findings between the control and experimental groups was determined by student's t-test as implemented by Microsoft Excel 2016 (*, P <0.05; ** P <0.01; and ***, P <0.001).

Results

Uev1A upregulates *CT45A* expression in a Ubc13-dependent manner

We performed a microarray analysis by comparing *UEV1A*-overexpressed and vector control MDA-MB-231 breast cancer cells, which revealed 47 genes upregulated by more than fivefold in *UEV1A*-overexpressed MDA-MB-231 cells (Supplementary Table S2). Interestingly, 16 out of 47 belong to cancer/testis antigens (CTAs), among which *CT45A* family members are most highly elevated in *UEV1A*-overexpressed MDA-MB-231 cells (Figure S1A). CTAs are tumor-associated and testis-derived specific immunogenic antigens closely associated with spontaneous immune responses in cancer patients [32, 33]. They are not expressed in nearly all normal tissues except testis after birth, but are highly expressed in various types of cancers [34-39]. Within CTAs, the *CT45A* gene family comprises 10 genes designated as *CT45A1* to *CT45A10*, which are distinct but highly conserved, as their amino-acid sequences exhibit more than 98% identity [40] (Figure S1B). To independently examine the role of Uev1A in upregulating *CT45A* expression and its biological implications, *UEV1A* was cloned into a pcDNA4.0/TO/HA(**+**) vector and then trasfected into MDA-MB-231-TR and MCF7 cells to construct stable cell lines as previously reported [19]. The level of *UEV1A* ectopic expression was monitored by western blot against the HA-tag after 10 μg/ml doxycyline (Dox) treatment (Figure S2A,B). Then *CT45A* expression was measured by qRT-PCR and found to be signifcantly upregulated in *UEV1A*-overexpressed MDA-MB-231-TR (Figure 1A) and MCF7 (Figure 1B) cells. It has been reported that *UEV1A* is upregulated in MDA-MB-231 and MCF7 cells by 2.8- and 4-fold, respectively [19]. To ask whether this moderate overexpression of *UEV1A* contributes to *CT45A* upregulation in breast cancer cells, we suppressed the endogenous *UEV1A* expression in MDA-MB-231 and MCF7 cells using shRNAs delivered by lentiviral particles as previously reported [19]. It was found that two independent shUEV1A constructs, shUEV1A-1 and shUEV1A-2, reduced *UEV1A* expression in MDA-MB-231 cells by 43% and 60% (Figure S3A), and in MCF7 cells by 71% and 85% (Figure S3B), respectively, compared to contral shRNA-treated cells. Meanwhile, the *CT45A* transcript levels were also reduced (Figure 1C, D). To further ask whether Uev1A upregulates *CT45A* expression in a Ubc13-dependent manner, we constructed stable MDA-MB-231-TR and MCF7 cell lines expressing Dox-inducible Uev1A-F38E mutant protein (Figure S2A,B), which is known to abolish physical interaction between Mms2/Uev1A and Ubc13 [7, 17, 19]. As expected, *UEV1A-F38E* failed to upregulate *CT45A* mRNA levels in both MDA-MB-231-TR (Figure 1E) and MCF7 (Figure 1F) cells. These observations collectively indicate that Uev1A upregulates *CT45A* expression in a Ubc13-dependent manner in breast cancer cells.

Uev1A positively regulates *CT45A* downstream gene expression in breast cancer cells

CT45A has been reported to act as a proto-oncogene through upregulating tumorigenic and metastatic genes [41]. We first measured the transcript level of several *CT45A* previously-reported [41] downstream genes thought to be involved in tumoregenesis, EMT, stemness and metastasis after *CT45A* ectopic expression. The expression of some tumoregenesis-associated genes, including those encoding RAS exchange factor (*RASGEF1A*), melanoma antigen family member (*MAGED4B*), homeobox B6 (*HOXB6* and *HOXD13*) was indeed significantly higher in *CT45A*-overexpressed MDA-MB-231 (Figure 2A) and MCF7 (Figure 2B) cells than their respective control cells. Expression of several EMT, stemness and metastasis related genes, including *TWIST1*, *KIT*, aldehyde dehydrogenase 1 family, member A1 (*ALDH1A1*), *CXCR4* and/or *SULF2* were also upregulated in *CT45A*-overexpressed MDA-MB-231 (Figure 2C) and MCF7 (Figure 2D) cells. Since *UEV1A* can upregulate *CT45A* expression, we asked whether *UEV1A* could also upregulate the expression of *CT45A* downstream genes in breast cancer cells. Indeed, the majority of *CT45A* downstream genes, including *HOXB6, HOXD13, RASGEF1A*, *MAGED4B*, *ALDH1A1*, *TWIST1*, *KIT*, *CXCR4* and *SULF2*, were upregulated in *UEV1A*-overexpressed MDA-MB-231 (Figure 2E, G) and MCF7 (Figure 2F, H) cells. Taken together, we conclude that *UEV1A* positively regulates *CT45A* downstream gene expression in breast cancer cells.

CT45A is a critical regulator for Uev1A-induced breast cancer cell migration

To ask whether an elevated *CT45A* level alone is indeed sufficient to promote breast cancer development and metastasis, *CT45A* was cloned into plasmid pcDNA4.0/TO/HA(**+**), transiently transfected into MDA-MB-231 and MCF7 cells and the level of *CT45A* ectopic expression after 200 μg/ml zeocin treatment was monitored by western blot against an HA-tag antibody (Figures 3A and 4A). The effects of *CT45A* ectopic expression on MDA-MB-231 (Figure 3) and MCF7 (Figure 4) cells were then assessed. The transwell without matrigel experiments show that overexpression of *CT45A* nearly tripled the MDA-MB-231 cell mobility compared with vector-transfected cells (Figure 3B,C). Similarly, after induction the migration of MCF7 *CT45A* transfectants was more than 2.3-fold higher than the control cells (Figure 4B,C), indicating that CT45A regulates breast cancer cell migration *in vitro*.

To ask whether Uev1A is a critical regulator for *CT45A*-induced migration, we successfully depleted *CT45A* by approximately 50% using siRNA in MDA-MB-231 (Figure S3C) and MCF7 (Figure S3D) cells. Similar effects were also observed in *UEV1A*-overexpressed MDA-MB-231 (Figure 3D) and MCF7 (Figure 4D) cells. The above treatment does not affect the expression of *UEV1A* (Figures 3E and 4E), but the moderate *CT45A* depletion in *UEV1A*-overexpressed cells markedly reduced cell migration as determined by a transwell assay without matrigel (Figures 3F,G and 4F,G). The above findings allow us to conclude that CT45A is a critical regulator for Uev1A-induced migration in breast cancer cells, as partial depletion of CT45A can reverse cell migration in *UEV1A*-overexpressed breast cancer cells.

CT45A promotes metastasis in other type of cancer cells

To ask whether *UEV1A* overexpression also increases *CT45A* expression in other cancer cells, we created stable *UEV1A*-transfected HCT116 colorectal cancer cell lines as previously described (Figure 5A) [21], in which *CT45A* was moderately upregulated upon *UEV1A* ectopic expression, and this upregulation was not

observed in the *UEV1A*-F38E-expressed HCT116 cells (Figure 5B). To ask whether this moderate overexpression of *UEV1A* contributes to *CT45A* upregulation in colorectal cancer cells, we suppressed the endogenous *UEV1A* expression in HCT116 cells by using shRNAs delivered by lentiviral particles as previously reported [21]. It was found that two independent shUEV1A constructs, shUEV1A-1 and shUEV1A-2, reduced *UEV1A* expression in HCT116 cells by 55% and 65%, respectively (Figure S4A), compared to control shRNA-treated cells. Meanwhile, the *CT45A* transcript levels were also reduced (Figure 5C). To ask whether ectopic expression of *CT45A* could promote metastasis in other types of cancer cells, HCT116 cells were transiently transfected with pcDNA4.0/TO/HA-CT45A and the CT45A expression was monitored by western blot analysis against HA-tagged CT45A (Figure 5D). The *CT45A* ectopic expression resulted in concommitant increase in HCT116 cell migration by sevenfold (Figure 5E,F), indicating that CT45A could also promote tumorigenesis and metastasis in other types of cancer cells. To further ask whether CT45A is a critical regulator for *UEV1A*-induced migration, we depleted CT45A by using siRNA in *UEV1A*-overexpressed HCT116 cells. As shown in Figure S4B, *CT45A* was depleted by 44%. The above treatment does not affect the expression of *UEV1A* (Figure S4C), but the moderate *CT45A* depletion in *UEV1A*-overexpressed HCT116 cells markedly reduced cell migration as determined by a transwell assay without matrigel (Figure 5G,H). The above findings indicate that Uev1A induces colorectal cancer cell migration through upregulating *CT45A* genes.

Depletion of *CT45A* can reverse EMT in *UEV1A*-overexpressed breast cancer cells

Epithelial-mesenchymal transition (EMT) is closely associated with cancer progression, cancer cell metastasis and drug resistance [42, 43]. Cells undergoing EMT display increased expression of mesenchymal genes including *N-cadherin*, *fibronectin* and *vimentin*, and decreased expression of epithelial genes including *E-cadherin*, *occulin* and *ZO-1* [44]. It was reported that overexpression of *CT45A* could induce breast cancer EMT, and thus foster cancer metastasis by upregulating EMT master gene *TWIST1* [41]. To further investigate the potential molecular mechanisms by which *CT45A* regulates breast cancer cell migration, we monitored alterations of EMT markers, including *N-cadherin* and *vimentin*, two well-characterized mesenchymal markers, and *E-cadherin*, a well-known epithelial marker [44, 45]. Consistent with breast cancer cell migration, increased mRNA levels of *N-cadherin* and *vimentin* and decreased *E-cadherin* were found upon *CT45A* overexpression in MDA-MB-231 (Figure 6A) and MCF7 (Figure 6B) cells. We also assessed the effect of CT45A on cellular N-cadherin and E-cadherin at protein levels. Firstly, we monitored cellular N-cadherin and E-cadherin levels in MDA-MB-231 and MCF7 cells and found that MDA-MB-231 and MCF7 cells produced detectable N-cadherin and E-cadherin, respectively (Figure 6C). Interestingly, ectopic expression of *CT45A* increased N-cadherin in MDA-MB-231 cells and decreased E-cadherin in MCF7 cells (Figure 6C,E,F), suggesting that cell migration stimulated by ectopic *CT45A* expression is likely due to the enhanced EMT in breast cancer cells. To address whether Uev1A is a critical upstream regulator of CT45A-induced EMT, we depleted *CT45A* by using siRNA in *UEV1A*-overexpressed MDA-MB-231 and MCF7 breast cancer cells (Figures 3D and 4D), which significantly increased E-cadherin protein levels in *UEV1A*-overexpressed MCF7 cells and decreased N-cadherin protein levels in *UEV1A*-overexpressed MDA-MB-231 cells (Figure 6D,G,H). Collectively, these results support a notion that Uev1A can serve as an important regulator for CT45A-induced EMT in breast cancer cells.

Uev1A regulates *CT45A* expression through the AKT signaling pathway

Since Uev1A has been reported to be associated with NF-κB activation [19-21] and AKT activation [6], we wish to investigate molecular mechanisms by which Uev1A regulates CT45A expression. To ask whether Uev1A regulates *CT45A* expression through the NF-κB pathway, MDA-MB-231 and HCT116 cells transiently overexpressing *UEV1A* were treated with the NF-κB pathway inhibitor Bay11-7082 [46] and its efficacy was measured by the nuclear P65 level (Figure S5A,C). The *CT45A* transcript level was not significantly reduced in *UEV1A*-ovexpressed MDA-MB-231 (Figure S5B) and HCT116 cells (Figure S5D) by treatment with Bay11-7082, indicating that Uev1A upregulation of *CT45A* expression is independent of the NF-κB pathway. To ask whether Uev1A regulates *CT45A* expression through the AKT signaling pathway in breast cancer cells, phosphorylation levels of both AKT-Thr308 and AKT-Ser473 in MDA-MB-231 and MCF7 cells transiently overexpressing *UEV1A* were first monitored by western blot and found to be increased (Figure 7A). In contrast, overexpression of *UEV1A-F38E* failed to induce AKT phosphorylation at both residues (Figure 7A), indicating that the effects of Uev1A on AKT is dependent on its interaction with Ubc13. These observations allow us to conclude that excessive Uev1A promotes the Uev1A-Ubc13 complex formation, which activates the AKT signaling pathway. To further address whether Uev1A promotes *CT45A* expression through the AKT signaling pathway, we examined effects of PI3K/AKT pathway inhibitor LY294002 [47] on MDA-MB-231 and MCF7 cells with ectopic *UEV1A* expression. As seen in Figure 7B, the AKT-Ser473 phosphorylation level was markedly decreased after LY294002 treatment in *UEV1A*-overexpressed MDA-MB-231 and MCF7 cells compared to those without the inhibitor treatment. We then examined *CT45A* expression and found that, compared to cells without LY294002 treatment, the *CT45A* transcript level was significantly reduced in *UEV1A*-overexpressed MDA-MB-231 (Figure 7C) and MCF7 (Figure 7D) cells after 10 μM LY294002 treatment. After 20 μM LY294002 treatment, the CT45A transcript further decreased to levels below the vector control cells without the inhibitor treatment (Figure 7C,D). It was previously reported that insulin-like growth factor (IGF-1) is an important activator of the PI3K/AKT signaling pathway [48, 49]. To further investigate whether *CT45A* is indeed a direct downstream gene of the AKT signaling pathway, we treated MDA-MB-231 (Figure 7E) and MCF7 (Figure 7F) cells with IGF-1, and found that the AKT-Ser473 phosphorylation level was dramatically increased after IGF-1 treatment compared to untreated cells. Under the above experimental conditions, the *CT45A* mRNA levels were significantly increased in MDA-MB-231 (Figure 7G) and MCF7 (Figure 7H) cells after IGF-1 treatment. Collectively, we conclude that Uev1A-Ubc13 regulates *CT45A* expression through the AKT signaling pathway in breast cancer cells.

Discussion

Previous reports have identified CT45A as a chemosensitivity mediator and immunotherapy target in ovarian cancer [40, 50]. In addion, CT45A has no detectable expression in normal tissues after birth, except for the testis, but it is closely associated with the progression and development of various cancers [41, 51-54]. In particular, it is highly expressed in cancer stem cells (CSCs), but not in differentiated cells [55], indicating that it is a promising biomarker for diagnosis and treatment of cancer patients. However, exactly how the *CT45A* family genes function in these processes remain unclear.

The *CT45A* family genes were brought to our attention based on our preliminary microarray data from which *CT45A* family genes were among the highest induced genes following *UEV1A* overexpression in MDA-MB-231 breast cancer cells. This obervation was independently confirmed in two breast cancer cell lines, although the levels of *CT45A* induction after *UEV1A* overexpression vary. In this study, we first investigated the correlation between CT45A and tumorigenesis using breast cancer cell models. At the beginning of our investigation, the *CT45A* gene family was thought to comprise six members (*CT45A1-CT45A6*) and their amino-acid sequences share more than 98% identity; hence we cloned one of them (*CT45A1*) to represent all members. Consistantly, siRNAs used in this study were designed to target all six *CT45A* family genes. Recently, the *CT45A* family has been updated to 10 genes in NCBI, and their amino-acid sequences still share more than 98% identity [40], making our initial experimental designs still valid. We overexpressed *CT45A* in MDA-MB-231 and MCF7 breast cancer cells and found that CT45A could promote cell migration, EMT and its downstream tumorigenic, EMT, stemness and metastatis related genes expression, indicating that CT45A plays an important role in breast cancer tumorigenesis.

A previous study showed that CT45A protein has a DEAD/H box which has RNA helicase activity and putative nucleic acid binding function [41]. RNA helicases of DEAD box family are required for gene expression and transcrition by interacting with RNA polymerase II (Pol II) [56], whether CT45A interacts with RNA Pol II or other transcription factors to promote tumorigenesis and metastasis remains to be further elucidated.

This study investigated the correlation between Uev1A and CT45A in breast cancer cell migartion and EMT. It was found that Uev1A upregulates *CT45A* expression in a Ubc13-dependent manner in one colorectal cancer and two breast cancer cell lines. In a reverse expreriment, depletion of *UEV1A* in the above three cancer cell lines significantly inhibited the upregulation of *CT45A*, indicating that Uev1A plays a critical role in the upregulation of *CT45A*. Siminarly, Uev1A positively regulates the expression of *CT45A* downstream tumorigenic, EMT, stemness and metastatis related genes in breast cancer cells. Moreover, under our experimental conditions, we found that N-cadherin was readily detectable in MDA-MB-231 but not MCF7 cells, while E-cadherin was detected in MCF7 but not MDA-MB-231 cells. Furthermore, ectopic expression of *CT45A* could increase N-cadherin in MDA-MB-231 cells and decrease E-cadherin in MCF7 cells, both of which are expected to promote tumorigenesis. Indeed, *CT45A* depletion in *UEV1A*-overexpressed cells reduced EMT and cell migration to a level comparable to that of control-transfected cells. These findings together indicate that Uev1A is a critical regulator of CT45A-induced cell migration and EMT in breast cancer.

In order to determine through which signaling pathway(s) Uev1A upregulates *CT45A* expression, we treated *UEV1A* ectopic expression cells with NF-κB and PI3K/AKT pathway inhibitors and found that inhibition of AKT markedly decreased CT45A expression, while inhibition of NF-κB activity had no observable effects. To further confirm that *CT45A* is a direct downstream gene of the AKT pathway, we treated breast cancer cells with the AKT pathway activator IGF-1 and found that the IGF-1 treatment leads to *CT45A* induction. The AKT signaling pathway is closely associated with many biological processes such as cell proliferation, migration and differentiation [24]. It has been reported that AKT undergoes the

TRAF6-triggered K63-linked polyubiquitination, which is critical for AKT membrane localization, phosphorylation and subsequent activation [57, 58]. Since Uev1A-Ubc13 is the only known E2 complex to regulate K63-linked polyubiquitination leading to the AKT pathway activation in breast cancer [6], this study reveals a novel Uev1A/Ubc13-AKT-CT45A axis to promote breast cancer cell migration and EMT (Figure 8). Given limited but consistent observations in a colorectal cancer cell line, the above signaling cascade may be expanded to other types of cancers.

Conclusions

Overexpression of *UEV1A* is sufficient to activate the AKT pathway in breast cancer cell lines, which in turn upregulates CT45A expression to promote breast cancer cell migration and EMT. These observations provide a potential therapeutic target in the treatment of breast cancer.

Abbreviations

CTA: cancer/testis antigen; DMEM: Dubecco's modified Eagle medium; Dox: doxycycline; EMT: epithelial-mesenchymal transition; FBS: fetal bovine serum; IGF: insulin-like growth factor; ORF: open reading frame; qRT-PCR: real-time reverse-transcription PCR; Ub: ubiquitin; UEV: Ubiquitin-conjugating enzyme variant.

Declarations

Ethics Approval and consent to participate

Not applicable

Consent for Publication

All authors agree with the the final version of the manuscript.

Availability of supporting data

Available upon request

Competing interests

The authors declare they have no competing interests.

Funding

This work was supported by a Capital Normal University Specia fund and the Canadian Breast Cancer Foundation research grant C7022 to WX. TN received a Capital Normal University visiting studentship.

Authors' contributions

TN and ZW participated in the project design and carried out all experiments. TN wrote the initial draft manuscript. WX conceived the study, participated in the project design, manuscript preparation and submission. All aothors read and approved the final manuscript.

Acknowledgements

The authors wish to thank mambers of the Xiao laboratory for helpful discussion and Michelle Hanna for proofreading the manuscript.

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Figures

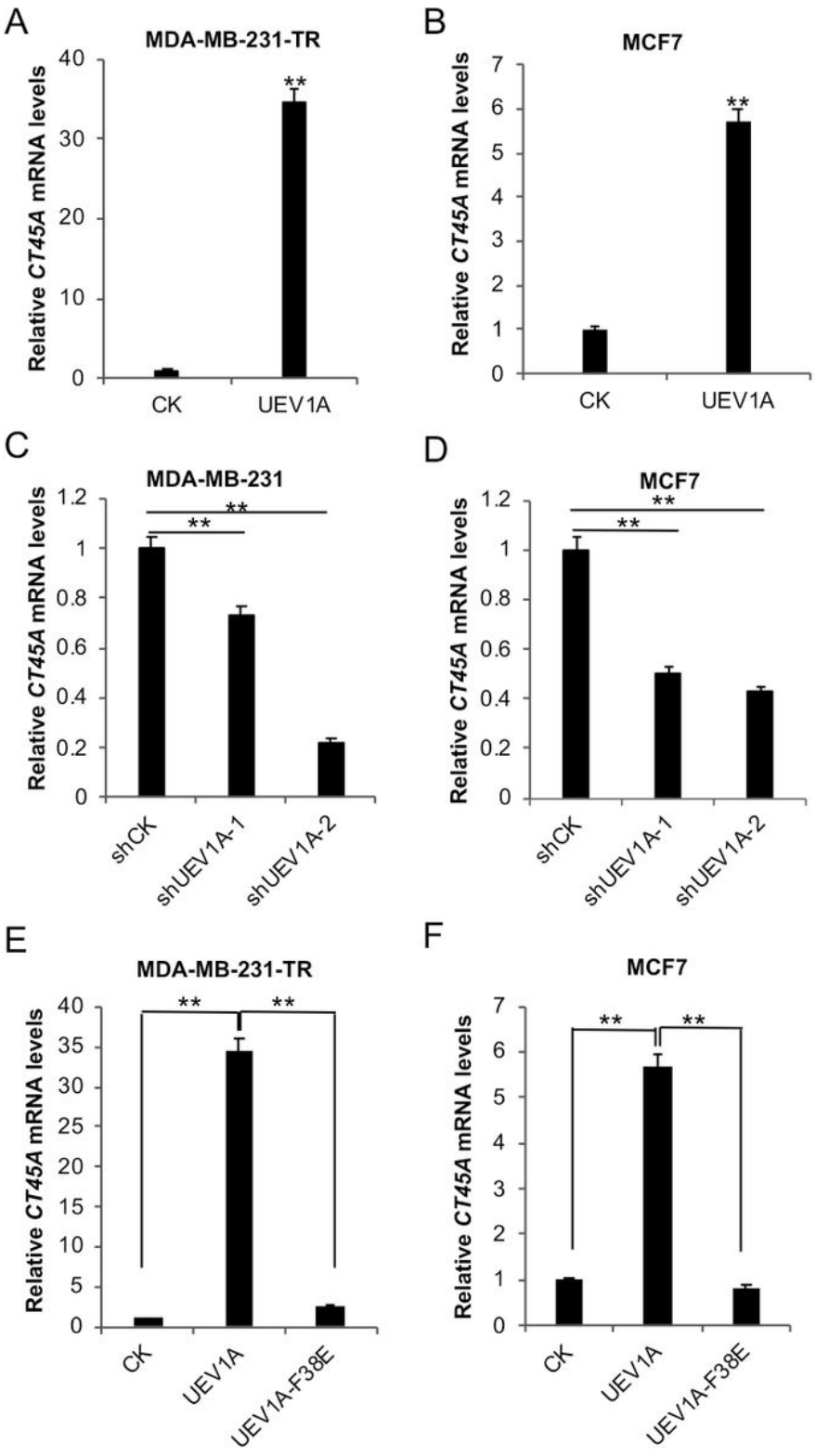
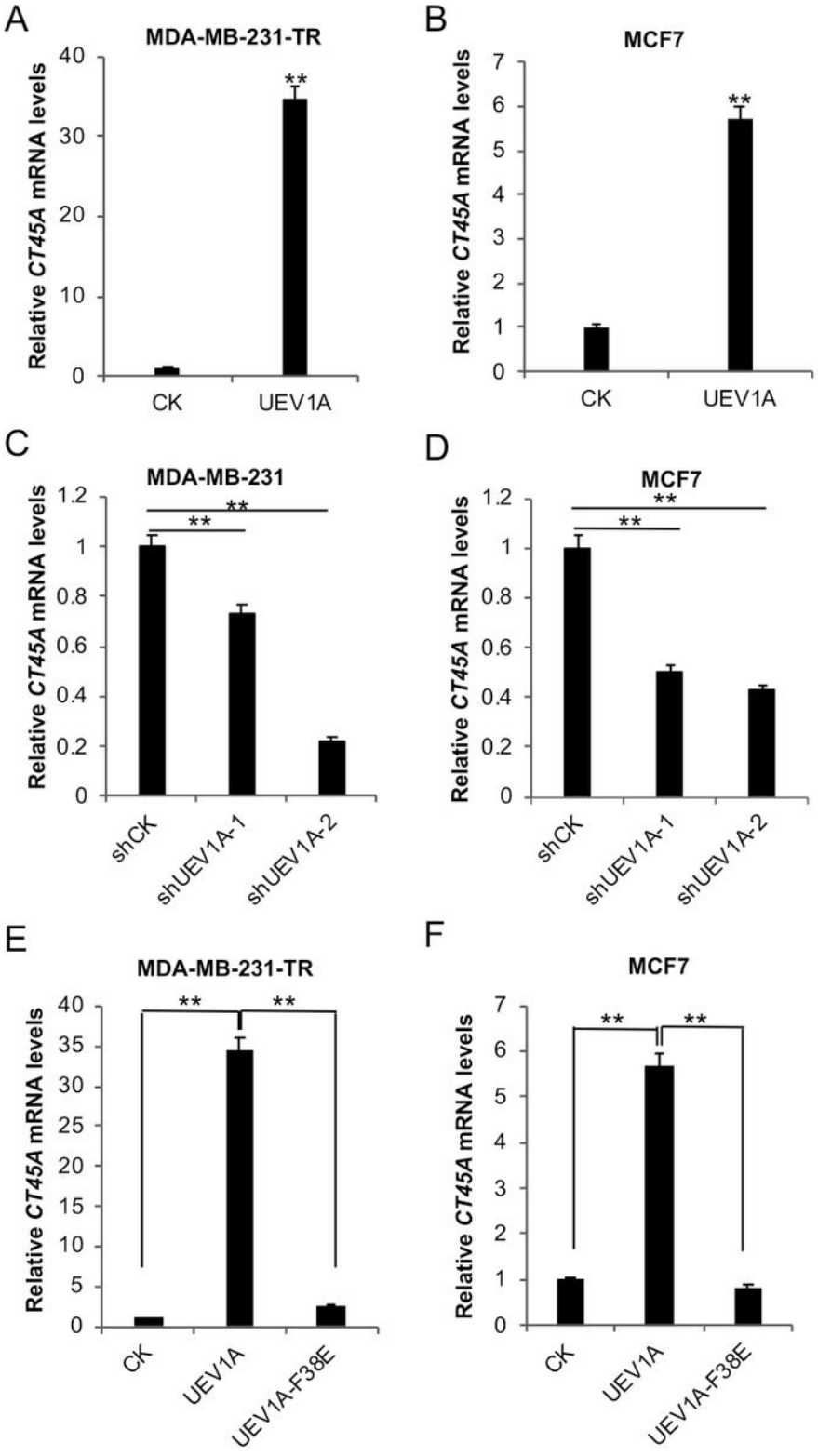


Figure 1

Uev1A upregulates CT45A expression in a Ubc13-dependent manner in breast cancer cells. (A, B) Relative CT45A transcript levels in UEV1A-overexpressed MDA-MB-231-TR (A) and MCF7 (B) cell lines were monitored by qRT-PCR. CK, control treatment. (C, D) MDA-MB-231 (C) and MCF7 (D) cells were transfected with shRNA lentiviral particles against UEV1A (shUEV1A) or non-specific target (shCK). shUEV1A-1 and shUEV1A-2 represent two independent stable shUEV1A cell lines. CT45A transcript levels

in shCK and shUEV1A cell lines were monitored by qRT-PCR. (E, F) Overexpressed UEV1A but not UEV1A-F38E upregulated CT45A expression in MDA-MB-231 (E) and MCF7 (F) cells, as determined by qRT-PCR. All experiments were performed in at least triplicate and the results are the average with standard deviation. **, P<0.01.



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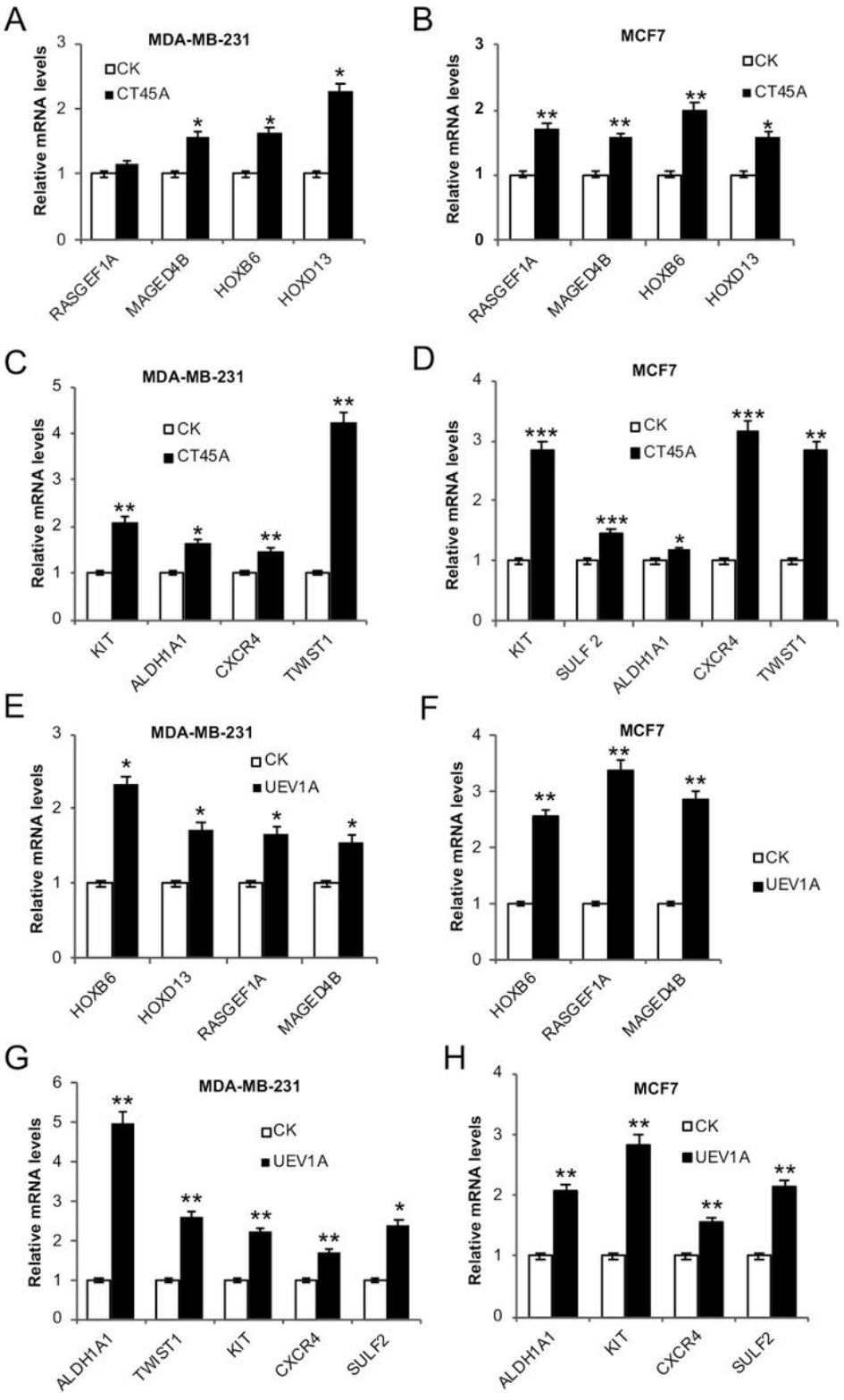
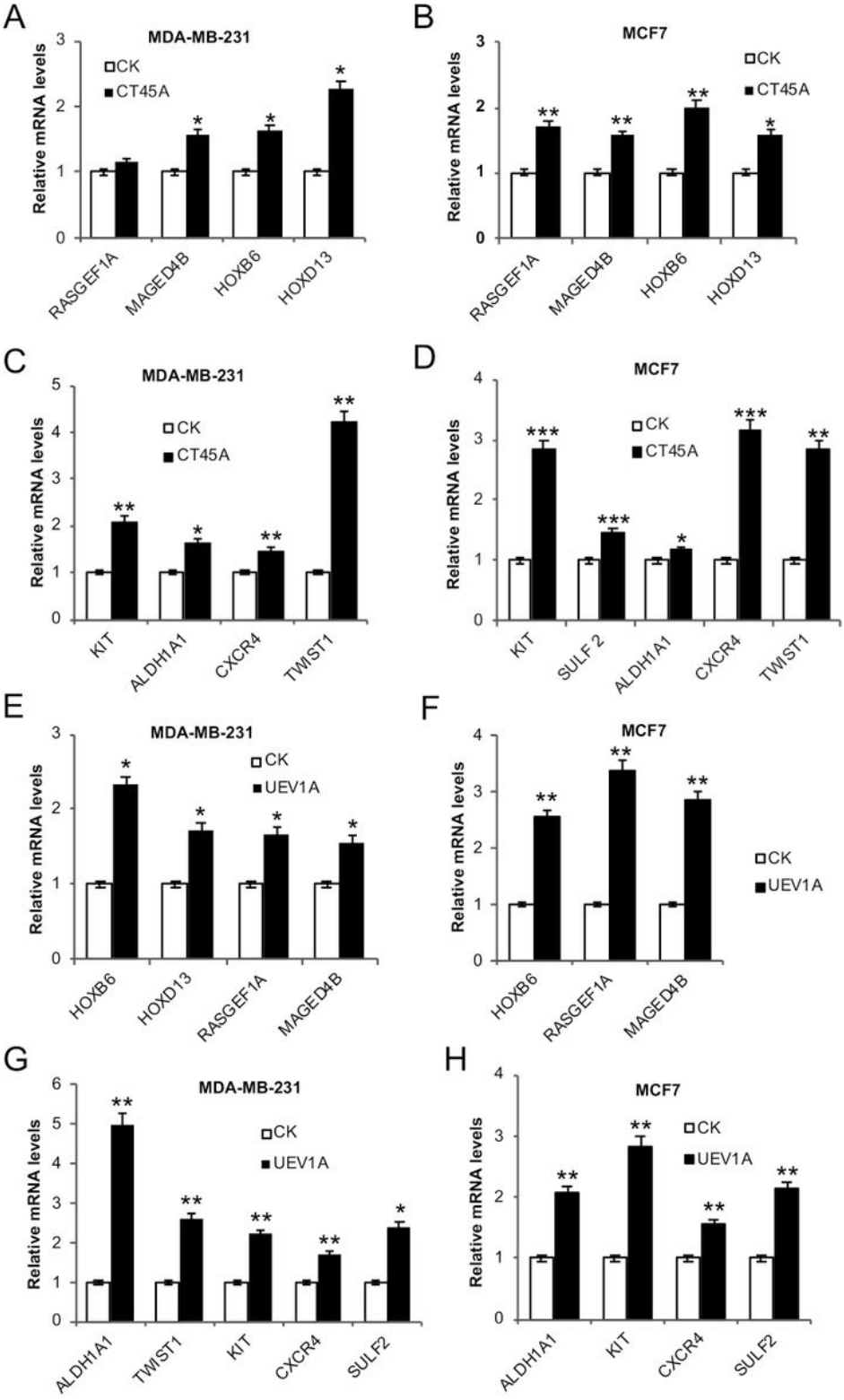


Figure 2

Uev1A positively regulates expression of CT45A downstream genes in breast cancer cells. (A, B) The transcript levels of tumorigenic genes, including RASGEF1A, MAGED4B, HOXB6 and HOXD13 in CT45A-overexpressed MDA-MB-231 (A) and MCF7 (B) cells were detected by qRT-PCR. (C, D) The transcript levels of EMT, stemness and metastatic genes, including KIT, ALDH1A1, CXCR4, TWIST1 and/or SULF2 in CT45A-overexpressed MDA-MB-231 (C) and MCF7 (D) cells were detected by qRT-PCR. (E, F) The

expression of CT45A downstream tumorigenic genes, including HOXB6, RASGEF1A, MAGED4B and/or HOXD13 in UEV1A transiently overexpressed MDA-MB-231 (E) and MCF7 (F) cells was monitored by qRT-PCR. (G, H) The expression of CT45A downsream EMT, stemness and metastasic genes, including ALDH1A1, KIT, CXCR4, SULF2 and/or TWIST1 was monitored in UEV1A transiently overexpressed MDA-MB-231 (G) and MCF7 (H) cells by qRT-PCR. All experiments were performed in at least triplicate and the results are the average with standard deviation. *, P<0.05; **, P<0.01; and ***, P<0.001.



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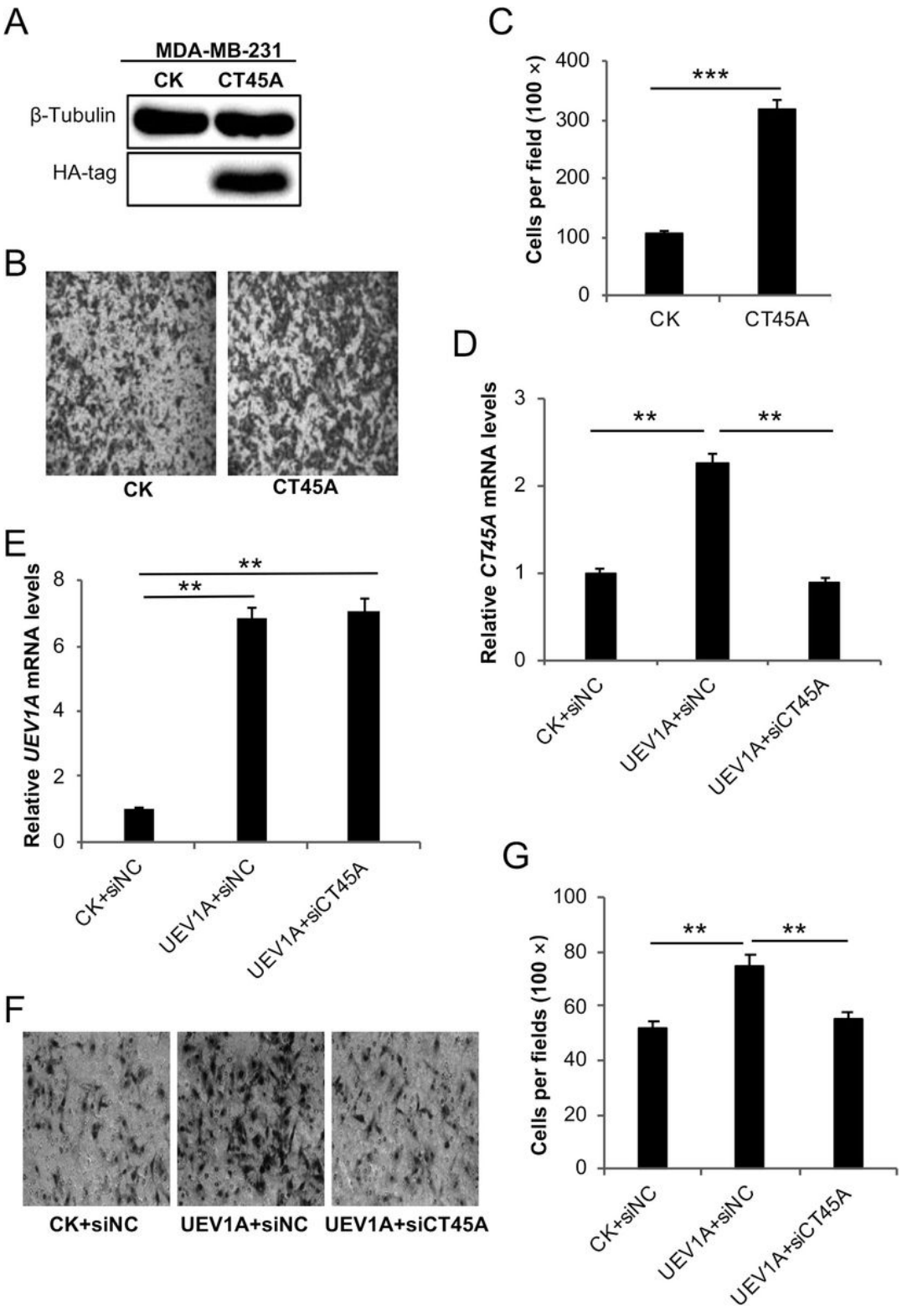
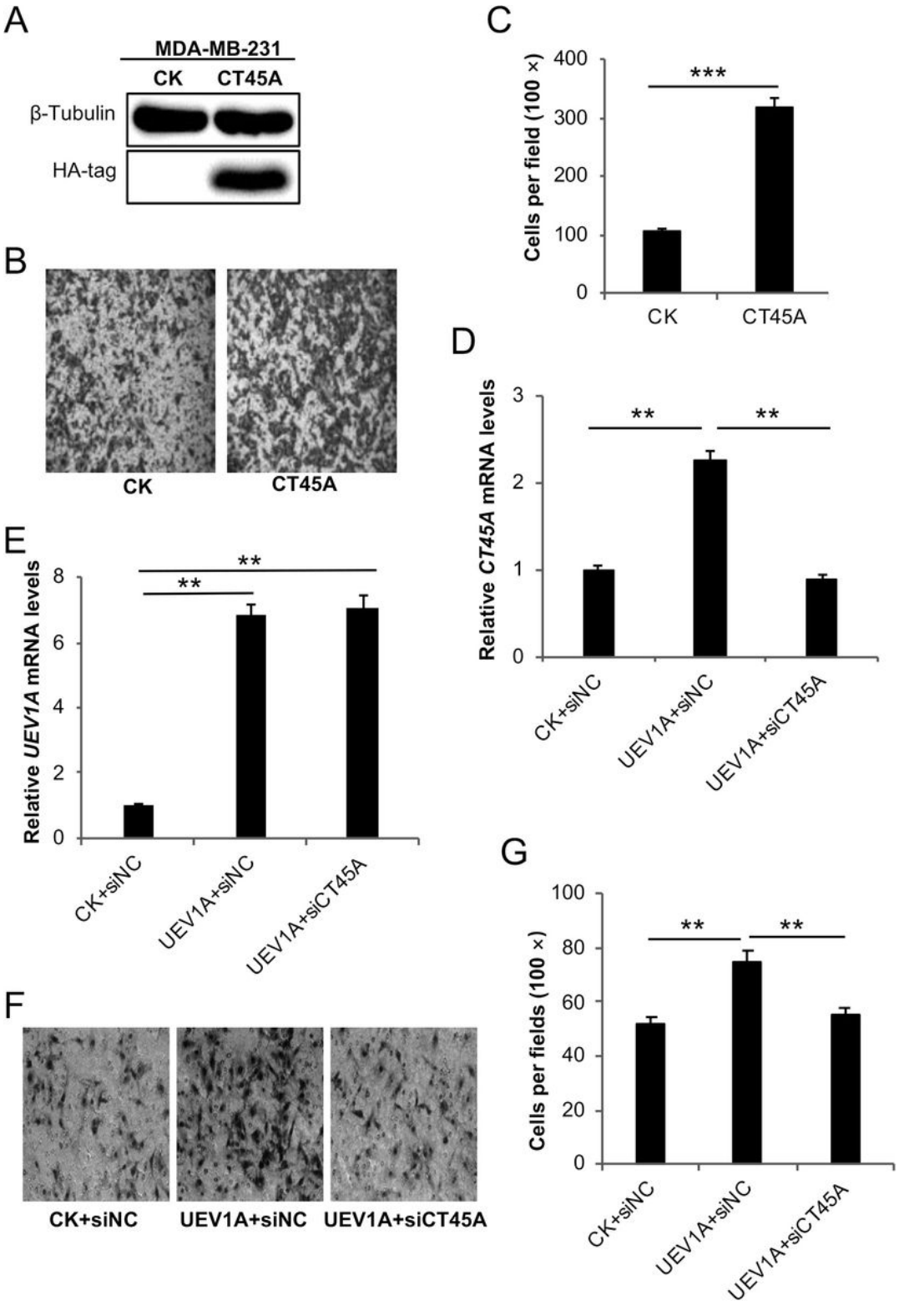


Figure 3

Effcts of manipulation of UEV1A and CT45A expression on MDA-MB-231 cell migration. (A) The ectopic CT45A expression was monitored by western blot against an HA-tag antibody. (B) Representative images of cell migration assay without Matrigel-coated transwell. (C) Statistical analysis of the cell migration assay data. Cells migrated to the lower surface of the filter were counted in five random fields under a light-microscope at 100 × magnification. (D, E) The relative expression of CT45A (D) and UEV1A (E) in

CT45A-depleted UEV1A transiently-overexpressed cells was monitored by qRT-PCR. (F) Representative images of cell migration ability without Matrigel-coated transwell. MDA-MB-231 cells transiently expressing UEV1A were depleted with CT45A and subjected to the transwell assay. siNC, control siRNA. (G) Statistical analysis of the cell migration assay data. Cells that migrated to the lower surface of the filter were counted in five random fields under a light-microscope at 100 × magnification. All experiments were performed in at least triplicate and the results are the average with standard deviation. **, P<0.01; and ***, P<0.001.



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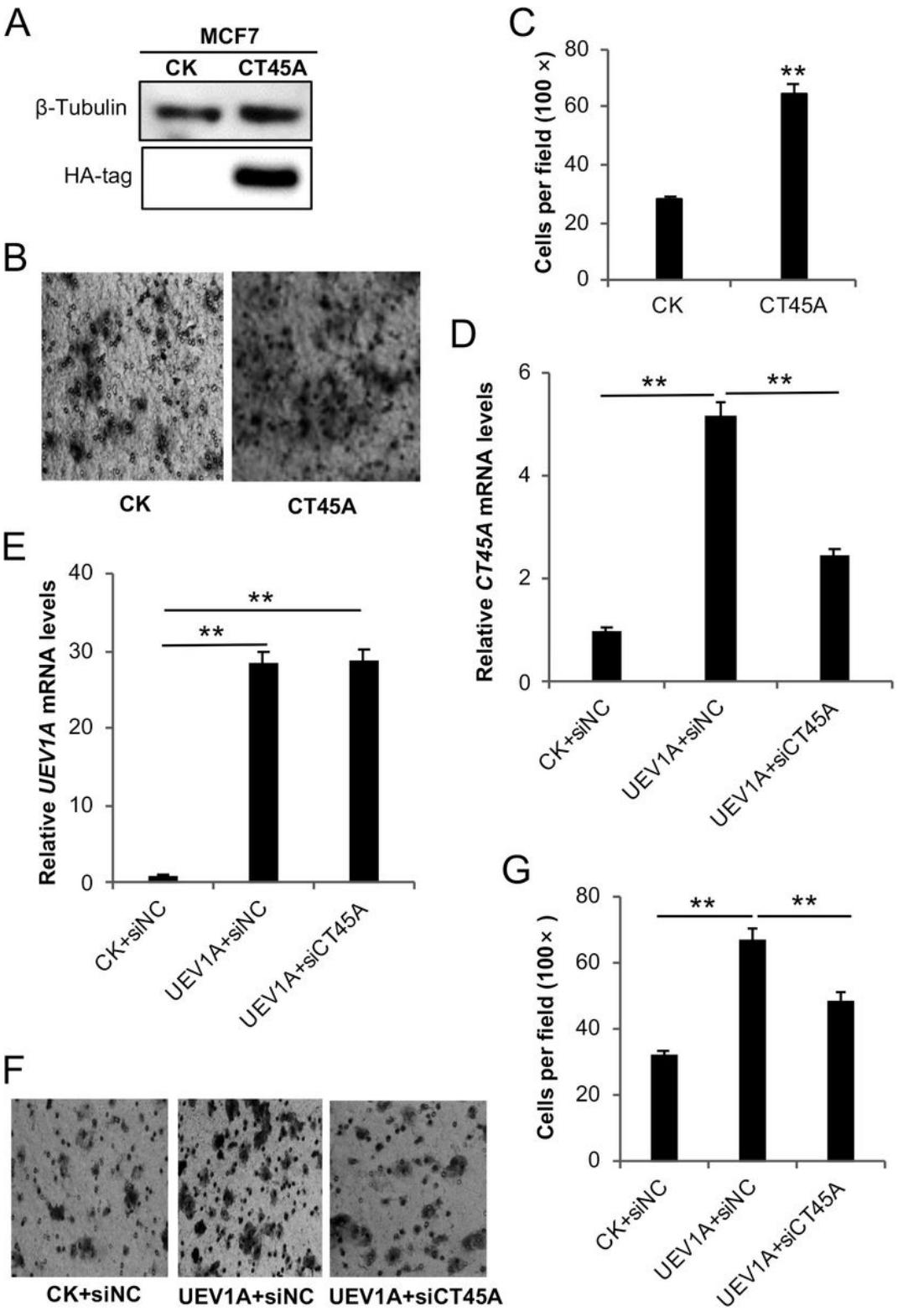

Figure 4

Effcts of manipulation of UEV1A and CT45A expression on MCF7 cell migration. (A) The ectopic CT45A expression was monitored by western blot against an HA-tag antibody. (B) Representative images of cell migration assay without Matrigel-coated transwell. (C) Statistical analysis of the cell migration assay data. (D, E) The relative expression of CT45A (D) and UEV1A (E) in CT45A-depleted UEV1A transiently-overexpressed cells was monitored by qRT-PCR. (F) Representative images of cell migration ability

without Matrigel-coated transwell. MCF7 cells transiently expressing UEV1A were depleted with CT45A and subjected to the transwell assay. siNC, control siRNA. (G) Statistical analysis of the cell migration assay data. Cells that migrated to the lower surface of the filter were counted in five random fields under a light-microscope at 100 × magnification. All experiments were performed in at least triplicate and the results are the average with standard deviation. **, P<0.01.

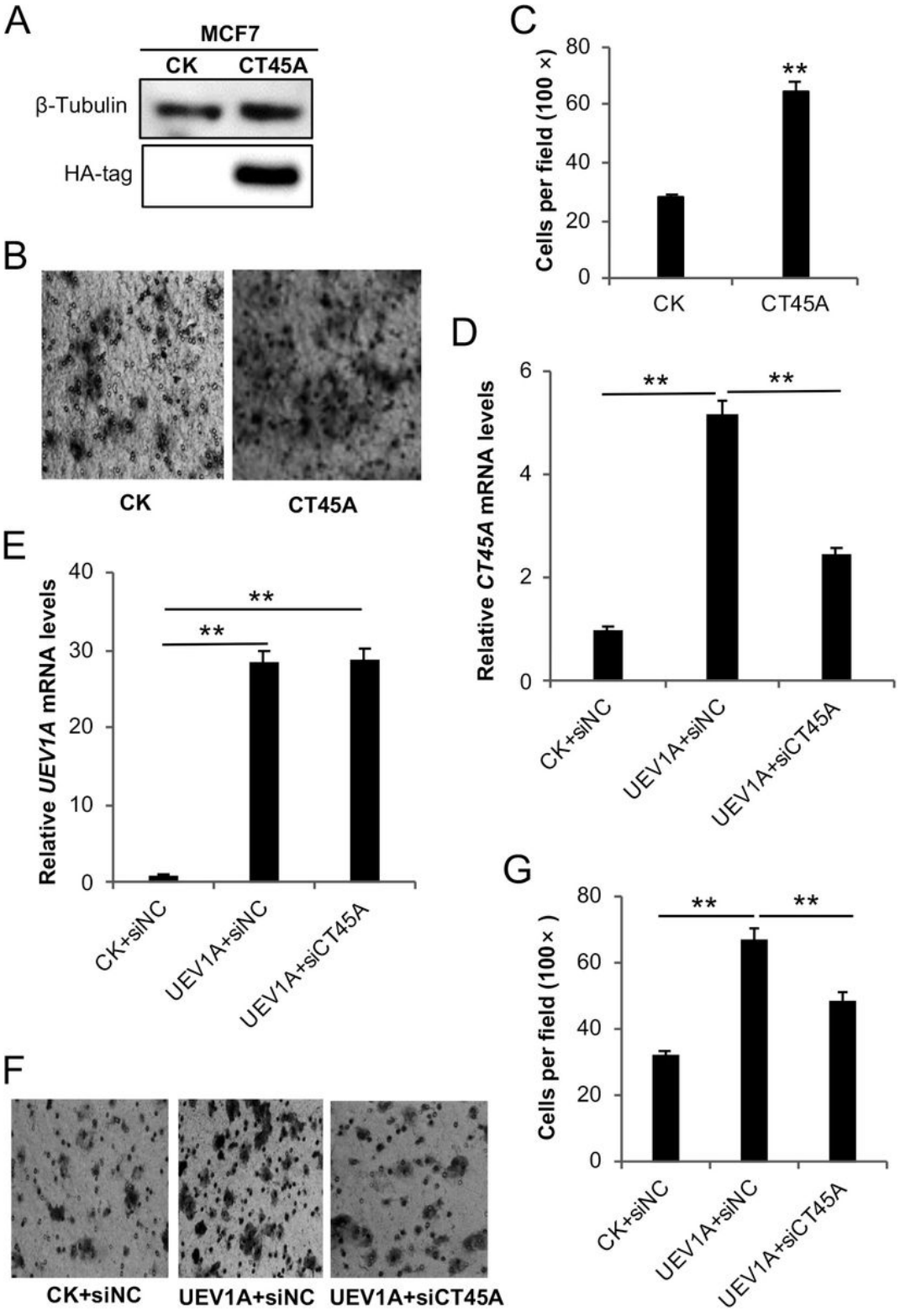

Figure 4

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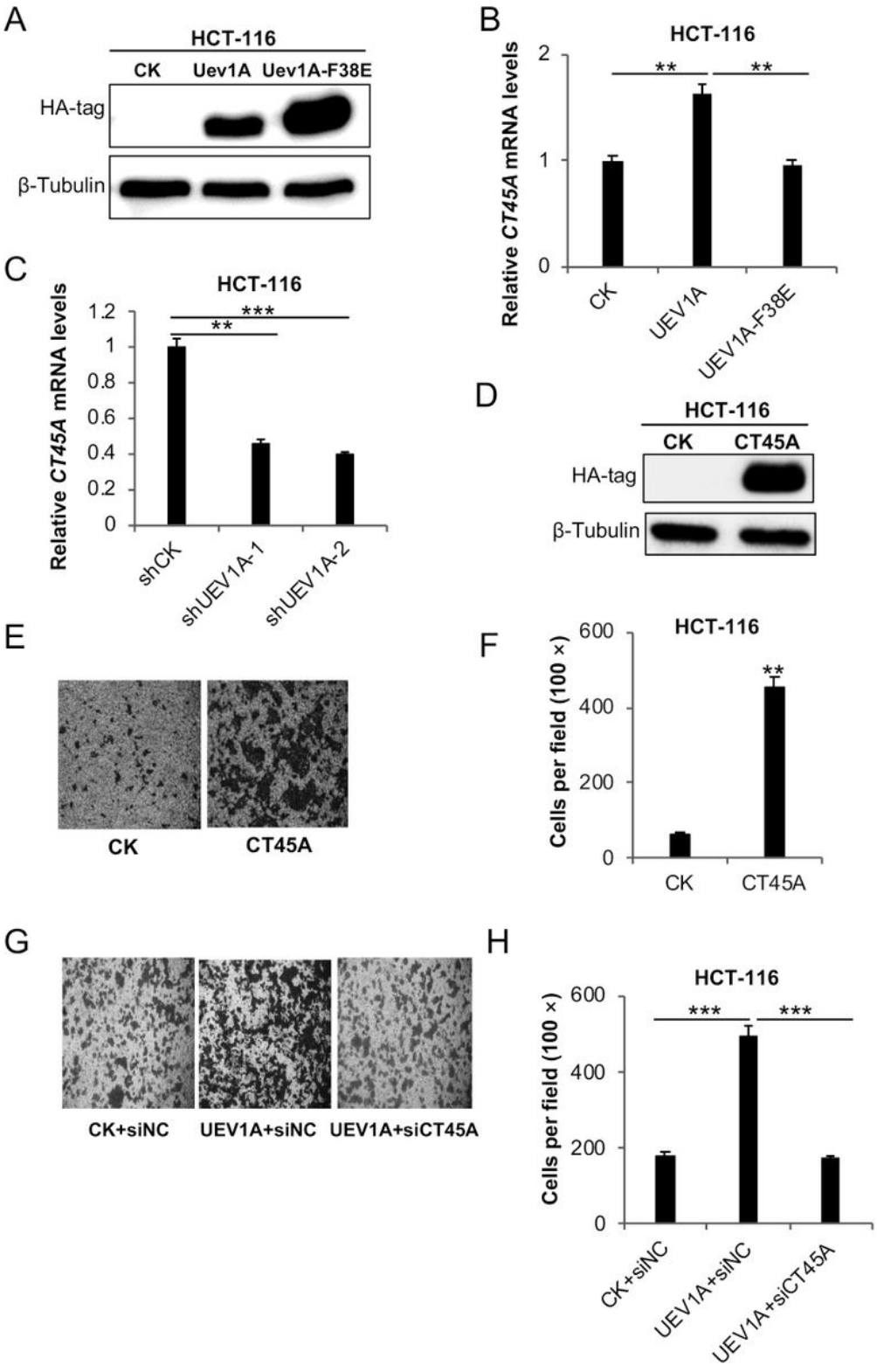

Figure 5

CT45A promotes metastasis in HCT116 colorectal cancer cells. (A) Cellular HA-tagged Uev1A and Uev1A-F38E were detected by western blot against HA-tag antibody. (B) Overexpressed UEV1A but not UEV1A-F38E upregulated CT45A expression in HCT116 colorectal cells. (C) HCT116 cells were transfected with shRNA lentiviral particles against UEV1A (shUEV1A) or non-specific target (shCK). CT45A transcript levels in shCK and shUEV1A cell lines were monitored by qRT-PCR. (D) Cellular HA-tagged CT45A was detected

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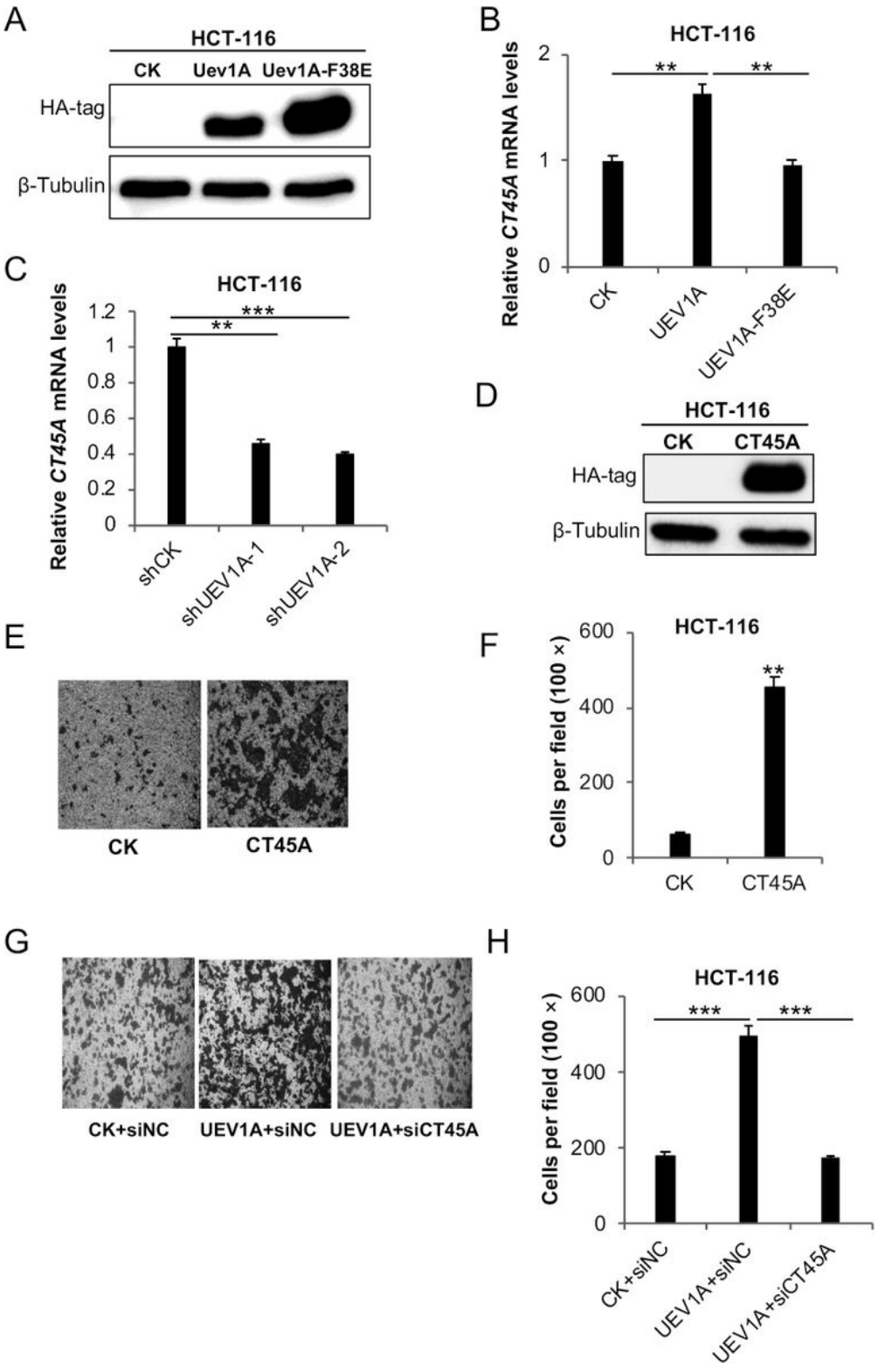

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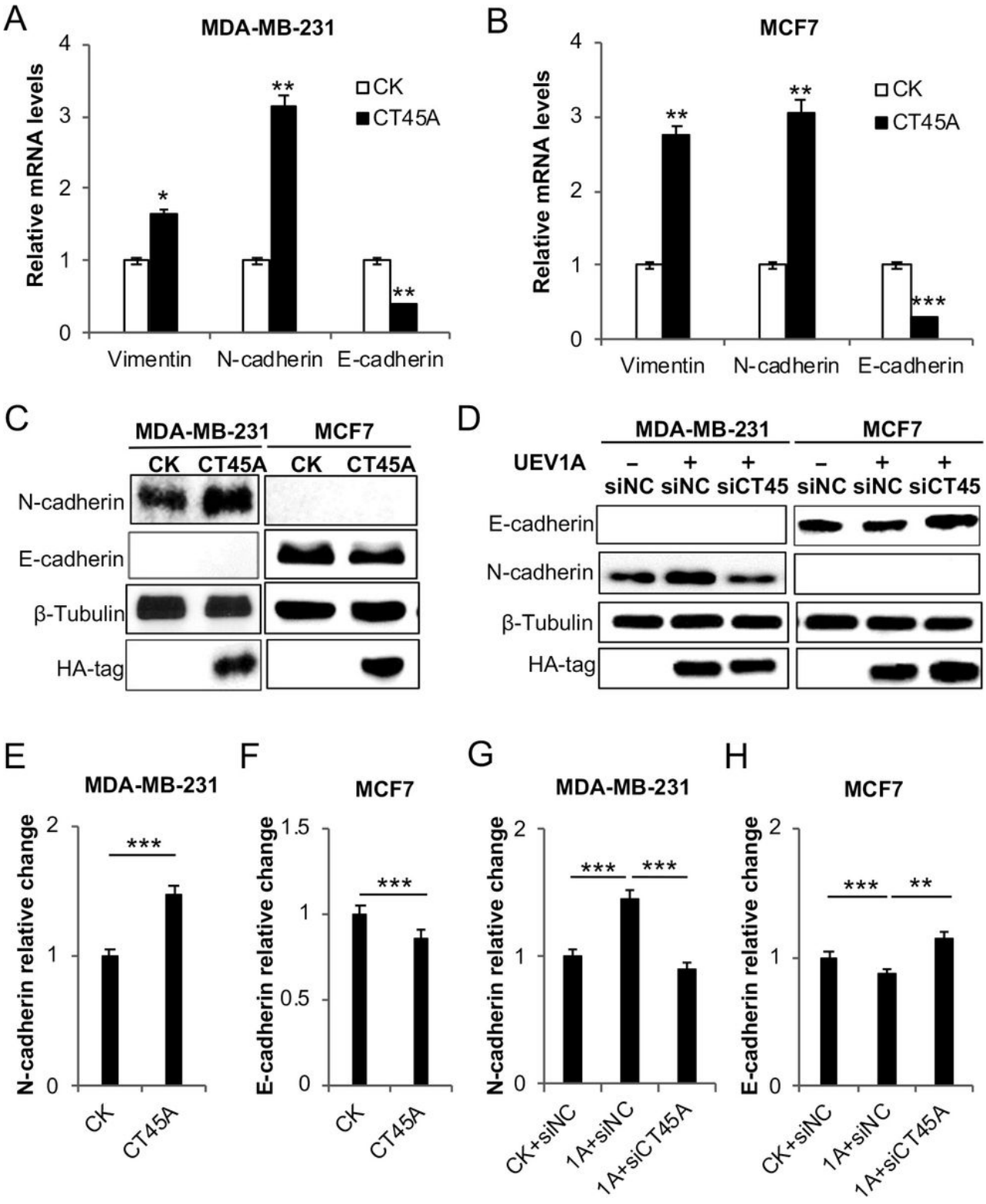

Figure 6

Effects of CT45A and UEV1A expression on EMT in breast cancer cells. (A, B) The relative transcript levels of EMT markers, including epithelial markers E-cadherin and mesenchymal markers N-cadherin, vimentin in CT45A-overexpressed MDA-MB-231 (A) and MCF7 (B) cells were determined by qRT-PCR. (C) The expression of N-cadherin and E-cadherin in CT45A-overexpressed MDA-MB-231 (left panel) and MCF7 (right panel) cells was detected by western blot against anti-N-cadherin and anti-E-cadherin antibodies.

(D) The expression of N-cadherin and E-cadherin in MDA-MB-231 (left panel) and MCF7 (right panel) cells transiently overexpressing UEV1A and depleted with CT45A was detected by western blot using anti-N-cadherin and anti-E-cadherin antibodies. (E-H) Statistical analyses of relative cellular N-cadherin and E-cadherin levels in MDA-MB-231 (E,G) and MCF7 (F,H) cells as indicated in the graphs. All experiments were performed in at least triplicate and the results are the average with standard deviation. **, P<0.01; and ***, P<0.001.

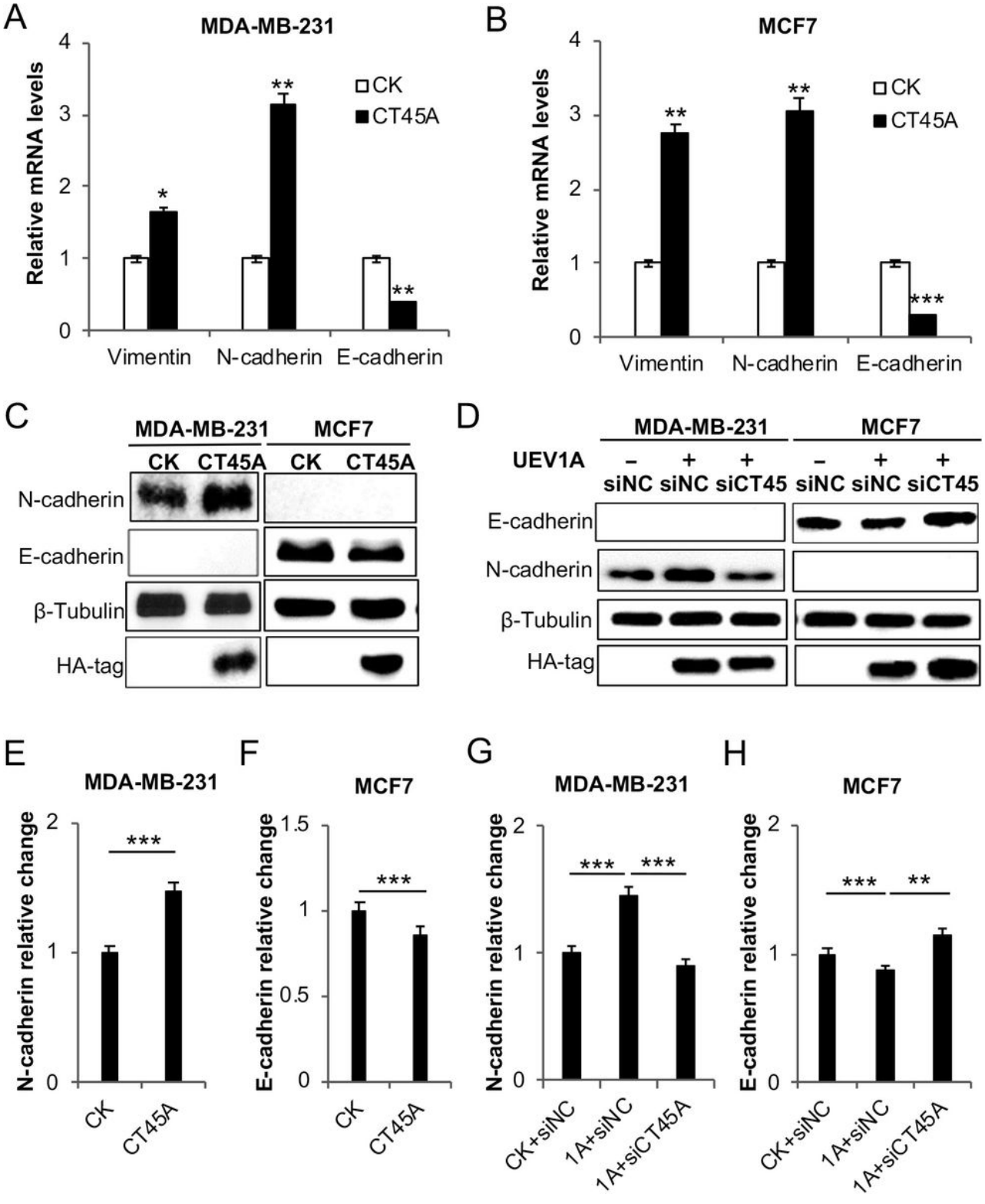

Figure 6

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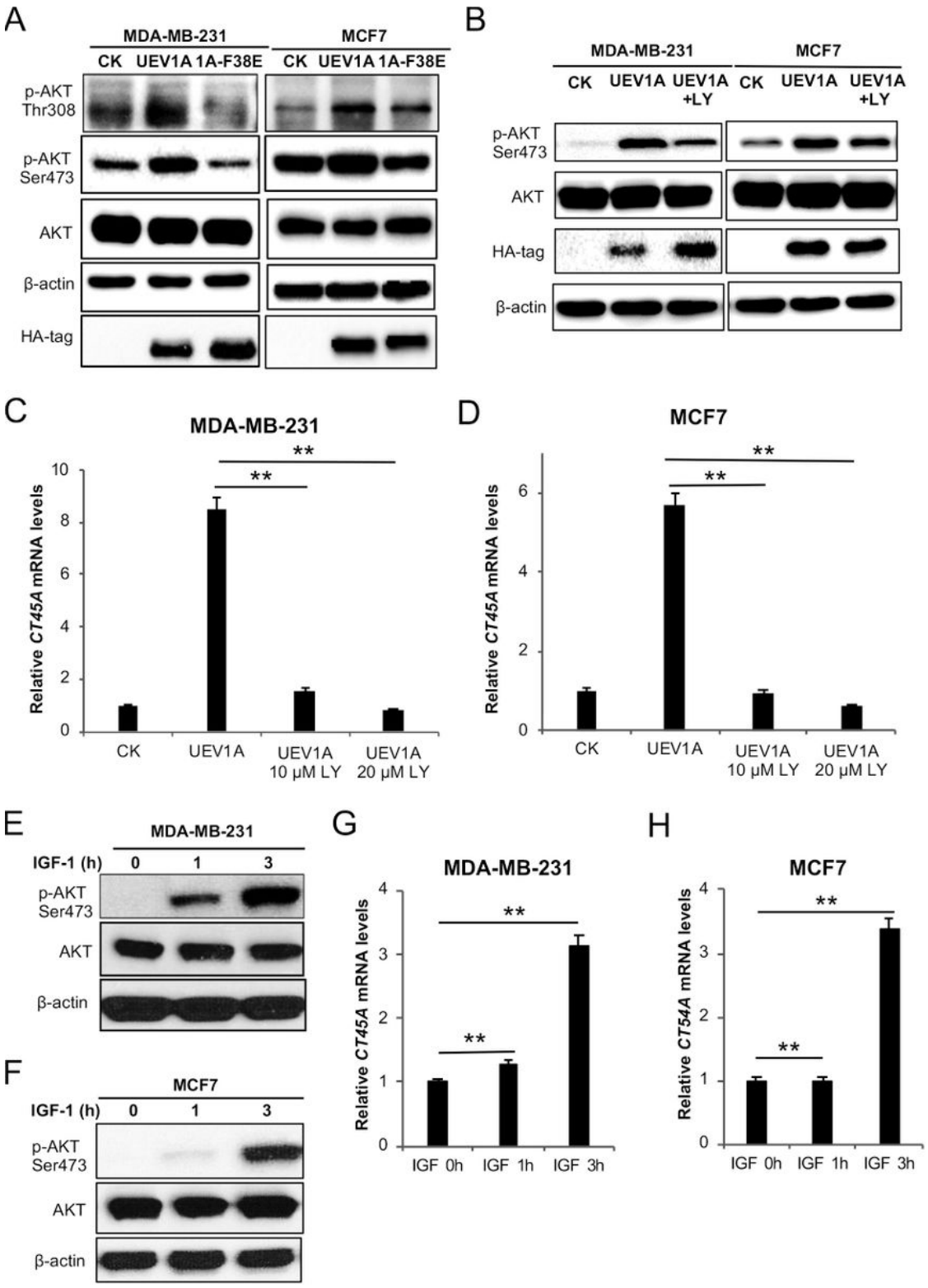
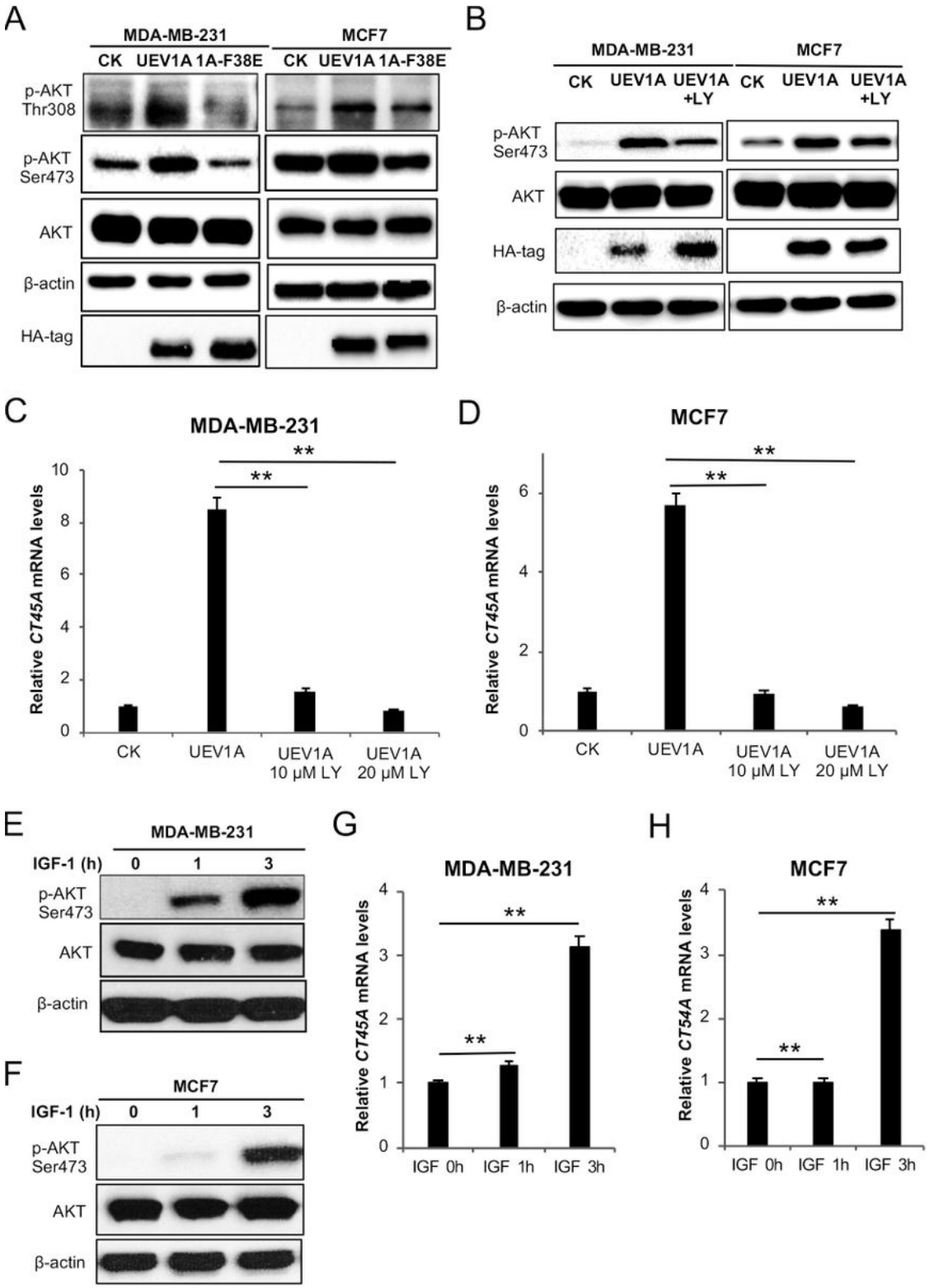


Figure 7

Uev1A regulates CT45A expression through the AKT signaling pathway. (A) The cellular AKT protein and its phosphorylation (p-AKT-Thr308, p-AKT-Ser473) levels in pcDNA4.0/TO/HA (+) vector (CK), UEV1A, UEV1A-F38E transiently-transfected MDA-MB-231 (left panel) and MCF7 (right panel) cells were monitored by western blot using anti-AKT, anti-p-AKT-Thr308 and anti-p-AKT-Ser473 antibodies. (B) The UEV1A transiently-transfected MDA-MB-231 (left panel) and MCF7 (right panel) cells were treated with 10

μM PI3K/AKT pathway inhibitor LY294002. After 24 h, the AKT and p-AKT-Ser473 levels were examined by western blot using anti-AKT, anti-p-Ser473 antibodies in cells transfected with vector, UEV1A with or without LY294002 treatment as indicated. Ectopic UEV1A expression was detected by an anti-HA-tag antibody. (C, D) Relative CT45A expression levels in MDA-MB-231 (C) and MCF7 (D) cells transfected with vector, UEV1A with or without LY294002 treatment as indicated, followed by qRT-PCR. (E, F) MDA- MB-231 (E) and MCF7 (F) cells were treated with IGF-1 over time as indicated and the cellular AKT and p-AKT-Ser473 proteins were monitored by western blot using anti-AKT and anti-p-AKT-Ser473 antibodies. (G, H) The transcript levels of CT45A in MDA-MB-231 (G) and MCF7 (H) cells treated with IGF-1 over time were monitored by qRT-PCR. All experiments were performed in at least triplicate and the results are the average with standard deviation. **, $P<0.01$.



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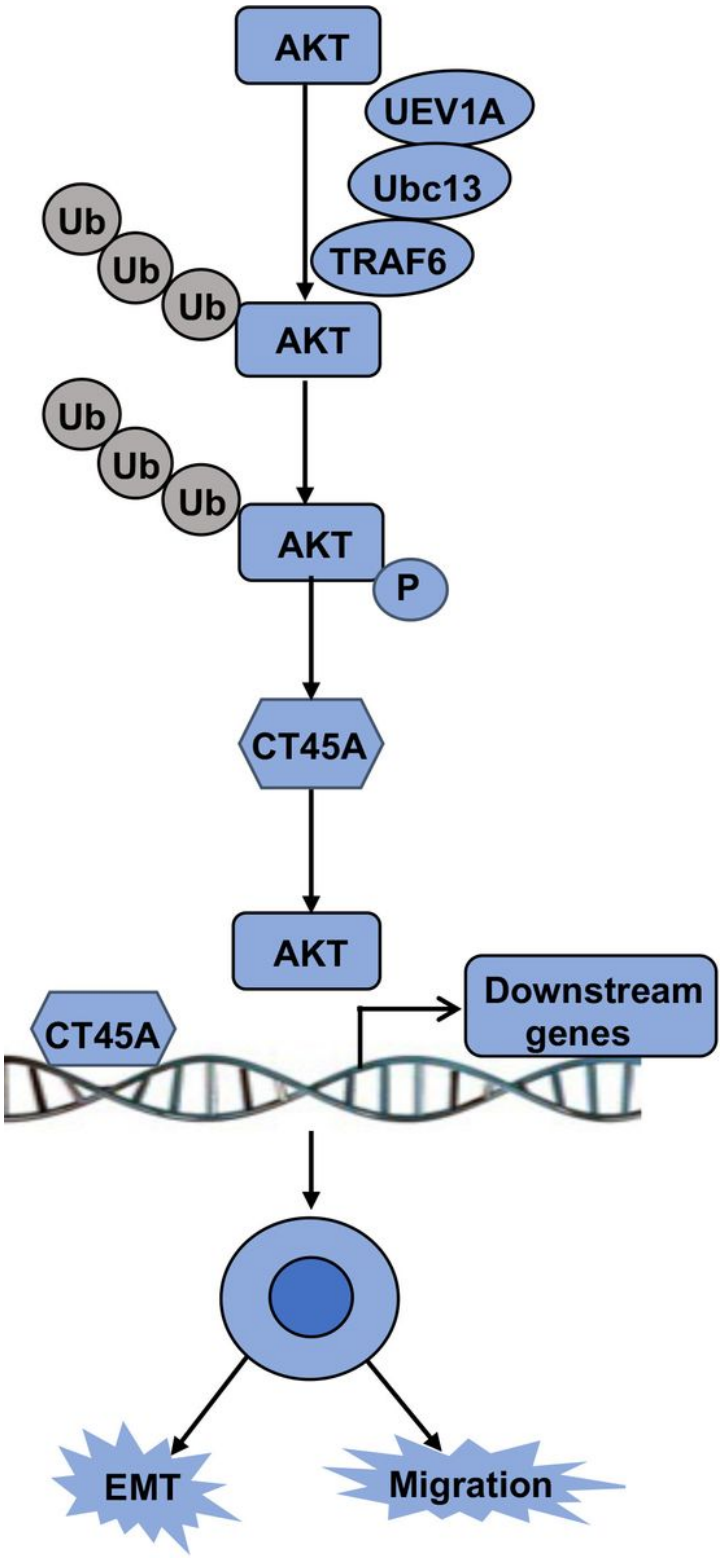
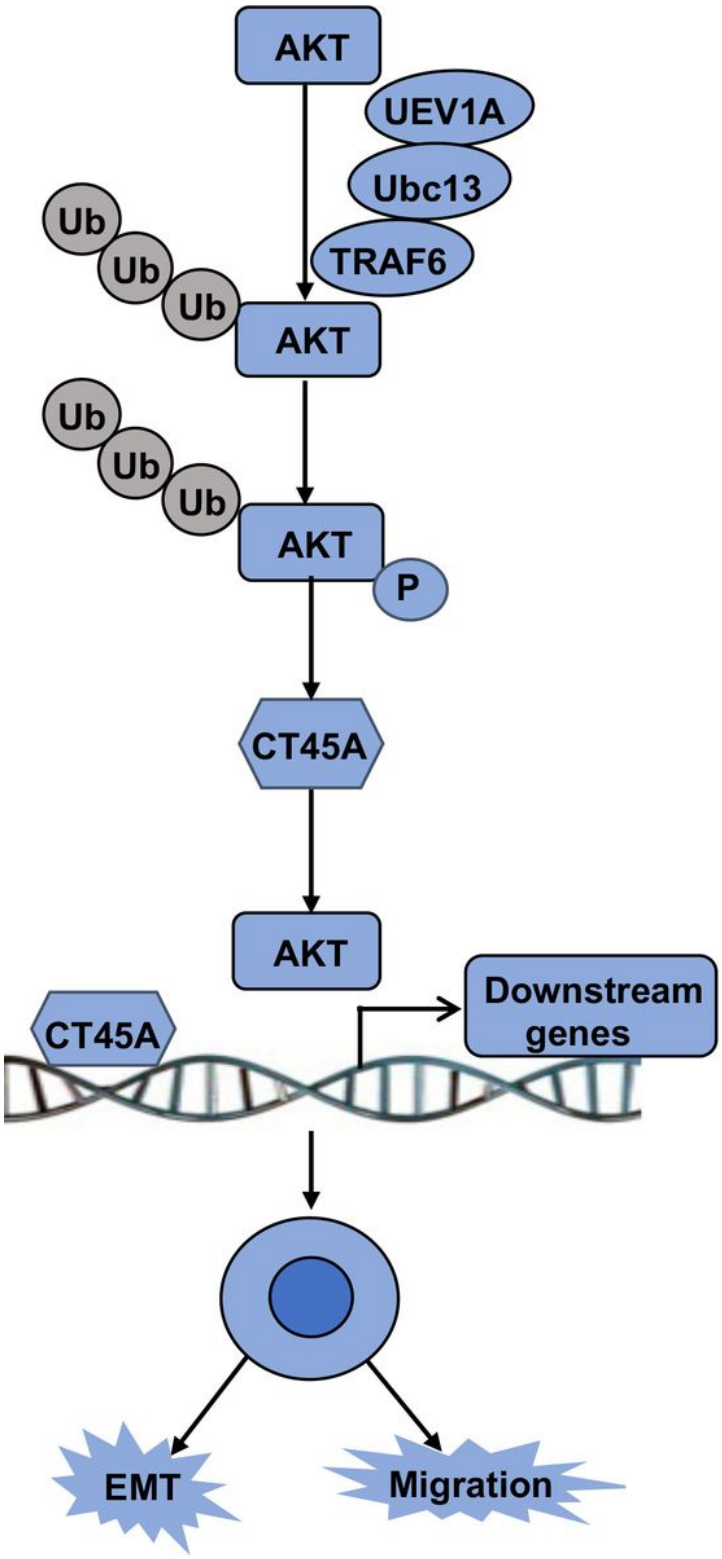


Figure 8

A proposed model in which Uev1A promotes cell migration and EMT through the Uev1A-AKT-CT45A axis in breast cancer. The Uev1A-Ubc13 complex (E2) together with TRAF6 (E3) ubiquitinate AKT, which is essential for AKT membrane localization, phosphorylation and activation. The phosphorylated and activated AKT positively regulates CT45A expression that in turn regulates the expression of the CT45A downstream genes, leading to increased cell migration and EMT in breast cancer.



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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- UEV1ACT45ASupplementary.docx
- UEV1ACT45ASupplementary.docx