Published in final edited form as:

Mol Carcinog. 2017 February ; 56(2): 722–734. doi:10.1002/mc.22528.

Zinc finger E-box binding homeobox-1 (Zeb1) drives anterograde lysosome trafficking and tumor cell invasion via upregulation of Na⁺/H⁺ Exchanger-1 (NHE1)

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Abstract

Tumor cell invasion through the extracellular matrix is facilitated by the secretion of lysosome-associated proteases. As a common mechanism for secretion, lysosomes must first traffic to the cell periphery (anterograde trafficking), consistent with invasive cells often containing lysosomes closer to the plasma membrane compared to non-invasive cells. Epithelial to mesenchymal transition (EMT) is a transcriptionally-driven program that promotes an invasive phenotype, and Zeb1 is one transcription factor that activates the mesenchymal gene expression program. The role of lysosome trafficking in EMT-driven invasion has not been previously investigated. We found that cells with increased levels of Zeb1 displayed lysosomes located closer to the cell periphery and demonstrated increased protease secretion and invasion in 3-dimensional (3D) cultures compared to their epithelial counterparts. Additionally, preventing anterograde lysosome trafficking via pharmacological inhibition of Na⁺/H⁺ exchanger 1 (NHE1) or shRNA depletion of ADP-Ribosylation like protein 8b (Arl8b) reversed the invasive phenotype of mesenchymal cells, thus supporting a role for lysosome positioning in EMT-mediated tumor cell invasion. Immunoblot revealed that expression of Na⁺/H⁺ exchanger 1 correlated with Zeb1 expression. Furthermore, we found that the transcription factor Zeb1 binds to the Na⁺/H⁺ exchanger 1 promoter, suggesting that Zeb1 directly controls Na⁺/H⁺ transcription. Collectively, these results provide insight into a novel mechanism regulating Na⁺/H⁺ exchanger 1 expression and support a role for anterograde lysosome trafficking in Zeb1-driven cancer progression.

Keywords

lysosome movement; NHE1; epithelial to mesenchymal transition; Zeb1; invasion

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The authors declare no conflict of interest.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer, and is the second leading cause of cancer-associated deaths in men over the age of 60 [1]. Organ confined disease is often readily treated; however, as with many solid tumors, the transition from organ confined disease to invasive carcinoma leading to metastatic PCa is often fatal. Therefore, elucidating the mechanisms that contribute to PCa invasion may identify potential therapeutic targets to slow cancer progression.

Tumor cell invasion consists of breaching the underlying extracellular matrix (ECM) and basement membrane, allowing tumor cells to gain access to the blood or lymph systems. The ECM contains collagen I, collagen IV, fibronectin, and other structural proteins and acts as a physical barrier that tumor cells must overcome in order to metastasize [2]. While tumor cells can undergo amoeboid, or protease independent invasion, protease-dependent ECM degradation and invasion is common [3]. The secretion of proteases into the extracellular space is overactive in invasive cancers, and the development of protease inhibitors as anti-cancer agents has been a large focus of pharmaceutical research. However, many protease inhibitors have failed clinical trials due to lack of target specificity leading to toxicity.

Lysosomes are acidic organelles that function as the terminal endpoint of the endocytic pathway. These organelles are rich in proteases and hydrolases, which confer on lysosomes their degradative activity. However, lysosomes are diverse organelles and can function as secretory vesicles in addition to their role in endocytosis. Secreted proteases, including lysosomal cathepsins and matrix metalloproteinases (MMPs) facilitate invasion by degrading the surrounding ECM, thus allowing tumor cells to escape the primary organ site [4,5]. Protease activity is often associated with the leading edge of invasive tumors, and published studies have shown that lysosomal cathepsin B is upregulated in invasive PCa compared to the normal surrounding tissue [6,7]. Additionally, cathepsin B can be found in the serum of PCa patients, suggesting that there is aberrant secretion of lysosomal proteases associated with this disease [8]. Collectively, these studies suggest that the release of lysosomal proteases into the extracellular space is important for PCa invasion.

Lysosome-mediated secretion is a complex process involving targeted organelle trafficking, membrane fusion, and release of lysosomal contents into the extracellular space. The trafficking and secretion of lysosomal contents and lysosome-like organelles is firmly established in many cellular processes, including T-cell degranulation and plasma membrane repair [9,10]. Lysosome secretion is also involved in tumor cell invasion and motility, and studies have shown that lysosomes traffic to the leading edge of motile cells and can be found in invadopodia, or invasive actin rich protrusions [11]. Lysosomes traffic along microtubules and actin filaments toward the cell periphery (anterograde) or toward the microtubule-organizing center (retrograde), and this movement is regulated by the activity of kinesin and dynein motors, respectively [12,13]. Kinesins and dyneins are recruited to lysosome membranes by the action of the lysosome-localized GTPases ADP-Ribosylation like protein 8b (Arl8b) and Rab7, respectively [13,14]. Previous works have found that the position of lysosomes in proximity to the plasma membrane influences tumor cell

invasiveness by facilitating protease secretion. Conversely, preventing anterograde lysosome trafficking results in a less invasive phenotype [15–18].

Lysosome positioning within cells is regulated, in part, by the activity of Na⁺/H⁺ exchangers (NHE). NHEs are trans-membrane ion pumps that use passive transfer to excrete hydrogen ions from cells in exchange for an extracellular sodium ion, thus acidifying the extracellular environment. Of the 9 NHE isoforms, NHE1 is the best characterized and is associated with invasive potential [19,20]. Prior studies suggest that the activity and expression of NHE family proteins influence lysosome positioning within the cell and inhibition of NHE activity results in juxtanuclear lysosome aggregation (JLA) [17,21]. The mechanism(s) by which NHE1 contributes to lysosome positioning are incompletely understood.

Previous studies identified common features of the extracellular microenvironment, including Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF) (Dykes and Cardelli, manuscript in preparation), as stimulators of anterograde lysosome trafficking [18]. These growth factors bind to their respective receptors resulting in cell proliferation and loss of cell-to-cell adhesions characteristic of epithelial to mesenchymal transition (EMT) [22]. EMT is a gene expression program that is upregulated during embryogenesis, wound healing, and in many invasive cancers. EMT is controlled by several transcription factors which are often overexpressed in invasive cancers and act as ‘master regulators’ of gene expression to confer a more motile and invasive phenotype [23]. Zinc finger E-box binding homeobox-1 (Zeb1) is one such ‘master regulator’. While Zeb1 can act as a transcriptional activator, it is most well characterized as a transcriptional repressor of E-cadherin [24–28]. The role of lysosome trafficking in Zeb1-mediated tumor invasion has not been previously investigated.

In this study, we describe several cancer cell lines that have been clonally selected to have an epithelial or mesenchymal phenotype. It was determined that mesenchymal clones had lysosomes positioned closer to the cell periphery as compared to their epithelial counterparts. Additionally, anterograde lysosome trafficking was associated with increased motility, invasion, and protease secretion in both transwell and 3-dimensional (3D) culture. Preventing peripheral lysosome distribution did not reverse EMT, but did inhibit cell invasion and protease secretion. Furthermore, we found that EMT-mediated anterograde lysosome trafficking is dependent on the activity of NHE1, and provide the first evidence suggesting that NHE1 expression is regulated by the EMT transcription factor Zeb1.

Materials and Methods

Cell Lines

DU145 epithelial and mesenchymal prostate cancer cells were generated from a parental DU145 cell line originally purchased from ATCC (Manassas, VA). The epithelial subclone (DU-E) was generated from DU145 prostate cancer cell lines, as a single cell-derived acini in 3-dimensional culture system. Mesenchymal DU145 (EM1) were generated from DU-E as a single cell-derived branching unit in 3D culture [29]. All DU145 cells were grown in RPMI (Mediatech) supplemented with 10% Fetal Bovine Serum (FBS). MCF10A breast

cancer cells were originally obtained from ATCC and were clonally selected from a pool parental population to have either an epithelial or mesenchymal phenotype in 2-dimensional culture. MCF10A cells were grown in DMEM/F12 media supplemented with 5% FBS, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 20 ng/mL EGF, and 0.1 µg/mL cholera toxin. ARCaP epithelial and mesenchymal prostate cancer cells were purchased from Novicure Biotechnology (Birmingham, AL) and cultured in MCaP media (Novicure) supplemented with 10% FBS [30]. All cells were grown at 37°C in the presence of 5% CO₂ and sub-cultured upon reaching 75% confluence. All shRNA expressing cells were maintained under selection with 3.6 µg/mL puromycin (Fisher Scientific, Waltham, MA).

Immunofluorescence

2-dimensional (2D) immunofluorescence was performed as previously described [31]. Briefly, cells were seeded at 50% confluence on glass coverslips. Cells were fixed with ice cold 4% paraformaldehyde (PFA) diluted in phosphate buffered saline (PBS) pH 7.2 for 20 minutes. Cells were then washed and incubated with primary antibody diluted 1:200 in 0.25% bovine serum albumin (BSA) and 0.1% saponin in PBS (BSP) for 1 hour. Following incubation, cells were washed in PBS and incubated for one hour with fluorescently conjugated secondary antibody diluted 1:200 in BSP. The actin cytoskeleton was detected using FITC-labeled phalloidin diluted 1:200 in BSP and incubated with cells for 20 minutes. Cells were then washed 3X and mounted with DAPI containing SlowFade Gold reagent (Molecular Probes, Eugene, Oregon). 2D microscopy was performed using an Olympus UPlanFl 40X/0.75 objective and an Olympus BX50 microscope, Roper Scientific Sensys Camera, and MetaMorph software. Images were pseudo-colored in ImageJ; scale bar represents 50 µm. For 3D immunofluorescence, cells grown on Matrigel-coated coverslips were incubated with 37°C 4% PFA pH 7.2 for 30 minutes, stained with phalloidin (1:200 in BSP) for 30 minutes at 37°C, washed 3X with PBS, and then mounted on slides with DAPI containing SlowFade Gold reagent. 3D images were taken using a HCX Plan Apo 63X/1.4-0.6 oil objective on the Leica TCS SP5 microscope and Leica LAS AF software.

Reagents and Antibodies

The Arl8a/Arl8b antibody was a generous gift from Dr. Michael Brenner (Harvard) and was used at a 1:500 dilution for immunoblot [32]. The Zeb1 antibody (Cell Signaling Technologies, Beverly, MA) was used at 1:500. The LAMP-1 (H4A3) antibody was used at 1:200 (Developmental Studies Hybridoma Bank at Iowa State University). Transforming growth factor beta (Sigma Aldrich, St. Louis, MO) was used at 5 ng/mL. Anti-tubulin was acquired from NeoMarkers (Fremont, CA) and used at 1:20,000. Anti-actin was obtained from Sigma-Aldrich and used at 1:5,000. E-cadherin and cathepsin B antibodies (Santa Cruz Biotechnology, Dallas, TX) were used at 1:1,000. The cathepsin D antibody was obtained from Calbiochem (Billerica, MA) and used at 1:1,000. NHE1, vimentin, cathepsin L, and β-catenin antibodies were purchased from BD Biosciences (San Jose, CA) and were used at 1:1,000. Secondary antibodies include Dylight 594 donkey anti-mouse (1:200) (Jackson ImmunoResearch, West Grove, PA) and HRP-conjugated anti-mouse and anti-rabbit (1:5,000) (GE Healthcare, Pittsburgh, PA). Phalloidin 488 and 633 (Invitrogen, Carlsbad, CA) were used at 1:200. Primary antibodies used for ChIP were purchased from Santa Cruz Biotechnology: Mouse IgG (CS-2025) and Zeb1 (H-102).

Lentivirus-delivered shRNA

Mission Lentivirus Transduction Particles were used to deliver shRNA targeting Non-Target (SHC202V), Zeb1 (a:TRCN0000369266; b:TRCN0000017565; c:TRCN0000364631), NHE1 (TRCN0000044651), and Arl8b (TRCN0000072857) according to the manufactures protocol (Sigma-Aldrich). Briefly, cells were incubated with 6 µg/mL polybrene and lentivirus for 48 hours prior to selection with puromycin.

3D Culture

3D culture methods were modified from previously described protocols [33,34]. Briefly, coverslips were coated with 100 µL Matrigel (Corning, Corning, NY) supplemented with 25 µg/mL DQ-collagen IV (Molecular Probes) and allowed to solidify at 37°C for 15 minutes. 1×10^5 cells were added to the Matrigel in complete media plus or minus drug treatments. Cells were maintained at 37°C and 5% CO₂ and grown for 4 days. Three independent experiments were performed and representative images are shown. After imaging, extracellular DQ-collagen IV fluorescence was assessed using Image J. Briefly, a mask was created encompassing the fluorescent signal in the actin channel. This masked area was subtracted from the corresponding fluorescent signal in the DQ-collagen IV channel using the Image Calculator tool. The remaining DQ-collagen IV fluorescent signal (extracellular) was detected as integrated density and displayed as arbitrary units.

Immunoblot

Whole cell lysates were collected directly in boiling Laemmli Buffer (0.125 M Tris-HCL, pH 6.8, 4% SDS, 0.13 mM bromophenol blue, 1M sucrose and 2% 2-mercaptoethanol) and boiled for 5 minutes. Lysates were run on polyacrylamide gel and transferred to PVDF. Membranes were blocked in 5% milk in TBST (20mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.5) and probed for the indicated proteins overnight at 4°C. Next, membranes were incubated with HRP-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA) for 1 hour and Pierce ECL2 Western Blotting Substrate (Life Technologies) was used for chemiluminescent detection on x-ray film (Phenix Research Products, Candler, NC). Representative blots are shown. Densitometry analysis of three independent immunoblots was performed using Image J software. Band intensity was normalized to actin or tubulin loading control and displayed as arbitrary units.

Reverse transcription PCR

Cells were homogenized in Trizol (Life Technologies) and total RNA was isolated according to the manufacture's protocol. The SuperScript First-Strand cDNA synthesis system (Life Technologies) was used to synthesize cDNA according to the manufacturer's protocol. The following primers were constructed and purchased from Integrated DNA Technologies (Coralville, IA): SLUG FP, 5'-GCA ACA GAG CAT TTG CAG ACA GGT-3' RP, 5'-AAC AAT GGC AAC CAG ACA ACC GAC-3'; SNAIL FP, 5'-GCT CGA AAG GCC TTC AAC TGC AAA-3' RP, 5'-AGG CAG AGG ACA CAG AAC CAG AAA-3'; TWIST FP, 5'-AGC TGA GCA AGA TTC AGA CCC TCA-3' RP, 5'-AGA ATG CAG AGG TGT GAG GAT GGT-3'; E-cadherin FP, 5'-TTC CCT CGA CAC CCG ATT CAA AGT-3' RP, 5'-TCC TTG GCC AGT GAT GCT GTA GAA-3'; GAPDH FP, 5'-AGC GAG ATC CCT CCA

AA-3' RP, 5'-CTT GAG GCT GTT GTC ATA CT-3'; NHE1 FP, 5'-AGC CTT CAC CTC CCG ATT TAC CT-3' RP, 5'-CAG GAA GTA TTT GAT GGT GGT GTG G-3'. CHIP primers for promoter regions include: E-cadherin: 5'-TGG CCG GCA GGT GAAC-3', 5'-Ggg ctg gag tct gaa ctg actt-3'; NHE1: 5'-ggc agc cct agt aag caa taca a-3', 5'-Aat gca ccc tgg cac att att ac-3'.

Chromatin Immunoprecipitation

Modified from a previously described protocol [35]. Briefly, confluent mesenchymal NT and Zeb1 KD cells were washed with PBS, trypsinized, and centrifuged at 1000rpm for 5 minutes to pellet the cells. Pellet was resuspended in 10ml RPMI supplemented with 10% FBS and rocked for 15 minutes at room temperature (RT) with addition of 270 μ l of 37% formaldehyde to cross-link protein-DNA complexes. 1 ml of 1.25M glycine was added and rocked for 5 minutes at RT. Cells were rinsed 3 times with cold PBS for 5 minutes at 4°C and resuspended in 1 $\times$ cell lysis buffer (Cell Signaling) + 10ul/ml PMSF to a final concentration of 1.0 $\times$ 10⁷ cells/ml. Chromatin was sonicated in 1.5 mL TPX tubes using a Biorupter sonicator (Diagenode, Sparta, NJ) for 30 minutes pulsing for 30 seconds on and off to shear DNA. Following sonication, the chromatin sample was centrifuged at 12,000 rpm in 4°C for 10 minutes to pellet cellular debris and then diluted in ND buffer containing protease inhibitors. Magnetic protein G Dynabeads (Invitrogen) was added and samples were incubated at 4°C for 1 hr with rotation to preclear chromatin. Primary antibody was added and samples rotated overnight at 4°C. Chromatin-antibody complexes were immunoprecipitated using magnetic protein G Dynabeads for 1–3 hrs at 4°C. Beads were washed four times for five minutes at 4°C as follows: two washes with ND buffer (20 mM Tris pH 8, 137 mM NaCl, 1% NP-40, 10% glycerol, and 2 mM EDTA) containing protease inhibitors, two washes with ND buffer containing 0.3 M NaCl, two washes with LiCl buffer (250 mM LiCl, 0.5% NP-40, 0.5% Sodium Deoxycholate), one wash with 1 $\times$ TE (10 mM tris-HCl pH 8.0, 1 mM EDTA) plus 0.2% Triton X-100, and one wash with 1 $\times$ TE buffer. Beads were resuspended in 1 $\times$ TE buffer containing 0.3% SDS, 200 mM NaCl, and 0.5 mg/ml proteinase K (Sigma) and incubated for 2 hrs at 45°C followed by 65°C overnight to reverse the crosslinks. Supernatants were removed and beads washed once with 100 μ l of 1 $\times$ TE containing 500mM NaCl. DNA from supernatant and wash were combined and purified using the MoBio PCR ultra clean Kit (MoBIO, Carlsbad, CA). Promoter enrichment was assessed using QPCR. Experiments were performed in triplicate and significance was assessed by one-tailed student's T-test.

Transwell Invasion and Motility Assays

For invasion assays, 50 μ L of a 1:5 dilution of Matrigel in serum free RPMI was plated on Costar Transwell Permeable Support inserts with 8 μ m pore size and allowed to solidify at 37°C for 2 hours. Matrigel was re-hydrated with 50 μ L serum free media for an additional 30 minutes at 37°C. Un-coated Transwell Inserts were used for motility assays. 600 μ L complete media containing vehicle control or drug was placed on the underside of the insert. 1 $\times$ 10⁴ cells diluted in a total volume of 100 μ L serum free media plus or minus drug treatments were seeded on top of the insert. Cells were allowed to migrate or invade for 48 hours then cells were fixed with 4% PFA for 20 minutes and stained with 0.1% crystal violet for 20 minutes. Cells and Matrigel remaining on the top of the Transwell insert were

removed with a cotton swab and cells on the underside of the Transwell insert were imaged using an Olympus CKX41 microscope with DPManager software. Representative 10X images are shown. Transwell invasion and motility assays were quantified by counting the number of cells on the underside of the insert per 10X field from three independent experiments.

Proliferation

Cells were seeded at 30% confluence and grown in RPMI supplemented with 10% FBS. Cell proliferation was measured at the indicated times with Cell Titer Blue (Promega, Madison, WI) according to the manufacture protocol. Graph represents the average of three independent experiments.

Lysosome Analysis

This protocol is previously described [16,17,21]. The average distance of lysosomes from the nucleus border was quantified using LysoTracker software, generously provided by Meiyappan Solaiyappan at Johns Hopkins University. Briefly, nuclear border was detected based on DAPI staining. Lysosomes were detected based on LAMP-1 staining and distance of each lysosome within a cell from the nucleus border was assessed. A total of 25 cells spanning 3 independent experiments for each condition were analyzed. Error bars represent SEM. Significance was determined using Two-Tailed Mann-Whitney T-test in GraphPad 3.0 software.

Results

Anterograde lysosome trafficking is associated with the mesenchymal phenotype

DU145 PCa cells displaying an either an epithelial or mesenchymal morphology were clonally selected from a parental population [29]. Epithelial clones were characterized as having cobblestone morphology while mesenchymal clones lost cell-to-cell adhesions and had a scattered appearance. We next assessed the expression of several genes that are characteristic of either epithelial or mesenchymal cell types. Like Zeb1, SNAIL, SLUG, and TWIST are transcription factors that promote the transition to a mesenchymal morphology and are often upregulated in invasive cancers. The increased expression of any one of these transcription factors will stimulate EMT. Conversely, E-cadherin is involved in cell-to-cell adhesions and is often lost when cells undergo EMT [23]. Reverse transcriptase PCR revealed that E-cadherin transcripts were lost in mesenchymal clones (Fig. 1A). Unexpectedly, transcripts from the EMT-promoter SNAIL were also lost in mesenchymal cells, and we observed no change in transcript levels of SLUG or TWIST. To further assess the EMT expression profile at the protein level, whole cell lysates from parental, epithelial, and mesenchymal DU145 cells were probed for EMT markers by immunoblot (Fig. 1B). The EMT transcription factor Zeb1 was increased and that E-cadherin was decreased in mesenchymal cells, consistent with Zeb1 driving EMT in DU145 mesenchymal clones. We next examined lysosome distribution in parental, epithelial, or mesenchymal clones. Cells were fixed and stained for lysosome associated membrane protein-1 (LAMP-1, red) as a marker for late endosomes and lysosomes, actin (green), or DAPI (blue) (Fig. 1C; quantified in 1D). Immunofluorescence microscopy revealed that parental and epithelial cells were

maintained in a cobblestone colony appearance characteristic of the epithelial state, and lysosomes were located in the peri-nuclear region. However, mesenchymal cells had a scattered morphology with lysosomes found located at the periphery in actin rich protrusions.

Activation of the growth factor receptors c-Met and EGFR can lead to cell scattering, indicative of EMT, and anterograde lysosome trafficking [18] (Dykes and Cardelli, manuscript in preparation). To evaluate whether aberrant growth factor receptor activation was responsible for cell scattering and anterograde lysosome trafficking in mesenchymal clones, we assessed c-Met, EGFR, and AKT activation by immunoblot. We found that mesenchymal cells did not have constitutive c-Met, EGFR, or AKT activation (Fig. S1). These data suggest that the cell scattering and peripheral lysosome distribution observed in mesenchymal clones is not the result of aberrant c-Met or EGFR activation.

We next asked whether lysosome distribution was altered in mesenchymal clones of other cell lines. MCF10A breast cancer cells and ARCaP prostate cancer cells were clonally selected from a single parental population to have either an epithelial or mesenchymal morphology [30]. To confirm EMT, we examined the expression profile of various proteins known to be associated with either the epithelial or mesenchymal state. E-cadherin is associated with epithelial gene expression, while vimentin and EMT transcription factors, such as Zeb1, are associated with mesenchymal gene expression. Immunoblot analysis of MCF10A breast cancer cells revealed that epithelial clones had high expression of E-cadherin and low expression of vimentin when compared to mesenchymal clones. ARCaP mesenchymal clones had increased Zeb1 and vimentin expression and a complete loss of E-cadherin when compared to ARCaP epithelial cells (Fig. S2A). Next, epithelial and mesenchymal MCF10A and ARCaP cells were fixed and stained to visualize the position of lysosomes (Fig. S2B). In both MCF10A and ARCaP cell lines, epithelial cells had a tight cluster of lysosomes adjacent to the nucleus, while mesenchymal cells contained lysosomes located near the cell periphery. Taken together, these data demonstrated that peripheral lysosome positioning correlated with mesenchymal gene expression. Additionally, EMT-driven anterograde lysosome trafficking is not cell line or tissue type specific.

The mesenchymal morphology and gene expression pattern does not depend on lysosome positioning

Lysosome positioning has recently been implicated as a critical regulator of gene expression via control of mammalian target of rapamycin (mTOR), suggesting that the position of lysosomes could impart a mesenchymal gene expression profile and morphology on cells [36]. Therefore, we asked whether inhibiting anterograde lysosome trafficking reversed EMT. To induce JLA, DU145 mesenchymal cells were transduced with lentivirus-delivered Non Target (NT) shRNA or shRNA targeting the ADP-Ribosylation factor like protein 8b (Arl8b), an Arf-like GTPase that recruits kinesin 1 to lysosomes. Depletion of Arl8b results in a tight clustering of lysosomes near the microtubule organizing center [32,37,38]. Immunoblot analysis confirmed knockdown (KD) of Arl8b (Fig. 2A). We also used the sodium proton exchanger (NHE) inhibitor 5-(N-ethyl-N-isopropyl)-Amiloride (EIPA) as an

additional method of reversing lysosome trafficking in mesenchymal cells, as NHE1 expression and activity is known to influence lysosome positioning [18,21]. Epithelial and mesenchymal cells were fixed and stained for LAMP-1 (red) to visualize lysosomes (Fig. 2B; quantified in 2C). While Arl8b KD and EIPA-treated mesenchymal cells contained lysosomes clustered in the perinuclear region, cells still maintained a scattered morphology characteristic of the mesenchymal state. Next, we harvested whole cell lysates from identical treatment conditions and probed for EMT markers by immunoblot (Fig. 2D). Zeb1, E-cadherin, and vimentin protein expression was maintained at similar levels in mesenchymal cells regardless of lysosome positioning, suggesting that lysosome clustering in the perinuclear region does not reverse EMT.

JLA prevents EMT-mediated invasion and protease secretion

Previous studies found that JLA prevented invasion driven by HGF, EGF, and acidic extracellular pH, stimuli commonly found in the tumor microenvironment [15–18]. However, HGF/c-Met and EGF/EGFR and downstream signaling through AKT was not activated in epithelial or mesenchymal clones (Fig. S1). To test whether JLA prevents EMT-mediated motility and invasion, mesenchymal cells were treated with EIPA or transduced with lentivirus encoding Arl8b shRNA. Epithelial cells and untreated NT mesenchymal cells were used as controls for all assays. Cells were placed in a Boyden chamber assay and allowed to migrate to the underside of the Transwell insert toward a serum gradient for 48 hours. Cells were then fixed and stained with crystal violet and the number of migrated cells was counted (Fig. 3A). Transwell motility was higher in mesenchymal NT cells compared to epithelial cells. Mesenchymal cells expressing Arl8b shRNA or treated with EIPA had similar motility compared to control-treated mesenchymal cells, suggesting that JLA does not affect overall cell motility. While tumor cell motility is necessary for cell invasion, not all motile cells are invasive. The formation of specialized leading edge structures- called invadopodia is necessary for tumor cell navigation of the ECM [39]. Additionally, proteolytic degradation and remodeling of the ECM facilitates tumor cell invasion. Using Matrigel-coated Boyden chambers and identical treatment conditions, we tested whether JLA prevented EMT-mediated Transwell invasion (Fig. 3B). Arl8b KD and EIPA treatment reduced invasion of mesenchymal cells to levels similar to epithelial cells.

While Boyden chamber assays are a good biological readout for *in vitro* invasion, these assays are limited in the interpretation of their results. Boyden chamber assays are assessed at a single endpoint and all non-invading cells are removed, leaving only a small population of cells for analysis. Thus, the researcher cannot make general conclusions about the invasive properties of the bulk of the cells in the assay. Recent advances in 3-dimensional (3D) culture techniques allow for the growth of tumor cells embedded in Matrigel or other ECM components. Using this method, whole colonies of tumor cells can be visualized, allowing researcher to make stronger conclusions about the behavior of the bulk of cells within a population. Thus, we assayed tumor invasion and protease secretion using a Dye-quenched (DQ)-collagen IV 3D culture assay. DQ collagen IV fluoresces upon proteolytic cleavage and is a measure for the activation of many proteases, including lysosomal cathepsins [33,34]. Epithelial, NT mesenchymal, NT mesenchymal cells in the presence of EIPA, or Arl8b KD mesenchymal DU145 cells were seeded in Matrigel supplemented with

DQ-collagen IV and grown for 4 days in complete media. Cells were fixed and stained for actin (red) (Fig. 3C). Control-treated epithelial DU145 cells formed spheroid colonies and had minimal protease activity, while control-treated mesenchymal DU145 cells formed highly invasive colonies and displayed robust protease activity indicated by ample DQ-collagen IV fluorescence (green). In contrast, mesenchymal cells treated with EIPA or Arl8b shRNA formed spheroid colonies with minimal protease activity similar to the epithelial cell control. One explanation for the smaller colony size of Arl8b KD cells compared to NT cells is that Arl8b knockdown results in slower cell proliferation; however, loss of Arl8b had no effect on the proliferation rate of mesenchymal cells (Fig. S3). In addition to having higher protease activity in 3D culture, immunoblot revealed that mesenchymal DU145 cells also had increased expression of lysosomal cathepsins B, D, and L compared to epithelial DU145 cells (Fig. S4), which may partially account for the increased matrix degradation observed in mesenchymal-control treated colonies. Collectively, these data indicated that peripheral lysosome positioning regulates EMT-mediated tumor cell invasion and protease secretion as assayed in both a Boyden chamber and 3D culture system.

Transforming growth factor beta (TGF β) is another common component of the tumor microenvironment known to induce EMT and invasive potential of cancer cells [40]. We queried whether TGF β would stimulate anterograde lysosome trafficking and invasion of the parental DU145 cells. Upon treatment with TGF β , parental DU145 prostate cancer cells lost cell-to-cell adhesions and lysosomes were redistributed from the perinuclear region to being diffuse throughout the cytoplasm (Fig. S5A). Furthermore, TGF β treatment resulted in increased invasion of parental DU145 cells in a Boyden chamber assay (Fig. S5B). Together, these data suggest that anterograde lysosome trafficking correlates with invasive potential in an inducible model of EMT.

NHE1 is necessary for EMT-mediated anterograde lysosome trafficking

NHE1 is often upregulated in invasive cancers and its activity is associated with anterograde lysosome trafficking and tumor cell invasion [17,21]. Immunoblot revealed that NHE1 protein levels were increased in DU145 mesenchymal cells compared to epithelial cells (Fig. 4A; quantified in 4B). We previously showed that treatment with EIPA, a broad NHE inhibitor, resulted in juxtanuclear lysosome aggregation (Fig. 2B). To test whether NHE1 was important in regulating EMT-mediated lysosome trafficking, we used lentivirus-delivered shRNA to generate NHE1 KD mesenchymal DU145 cells. Immunoblot revealed that NHE1 levels were depleted by greater than 90%, and that NHE1 KD cells maintained a loss of E-cadherin, suggesting that NHE1 KD does not reverse EMT (Fig. 4C). Next, lysosomes (red) were visualized by immunofluorescence microscopy and this revealed that shRNA-depletion of NHE1 resulted in JLA (Fig. 4D; quantified in 4E). These data suggested that NHE1 regulates EMT-mediated anterograde lysosome trafficking.

Zeb1 regulates NHE1 expression and lysosome positioning in mesenchymal cells

We hypothesized that EMT transcription factor Zeb1 was driving the expression of NHE1 in mesenchymal cells. To test this, we depleted Zeb1 in mesenchymal DU145 cells using lentiviral delivered shRNA. Immunoblot confirmed knockdown of Zeb1 and revealed that NHE1 levels were reduced in Zeb1 KD cells compared to NT mesenchymal cells. E-

cadherin protein expression was also rescued in Zeb1 KD cells, confirming that Zeb1 is regulating EMT in our system (Fig. 4F; quantified in 4G). Reverse Transcriptase PCR revealed that NHE1 mRNA levels were reduced in Zeb1 KD cells (Fig. S6A), and we found that NHE1 protein levels correlated with varying degrees of Zeb1 depletion by immunoblot (Fig. S6B). Zeb1 binds to the E-box sequence (CACCTG/CAGGTG) in the promoter of target genes and can influence gene expression. Interestingly, an E-box motif exists in the human NHE1 gene promoter (nucleotides –463 to –458 with respect to the TATA Box; accession number L25272). To assess whether Zeb1 associated with the NHE1 promoter, we performed chromatin immunoprecipitation assays. Zeb1 was immunoprecipitated, and binding to the NHE1 and E-cadherin promoters was determined in mesenchymal NT and Zeb1 KD cells (Fig. 4H). These experiments revealed that Zeb1 was detectable on the NHE1 and the E-cadherin promoters. Additionally, in the presence of Zeb1-targeted shRNA, Zeb1 is no longer detectable on the NHE1 and E-cadherin promoters. Next, we performed immunofluorescence microscopy for LAMP1+ vesicles in NT and Zeb1 KD mesenchymal cells, and found that Zeb1 depletion resulted in a return to a cobblestone morphology characteristic of an epithelial state and lysosomes (red) clustered in the perinuclear region (Fig. 4I; quantified in 4J). Together, these data suggested that Zeb1 binds directly to the NHE1 promoter and controls the expression of NHE1 at the transcriptional level. Overall, these studies suggest that NHE1 is necessary for Zeb1-mediated lysosome positioning.

Discussion

We have presented data that suggests a role for lysosome trafficking in EMT-mediated tumor cell invasion. Our findings suggest that EMT drives peripheral lysosome trafficking via increased expression of NHE1, and that a peripheral lysosome distribution is necessary for EMT-mediated tumor cell invasion and protease secretion. Additionally, the present study is the first to propose that Zeb1 regulates NHE1 expression and that NHE1 is critical to EMT-mediated tumor cell invasion.

Previous studies identified select stimuli commonly found in the tumor microenvironment as promoters of anterograde lysosome trafficking, and demonstrated that JLA inhibited growth factor-mediated invasion and protease secretion [15–18]. However, many tumors are diagnosed after cancer cells have undergone EMT and begun to invade. Therefore, it is important to determine whether reversing anterograde lysosome trafficking in cells that have already acquired a mesenchymal phenotype can block subsequent invasion. Thus, the present study utilized clonal cell lines to determine the role of lysosome trafficking in EMT-mediated invasion. We found that lysosomes are located closer to the cell periphery in mesenchymal cells and that mesenchymal cells are more invasive and proteolytically active compared to their epithelial counterparts. The secretion of lysosomal proteases is well documented in a wide array of invasive solid tumors; however, the use of protease inhibitors as anti-cancer therapeutics has not been clinically successful.

EMT-driven tumors are often highly aggressive [41], and we propose anterograde lysosome trafficking inhibition as a novel therapeutic approach to slow EMT-mediated protease-dependent tumor cell invasion. Two independent methods were used to block EMT-mediated anterograde lysosome trafficking, and the resulting JLA prevented protease secretion in 3D

culture. Strikingly, JLA completely prevented the formation of invasive outgrowths in mesenchymal cells, resulting in spheroid colony morphology similar to epithelial cells; however, overall cell motility was not affected. The ability to re-model the ECM is necessary for invasion, but not motility, and this may explain the differences in the data trend when comparing the invasion and motility assays (Fig 3). Collectively, these data highlight the importance of peripheral lysosome trafficking in the release of proteases involved in cancer invasion, and support the notion that targeting organelle movement is a viable anti-cancer target for the development of novel anti-cancer therapeutics. Lysosome trafficking likely contributes to EMT-mediated tumor cell invasion in several ways: 1) lysosomes are a storehouse of active proteases, which contribute to ECM degradation [11], 2) lysosomes function to control cancer cell proliferation and invasion via regulating growth factor receptor and integrin recycling [42,43], 3) lysosomes influence leading edge and invadopodia formation [44], and 4) lysosomes influence cancer cell survival in low nutrient conditions through control of the autophagy pathway [36].

The data presented here suggests that NHE1 regulates anterograde lysosome trafficking in mesenchymal cells. Currently, the precise mechanism by which NHEs regulate lysosome positioning is still under investigation; however, several studies hypothesize that the exchanger activity is necessary for anterograde lysosome trafficking [17,21]. Additionally, NHEs are known to interact with the cytoskeleton [45], and this association may influence kinesin or dynein binding to the cytoskeleton and thus impact lysosome positioning. Recently, NHE1 was found to interact with calcineurin homologous protein (CHP) [46]. CHP binds to members of the kinesin family of proteins and can regulate membrane docking and fusion [47,48]. The connection between NHE1, CHP, and kinesin proteins suggests that NHE1 may indirectly regulate lysosome trafficking through CHP. Elucidating the precise mechanisms of how NHE1 regulates lysosome movement will be the focus of future studies.

In addition to their control of lysosome trafficking, the role of NHEs in cancer progression is multifaceted. Overactive NHEs confer an alkaline intracellular pH, which is favorable for many metabolic processes and promote tumor cell survival, and previous work suggests that NHE1 influences tumor cell motility and invasion through regulation of invadopodia formation [20,49]. Many proteases, including lysosomal cathepsins, are optimally active in acidic environments, and NHE1 recruitment to invadopodia provides a hospitable environment for targeted extracellular protease activity [50,51]. Thus, multiple lines of evidence support the hypothesis that NHEs are critical for tumor cell invasion.

The EMT transcription factor Zeb1 is known to be a repressor of E-cadherin [52]; however, Zeb1 is also reported to upregulate the expression of mesenchymal genes involved in invasion. Recently, Sánchez-Tilló *et al.* reported that Zeb1 activates expression of urokinase plasminogen activator (uPA), a protein that processes plasminogen into plasmin, which can then cleave ECM components [53]. Thus, Zeb1 may contribute to tumor cell invasion by facilitating proteolytic degradation of the ECM. We found that protein levels of the lysosomal cathepsins B, D, and L were increased in mesenchymal cells (Fig. S4), but further studies are needed to investigate whether Zeb1 also controls expression of these proteases.

This study is the first to propose a link between Zeb1 and regulation of NHE1 expression. While both EMT and an increase in NHE1 protein expression are associated with tumor progression, it was not known whether Zeb1 influences NHE1 expression. We found that shRNA depletion of Zeb1 resulted in lower NHE1 protein and mRNA levels (Fig. 4F and 4G; S4), suggesting that Zeb1 may regulate NHE1 expression at the transcriptional level. Zeb1 promotes EMT by binding to the E-box sequence (CACCTG/CAGGTG) in the promoters of target genes, thereby regulating gene expression [54]. The human NHE1 promoter does contain an E-box sequence, and we found that Zeb1 binds to the NHE1 promoter (Fig. 4H). Zeb1 is known to be both an activator and a repressor of transcription when it binds to the E-box sequence, depending on its association with specific co-factors [55]. This manuscript presents the first evidence suggesting that Zeb1 directly binds to and activates transcription of the NHE1 gene. Zeb1-mediated regulation of proteins that stabilize cellular pH is not unprecedented. A recent paper demonstrated that Zeb1 transcriptionally upregulated expression of carbonic anhydrase9 (CA9), a protein that like NHE1, regulates pH [56].

In conclusion, the data presented in this study highlight the importance of organelle trafficking in EMT-mediated tumor cell invasion. We have demonstrated that reversing lysosome positioning inhibits the invasion and protease secretion of highly invasive mesenchymal cells. Finally, we are the first to suggest that the EMT transcription factor Zeb1 drives invasion through regulation of NHE1, which in turn drives anterograde lysosome movement. Overall, our data suggest that targeting lysosome traffic and NHE activity is a viable treatment for EMT-driven invasive carcinomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Christine Birdwell and Min Chu for technical assistance, Dr. Meiyappan Solaiyappan for the generous gift of the lysosome quantification software, and Drs. Rona Scott, Michelle Arnold, and David Coleman for the critical reading of this document.

Funding: This work was supported by a Carroll Feist pre-doctoral fellowship awarded to SSD Grant (#148741195A).

Abbreviations

PCa	prostate cancer
ECM	extracellular matrix
MMP	matrix metalloproteinases
NHE	sodium hydrogen exchanger
JLA	juxtannuclear lysosome aggregation
HGF	hepatocyte growth factor

EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
Zeb1	zinc finger E-box binding homeobox-1
LAMP-1	lysosome associated membrane protein-1
Arl8b	ADP-Ribosylation factor like protein 8b
EIPA	5-(N-ethyl-N-isopropyl)-Amiloride
NT	non target
KD	knockdown
TGFβ	transforming growth factor beta

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Figure 1. Cells that have undergone EMT display anterograde lysosome trafficking
(A) Reverse Transcriptase PCR showing mRNA levels of the indicated products in Parental, Epithelial, and Mesenchymal clones; N=3 **(B)** Whole cell lysates were collected from Parental, Epithelial, and Mesenchymal DU145 clones. The indicated proteins were detected via immunoblot; N=3. **(C)** Parental, Epithelial, and Mesenchymal DU145 cells were stained for LAMP-1 (red), Actin (green), and DAPI (blue); N=3 **(D)** Represents mean lysosome distribution of 25 cells for each condition; P<0.05 compared to Parent.

Figure 2. Juxtannuclear lysosome aggregation does not reverse EMT

(A) DU145 Mesenchymal cells were transfected with lentiviral delivered scrambled or Arl8b shRNA and knockdown was determined by immunoblot. (B) DU145 Epithelial, Mesenchymal Non Target, or Mesenchymal Arl8b KD were treated with 25 μM EIPA or vehicle control and fixed and stained for LAMP-1 (red), phalloidin (green), and DAPI (blue); N=3. (C) Represents mean lysosome distribution of 25 cells. *=P<0.05 compared to Mesenchymal NT. (D) DU145 Epithelial, Mesenchymal Non Target, or Mesenchymal Arl8b KD were treated for 24 hours with 25 μM EIPA or vehicle control. Whole cell lysates were analyzed by immunoblot for the indicated proteins; N=3.

Figure 3. Juxtanuclear lysosome trafficking inhibits EMT-mediated invasion and protease secretion

(A) DU145 Epithelial, Mesenchymal Arl8b KD, Mesenchymal NT, or Mesenchymal NT treated with 25 μ M EIPA were allowed to migrate through transwell chambers for 48 hours. Migrated cells were fixed and stained with crystal violet. Images represent 10X fields. Graph represents analysis of three independent experiments. $\ast = p < 0.05$ compared to Epithelial. (B) DU145 Epithelial, Mesenchymal Arl8b KD, Mesenchymal NT, or Mesenchymal NT treated with 25 μ M EIPA were plated on top of Matrigel coated transwell inserts. Cells were

allowed to invade for 48 hours and invaded cells were fixed and stained with crystal violet. Images represent 10X fields. Graph represents analysis of three independent experiments. $*=p<0.05$ compared to Epithelial. (C) DU145 Epithelial, Mesenchymal Arl8b KD, Mesenchymal NT, or Mesenchymal NT treated with 25 μ M EIPA were cultured in Matrigel in the presence of DQ-collagen IV. Cells were cultured for 4 days then fixed and stained for phalloidin (red); N=3. Green indicates cleaved DQ-collagen IV as a read out for protease activity. Graph represents analysis from three independent experiments. $*=p<0.05$ compared to Epithelial.

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Figure 4. Sodium Proton Exchanger 1 protein expression is increased in Mesenchymal cells, regulated by Zeb-1, and is necessary for anterograde lysosome trafficking
(A) Whole cell lysates from DU145 Epithelial or Mesenchymal cells were analyzed by immunoblot; N=3. **(B)** Represents densitometric analysis of three independent immunoblots; *= $p < 0.05$. **(C)** DU145 Mesenchymal cells were transduced with NT or NHE1 shRNA and immunoblot confirmed knockdown. **(D)** DU145 Mesenchymal cells expressing non target or NHE1 shRNA were fixed and stained for LAMP-1 (red), phalloidin (green), and DAPI (blue); N=3. **(E)** Represents mean lysosome distribution of 25 cells for each condition;

= $p<0.01$ compared to NT. **(F) DU145 Mesenchymal cells were transduced with lentiviral delivered NT or Zeb1-c shRNA. Whole cell lysates were collected and probed by immunoblot; N=5. **(G)** Represents densitometric analysis of 5 independent immunoblots; **= $p<0.05$. **(H)** Chromatin immunoprecipitation (ChIP) assays were performed to analyze Zeb1 binding to the E-cadherin and NHE1 promoters in NT or Zeb1 KD Mesenchymal DU145 cells. Chromatin was immunoprecipitated with control rabbit IgG or Zeb1 antibodies. Q-PCR was performed for E-cadherin and NHE1 promoters. *= $p<0.02$ and **= $p<0.01$. **(I)** DU145 Mesenchymal NT or Zeb1-c KD cells were fixed and stained for LAMP-1 (red), phalloidin (green), and DAPI (blue); N=3. **(J)** Represents mean lysosome distribution of 25 cells for each condition; **= $p<0.01$ compared to NT.