The Role of Alcohol-Induced Golgi Fragmentation for Androgen Receptor Signaling in Prostate Cancer

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Abstract

Multiple epidemiologic observations and meta-analysis clearly indicate the link between alcohol abuse and the incidence and progression of prostate cancer; however, the mechanism remains enigmatic. Recently, it was found that ethanol (EtOH) induces disorganization of the Golgi complex caused by impaired function of the largest Golgi matrix protein, giantin (GOLGB1), which, in turn, alters the Golgi docking of resident Golgi proteins. Here, it is determined that in normal prostate cells, histone deacetylase 6 (HDAC6), the known regulator of androgen receptor (AR) signaling, localizes in the cytoplasm and nucleus, while its kinase, glycogen synthase kinase β (GSK3β), primarily resides in the Golgi. Progression of prostate cancer is accompanied by Golgi scattering, translocation of GSK3β from the Golgi to the cytoplasm, and the cytoplasmic shift in HDAC6 localization. Alcohol dehydrogenase-generated metabolites induces Golgi disorganization in androgen-responsive LNCaP and 22Rv1 cells, facilitates tumor growth in a mouse xenograft model and activates anchorage-independent proliferation, migration, and cell adhesion. EtOH-treated cells demonstrate reduced giantin and subsequent cytoplasmic GSK3β. This phenomenon was validated in giantin-depleted cells. Redistribution of GSK3β to the cytoplasm results in phosphorylation of HDAC6 and its retention in the cytoplasm, which, in turn, stimulates deacetylation of HSP90, AR import into the nucleus, and secretion of prostate-specific antigen (PSA). Finally, the relationship between Golgi morphology, HDAC6 cytoplasmic content, and clinicopathologic features was assessed in human prostate cancer patient specimens with and without a history of alcohol dependence.

Implications: This study demonstrates the importance of alcohol-induced Golgi fragmentation in the activation of AR-mediated proliferation.

Introduction

In the United States, prostate cancer is among the leading causes of cancer-related death in men (1). A growing number of epidemiologic studies indicate a connection between frequent alcohol consumption and the incidence of prostate cancer, as well as to the high rate of prostate cancer mortality in a cohort of patients with a discharge diagnosis of alcoholism (2–4). Nevertheless, despite the evidence of recent, multiple meta-analyses (5–7), the precise mechanism of the effect of alcohol on the growth and progression of prostate tumors remains unknown.

Androgens and androgen receptor (AR) signaling pathways are commonly considered the main oncogenic drivers of prostate carcinogenesis (8). AR belongs to the nuclear receptor superfamily and is activated by steroid hormones, including testosterone and dihydrotestosterone (DHT). In normal prostate tissues, the binding of hormones to AR determines the translocation of AR to the nucleus, where it can activate the expression of several genes related to the maintenance of prostate homeostasis. In prostate adenocarcinoma, AR activity is switched from guiding cell differentiation to the induction of cell proliferation. In the earliest stages of the disease, the proliferation of prostate cancer cells is dependent on AR, and the most effective therapy is androgen deprivation. However, in later stages, in castration-resistant prostate cancer (CRPC), cells can proliferate even with reduced levels of testosterone in the blood. Mutations occurring in the AR gene and intratumoral production of androgens by unknown mechanisms are able to maintain AR activation, sustaining proliferation of tumor cells and their metastasis (9). Thus, AR signaling seems critical not only for the growth of androgen-dependent prostate cancer, but for development of CRPC.

It is known that the molecular chaperone HSP90 is required for the stability, maturation, and function of AR (10, 11). HSP90 inhibition prevents DHT-dependent nuclear translocation of AR, suggesting a virtual role for HSP90 in AR-dependent signaling (12). HSP90 undergoes various posttranslational modifications, including acetylation at K294. Deacetylation of K294 plays an important role in regulating the HSP90 chaperone cycle and its binding to client proteins, including AR (13). In prostate cancer...
cells, HSP90 hyperacetylation at K294 after treatment with inhibitors of histone deacetylases (HDAC) or HDAC6 siRNAs results in dissociation of HSP90 from AR, with consequent proteasomal degradation and attenuation of AR signaling (14–16). Thus, HDAC6 influences androgen signaling in prostate cancer via deacetylation of HSP90 and the subsequent maturation of AR and its intranuclear translocation (8, 10).

In recent years, several independent studies have shown that the activity of HDACs is linked to the initiation and progression of various cancers (17). HDAC6 distributes over the nucleus and cytoplasm, with predominant cytoplasmic localization in proliferative cells, while differentiation and arrest of the cell cycle correspond to its higher concentration in the nucleus (18). Currently, however, the mechanism of cytoplasmic retention of HDAC6 is poorly understood. It has also been assumed that the deacetylase activity of HDAC6 is linked to its phosphorylation (19). Particularly, Ser22 of HDAC6 is a target of a Ser/Thr kinase, glycogen synthase kinase β (GSK3β), which acts as a cytoplasmic-specific activator of the deacetylation activity of HDAC6 in neurons (20).

It has been demonstrated that EtOH activates GSK3β through the dephosphorylation at Ser 9 (21). GSK3β has also been implicated in prostate cancer progression because the elevated level of GSK3β was found to be correlated with hormone-independent, but AR-mediated, gene expressions (22, 23). Different groups found that GSK3β predominantly and intriguingly localizes in the cis- and trans-faces of the Golgi apparatus. Knockdown (KD) of GSK3β also caused Golgi disorganization and altered trafficking in both HeLa and neuronal cells (24, 25). Therefore, under normal circumstances, GSK3β and its client protein, HDAC6, do not overlap with each other, because they occupy distinct spaces: GSK3β in the Golgi, and HDAC6 in the cytoplasm and nucleus. Given the grand role for HDAC6 and its phosphorylation in the progression of prostate cancer, it is logical to raise a mechanistic question: whether GSK3β translocates to the cytoplasm in advanced prostate cancer cells, and whether the EtOH link to the progression of prostate cancer is mediated inter alia via a GSK3β/HDAC6-dependent pathway.

We recently showed that in hepatocytes, alcohol-induced Golgi disorganization is caused by inactivation of Sar1a GTPase, the protein initiator for the formation of COPII vesicles (26). This process interrupts dimerization of the largest Golgi matrix protein, giantin, which controls the structural integrity of Golgi (27).

It is important to note that the giantin dimer is required for successful Golgi targeting of different proteins (28, 29) and that EtOH-induced giantin dimerization and its subsequent degradation results in mislocalization of giantin-sensitive proteins, from the Golgi to the cytoplasm (30, 31). We were intrigued to observe the identical phenomenon in prostate cancer cells. In androgen-responsive low-passage LNCaP cells, Golgi appears as a compact structure that localizes in juxtapanelux space; however, in androgen-restrictive PC-3 and DU145 cells, Golgi is disorganized (32). This leads to mistargeting of key core-2 O-glycosylation enzymes, cardinally changes sugar modifications and results in the formation of pro-oncogenic glycan epitopes (32, 33). Thus, we hypothesize that alcohol-induced phosphorylation of HDAC6 may arise from Golgi disorganization and the subsequent translocation of GSK3β to the cytoplasm.

Here, we observed that Golgi fragmentation in tissue sections of prostate cancer is more prominent in alcohol-dependent persons than in nondrinkers. Using different approaches, we found that Golgi localization for GSK3β is controlled by giantin. EtOH administration reduces giantin and induces robust redistribution of GSK3β to the cytoplasm. The same phenomenon was detected in high-grade prostate cancer tissues, particularly in patients who abuse alcohol, and was seen to accelerate phosphorylation of HDAC6, thus blocking its import into the nucleus. HDAC6-P deacetylates HSP90, and the latter activates AR intranuclear translocation. Thus, we provide the link between the structural organization of Golgi and HDAC6/AR-mediated carcinogenesis, which is aggravated by alcohol treatment.

Materials and Methods

Antibodies

The primary antibodies used were: (i) rabbit polyclonal – giantin (Novus Biologicals, NB22-22321), giantin (Abcam, ab24586 and ab93281), HDAC6 (Abcam, ab117516), HDAC6-P (Abcam, ab1058, and Sigma, SAB4504196), GSK3β (Abcam, ab107166, and Fitzgerald, 20R-2187), AR (androgen receptor; Santa Cruz Biotechnology, sc-816), Lamin B1 (Abcam, ab16048), HSP90-α acetyl-specific K294 (Rockland Immunocchemicals, 600-401-981), and Ki67 (Abcam, ab15580); (ii) rabbit monoclonal – GM130 (Abcam, ab25649), GRASPE65 (Abcam, ab174834), and ADH1 (Abcam, ab108203); (iii) mouse monoclonal – GRASPE65 (Santa Cruz Biotechnology, sc365434), β-actin (Sigma, A2282), giantin (Abcam, ab37266), and HSP90 (Abcam, ab58950); (iv) mouse polyclonal – GM130 (Abcam, ab169276). The secondary antibodies (Jackson ImmunoResearch) were: (i) HRP-conjugated donkey anti-rabbit and donkey anti-mouse for Western blotting (W-B; 711-035-152 and 715-035-151, respectively); (ii) donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (115-546-003 and 711-585-152, respectively).

Cell culture and drug treatment

LNCaP and 22Rv1 cells are purchased from ATCC. Cells were grown in phenol red-free RPMI medium with 11 mmol/L glucose, 10% FBS, 2 mmol/L glutamine, nonessential amino acids, and 100 U/mL of penicillin plus streptomycin. Given their androgen responsiveness, cells were cultured under treatment with 10 nmol/L dihydrotestosterone (DHT). The immortalized human prostate cell line RWPE-1 was from ATCC, and cells were grown in keratinocyte serum-free medium, which contains 50 μg/mL of BPE and 5 ng/mL EGF, plus an antibiotic/antimycotic mixture (penicillin, 100 U/mL, streptomycin 100 μg/mL, and fungizone, 25 μg/mL). Twenty-four hours after seeding cells at ~75% confluence, culture media were changed for one containing 50 mmol/L EtOH for another 96 hours. The medium was replaced every 48 hours to maintain a constant EtOH concentration. Control cells were seeded at the same time as treated cells and maintained in the same medium; EtOH was replaced by the appropriate volume of medium to maintain similar caloric content. To investigate whether EtOH metabolites are responsible for the Golgi disorganization, cells were exposed for up to 48 hours to the acetaldehyde-generating system (AGS) added directly to the culture medium. The AGS included yeast ADH (0.02 U/mL), 2 mmol/L NAD and 50 mmol/L EtOH. The generation of acetaldehyde measured by gas chromatography in the medium corresponds to the physiologic concentrations observed in the liver of alcohol consumers (34). 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from...
In vivo Western Iowa HCS, and the University of Nebraska Medical Center (Omaha, NE). This protocol was approved by the Institutional Animal Care and Use Committee (Omaha, NE).

In vivo xenograft model
Male athymic nude mice (BALB/c nu/nu, 20–22 g, 5–6 weeks old; Jackson Laboratory) were individually housed in filter-top cages at the UNMC animal facility and consumed food and fresh tap water ad libitum. Food consumption and body weights were recorded weekly. The animals received alcohol orally in drinking water. The amount of alcohol was increased gradually from 4% to 14% within one week (36). Control mice received water with the appropriate isocaloric amount of sucrose. On the first day of 14% EtOH administration, both groups were inoculated with LNCaP (c-28) cells by subcutaneous injection in the flank 2 × 10³ cells in 50 μL of plain RPMI medium plus 50 μL Matrigel (Corning). Tumor volume (mm³) was measured weekly throughout the study. This protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (Omaha, NE).

Immunoprecipitation and transfection
For the identification of proteins in the complexes pulled down by immunoprecipitation (IP), confluent cells grown in a 175 flask were washed three times with 6 mL PBS each, harvested by trypsinization, and neutralized with the FBS-containing medium. IP steps were performed using antibodies that are covalently coupled to Dynabeads M-270 Epoxy beads (Pierce) according to the manufacturer’s instructions. All cell lystate samples for IP experiments were normalized by appropriate proteins. To determine whether the target protein was loaded evenly, the input samples were preliminarily run on a separate gel with different dilutions of control samples versus treated, then probed with anti-target protein Abs. The intensity of obtained bands was measured as the mean of integrated fluorescence intensity ± SD (arbitrary units, a.u.).

Confocal immunofluorescence microscopy
Staining of cells was performed by methods described previously (37). Normal human prostate and prostate adenocarcinoma tissue arrays were purchased from US Biomax and Novus Biologicals. After deparaffinization and dehydration, tissues were blocked in 1% donkey serum for 1 hour at room temperature and incubated with the primary antibodies for 3 hours at room temperature. After washing with PBST three times, the slides were incubated with Alexa Fluor secondary antibodies for 1 hour at room temperature. The nuclei of the tissues were counterstained with DAPI (Invitrogen). Slides were examined under an LSM 800 Zeiss Airyscan microscope performed at the Advanced Microscopy Core Facility of the University of Nebraska Medical Center (Omaha, NE). Images were analyzed using ZEN 2.1 software and IMARIS versions 7.2.2–7.6.0 (Bitplane Scientific). For some figures, image analysis was performed using Adobe Photoshop and ImageJ. Statistical analysis of colocalization was performed by ImageJ, calculating the Pearson correlation coefficient. To assess the fluorescence of HDAC6 in nuclei, the area of nuclei was highlighted in the ImageJ followed by their cropping and quantification of fluorescence signal (Supplementary Fig S1), which was measured as the mean of integrated fluorescence intensity ± SD (arbitrary units, a.u.).

Three-dimensional structured illumination (3D-SIM) microscopy and image analysis
SIM imaging of Golgi ribbons was performed on a Zeiss ELYRA PS.1 superresolution scope (Carl Zeiss Microscopy) using a Pco. Edge 5.5 camera equipped with an MA 1.4 oil objective. Optimal grid sizes for each wavelength were chosen according to manufacturer’s recommendations. For 3D-SIM, stacks with a step size of 110 nm were acquired sequentially for each fluorophore, and each fluorescent channel was imaged with three pattern rotations with three translational shifts. The final SIM image was created using modules built into the Zen Black software suite accompanying the imaging setup. Analyses were undertaken on 3D-SIM data sets in 3D using IMARIS versions 7.2.2–7.6.0 (Bitplane Scientific). The 3D mask was obtained by applying a Gaussian filter to merged channels, thresholding to remove low-intensity signals, and converting the obtained stack into a binary file that mapped all voxels of interest for coefficient calculation. For colocalization, the module “Colocalization Module” was used. To avoid subjectivity, all thresholds were automatically determined using algorithms based on the exclusion of intensity pairs that exhibit no correlation (31). 3D animation was also generated using IMARIS “Animation Module.”

Soft agar assay for colony formation
For the anchorage-independent assay, LNCaP cells were seeded in 60-mm Petri dishes, and a base of 0.5% agar + 1× RPMI + 10% FBS was prepared together with a top of 0.3% agar + 2× RPMI + 20% FBS. Cells were incubated at 37°C in a humidified incubator for 10 days and treated with or without EtOH. After 10 days, colonies were observed in microscope, and the number of colonies was quantified in 20 randomly selected areas. To count all the colonies seeded at the different levels of agar, the focal depth of the microscope was adjusted manually for each area. Then, the colonies were stained with 0.5% crystal violet in 25% methanol for 10 minutes and washed extensively with water until colonies became visible.

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Cell adhesion assay

Adhesion of cancer cells was analyzed with CytoSelect Tumor-Endothelium Adhesion Assay (Cell Biolabs, INC.). A total of 5 × 10^4–1 × 10^5 endothelial cells were seeded on gelatin to each well of 96-well tissue culture plate and incubated until the formation of a monolayer. LNCaP cells treated with or without EtOH were harvested, labeled with CytoTracker, added to endothelial cells, and incubated for 1 hour at 37°C. Then, the wells were washed for three times to remove non-adherent cells; the adherent cells were lysed and incubated for 5 minutes in shaking at room temperature. The fluorescence was determined using a microplate reader (Synergy H4, BioTek) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Cell migration assay

For assay cell migration, 2 × 10^4 cells suspended in 200 μL RPMI1640 medium without FBS were seeded onto the fibronectin-coated polycarbonate membrane of a Transwell insert (Corning). For alcohol treatment, 50 mmol/L EtOH was added to the medium in presence or absence of 5 mmol/L pyrazole; medium was replaced every 24 hours. A volume of 600 μL RPMI1640 with 10% FBS was added as a chemoattractant in the bottom chamber. Following incubation for 72 hours at 37°C in a 5% CO₂ atmosphere, the Transwell insert was washed with PBS and the cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with ice-cold 100% methanol for 10 minutes, washed with PBS for three times, stained with DAPI, and finally air-dried. The migrated cells were counted using the EVOS AMF-4300 microscope (AMG) microscope, and the representative phase-contrast digital images captured with a 20× lens.

Miscellaneous

Protein concentrations were determined with the Coomassie Plus Protein Assay (Pierce Chemical Co). The results shown are representative of three independent experiments. Data are expressed as mean ± SEM. The analysis was performed using a two-sided t test. A value of P < 0.05 was considered statistically significant.

Results

The effect of alcohol on Golgi and tumor development in vitro and in vivo

It is known that EtOH administration alters the structure of the Golgi apparatus in different organs, including the prostate (38). To validate the effect of chronic alcohol administration on rat prostate, we used the protocol of EtOH containing Lieber-DeCarli diet for 5–7 weeks, described in Materials and Methods and widely established by our groups (39). EtOH-exposed rats show histologic evidence of Golgi disorganization: in the ventral prostate lobes of control rats, Golgi (stained by giantin) appear as a compact perinuclear structure (Fig. 1D, control; Supplementary Movie S1), in EtOH-treated cells, multiple fragments of Golgi were detected (Fig. 1D, EtOH; Supplementary Movie S2) resembling the disorganized Golgi in PC-3 cells (32). The similar Golgi phenotype was observed in cells exposed to the acetaldehyde (Ach)-generating system (AGS; ref. 34; Fig. 1D, AGS). However, when EtOH treatment was carried out in the presence of 5 mmol/L pyrazole, an ADH inhibitor, the degree of Golgi fragmentation was significantly reduced and identical to control (Fig. 1D, EtOH + pyrazole). Statistical analysis of the number of Golgi fragments indicates that EtOH metabolite Ach is the primary contributor of EtOH-induced Golgi fragmentation in prostate cells (Fig. 1E).

To assess whether the growth of tumors is associated with the ability of cells to metastasize, we performed soft agar assay for anchorage-independent analysis of LNCaP cells treated with EtOH. Given that we kept EtOH treatment no more than 10 days, we were able to detect only those colonies with a diameter of less than 200 μm (Fig. 2A). As shown in Fig. 2B, EtOH-treated cells produced a significantly higher number of colonies (35 ± 3 ± 3.2) than control cells (22.3 ± 1.5). Next, to check whether EtOH treatment accelerates extravasation of prostate cancer cells, we employed the cell adhesion assay. We quantified adherence of LNCaP (c-18) cells to the monolayer of the human umbilical vein endothelial cells (HUVEC). In control cells, the scan of relative fluorescence units (RFI) showed within the range of 1.3 ± 0.3. In contrast, in EtOH-treated cells, this parameter was significantly increased to 2.1 ± 0.7 (Fig. 2C). To check the universality of these findings, we tested the migration of another androgen-responsive cell line 22Rv1 (40) using Transwell migration assay. As shown in Fig. 2D, these cells express ADH1, which level was slightly enhanced after EtOH treatment. Also, similarly to LNCaP cells, EtOH-treated 22Rv1 cells demonstrated Golgi fragmentation (Supplementary Fig. S2). Cell counts of the lower surfaces of the Transwell membranes revealed that the migration of 22Rv1 cells was significantly increased following EtOH treatment when compared with control cells or EtOH-treated cells in presence of pyrazole (55 ± 18 for control, 220 ± 39 for EtOH, and 67 ± 11 for EtOH+pyrazole; Fig. 2E and F).

Alcohol-induced cytoplasmic retention of HDAC6 is associated with its phosphorylation

Recent observations suggest that HDAC6 is required for efficient oncogenic transformation, anchorage-independent proliferation, tumor growth, and cell adhesion (41, 42). Given the grand role for cytoplasmic HDAC6 in AR-dependent carcinogenesis (14–16), we might ask whether highly aggressive prostate cancer is characterized by the shift of HDAC6 from the nucleus to the cytoplasm, and if so, whether this is associated with phosphorylation of this histone deacetylase. To test this possibility, we first analyzed fluorescence intensity (FI) of HDAC6 in
Alcohol Is Critical for AR Signaling

Figure 1.
The effect of alcohol on prostate cells in vitro and in vivo. A, Immunostaining of Golgi (giantin, white) in prostate tissue from the control or EtOH-fed rats. Nucl. were counterstained with DAPI (gray); bars, 5 μm. B, Statistical analysis of cells with fragmented Golgi in cells presented in A; means ± SD; ***, P < 0.001, n = 2 rats for each series of experiments. C, ADH Western blot analysis of the cell lysates from RWPE-1 and LNCaP (c-26) cells before and after EtOH treatment (50 mmol/L for 96 hours). Lysses were normalized by β-actin. D, Immunostaining of giantin (white) in LNCaP (c-26) cells: control, treated with EtOH (50 mmol/L, for 96 hours), exposed to AGS, and treated with EtOH in the presence of 5 mmol/L pyrazole; scale bars, 10 μm. E, Quantification of cells with fragmented Golgi; means ± SD; ***, P < 0.001, n = 60 cells for each series of experiments. All confocal images were acquired with the same imaging parameters. F, EtOH administration induces growth in LNCaP tumor xenograft. Mice received 14% EtOH, the control group received water with the appropriate isocaloric amount of sucrose. G, Statistical analysis of the growth of the tumor; means ± SD; ***, P < 0.001, n = 4 mice from each group were observed.

Tissue from the ex vivo human prostate cancer specimens. We were attempting to compare HDAC6 FI in cytoplasm versus nucleus and found that, due to well differentiation, in the normal prostate and low-grade prostate cancer, visualization of the individual luminal cells was effortless. However, in high-grade, poorly differentiated prostate cancer, despite the high cytoplasmic content of HDAC6, the calculation of FI in the individual tumor cells was unfeasible. We have thus decided to measure HDAC6 FI only in the nuclei (Fig. 2C). Prostate cancer with Gleason scores of 5 to 6 were considered as moderately differentiated; 7 as moderate to poor; and 8–10 as poor (43). However, the intranuclear signal of HDAC6 in patients with Gleason 8 was comparable with Gleason 6 and 7, and no significant difference was detected between these groups and normal tissue. In the meantime, the nonparametric two-sided Wilcoxon rank-sum test showed that HDAC6 FI in Gleason 6–8 and Gleason 9–10 groups are significantly different (Fig. 2H). Our data fit well with recent clinical analysis, which indicates that patients with prostate cancer biopsy Gleason 9 and 10 have a significantly worse prognosis than do patients with Gleason 8 (44). Thus, these data suggest that clinical manifestation of prostate cancer is associated with reduced nuclear HDAC6, and this may serve as a prognostic factor.

As a second step toward addressing the question of the link between cytoplasmic HDAC6 and the progression of prostate cancer, we investigated tissue sections obtained from the tumor foci of patients with prostate cancer provided by the Department of Pathology and Microbiology (IRB protocol #304-16-EP) at the University of Nebraska Medical Center. We compared the nuclear HDAC6 in patients with prostate cancer with the same grade and Gleason score: nondrinking patients versus patients who frequently consume alcohol (more than 40 years of alcohol consumption, frequency, and dosage equaling 12 oz. beer 1–2 × daily plus 4 oz. glass of wine weekly; Fig. 3A). As presented in Fig. 3B, we could not detect significant differences in intranuclear HDAC6 between normal prostate and tissues of nondrinkers with Gleason 7 (1.4 ± 0.3 and 1.2 ± 0.3, accordingly). However, in the prostate cancer tissue from the alcohol-dependent patients, with the same Gleason grade and score, the fluorescence signal of intranuclear HDAC6 was robustly reduced (0.5 ± 0.1).

To validate these observations, we performed in vitro alcohol administration. To understand the basis of EtOH-induced carcinogenesis, it is important to examine the distribution of HDAC6 in nonmalignant prostate cells, such as the human prostate epithelial RWPE-1 cell line, as a baseline to which the EtOH insult can be compared. Of interest, in the control RWPE-1 cells, HDAC6 was predominantly detected in the nucleus; the ratio of nucleus/cytoplasm for HDAC6 FI was 3.6 ± 0.2 (Fig. 3C and D). Whereas in control LNCaP (c-26) cells, this parameter was reduced to 2.5 ± 0.3, it still demonstrated the prevalent intranuclear localization of HDAC6 (Fig. 3C and D). However, in both RWPE-1 and LNCaP cells treated with 50 mmol/L EtOH for 96 hours, HDAC6 shifted from nucleus to cytoplasm, as indicated by the significant reduction of ratio nucleus/cytoplasm (0.5 ± 0.2 and 0.3 ± 0.15 in RWPE-1 and LNCaP, respectively; Fig. 3C and D). The HDAC6 and phosphorylated HDAC6 (Ser 22, HDAC6-P) Western blot of cytoplasmic and nuclear fractions isolated from the control and EtOH-treated LNCaP (c-26) cells indicate that the increase of total cytoplasmic HDAC6 in EtOH-treated samples is
Figure 2.
EtOH treatment stimulates anchorage-independent growth, adherence, and migration in low-passage LNCaP and 22Rv1 cells. **A**, Anchorage-independent colony formation was measured by the soft agar assay for LNCaP cells treated with or without 50 mmol/L of EtOH, as described in Materials and Methods. After 10 days, colonies were observed at EVOS AMF-4300 microscope (AMG) microscope, and the representative phase-contrast digital images captured with a 20× lens; scale bars, 200 μm. **B**, Quantification of the number of colonies in 20 randomly selected areas for control and EtOH-treated LNCaP cells. Data were collected from three independent experiments of soft agar assay and expressed as a mean ± SD; *, *P* < 0.01. **C**, Quantification of adherent LNCaP cells after cultivation on HUVEC monolayers. Cells were incubated for 1 hour on the monolayer and nonadherent cells were washed away before lysis of adherent cells. Relative fluorescence unit (RFU) was quantified by the microplate reader at 480–520 nm. Data presented as means ± SD from three independent experiments; **,** *P* < 0.001. **D**, ADH1 Western blot analysis of the cell lysates from 22Rv1 cells before and after EtOH treatment (50 mmol/L for 96 hours). Lysates were normalized by β-actin. **E**, Migration of 22Rv1 cells: control, treated with 50 mmol/L EtOH for 96 hours, and treated with EtOH in presence of 5 mmol/L pyrazole; scale bars, 200 μm. Cell migration was measured via the Transwell chamber assay, as described in Materials and Methods. **F**, Quantification of the migrated cells for the cells presented in **E**. Data expressed as a mean ± SD; **,** *P* < 0.001. **G**, Immunostaining of HDAC6 (red) in the representative normal prostate tissues and prostate cancer tissues with different Gleason scores. Slides were stained with anti-HDAC6 antibody and analyzed as indicated in Materials and Methods. White boxes indicate nuclear areas enlarged and shown at the right side. Nuclei were counterstained with DAPI (blue). All confocal images were acquired with the same imaging parameters; scale bars, 10 μm. **H**, Quantification of integrated fluorescence intensity (a.u.) of HDAC6 in nuclei from the tissue samples: normal, prostate cancer with a Gleason score of 6–8, and prostate cancer with a Gleason score of 9–10. Groups were tested for normality with the Lilliefors test, and the normality of values was rejected. The nonparametric two-sided Wilcoxon rank-sum test was used to test for difference of medians between groups; means ± SD, *, *P* < 0.05.
mainly determined by HDAC6-P (Fig. 3E and F). Moreover, we noted that HDAC6-P could not be detected in the nuclear fraction. Conversely, the AR content in the nucleus is increased in the EtOH-treated sample. Further densitometric analysis indicates that this shift is associated with the robust reduction of AR in the cytoplasm (Fig. 3F), confirming that HDAC6 is the client protein...
for GSK3β (20). Importantly, when LNCaP cells were exposed to EtOH in medium lacking DHT, we could not detect any significant difference in the cytoplasmic or nuclear AR (Supplementary Fig. S3A), indicating that alcohol-induced translocation of AR into the nucleus is mediated through its interaction with DHT. Next, in LNCaP cells exposed to alcohol, we observed an increased complex between HSP90 and AR (Fig. 3H), and, predictably, decreased acetylated HSP90 (Fig. 3I). Importantly, cotreatment with pyrazole blocks alcohol-induced deacetylation of HSP90, suggesting the critical role of the ADH-generated metabolites for the activation of HDAC6 pathway (Fig. 3J). Similarly, when EtOH administration of LNCaP cells was performed in presence of HDAC6 siRNAs, the level of HDAC6-P was subsequently reduced, but the expression of acetylated HSP90 was identical to the control cells (Fig. 3J). Thus, these experiments imply that alcohol stimulates cooperation between AR and HSP90, and such interaction is directly regulated by the GSK3β-mediated phosphorylation of HDAC6. To validate this assumption, we cotreated alcohol-exposed LNCaP cells with 17-AAG, the drug that induces hyperacetylation of HSP90 (45). We found that treatment with 25 nmol/L 17-AAG blocks EtOH-induced nuclear shift of AR (Fig. 3K). Notably, this reduction was not induced by apoptosis, because the level of cleaved caspase-3 remained unchanged under treatment with this dosage of 17-AAG (Supplementary Fig. S3B). Finally, to examine the EtOH effect on the AR transactivation, we assayed the expression of prostate-specific antigen (PSA), because AR is the primary regulator of PSA expression through androgen response elements located in the PSA promoter (46). As shown in Fig. 3L, a robust increase of PSA level was detected in LNCaP cells exposed to either EtOH or AGS.

Relocation of GSK3β in the advanced prostate cancer and after alcohol administration

The intra-Golgi position of GSK3β and cytoplasmic/nuclear localization of HDAC6 in normal cells and cells with low proliferation (24, 25) prompts us to suggest that EtOH-induced phosphorylation of HDAC6 is caused by the relocation of GSK3β, which is highly likely a direct consequence of Golgi disorganization described above. To check this possibility, we first analyzed the Golgi morphology in the prostate cancer patients with the history of heavy alcohol consumption and patients that has no alcohol dependence, as described above. The number of cells with fragmented Golgi was assessed by quantifying cells with more than two Golgi fragments for five fields of view. As shown in Fig. 4A–C, the number of cells (four patients in each group) with the same clinical stages) with fragmented Golgi was significantly higher in prostate cancer cells of drinkers than nondrinkers, confirming that alcohol consumption may be a critical factor contributing to Golgi disorganization of prostate cancer cells.

We next compared the intracellular localization of GSK3β in the prostate cancer tissue with a different Gleason score. Predictably, in the luminal cells of the normal prostate tissue, we detected the strong colocalization of GSK3β with giantin, convincing us that under normal circumstances this kinase is a residential Golgi protein (Fig. 5A and B, normal). Contrary to the compact perinuclear signal in control, the fluorescence signal of GSK3β in prostate cancer tissue was distributed across a much higher number of objects (Fig. 5A, Gleason 6–10). In groups with Gleason 6, 7, or 8, we were still able to detect a moderate colocalization with giantin; however, in groups with Gleason 9 and 10, the overlay of GSK3β with giantin was significantly reduced. Quantification of Pearson coefficient revealed that, regardless of Gleason score, all cases of prostate cancer demonstrated a strong cytoplasmic signal for GSK3β (Fig. 5B). However, similar to HDAC6 (Fig. 2G and H), the difference in this parameter among patients with prostate cancer became evident when we combined patients with a Gleason of 6–8 and those with a Gleason of 9–10 in the two separate groups (Fig. 5C). The Pearson coefficient was as follows: 0.9 ± 0.06 in normal, 0.6 ± 0.13 in Gleason 6–8, and 0.45 ± 0.18 in Gleason 9–10. In sum here, these data clearly indicate that progression of prostate cancer is accompanied by mislocalization of GSK3β.

To address the mechanism of GSK3β translocation into the cytoplasm, the roles of critical Golgi matrix dimeric proteins, giantin, GM130, and GRASP65, were investigated. In our previous publications, we found that giantin and GM130-GRASP65 represent different Golgi docking sites (29, 31, 32). Of note, in hepatocytes, alcohol induces dedimerization of giantin and...
reduces its level; however, it has no significant impact on the GM130–GRASP65 complex (26). This could potentially block the Golgi targeting of giantin-sensitive proteins. In light of this, it seems reasonable to classify GSK3β as a protein that requires giantin for trafficking to the Golgi and for retention. Indeed, in control untreated LNCaP (c-14-16) cells, GSK3β resides in the Golgi, but in EtOH-treated cells, Golgi disorganization is affiliated with a scattering of GSK3β signal and segregation with giantin (Fig. 6A, B, and K). In these cells and similarly to hepatocytes (26), EtOH treatment notably results in the reduction of giantin level (Fig. 6G). Hence, we next analyzed the distribution of GSK3β in giantin-depleted cells. As shown in Fig. 6C and D, in cells lacking detectable giantin after GOLGB1 siRNAs treatment, GSK3β begins to lose its compact signal, although giantin knockdown has no significant impact on the perinuclear position of Golgi (refs. 29, 32; Fig. 6C, D, H, and L). We calculated GSK3β in the cytoplasm versus the perinuclear area, as described previously (37), and found that in giantin-depleted cells, this kinase is highly present in the cytoplasm. Also, as we have shown previously (29) and confirmed here (Fig. 6I), giantin knockdown upregulates the level of both GRASP65 and GM130. We therefore next examined whether the GM130–GRASP65 complex could regulate GSK3β localization. We treated LNCaP cells with GRASP65 or GM130 siRNAs and found that despite the lack of either GRASP65 (Fig. 6F and I) or GM130 (Supplementary Fig. S4), the perinuclear and compact signal of GSK3β was indistinguishable from that in control cells (Fig. 6L). Finally, the intimate relationship between giantin and GSK3β was confirmed by giantin IP, which is able to pull down GSK3β (Fig. 6J). Altogether, these data clearly show that giantin is essential for the localization of GSK3β in the Golgi and that mislocalization of GSK3β in the EtOH-treated cells is induced by downregulation of giantin.

**Discussion**

The interference of alcohol with prostate cancer is an area of great importance, given that an increasing number of observations indicate the high rate of incidence and mortality of prostate cancer in alcohol-dependent patients (3, 5–7). Here, for the first time, we show that AR-dependent carcinogenesis is clearly influenced by alcohol’s disorganizing effect on the structure and function of Golgi. We provide the existence
of at least two events resulting in the progression of prostate cancer. First, alcohol-induced Golgi fragmentation results in altered Golgi targeting for GSK3β, thereby relocating this kinase to the cytoplasm and facilitating phosphorylation of HDAC6. This process, secondly, blocks export of HDAC6 to the nucleus and retains this enzyme in the cytoplasm. Finally, HDAC6-P deacetylates HSP90 followed by AR maturation and its translocation into the nucleus (Fig. 7).

The large group of kinases has been shown to be involved in the progression of prostate cancer, particularly in the development of metastatic CRPC (4, 47). These findings naturally suggest kinase inhibitors as an attractive and promising therapeutic intervention.
The relationship between different classes of kinases and Golgi is considering them as acting in close cooperation. On the one hand, Golgi serves as a hub of phosphorylation (48, 49), yet on the other, Golgi morphology and intra-Golgi trafficking are controlled by multiple protein kinase pathways, including those that are regulated by GSK3β (25, 50–52). Here, we described the phenomenon of GSK3β translocation from its primary residence within the Golgi to the cytoplasm. Similar behavior was observed with interest for other Ser/Thr protein kinases. For instance, the catalytic subunit of PKA (c-AMP-dependent protein kinase) is also imported to the cytoplasm en route to the nucleus (54). We found that Golgi localization of GSK3β requires giantin, because: (i) giantin forms a complex with GSK3β, and (ii) the knockdown of giantin significantly enhances cytoplasmic pattern of GSK3β.

How exactly does GSK3β reside in the Golgi membranes? We and others have shown that the cytoplasmic tail of glycosyltransferases is responsible for their Golgi localization (55–57). However, contrary to most Golgi resident and matrix proteins, GSK3β has neither a cytoplasmic nor transmembrane domain. There are not many possibilities that can be considered here. The first, and most simple, is that GSK3β is not only targeted to the Golgi via giantin but that it is also retained via this golgin. Another avenue to consider is that recruitment of GSK3β to the Golgi membranes requires an active form of Rab GTPases. Neither model rules out the other, but both require rigorous study.

Of note, in both EtOH-treated LNCaP cells and low-grade prostate cancer tissues, GSK3β, despite the shift to the cytoplasm, partially maintains residency in the Golgi. This implies that in these cells, some Golgi membranes are still maintained by the dimeric giantin. However, the amount of GSK3β that is distributed into the cytoplasm seems sufficient for the phosphorylation of HDAC6. In high-grade prostate cancer, the presence of GSK3β in the cytoplasm is robustly increased, while in the Golgi it is negligible, and the number of Golgi fragments is much higher than that found in low-grade prostate cancer. Our data are echoing the large clinical analysis of prostate cancer surgical specimens from 499 patients, which observed that increased cytoplasmic accumulation of GSK3β is positively correlated with aggressive clinicopathologic parameters, including late clinical stage, lymph node metastasis, extracapsular extension, high Gleason score, and 2-fold shorter recurrence-free survival, with up to 12-year follow-up (58). It is also important to note that knockdown of GSK3β alters the Golgi architecture, suggesting that the function of this kinase is required for the maintenance of Golgi architecture (24, 25). Therefore, GSK3β deficiency in the Golgi, induced by the alcohol’s effect on its Golgi partner giantin, aggravates Golgi disorganization. Moreover, it appears that the effect EtOH has on the Golgi imitates the Golgi phenotype in advanced prostate cancer cells. Thus, alcohol could potentially accelerate Golgi fragmentation and play a crucial role in the development of metastatic CRPC. While this concept needs further validation, our data from anchorage-independent growth, adhesion, and migration assays make this a likely scenario.

Here, our analysis revealed that EtOH induces HDAC6 phosphorylation, and that prevents the transport of HDAC6 to the nucleus. Other members of HDAC family were also shown to be remarkably compartmentalized after phosphorylation. In particular, HDAC4, HDAC5, and HDAC7 were found to be retained in the cytoplasm after phosphorylation in several phosphorylation sites, followed by the enhanced binding to their client proteins (59–62). However, we found for the first time that accumulation of HDAC6 in the cytoplasm, and accordingly, its lack in the nucleus, are more prominent in patients with a high Gleason score. The lack of HDAC6 in the nucleus was significantly visible in the tissue of alcoholic patients with prostate cancer, implying that the intranuclear signal of HDAC6, in addition to the level of Golgi fragmentation, could serve as a reliable prognostic factor for aggressiveness in prostate cancer.

We have recently shown that among other motor proteins closely associated with Golgi, the nonmuscle Myosin IIA (NMIIA) interacts directly with Golgi resident proteins (63). We and others have also reported that NMIIA is tethered to the Golgi membranes under various stress conditions, such as treatment with Brefeldin A, heat shock, or the inhibition
of HSPs, and by depletion of beta-COP (37, 63, 64). Depletion or inhibition of NMIIA by Blebbistatin prevents Golgi disorganization and restores compact Golgi morphology in highly metastatic prostate and colon cancer cells (32, 37). Moreover, restoration of compact Golgi morphology makes PC-3 and DU145 androgen-negative cells more susceptible to galexin-1-induced apoptosis due to the recovery in Golgi core-2 O-glycosylation enzyme (32). Understanding that the actomyosin complex forces EtOH-induced Golgi disorganization (30), we suggest that targeting NMIIA may be important for preventing the damaging effects of alcohol metabolism on the prostate cancer cell and preventing disease progression. So far, it has been shown that targeting NMIIA by Blebbistatin may block invasiveness of both breast cancer cells and pancreatic adenocarcinoma cells (65, 66). Our unpublished observations also indicate that inhibition or knockdown of NMIIA blocks alcohol-mediated Golgi fragmentation.

In sum, our data are among the very first detailed studies correlating the mechanisms of alcohol-induced Golgi disorganization within the progression of prostate cancer. These results explore the basic biology of prostate cancer carcinogenesis and elucidate the role of the Golgi complex in the development and progression of prostate cancer. They also establish a link between alcohol consumption and the functional activation of proncogenic proteins.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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