Tumor-secreted Hsp90 Subverts Polycomb Function to Drive Prostate Tumor Growth and Invasion*

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Prostate cancer remains the second highest contributor to male cancer-related lethality. The transition of a subset of tumors from indolent to invasive disease is associated with a poor clinical outcome. Activation of the epithelial to mesenchymal transition (EMT) genetic program is a major risk factor for cancer progression. We recently reported that secreted extracellular Hsp90 (eHsp90) initiates EMT in prostate cancer cells, coincident with its enhanced expression in mesenchymal models. Our current work substantially extended these findings in defining a pathway linking eHsp90 signaling to EZH2 function, a methyltransferase of the Polycomb represser complex. EZH2 is also implicated in EMT activation, and its up-regulation represents one of the most frequent epigenetic alterations during prostate cancer progression. We have now highlighted a novel epigenetic function for eHsp90 via its modulation of EZH2 expression and activity. Mechanistically, eHsp90 initiated sustained activation of MEK/ERK, a signal critical for facilitating EZH2 transcriptional up-regulation and recruitment to the E-cadherin promoter. We further demonstrated that an eHsp90-EZH2 pathway orchestrates an expanded repertoire of EMT-related events including Snail and Twist expression, tumor cell motility, and anoikis resistance. To evaluate the role of eHsp90 in vivo, eHsp90 secretion was stably enforced in a prostate cancer cell line resembling indolent disease. Remarkably, eHsp90 was sufficient to induce tumor growth, suppress E-cadherin, and initiate localized invasion, events that are exquisitely dependent upon EZH2 function. In summary, our findings illuminate a hitherto unknown epigenetic function for eHsp90 and support a model wherein tumor eHsp90 functions as a rheostat for EZH2 expression and activity to orchestrate mesenchymal properties and coincident aggressive behavior.

Prostate cancer is the most commonly diagnosed cancer in men and the second leading cause of cancer mortality (1). Although the majority of patients with organ-confined disease have a favorable prognosis, tumor metastasis is the primary cause of prostate cancer lethality (2, 3). Tumor invasion is a major risk factor for progression, and distinguishing lethal tumors from the indolent majority remains a major clinical challenge (4). Pathological reactivation of the developmental genetic program epithelial to mesenchymal transition (EMT) is associated with increased tumorigenesis and invasive behavior and is considered a primary culprit for metastasis and cancer-associated lethality (5–7). In prostate cancer, EMT activation is correlated independently with a high Gleason score, metastatic recurrence following surgery, and transition to invasive carcinoma in relevant animal models (8–11). Diminished expression of the adherens junction protein E-cadherin, a principal gatekeeper of tumor invasion, is a preserved and fundamental hallmark associated with early EMT events (6, 12, 13). In clinical prostate cancer, measures of progression correlate with increased expression of the core EMT transcription factors and diminished expression of E-cadherin (10, 14–16). Despite these well characterized genetic events, comparatively less is known regarding the clinically relevant signaling mediators that trigger this invasive program to facilitate prostate cancer progression.

Tumor-secreted extracellular Heat shock protein 90 (eHsp90) has been identified as a widespread regulator of cancer cell motility, invasion, and metastasis (as recently reviewed in Ref. 17). We demonstrated a unique role for eHsp90 as an initiator of EMT events in cell-based models of prostate cancer (18), thereby providing mechanistic relevancy for its tumorigenic function.

* This work was supported, in whole or in part, by National Institutes of Health Grants F31 CA177015 (to K.D.N.) and 1U01 CA151924 (to S.W.H.). This work was also supported by Department of Defense Grant W81XWH-12-1-0324 (to J.S.I.) and American Cancer Society Grant 124154-PF-13-024-CSM (to M.W.H.).

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5 The abbreviations used are: EMT, epithelial to mesenchymal transition; eHsp90, extracellular heat shock protein 90; NPGA, nonpermeable geldanamycin; qRT-PCR, quantitative reverse transcriptase PCR; EZH2, enhancer of Zeste homolog 2; H3K27m3, histone 3 lysine 27 trimethylation; H3K27Ac, histone 3 lysine 27 acetylation; ANOVA, analysis of variance.
Subsequent corroboration of eHsp90 as an EMT inducer in colon cancer (19) lends support for a conserved role for eHsp90 in malignancy. Moreover, several reports have documented elevated Hsp90 in patient serum relative to cancer-free controls (20–23), implicating a conserved clinical role for eHsp90. Of particular interest, patients with metastatic disease, including those with prostate cancer, exhibit the highest levels of serum Hsp90 (20, 21). We reported previously the presence of surface Hsp90 in human prostatectomy specimens, further supporting a clinical role for eHsp90. Given that tumor surface Hsp90 correlates with elevated expression of transcripts encoding several key drivers of EMT (18), eHsp90 is emerging as a clinically relevant mediator of tumorigenic progression. Despite its growing importance, the accessory players cooperating with eHsp90 in malignancy, and particularly in prostate cancer, remain largely undefined.

Prior studies have attempted to discern eHsp90 function by employing eHsp90 blocking strategies in metastatic cancer models (21, 24, 25). Our current study is unique in several respects. First, utilizing a molecular approach for enforced Hsp90 secretion in a minimally invasive prostate cancer model, we demonstrate that eHsp90 is sufficient to promote prostate tumor growth and invasion in vivo. Second, to our knowledge, this is the first study to evaluate the tumorigenic effects of eHsp90 in a cancer model representative of early disease, as well as in prostate cancer. The present study also reveals several novel mechanistic aspects of eHsp90 action. We now ascribe a previously unrecognized epigenetic function to eHsp90 as a conserved modifier of Polycomb group (PcG) protein activity. The methyltransferase enhancer of Zeste homolog 2 (EZH2) is the catalytic component of the repressive Polycomb complex 2 (PRC2) that initiates gene silencing by inducing histone H3-K27 trimethylation (26–29). We show that eHsp90 initiates MEK/ERK signaling, which in turn modulates EZH2 expression and repressive activity. Notably, an eHsp90-ERK axis was critical for the recruitment of EZH2 to the E-cadherin promoter and for subsequent suppression of E-cadherin expression. These molecular relationships were validated in vivo, wherein EZH2 was an essential effector of eHsp90 tumorigenic and invasive activity. Collectively, these data support a model wherein tumor eHsp90 functions as an upstream rheostat for EZH2 expression and activity to orchestrate mesenchymal properties and coincident aggressive behavior. Collectively, our findings lend credence to the premise that eHsp90 may subvert EZH2 function to promote the transition from localized to invasive disease.

**MATERIALS AND METHODS**

**Reagents**—Recombinant Hsp90α protein was purchased from Enzo Life Sciences (ADI-SPP-776). Non-permeable geldanamycin (NPGA), also known as DMAG-N-oxide-modified, was synthesized by Chris Lindsey and Craig Beeson (Drug Discovery, Medical University of South Carolina). MEK inhibitor U0126 was purchased from Promega (V112A). EZH2 inhibitor GSK343 was obtained from the Structural Genetics Consortium, and TO-PRO-3 stain (T 3605) was obtained from Molecular Probes.

**Cell Culture, Plasmids, and Transfections**—The ARCaPE and ARCaPM cell pair was purchased from Novicure Biotechnologies with sequences as follows: CDH1 sense, 5'-AATCCGTTGACTCCGACCTT-3'; EZH2 sense, 5'-GGCTTCCCAATAACAGT-3'; and GAPDH sense, 5'-GGGATGCCAAGCTT-3'. All quantitative real-time PCR reactions were performed in technical triplicates from at least two biological replicates. The data shown are presented as mean ± S.D. with differences in treatment groups defined as statistically significant at p < α = 0.05, as calculated from Student’s t test.

**Western Blot and Antibodies**—Cell extracts for Western blot analysis were prepared and performed as described (18, 30). Nuclear fractionation, where indicated, was performed as described previously (31). All blots are representative of a minimum of two independent experiments. Antibodies for E-cadherin (catalog No. 3195), EZH2 (5246), P-ERK1/2 (4370), ERK1/2 (4695), Snail (3895), Myc tag (2276), and histone H3 (4620) were from Cell Signaling. Zeb1 (NBP-05987) was from Novus Biologicals. Histones for immunoblot analysis were extracted via the acid extraction method as described previously (32), and antibodies for H3K27me3 (catalog No. 39536) and H3K27Ac (39133) were purchased from Active Motif. The antibody to α-tubulin (T6074) was from Sigma, and Ki67 antibody (catalog No. 16667) was from Abcam. Where indicated, relative fold changes in protein expression were quantified with ImageJ.

**RNA Isolation and Real-time PCR Analysis**—RNA purification from cells was performed following a TRIzol/chloroform extraction procedure according to the manufacturer’s recommendations (Qiagen miRNeasy kit 217004). Isolated mRNA was converted into complementary DNA (cDNA) (Bio-Rad iScript cDNA synthesis kit, catalog No. 170-8891) and amplified. Primers were purchased from Integrated DNA Technologies with sequences as follows: CDH1 sense, 5'-TGGGCCAG-GAAATCACATCCTACA-3'; and antisense, 5'-TTGGCAG-TGTCTCTCTCAATCGGA-3'; EZH2 sense, 5'-AGAGAAC-GGTCTCCAATACAGT-3'; and antisense, 5'-TTAGCTC-CCTGCTTTCCATACACT-3'; and GAPDH sense, 5'-TCGAC-GTCAGCGCGCATCTTTTT-3'; and antisense, 5'-ACCA-ATCCGGTTGACTCGGACCCTT-3'. All quantitative real-time PCR reactions were performed in technical triplicates from at least two biological replicates. The data shown are presented as mean ± S.D. with differences in treatment groups defined as statistically significant at p < α = 0.05, as calculated from Student’s t test.
Chromatin Immunoprecipitation and qPCR—Cells for chromatin immunoprecipitation (ChIP) were collected at ~80–85% confluency, and cell numbers were quantified. Chromatin was then harvested using the enzymatic ChIP kit from Cell Signaling (catalog No. 9003) following the manufacturer’s instruction. Briefly, cells were fixed in 1% formaldehyde for 10 min, quenched, and enzymatically digested for 20 min. The digested chromatin was then briefly sonicated to lyse nuclear membranes and stored at −80 °C. Approximately 1 × 10^6 cells (about 10 μg of DNA) was used for all immunoprecipitations. All immunoprecipitations were performed using magnetic beads according to the manufacturer’s instructions (Cell Signaling). The following Active Motif antibodies were used for ChIP: H3K27m3 (catalog No. 39155), H3K27Ac (39133), and EZH2 (39875). Control IgG antibodies were provided with the ChIP kit. Immunoprecipitated (ChIPed) DNA was then amplified via quantitative PCR utilizing primers flanking a validated EZH2 binding site on the E-cadherin promoter (−1.4 kb) (33). Primer sequences (Integrated DNA Technologies) for the E-cadherin promoter and GAPDH control promoter were as follows: CDH1 sense, 5’-ACCATGCTGCGCTATGTTACT-3’, and antisense, 5’-ATGTCTCCATGCTTGTGGAGA-3’; and GAPDH sense, 5’-TACTACGGTTTTACGCG-3’, and antisense, 5’-TCGACAGGAGGACGAGA-GCGA-3’. The data presented are from technical triplicates representing at least two biological replicates and are presented as mean ± S.E. with statistical significance defined as p ≤ α = 0.05, as calculated from Student’s t test.

Anoikis and Proliferation Assays—Cells were trypsinized and resuspended, and equivalent numbers (5 × 10^5) were added to either 6-well Corning Ultra-low attachment plates or standard tissue culture-treated plates. Cells were harvested at 1, 3, and 5 days, and live cells were either quantified by counting trypan blue-negative cells with a hemocytometer or measured using CellTiter-Blue (Promega).

Cell Motility Assays—Wounding assays were performed as described previously (18). Briefly, a thin sterile pipette tip was used to create a scratch wound in confluent cell monolayers. Mitomycin C (5 μg/ml, Sigma) was added just prior to wounding to suppress proliferation and was replenished with the medium. At 0 and 20 h after wounding, images were captured with an inverted Nikon eclipse TE 2000-S microscope with ×10 magnification. The extent of migration was calculated by measuring the gap area using ImageJ software.

Immunofluorescence—Equivalent cell numbers (2.5 × 10^6) were plated overnight on coverslips. Cells were then treated as indicated, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS as described (18). Images obtained with an Olympus FV10i were processed in Photoshop.

Animal Studies—Equivalent cell numbers (1 × 10^6) from each experimental group were resuspended in 50 μl of type I rat collagen as described previously (34). Collagen plugs were incubated overnight at 37 °C and grafted under the kidney capsule of adult male SCID mice (Harlan Sprague-Dawley, Indianapolis, IN), as described (34). Two replicates/kidney from each experiment were xenografted in three mice (total of six replicates). Mice were sacrificed after 7–8 weeks, and grafts were harvested. Pictures of the grafts before and after sagittal sectioning were taken followed by formalin fixation and processing for paraffin embedding. Histological assessment of xenografts was performed by H&E staining. Calculation of xenograft volumes was performed using the following formula: volume = width × length × depth × π/6. Data are shown as means ± S.D. A multiple group comparison was performed by one-way ANOVA followed by the Kruskal-Wallis procedure for comparison of means. p < 0.05 was considered significant.

Immunohistochemistry—Immunohistochemical analysis was performed on 5-μm sections from paraffin blocks using a previously optimized protocol (35). The following dilutions were used: E-cadherin (1:400), EZH2 (1:200), and Ki67 (1:200). Ki67 values were quantified according to the ImmunoRatio plugin with ImageJ image analysis software. Statistical significance for comparisons between groups was determined using ANOVA. In all cases, p < 0.05 was considered statistically significant.

Statistical Analysis—Quantitative results are means ± S.E. for at least three replicate determinations for each data point, and significant (p < 0.05) induction of a response compared with untreated controls, as determined by Student’s t test, is indicated.

RESULTS
eHsp90 Regulates EZH2 Expression via an ERK-dependent Pathway—We recently reported that eHsp90-initiated ERK signaling elicits the transcriptional repression of E-cadherin in prostate cancer cells (18). To further interrogate the mechanistic basis for this suppression, we explored the potential involvement of EZH2, an epigenetic repressor of E-cadherin (33, 37). To answer this question, we first investigated whether eHsp90 modulates EZH2 expression. Hence, EZH2 expression was assessed in two pairs of prostate epithelial cells, with each matched pair composed of an epithelial cell and its mesenchymal counterpart. We demonstrated previously that eHsp90 is more highly expressed in the mesenchymal derivative relative to the matched epithelial counterpart (18). Of note, EZH2 expression was modestly elevated (1.5-fold), and E-cadherin correspondingly repressed, in each of the mesenchymal cell lines (ARCaPM and M12) relative to the matched epithelial ARCaPE and P69 cell lines (Fig. 1A). Consistent with our prior findings, basal P-ERK1/2 levels were significantly elevated in the eHsp90-expressing mesenchymal lines. To evaluate whether an eHsp90-ERK signaling pathway played a regulatory role in modulating EZH2 expression, ARCaPM was treated with either the small molecule eHsp90 inhibitor NPGA (18, 25) or with the MEK/ERK inhibitor UO126. As shown, blockade of eHsp90 or MEK/ERK diminished both P-ERK and EZH2 expression, concomitant with an increase in E-cadherin (Fig. 1B). Although both UO126 and NPGA restored E-cadherin, there were obvious differences in the kinetics of this restoration. Whereas UO126 restored E-cadherin between 3 and 5 days, E-cadherin was not detectable prior to 10 days of NPGA treatment. These results are consistent with the more robust and earlier effects of UO126 upon both P-ERK and EZH2 relative to NPGA, further supporting the involvement of an eHsp90-ERK pathway in the regulation of EZH2 expression.

To validate eHsp90 as a primary effector of EZH2 expression, we utilized our previously reported lentiviral approach to
enforce eHsp90 expression in ARCaPE, thereby creating an isogenic pair consisting of a LacZ-transduced control (ARCaPE-LacZ) and an eHsp90-expressing counterpart (ARCaPE-eHsp90) (18). As shown, P-ERK was strongly up-regulated and EZH2 modestly increased (1.5-fold) in ARCaPE-eHsp90 relative to ARCaPE-LacZ (Fig. 1C), supporting the notion that eHsp90 is sufficient to induce EZH2 protein expression. To validate these trends, we tested whether NPGA and UO126 would antagonize the effects of eHsp90. As expected, treatment with NPGA or UO126 restored E-cadherin expression in tandem with diminished P-ERK and EZH2 (Fig. 1D). Consistent with findings in ARCaPM, UO126 elicited a more rapid restoration of E-cadherin, congruous with its earlier inhibition of P-ERK and loss of EZH2 compared with NPGA. To further evaluate whether eHsp90-ERK signaling may be a conserved pathway for EZH2 regulation in prostate cancer, we extended our analysis to additional cell lines, including mesenchymal M12 cells (38) as well as the established DU145. As shown, NPGA and UO126 each increased E-cadherin and inversely diminished EZH2 and P-ERK in both cell models (Fig. 1, E and F). Strikingly, NPGA elicited robust effects upon P-ERK, EZH2, and E-cadherin in these models that were comparable...
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MARCH 27, 2015 • VOLUME 290 • NUMBER 13
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to U0126, indicating an enhanced reliance upon eHsp90-ERK signaling. Although cell context-dependent variability in eHsp90 signaling likely exists, our findings convincingly demonstrate that an eHsp90-ERK axis is a prominent regulatory node for the regulation of EZH2 expression in prostate cancer.

eHsp90-ERK Signaling Elicits Transcriptional Regulation of E-cadherin and EZH2—We next assessed whether eHsp90 functions as a general modulator of E-cadherin transcription. As expected, eHsp90 diminished E-cadherin transcription in the ARCaPE model (Fig. 2A), whereas E-cadherin message was restored by NPGA treatment (Fig. 2B). A similar restoration was evident in ARCaPM and M12 cells following NPGA treatment (Fig. 2C and D). Although E-cadherin protein expression was undetectable in ARCaPM following 5 days of NPGA treatment, E-cadherin transcript levels were visibly increased by 3 days. This indicates that E-cadherin is highly repressed in ARCaPM and that prolonged exposure to NPGA is required to reach a threshold for detection of E-cadherin protein expression. Given the apparent inverse expression of E-cadherin and EZH2, we next assessed whether eHsp90 also exerted transcriptional control over EZH2. As shown, eHsp90 modestly increased EZH2 transcript expression in the ARCaPE model (Fig. 2E). As expected, NPGA treatment reduced EZH2 tran-

FIGURE 2. eHsp90-ERK signaling elicits transcriptional regulation of E-cadherin and EZH2. A, E-cadherin (CDH1) transcript expression in LacZ and eHsp90 ARCaPE cells, with GAPDH as the internal control. B–D, CDH1 transcript levels following a 1-, 3-, and 5-day treatment with NPGA (1 μM) in ARCaPE-eHsp90 (B), ARCaPM (C), and M12 cells (D). UT, untreated control. E, EZH2 transcript expression in LacZ and eHsp90 ARCaPE cells, with GAPDH as the internal control. F–H, EZH2 transcript levels following a 1-, 3-, and 5-day treatment with NPGA (1 μM) in ARCaPE-eHsp90 (F), ARCaPM (G), and M12 (H). I and J, transcript levels of CDH1 and EZH2 following a 5-day treatment with U0126 (10 μM) in ARCaPE-eHsp90 (I) and M12 (J). Error bars = S.D.; *, p < 0.05; **, p < 0.01.
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script expression in ARCaPE-eHsp90 (Fig. 1F). Similar trends upon EZH2 expression were observed in ARCaPM and M12 (Fig. 2, G and H). We next sought to confirm the involvement of ERK signaling in the eHsp90-dependent regulation of E-cadherin and EZH2 transcription. As shown, treatment of ARCaPE-eHsp90 and M12 cells with UO126 robustly increased E-cadherin while diminishing EZH2 transcript expression (Fig. 2, I and J). These findings support a previously unappreciated role for eHsp90 as a transcriptional effector of EZH2.

EZH2 Is Essential for eHsp90-mediated E-cadherin Suppression—Our findings thus far supported the premise that an eHsp90-ERK pathway inversely regulates E-cadherin and EZH2 expression. We next evaluated whether EZH2 might directly participate in eHsp90 suppression of E-cadherin, given the known ability of EZH2 to function as a repressor of E-cadherin (33, 37). To functionally evaluate the significance of eHsp90-mediated EZH2 up-regulation within the context of E-cadherin suppression, ARCaPE-eHsp90 was treated with the highly potent specific EZH2 methyltransferase inhibitor GSK343 (39). As shown, GSK343 antagonized eHsp90-mediated E-cadherin suppression in ARCaPE-eHsp90 (Fig. 3A).

Similar to the effects of NPGA, GSK343 elicited a time-dependent increase in the E-cadherin message (Fig. 3B). To mitigate the remote possibility of off-target drug effects, EZH2 activity
was also blunted either with a shRNA approach or via transduction with a functionally inactive Myc-tagged EZH2 mutant protein (EZH2-H694L) (40). These EZH2 blocking strategies similarly increased E-cadherin expression at the protein (Fig. 3C) and transcriptional levels (Fig. 3D). A similar analysis in ARCaPM (Fig. 3, E–G) confirmed that blockade of EZH2 activity was required for the restoration of E-cadherin protein and transcript expression. We next extended our interrogation of EZH2 function in DU145 and M12, as eHsp90-ERK signaling regulated both E-cadherin and EZH2 expression in these cell models. As shown, GSK343 treatment increased E-cadherin protein expression in both DU145 and M12. These findings collectively support the notion that EZH2 cooperates with eHsp90 to direct E-cadherin repression. Of note, the inability of GSK343 to suppress P-ERK (Fig. 3, A and H) indicates that its restoration of E-cadherin is not incompatible with P-ERK expression, further depicting EZH2 as a downstream target of eHsp90-ERK signaling.

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**FIGURE 4. eHsp90-ERK signaling regulates EZH2 activity and recruitment to the E-cadherin promoter.** A, immunoblot analysis of basal H3K27m3 expression from extracted histones in ARCaPE and ARCaPM (left) and in ARCaPM following NPGA treatment (3 days, right). B, similar analysis of basal H3K27m3 expression in the ARCaPE-LacZ and ARCaPE-eHsp90 pair (left) and in ARCaPE-eHsp90 following a 3-day treatment with the indicated agents (right). C, ChIP analysis of EZH2, H3K27m3, and H3K27Ac within −1.4 kb proximal of the CDH1 transcriptional start site in LacZ and eHsp90 ARCaPE. GAPDH was used as an internal control. D, similar ChIP analysis in ARCaPE-eHsp90 treated with U0126 as indicated. E, similar ChIP analysis in DU145 following a 3-day treatment of NPGA. Error bars = S.D.; *, p < 0.05; **, p < 0.01. UT, untreated control.

We next evaluated whether these molecular trends were evident at the E-cadherin promoter, thereby serving as a putative regulatory mechanism for E-cadherin transcription. Initially, to assess EZH2 activity, we evaluated the basal cellular levels of histone H3-K27 methylation (H3K27m3) in ARCaPE and ARCaPM. As shown, the repressive mark was up-regulated (2.4-fold) in ARCaPM relative to ARCaPE (Fig. 4A). Strikingly, a 3-day exposure to NPGA obliterated the repressive mark in ARCaPM, although moderate EZH2 protein was detected at this time point (see Fig. 1B). To further validate eHsp90 as an effector of EZH2 activity, H3K27m3 expression was evaluated in the isogenic LacZ- and eHsp90-expressing ARCaPE. As shown, eHsp90 increased (6-fold) the repressive mark, whereas NPGA, U0126, and GSK343 abrogated H3K27m3 expression (Fig. 4B). These findings indicate that eHsp90-ERK signaling globally increases the repressive activity associated with EZH2.

We next evaluated whether these molecular trends were evident at the E-cadherin promoter, thereby serving as a putative regulatory mechanism for E-cadherin transcription. EZH2 recruitment and activity at the E-cadherin promoter was assessed by performing ChIP in the isogenic ARCaPE-LacZ and ARCaPE-eHsp90 pair. As shown, EZH2 and its repressive mark exhibited a 2-fold enrichment at the E-cadherin promoter in
ARCaPE-eHsp90 relative to ARCaPE-LacZ (Fig. 4C). Although active gene transcription frequently correlates with increased promoter acetylation (H3K27Ac), only a minor decrease in this mark was observed, indicating that eHsp90 primarily regulates EZH2-directed repressive activity at the E-cadherin promoter in this model. We next evaluated whether MEK/ERK signaling diminished EZH2 recruitment to the E-cadherin promoter, an expected result given the ability of UO126 to restore E-cadherin expression. As shown, UO126 exposure evicts EZH2 from the E-cadherin promoter, with a corresponding decrease in the inhibitory H3K27m3 mark (Fig. 4D). We additionally evaluated the relation between eHsp90 and EZH2 recruitment in DU145. Although the decrease in EZH2 recruitment did not reach statistical significance, loss of the repressive mark was significant, as was acquisition of the H3K27Ac mark. Collectively, these results support the premise that an eHsp90-ERK pathway facilitates EZH2 repressive activity at the E-cadherin promoter.

**EZH2 Is a Major Effector of eHsp90-dependent EMT Events**—We demonstrated previously that eHsp90 initiates several events consistent with EMT activation (18). As our current findings support a functional cooperation between eHsp90 and EZH2 in the suppression of E-cadherin, we assessed the functional role of EZH2 within the broader context of eHsp90 and EMT activation. We demonstrated previously that eHsp90 disrupts epithelial morphology and promotes a scattered phenotype concomitant with aberrant localization of E-cadherin (18). Remarkably, the inhibition of EZH2 either by treatment with GSK343 or by introducing mutant EZH2-H694L relocalized the E-cadherin to adherens junctions and restored the epithelial phenotype, mirroring the effects elicited by UO126 (Fig. 5A). We demonstrated previously that eHsp90 induces the expression of several core EMT transcription factors (18). We now show that EZH2 blockade via GSK343 treatment or the introduction of EZH2-H694L similarly reduced Twist and Snail expression, whereas Zeb1 was unaffected (Fig. 5B).

Given that increased cell motility is a hallmark of EMT and that eHsp90 elicits a pro-motile phenotype in this model (18), we next evaluated the role of EZH2 in eHsp90-directed cell motility. As shown, EZH2 targeting profoundly inhibited the motogenic action of eHsp90 (Fig. 5C). We also evaluated the role of EZH2 within the context of cell proliferation and survival. Under adherent conditions, ARCaPE-eHsp90 exhibit a minimal increase in proliferative capacity relative to ARCaPE-LacZ, results that were confirmed via a quantitative fluoromet-
EZH2 Is Required for eHsp90-driven Tumorigenesis and Invasion—We next interrogated the relation between eHsp90 and EZH2 within a more physiologically relevant context. Implantation of prostate cancer cells into the kidney capsule is an effective approach to assessing tumorigenicity and invasive behavior (35). Although ARCaPE has not, to our knowledge, been evaluated in this model, ARCaPE is poorly metastatic in orthotopic models (9) and therefore resembles localized disease. Consistent with this notion, resultant ARCaPE-LacZ tumors grew poorly in the renal model (Fig. 6A). Of note, eHsp90 expression significantly increased tumorigenicity, an effect that was completely dependent upon EZH2 function. Although suppression of tumor growth was attained by either shEZH2 or the introduction of EZH2-H694L, the latter more potently abolished tumor growth, a difference likely due to a more robust ablation of EZH2 activity. Consistent with the supportive effects of eHsp90 upon tumorigenicity, the resultant tumor tissue exhibited increased proliferation, an effect that was also dependent upon EZH2 activity (Fig. 6B). There was no apparent effect of eHsp90 upon cell death as assessed by TUNEL (not shown).

H&E analysis of tumor sections indicated that ARCaPE-LacZ typically formed non-invasive tumors with relatively well indicated tumor-stromal boundaries (Fig. 6C, highlighted by dashed white line in upper left panel). In contrast, eHsp90 expression was sufficient to stimulate frank tumor invasion into the kidney parenchyma. Remarkably, this eHsp90-dependent invasive activity was completely antagonized by EZH2 suppression. The relative expression of EZH2 in these corresponding tumors is shown (Fig. 6C, middle row). We next evaluated whether tumor invasive activity was accompanied by respective changes in E-cadherin expression. Although strong E-cadherin staining was evident in ARCaPE-LacZ tumors (Fig. 6C, bottom row), ARCaPE-eHsp90 derived tumors demonstrated significant loss of E-cadherin, a trend especially apparent at the tumor-stromal interface. Strikingly, suppression of EZH2 uniformly restored E-cadherin, despite the enforced expression of eHsp90. These data convincingly demonstrate that EZH2 is a major effector of eHsp90-mediated E-cadherin repression in vivo, in accordance with our in vitro findings, and that an eHsp90-EZH2 axis is critical for regulating tumor invasive function. These molecular relationships are depicted schematically in Fig. 6D.

DISCUSSION

It is becoming an accepted paradigm that an intimate and dynamic relationship exists between signaling mediators and the epigenetic machinery (42). Within this framework, eHsp90 is emerging as an effector of epigenetic cross-talk, linking ERK activation with EZH2 activation. We have convincingly demonstrated that an eHsp90-ERK signaling axis governs EZH2 transcription, recruitment to the E-cadherin promoter, and deposition of repressive marks. These trends were confirmed by enforced expression of eHsp90 in minimally invasive cells, as well as by pharmacological blockade of eHsp90 function in aggressive lines. Moreover, the functional relevance of an eHsp90-ERK-EZH2 pathway was observed in a broad panel of prostate cancer models, highlighting the conserved nature of this regulation. Mechanistically, we demonstrate that EZH2 is a downstream target of eHsp90-ERK signaling, a hierarchy that differs from the recently reported bidirectional regulation between ERK and EZH2 in murine embryonic stem cells (43). In our models, a blockade of either eHsp90 or ERK consistently reduced EZH2 expression and activity. EZH2 targeting antagonized eHsp90 action, despite maintenance of eHsp90-activated ERK, further supporting EZH2 as a downstream effector of eHsp90 action. Notably, EZH2 was obligate for eHsp90 suppression of E-cadherin in vitro and in vivo and for coincident invasive activity in vivo. These collective findings reinforce the idea that eHsp90-EZH2 dependent modulation of E-cadherin represents an important step in prostate cancer progression. Although additional eHsp90-regulated mechanisms may conspire to down-regulate E-cadherin, our results support a role for EZH2 as a primary effector of eHsp90 action.

The relationship among eHsp90, ERK, and EZH2 is complex. Although a precedent exists for ERK to function as a transcriptional regulator of EZH2 via an ELK1 pathway (44), our preliminary findings (not shown) do not support ELK1 as a key effector, leaving open the question of the precise mechanism for eHsp90-ERK-mediated EZH2 up-regulation. It is also conceivable that post-transcriptional mechanisms such as microRNAs may be involved, a possibility currently under investigation. The regulation of E-cadherin by eHsp90-ERK signaling is likely to involve both EZH2-dependent and -independent mechanisms. The latter premise is supported by our findings that in some instances, ERK inhibition elicited a more robust restoration of E-cadherin relative to eHsp90 targeting, without eliciting an accompanying larger suppression of EZH2 expression. This differential may be explained by the expanded epigenetic functions of ERK. In addition to functioning as an upstream regulator of EZH2, ERK has recently been shown to co-occupy a cohort of EZH2 target sites and function as a repressive cofactor (43, 44). Therefore, additional studies are warranted to further define whether ERK and EZH2 may be co-recruited to eHsp90-repressed targets such as E-cadherin. Interestingly, Tee et al. and others (43, 44) have demonstrated the profound inhibition of EZH2 repressive activity following ERK targeting. Consistent with this notion, we demonstrate that the inhibition of either ERK or eHsp90 ablates the EZH2 repressive mark in histone lysates, further reinforcing an eHsp90-ERK regulatory node for EZH2 function. As EZH2 cooperates with a panoply of
cofactors to elicit the repressive function (37, 45–47), further work will be required to delineate the accessory factors mediating EZH2 recruitment to E-cadherin, as well as how eHsp90-ERK signaling may impact upon these associations.

We recently reported that eHsp90 is an initiator of EMT events in prostate cancer (18). EZH2 has also gained recognition as an effector of the EMT program (46, 48), and our current findings reinforce the premise that EZH2 is critical for executing a number of eHsp90 EMT-related events. In addition to facilitating eHsp90-mediated E-cadherin repression, EZH2 is essential for motile and tumor invasive actions of eHsp90. Moreover, EZH2 conferred eHsp90-mediated anoikis resistance, a central hallmark of EMT and a prerequisite for tumor metastasis (41). Furthermore, EZH2 participated in the eHsp90-mediated induction of Snail and Twist, a function consistent with the reported ability of EZH2 to modulate H3K27m3 status at these respective promoters (46, 48). Interestingly, EZH2 regulation of EMT transcription factors exhibits cell context-dependent variability (48), and our

FIGURE 6. EZH2 is required for eHsp90-driven tumorigenesis and invasion. A, top, representative gross images of renal capsule xenograft tumors originating from the indicated ARCaPE derivatives 7–8 weeks after implantation. Bottom, quantified analysis of corresponding tumors (n = 5). ARCaPE-eHsp90 transduced with an EZH2-H694L expression construct did not produce measurable tumors. B, quantified analysis of tumor proliferation assessed by Ki67 staining. Significance was determined by one-way ANOVA with the Kruskal-Wallis test. C, H&E staining of tumors shown in A, along with immunohistochemical analysis of EZH2 and E-cadherin. H&E images are at ×20 magnification and immunohistochemical images at ×40. D, schema depicting proposed mode of action for eHsp90. eHsp90 signaling may occur via several effectors, such as the low density lipoprotein receptor-related 1 protein (LRP1), to sustain ERK activation. eHsp90-ERK signaling promotes the transcriptional up-regulation of EZH2 with subsequent EZH2 recruitment to the E-cadherin promoter, resulting in E-cadherin repression and conditions supporting tumorigenicity and invasion.

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finding that Zeb1 is relatively unaffected by EZH2 expression is consistent with this observation. Given that eHsp90 also induces Zeb1 expression (18), additional effectors likely contribute to fully execute the eHsp90 EMT program. Although our current study has focused on the EZH2-dependent suppression of E-cadherin, EZH2 may also augment tumor invasion by silencing additional tumor suppressor targets (49). Hence, studies are under way to identify the expanded cohort of EZH2 targets that may support EMT activation and tumor invasion in response to eHsp90 signaling. As ERK signaling may also serve as a prominent inducer of EMT (50, 51), it is of interest to further dissect the potentially broader role of ERK in relation to eHsp90-directed EMT events.

The importance of EZH2 in prostate cancer progression is underscored by its frequent overexpression in tumors, particularly in hormone refractory metastatic disease, as well as its correlation with Gleason score, prostate cancer recurrence, metastasis, and treatment failure (52–54). Although genetic alterations such as the TMPRSS2-ERG gene fusion are key drivers of EZH2 up-regulation (36, 55), fusion-independent mechanisms may also contribute to aberrant EZH2 expression and activity (36, 55). Our demonstration of a novel epigenetic role for eHsp90 further highlights the ability of signaling mediators to govern EZH2 expression and function. Our findings raise the intriguing possibility that EZH2 may be a key downstream effector of eHsp90-ERK activity in diverse tumors. This notion is consistent with the demonstrated elevation of eHsp90 expression in mesenchymal and aggressive subtypes in breast and prostate models (18, 21), a trend now shown to correlate directly with eHsp90-ERK-dependent EZH2 axis has the dual capacity to drive early cancer invasion while also enforcing mesenchymal properties in cell models representative of later stage disease. Through its defined cross-talk with EZH2, our molecular and functional data unequivocally place eHsp90 within the context of an epigenetic modifier, a role inextricably linked with its support of disease progression. In summary, although mitigating EMT activation and tumor invasion is a challenging prospect, our study provides new mechanistic insights for a primary instigator of this central program, opening the door to unique treatment strategies.

Acknowledgments—We thank Joy Ware for the P69 and M12 cells, Chris Lindsey and Craig Beeson for synthesis of NPGA, and Maarten van Lohuizen for the EZH2-H694L expression plasmid. The imaging facilities for this research were supported in part by Cancer Center Support Grant P30 CA138313 to the Hollings Cancer Center, Medical University of South Carolina.

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