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

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Abstract

Multiple epidemiological observations and meta-analysis clearly indicate the link between alcohol abuse and the incidence and progression of prostate cancer (PCa); 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 (GSK3 β), primarily resides in the Golgi. Progression of PCa is accompanied by Golgi scattering, translocation of GSK3 β 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 GSK3 β ; this phenomenon was validated in giantin-depleted cells. Redistribution of GSK3 β 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 PCa 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.

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Keywords

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Introduction

In the United States, prostate cancer (PCa) is among the leading causes of cancer death in men (1). A growing number of epidemiological studies indicate a connection between frequent alcohol consumption and the incidence of PCa, as well as to the high rate of PCa 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 PCa 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 PCa, but for development of CRPC.

It is known that the molecular chaperone heat shock protein 90 (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 post-translational 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 PCa 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 PCa 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 (GSK3 β), which acts as a cytoplasmic-specific activator of the deacetylation activity of HDAC6 in neurons (20).

It has been demonstrated that EtOH activates GSK3 β through the de-phosphorylation at Ser 9 (21). GSK3 β has also been implicated in PCa progression because the elevated level of GSK3 β was found to be correlated with hormone-independent, but AR-mediated, gene expressions (22,23). Different groups found that GSK3 β predominantly and intriguingly localizes in the *cis*- and *trans*-faces of the Golgi apparatus. Knockdown (KD) of GSK3 β also caused Golgi disorganization and altered trafficking in both HeLa and neuronal cells (24,25). Therefore, under normal circumstances, GSK3 β and its client protein, HDAC6, do not overlap with each other, since they occupy distinct spaces: GSK3 β in the Golgi, and HDAC6 in the cytoplasm and nucleus. Given the grand role for HDAC6 and its phosphorylation in the progression of PCa, it is logical to raise a mechanistic question: whether GSK3 β translocates to the cytoplasm in advanced PCa cells, and whether the EtOH link to the progression of PCa is mediated *inter alia* via a GSK3 β /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 de-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 PCa cells. In androgen-responsive low-passage LNCaP cells, Golgi appears as a compact structure that localizes in juxtanuclear 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 GSK3 β to the cytoplasm.

Here, we observed that Golgi fragmentation in tissue sections of PCa is more prominent in alcohol-dependent persons than in non-drinkers. Using different approaches, we found that Golgi localization for GSK3 β is controlled by giantin. EtOH administration reduces giantin and induces robust redistribution of GSK3 β to the cytoplasm. The same phenomenon was detected in high-grade PCa 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 heat shock protein 90 (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: a) rabbit polyclonal – giantin (Novus Biologicals, NBP2–22321), giantin (Abcam, ab24586 and ab93281), HDAC6 (Abcam, ab117516), HDAC6-P (Abcam, ab61058, and Sigma, SAB4504196), GSK3 β (Abcam, ab107166, and Fitzgerald, 20R–2187), AR (androgen receptor) (Santa Cruz Biotechnology, sc-816), Lamin B1 (Abcam, ab16048), HSP90- α acetyl specific K294 (Rockland Immunochemicals, 600–401–981), and Ki67 (Abcam, ab15580); b) rabbit monoclonal – GM130 (Abcam, ab52649), GRASP65 (Abcam, ab174834), and ADH1 (Abcam, ab108203); c) mouse monoclonal – GRASP65 (Santa Cruz Biotechnology, sc365434), β -actin (Sigma, A2228), giantin (Abcam, ab37266), and HSP90 (Abcam, ab58950); d) mouse polyclonal – GM130 (Abcam, ab169276). The secondary antibodies (Jackson ImmunoResearch) were: a) HRP-conjugated donkey anti-rabbit and donkey anti-mouse for Western-blotting (W-B) (711–035-152 and 715–035-151, respectively); b) 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 mM glucose, 10% FBS, 2mM glutamine, non-essential amino acids and 100U/ml of Penicillin plus Streptomycin. Given their androgen responsiveness, cells were cultured under treatment with 10 nM 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 μ g/ml of BPE and 5 ng/ml EGF, plus an antibiotic/antimycotic mixture (Penicillin, 100 U/ml, Streptomycin 100 μ g/ml and Fungizone, 25 μ g/ml). Twenty-four hours after seeding cells (at ~75% confluence), culture media were changed for one containing 50 mM EtOH for another 96 h. The medium was replaced every 48 h 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 h to the acetaldehyde generating system (AGS) added directly to the culture medium. The AGS included yeast ADH (0.02 U/ml), 2 mM nicotinamide adenine dinucleotide and 50 mM EtOH. The generation of acetaldehyde measured by gas chromatography in the medium corresponds to the physiological concentrations observed in the liver of alcohol consumers (34). 17-Allylamino-17-demethoxygeldanamycin (17AAG) was purchased from InvivoGen and dissolved in DMSO for the working concentration of 10 nM. Pyrazole was obtained from Sigma and dissolved in DMSO for the working concentration of 5 mM. Cells treated with the corresponding amount of DMSO served as a control.

Alcohol *in vivo* administration

Rats weighing 140–160 g were purchased from Charles River Laboratories. Initially, animals were fed Purina chow diet and allowed to acclimate to their surroundings for a period of 3 days. Then the rats were paired according to weight and fed either control or EtOH containing (35% fat, 18% protein, 11% carbohydrates, 36% EtOH) Lieber-DeCarli diet for

5–7 weeks (Dyets, Inc) (35). The control diet was identical to the EtOH diet except for the isocaloric substitution of EtOH with carbohydrates. This protocol was approved by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs, Nebraska Western Iowa HCS, and the University of Nebraska Medical Center.

***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 injection s.c. in the flank 2×10^6 cells in 50 μ l of plain RPMI medium plus 50 μ l Matrigel (Corning). Tumor volume (mm^3) 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.

Immunoprecipitation (IP) and transfection

For the identification of proteins in the complexes pulled down by IP, confluent cells grown in a T75 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 manufacturer's instructions. All cell lysate 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 vs. treated, then probed with anti-target protein Abs. The intensity of obtained bands was analyzed by ImageJ software, and samples with identical intensity were subjected to IP. Mouse or rabbit non-specific IgG was used as non-specific controls. Proteins were separated on SDS PAGE on mini-gels with various acrylamide % specified for each experiment (Bio-Rad). HDAC6, GOLGB1 (giantin), GOLGA2 (GM130), GORASP1 (GRASP65), and scrambled ON-TARGET *plus* smartpool siRNAs were purchased from Santa Cruz Biotechnology. All products consisted of pools of three target-specific siRNAs. Cells were transfected with 100–150 nM siRNAs using Lipofectamine RNAi MAX reagent (Life science technologies).

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 RT° and incubated with the primary antibodies for 3 hours at RT°. After washing with PBST three times the slides were incubated with Alexa Fluor secondary Abs for 1 hour at RT°. 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. 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 (Fig. S1), which was measured as the mean of integrated fluorescence intensity $\pm$ 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, Germany) using a PCO.Edge 5.5 camera equipped with a Plan-Apochromat 63 $\times$ 1.4 oil objective. Optimal grid sizes for each wavelength were chosen according to manufacturer 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 studies, IMARIS “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 + 1X RPMI + 10% FBS was prepared together with a top of 0.3% agar + 2X 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. In order 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.

Cell adhesion assay

Adhesion of cancer cells was analyzed with CytoSelect™ Tumor-Endothelium Adhesion Assay (Cell Biolabs, INC.). 50,000–100,000 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 h at 37° C. Then, the wells were washed for three times to remove nonadherent cells; the adherent cells were lysed and incubated for 5 minutes in shacking at RT°. 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 μ l RPMI-1640 medium without FBS were seeded onto the fibronectin-coated polycarbonate membrane of a Transwell® insert (Corning). For alcohol treatment, 50 mM EtOH was added to the medium in presence or absence of 5 mM pyrazole; medium was replaced every 24 h. A volume of 600 μ l RPMI-1640 with 10% FBS was added as a chemoattractant in the lower chamber. Following incubation for 72 h 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 min, 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 20x 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 $\pm$ SEM. The analysis was performed using a 2-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 histological evidence of Golgi disorganization: in the ventral prostate lobes of control rats, Golgi (stained by giantin) appear as a compact structure. However, in EtOH-fed rats, they are fragmented (Fig. 1A, B).

The alcohol dehydrogenase (ADH)-catalyzed oxidation of EtOH is a major pathway for EtOH disposition in the body. We found that similarly to hepatocytes, both normal prostate epithelial RWPE-1 cells and LNCaP cells express ADH, which level is increased after EtOH treatment (Fig. 1C). To examine the metabolic basis of EtOH-induced cytotoxicity, we then analyzed Golgi morphology in low-passage (c-26) LNCaP cells treated with 50 mM EtOH for 96 h. For imaging of cells, we employed structured illumination superresolution microscopy (SIM), allowing us to achieve two-color 3D imaging at ~ 110 nm resolution. Whereas in control cells Golgi appeared as a compact perinuclear structure (Fig. 1D, control, and Movie S1), in EtOH-treated cells, multiple fragments of Golgi were detected (Fig. 1D, EtOH, and 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) (34) (Fig. 1D, AGS). However, when EtOH treatment was carried out in the presence of 5 mM 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). Next, we examined the effect of alcohol on LNCaP (c-24) tumor xenografts in athymic male mice. In mice that received water with EtOH, palpable tumors appeared around day 22 after injection. In control, by contrast, tumors were palpable only around day 37 (Fig. 1F, highlighted by black circles). Subsequently, in EtOH-treated mice, tumor volume was significantly larger throughout the observation period (Fig. 1G).

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 PCa 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 (HUVECs). 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 (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 PCa 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 tissue from the *ex vivo* human PCa 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 PCa, visualization of the individual luminal cells was effortless. However, in high-grade, poorly differentiated PCa, despite the high cytoplasmic content of HDAC6, the calculation of FI in the individual tumor cells was unrealizable. We have thus decided to measure HDAC6 FI only in the nuclei (Fig. 2G). PCa with Gleason scores of 5 to 6 consider as moderately differentiated; 7 as moderate to poor; and 8 to 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 non-parametric 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

PCa 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 PCa 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 PCa, we investigated tissue sections obtained from the tumor foci of PCa patients 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 PCa patients with the same grade and Gleason score: non-drinking patients versus patients who frequently consume alcohol (more than 40 years of alcohol consumption, frequency, and dosage equaling 12 oz. beer 1–2x 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 non-drinkers with Gleason 7 (1.4 ± 0.3 and 1.2 ± 0.3 , accordingly). However, in the PCa 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 non-malignant 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 nucleus/cytoplasm for HDAC6 FI was 3.6 ± 0.2 (Fig. 3C, 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, D). However, in both RWPE-1 and LNCaP cells treated with 50 mM EtOH for 96 h, 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, D). The HDAC6 and phosphorylated HDAC6 (Ser 22, HDAC6-P) W-B 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 mainly determined by HDAC6-P (Fig. 3E, 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). Next, the siRNAs-mediated KD of GSK3 β significantly and proportionally reduced the level of HDAC6-P (Fig. 3G), confirming that HDAC6 is the client protein for GSK3 β (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 (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, co-treatment with pyrazole blocks alcohol-induced deacetylation of HSP90, suggesting the critical role of the ADH-generated metabolites for the activation of HDAC6 pathway (Fig. 3I). 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. Thus, these experiments

imply that alcohol stimulates cooperation between AR and HSP90, and such interaction is directly regulated by the GSK3 β -mediated phosphorylation of HDAC6. To validate this assumption, we co-treated alcohol-exposed LNCaP cells with 17-Allylamino-17-demethoxygeldanamycin (17AAG), the drug that induces hyperacetylation of HSP90 (45). We found that treatment with 25 nM 17AAG blocks EtOH-induced nuclear shift of AR (Fig. 3K). Notably, this reduction was not induced by apoptosis, since the level of cleaved caspase-3 remained unchangeable under treatment with this dosage of 17AAG (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 GSK3 β in the advanced PCa and after alcohol administration.

The intra-Golgi position of GSK3 β 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 GSK3 β , which is highly likely a direct consequence of Golgi disorganization described above. To check this possibility, we first analyzed the Golgi morphology in the PCa 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 PCa cells of drinkers than non-drinkers, confirming that alcohol consumption may be a critical factor contributing to Golgi disorganization of PCa cells.

We next compared the intracellular localization of GSK3 β in the PCa tissue with a different Gleason score. Predictably, in the luminal cells of the normal prostate tissue, we detected the strong co-localization of GSK3 β with giantin, convincing us that under normal circumstances this kinase is a residential Golgi protein (Fig. 5A, B, normal). Contrary to the compact perinuclear signal in control, the fluorescence signal of GSK3 β in PCa 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 GSK3 β with giantin was significantly reduced. Quantification of Pearson's coefficient revealed that, regardless of Gleason score, all cases of PCa demonstrated a strong cytoplasmic signal for GSK3 β (Fig. 5B). However, similar to HDAC6 (Fig. 2G, H), the difference in this parameter among PCa patients became evident when we combined patients with a Gleason of 6 to 8 and those with a Gleason of 9 to 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 PCa is accompanied by mislocalization of GSK3 β .

To address the mechanism of GSK3 β 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 de-dimerization 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 GSK3 β as a protein that requires giantin for trafficking to the Golgi and for retention. Indeed, in control untreated LNCaP (c-14–16) cells, GSK3 β resides in the Golgi, but in EtOH-treated cells, Golgi disorganization is affiliated with a scattering of GSK3 β signal and segregation with giantin (Figs. 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 GSK3 β in giantin-depleted cells. As shown in Fig. 6C and D, in cells lacking detectable giantin after *GOLGB1* siRNAs treatment, GSK3 β begins to lose its compact signal, although giantin KD has no significant impact on the perinuclear position of Golgi (29,32) (Figs. 6C, D, H, and L). We calculated GSK3 β 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 KD upregulates the level of both GRASP65 and GM130. We therefore next examined whether the GM130-GRASP65 complex could regulate GSK3 β 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 (Fig. S4), the perinuclear and compact signal of GSK3 β was indistinguishable from that in control cells (Fig. 6L). Finally, the intimate relationship between giantin and GSK3 β was confirmed by giantin IP, which is able to pull down GSK3 β (Fig. 6J). Altogether, these data clearly show that giantin is essential for the localization of GSK3 β in the Golgi and that mislocalization of GSK3 β in the EtOH-treated cells is induced by downregulation of giantin.

Discussion

The interference of alcohol with PCa is an area of a great importance, given that an increasing number of observations indicate the high rate of incidence and mortality of PCa 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 PCa. Firstly, alcohol-induced Golgi fragmentation results in altered Golgi targeting for GSK3 β , thus 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 PCa, 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 GSK3 β (25,50–52). Here, we described the phenomenon of GSK3 β 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) was shown to translocate from Golgi to the nucleus after EtOH treatment, inducing phosphorylation of a cAMP-response element-binding protein (53). In addition, after Brefeldin A or nocodazole treatment, the Golgi resident kinase STK16 (PKL12) is also imported to the cytoplasm en route to the nucleus (54). We found that Golgi localization of GSK3 β requires giantin, because: (a) giantin forms a complex with GSK3 β , and (b) the KD of giantin significantly enhances cytoplasmic pattern of GSK3 β . How exactly does GSK3 β 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, GSK3 β has neither a cytoplasmic nor transmembrane domain. There are not many possibilities that can be considered here. The first, and most simple, is that GSK3 β 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 GSK3 β 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 PCa tissues, GSK3 β , 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 GSK3 β that is distributed into the cytoplasm seems sufficient for the phosphorylation of HDAC6. In high-grade PCa, the presence of GSK3 β 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 PCa. Our data are echoing the large clinical analysis of PCa surgical specimens from 499 patients, which observed that increased cytoplasmic accumulation of GSK3 β is positively correlated with aggressive clinicopathological 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 KD of GSK3 β alters the Golgi architecture, suggesting that the function of this kinase is required for the maintenance of Golgi architecture (24,25). Therefore, GSK3 β 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 PCa 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

PCa patients, 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 PCa.

We have recently shown that among other motor proteins closely associated with Golgi, the non-muscle 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 heat shock proteins (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 galectin-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 PCa 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 KD 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 PCa. These results explore the basic biology of PCa carcinogenesis and elucidate the role of the Golgi complex in the development and progression of PCa. They also establish a link between alcohol consumption and the functional activation of pro-oncogenic proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

PCa	prostate cancer
EtOH	ethanol
AR	androgen receptor
HDAC6	histone deacetylase 6
GSK3β	glycogen synthase kinase β

PSA	prostate-specific antigen
KD	knockdown

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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. Nuclei were counterstained with DAPI (gray); bars, 5 μ m. (B) Statistical analysis of cells with fragmented Golgi in cells presented in A; means $\pm$ SD; ***- $p < 0.001$, $n = 2$ rats for each series of experiments. (C) ADH1 W-B of the cell lysates from RWPE-1 and LNCaP (c-26) cells before and after EtOH treatment (50 mM for 96 h). Lysates were normalized by β -actin. (D) Immunostaining of giantin (white) in LNCaP (c-26) cells: control, treated with EtOH (50 mM for 96 h), exposed to acetaldehyde (Ach)-generating system (AGS), and treated with EtOH in the presence of 5 mM pyrazole; bars, 10 μ m. (E) Quantification of cells with fragmented Golgi; means $\pm$ 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 $\pm$ SD; ***- $p < 0.001$; n = 4 mice from each group were observed.

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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 mM 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 20x lens; bars, 200 μ m. (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 $\pm$ 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 non-adherent 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 $\pm$ SD from three independent experiments; *** - $p < 0.001$. (D) ADH1 W-B of the cell lysates from 22Rv1 cells before and after EtOH

treatment (50 mM for 96 h). Lysates were normalized by β -actin. **(E)** Migration of 22Rv1 cells: control, treated with 50 mM EtOH for 96 h, and treated with EtOH in presence of 5 mM pyrazole; bars, 200 μ m. 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 $\pm$ SD; *** - $p < 0.001$. **(G)** Immunostaining of HDAC6 (red) in the representative normal prostate tissues and PCa tissues with different Gleason scores. Slides were stained with anti-HDAC6 Ab 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; bars, 10 μ m. **(H)** Quantification of integrated fluorescence intensity (a.u.) of HDAC6 in nuclei from the tissue samples: normal, PCa with a Gleason score of 6–8, and PCa with a Gleason score of 9–10. Groups were tested for normality with the Lilliefors test, and the normality of values was rejected. The non-parametric two-sided Wilcoxon rank sum test was used to test for difference of medians between groups; means $\pm$ SD, * - $p < 0.05$.

Figure 3. The effect of alcohol on the distribution of HDAC6.

(A) Immunostaining of HDAC6 (white) with DAPI (grey color, right panel) and without DAPI (left panel) in normal prostate and tissue section from PCa patient consuming alcohol (DR) and non-alcoholic patient (Non-DR). All confocal images were acquired with the same imaging parameters; bars, 5 μ m. (B) Quantification of HDAC6 IF in nucleus vs. cytoplasm in four patients for each group; mean $\pm$ SD; * - $p < 0.001$. All patients were selected with grade 3 and Gleason score 7. (C) Immunostaining of HDAC6 with DAPI (grey color, right panel) and without DAPI (left panel) in RWPE-1 and LNCaP cells: control and treated with 50 mM EtOH for 96 h. (D) Quantification of HDAC6 IF in nucleus vs. cytoplasm in the cells presented in C; means $\pm$ SD, *** - $p < 0.001$. (E) The total HDAC6, HDAC6-P, and AR W-B of the cytoplasmic and nuclear fractions isolated from LNCaP (c-18) cells: control and treated with 50 mM EtOH for 96 h. Cytoplasmic and nuclear fractions were normalized by β -actin and Lamin B1, accordingly. (F) Graphs show the density of protein bands

normalized to either β -actin (cytoplasmic HDAC6 and AR) or Lamin B1 (nuclear HDAC6 and AR/). (G) GSK3 β and HDAC6-P (Ser 22) W-B of the cell lysate of LNCaP (c-24) cells treated with scramble or 100 nM GSK3 β for 72 h; β -actin was used as a loading control, and the densitometry of ratios Scr/KD normalized to the β -actin are presented at the right. (H) AR W-B of the HSP90 IP from the cell lysate of LNCaP (c-24) cells: control and treated with 50 mM EtOH for 96 h. Input was normalized to the HSP90; the non-specific mouse IgG IP was used as a control. (I) HSP90-Ac W-B of the cell lysate of LNCaP (c-24) cells: control, treated with 50 mM EtOH for 96 h, and treated with EtOH in presence of 5 mM pyrazole; β -actin was used as a loading control. (J) HDAC6-P and HSP90-Ac W-B of the cell lysate of LNCaP (c-24) cells: control, treated with 50 mM EtOH for 96 h, and treated with EtOH in presence of 100 nM of HDAC6 siRNAs for 96 h; β -actin was used as a loading control. (K) AR W-B of the nuclear fractions isolated from LNCaP (c-24) cells: control, treated with 50 mM EtOH for 96 h, and treated with EtOH in presence of 10 nM 17-AAG; Lamin B1 was used as a loading control. (L) PSA W-B of the cell lysate of LNCaP (c-19) cells: control, and treated with 50 mM EtOH for 96 h; β -actin was used as a loading control.

Figure 4. The effect of alcohol on Golgi morphology in PCa tissues.

(**A, B**) Immunostaining of Golgi (giantin, white) in tumor foci sections from PCa patients consuming alcohol (DR) and non-alcoholic patients (Non-DR). Images were analyzed within the patients with same Gleason score. All confocal images were acquired with the same imaging parameters; bars, 5 μ m. (**C**) Quantification of cells with fragmented Golgi in four patients for each group; means $\pm$ SD; * - $p < 0.05$; ** - $p < 0.01$.

Figure 5. Mislocalization of GSK3 β in advanced PCa.

(A) Immunostaining of giantin (green) and GSK3 β (red) in the representative normal prostate tissues and PCa tissues with different Gleason scores. White boxes indicate perinuclear areas enlarged and shown at the right side. Nuclei were counterstained with DAPI (blue). All confocal images were acquired with the same imaging parameters; bars, 5 μ m. (B, C) Quantification of Pearson's overlap coefficient of giantin and GSK3 β in the normal prostate tissue vs. sections from PCa patients with Gleason score 6, 7, 8, 9, and 10 (B), and normal vs. PCa with a Gleason score of 6–8, and PCa with a Gleason score of 9–10 (C); means $\pm$ SD; *** - $p < 0.001$; ** - $p < 0.01$.

Figure 6. Localization of GSK3 β in the Golgi requires giantin. **(A-D)** Immunostaining of giantin (green) and GSK3 β (red) in LNCaP cells (c-16): control and treated with 50 mM EtOH for 96 h (A and B, respectively); treated with scramble or giantin siRNAs (C and D, respectively). **(E, F)** Immunostaining of GRASP65 (green) and GSK3 β (red) in LNCaP cells (c-16): treated with scramble or GRASP65 siRNAs (E and F, respectively). White boxes indicate GSK3 β -stained perinuclear areas enlarged and shown at the right side. Nuclei were counterstained with DAPI (blue). All confocal images were acquired with the same imaging parameters; bars, 10 μ m. **(G)** Giantin, GM130, and GRASP65 W-B of the lysate of LNCaP (c-16) cells: control and treated with 50 mM EtOH for 96 h; β -actin was used as a loading control. Giantin dimer is indicated by the arrow. **(H)** Giantin W-B of the lysate of LNCaP (c-16) cells: treated with scramble or giantin siRNAs; β -actin was used as a loading control. **(I)** GM130 and GRASP65 W-B of the lysate of LNCaP (c-16) cells: treated with scramble, giantin, GM130, and GRASP65 siRNAs; β -actin

was used as a loading control. **(J)** Giantin and GSK3 β W-B of the giantin IP from the cell lysate of non-treated LNCaP (c-24) cells. The IP by non-specific mouse IgG was used as a control; giantin Ab was loaded separately to validate the IgG-corresponding band. Arrows indicate the difference in mobility between GSK3 β and IgG-corresponding bands. **(K)** Quantification of Pearson's overlap coefficient of giantin and GSK3 β from the cells presented in A-B. **(L)** Quantification of GSK3 β integrated fluorescence intensity (cytoplasm/perinuclear area) from the cells presented in C-F and S4. Data are collected from three independent experiments, results expressed as a mean $\pm$ SD; *** - $p < 0.001$.

Figure 7. The working model of alcohol's effect on phosphorylation of HDAC6.

(A) In low-aggressive PCa cells, HDAC6 is shuttling between the nucleus and the cytoplasm with predominant disposition in the nucleus. In cells with low proliferation, phosphorylation of HDAC6 is moderate because GSK3 β localizes to the Golgi. In addition, acetylated HSP90 is unable to bind to the AR, and deacetylation of HSP90 is reduced due to the low level of HDAC6-P. (B) EtOH treatment results in Golgi fragmentation and translocation of GSK3 β to the cytoplasm (1) followed by phosphorylation of HDAC6 (2) and subsequent deacetylation of HSP90 (3). This, in turn, results in AR conformational maturation (4), its subsequent binding to DHT, and translocation to the nucleus (5).