Aurora B Kinase Promotes CHIP-Dependent Degradation of HIF1α in Prostate Cancer Cells

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ABSTRACT

Hypoxia is a major factor in tumor progression and resistance to therapies, which involves elevated levels of the transcription factor HIF1α. Here, we report that prostate tumor xenographs express high levels of HIF1α and show greatly enhanced growth in response to knockdown of the E3 ligase CHIP (C-terminus of Hsp70-interacting protein). In multiple human prostate cancer cell lines under hypoxia, taxol treatment induces the degradation of HIF1α, and this response is abrogated by knockdown of CHIP, but not by E3 ligase VHL or RACK1. HIF1α degradation is accompanied by loss of function, evidenced by reduced expression of HIF1α-dependent genes. CHIP-dependent HIF1α degradation also occurs in cells arrested in mitosis by nocodazole instead of taxol. Mitotic kinase Aurora B activity is required for taxol-induced HIF1α degradation. Purified Aurora B directly phosphorylates HIF1α at multiple sites, and these modifications enhance its polyubiquitination by CHIP in a purified reconstituted system. Our results show how activation of Aurora B promotes CHIP-dependent degradation of HIF1α in prostate cancer cells. This new knowledge may affect the use of mitotic kinase inhibitors and open new approaches for treatment of hypoxic prostate tumors.

Introduction

It is now established that hypoxia occurs in most solid tumors and has a major influence on response to therapies (1–3). Under normal oxygen levels, hypoxia-inducible factors (HIF) are constantly hydroxylated by prolyl hydroxylase (PHD) enzymes that use oxygen as a substrate. The hydroxylated HIFs are recognized by the von Hippel–Lindau (VHL) protein, a component of an E3-ubiquitin ligase that initiates degradation through the proteasome (4). At low levels of oxygen (hypoxia), the PHD enzymes have insufficient substrate; HIF1α is not hydroxylated and therefore escapes degradation. In response to hypoxia, nonhydroxylated HIF1α translocates to the nucleus where it partners with constitutively expressed HIF1β subunit to form a dimer and upregulate the expression of sets of response genes (2, 4). HIF-independent prosurvival responses also exist and include mTOR, p38 MAPK, and NF-κB pathways (5–7). Thus, a complex network of cellular and molecular signaling occurs in cells exposed to hypoxic stress. Recent reports demonstrate O2/PHD/VHL-independent regulation of HIF1α activity through various proteins, including receptor of activated protein kinase C 1 (RACK1; ref. 8), carboxyl terminus of Hsp70-interacting protein (CHIP; ref. 9), Cullin-5, B-cell lymphoma 2 (Bcl2), and factor inhibiting HIF1 (FIH-1; refs. 10, 11).

Various studies demonstrate that the oxygen level in tissues can range from approximately 4% to 6% oxygen. The prostate has one of the lowest reported median oxygen levels (~4%; ref. 12). Physiologic stress responses to low level of oxygen occur between 1% and 3% of oxygen. In solid tumors, oxygen levels are typically below 1%, indicating severe hypoxic stress. Not surprisingly, HIFs are overexpressed in ~70% of human tumors relative to adjacent normal tissue (13), making those factors attractive targets for anticancer therapies. Prostate tumors can be very hypoxic (~0.3% oxygen), more than 10 times lower than oxygen levels found in the normal prostate (12, 14). Prostate tumor hypoxia has been implicated as a driver of malignant progression, genetic instability, endothelial-to-mesenchymal transition (15, 16). Hypoxia provides selective pressure for more resistant cells with greater invasive potential. The changes in tumors induced by hypoxia have significant implications for cancer treatment.

We previously reported that treatment of prostate cancer cells with the naturally occurring estrogen metabolite 2-methoxyestradiol (2-ME) activated the E3 ligase CHIP (C-terminus of Hsp70-interacting protein) and increased proteasomal degradation of the androgen receptor (AR; ref. 17). CHIP interacts with Hsp70 and Hsp90 while mediating the ubiquitination and degradation of multiple chaperone-associated client proteins, including HIF1α (9). Here, we demonstrate that knockdown of CHIP in prostate cancer cells promotes the growth of xenograft tumors. These tumors had high levels of HIF1α. Taxol is used for therapy with castrate-resistant prostate cancer (18, 19). Therefore, we investigated taxol effects on HIF1α expression in prostate cancer cells grown under hypoxic conditions. Our data reveal a new taxol–Aurora B–HIF1α connection that provides new insights to cellular responses to taxol and hypoxia.

Methods and Materials

Cell culture and reagents

Androgen-dependent human prostate carcinoma, LNCaP, and androgen-independent human prostate carcinoma C4-2 and 22Rv1 cells (American Type Culture Collection) were maintained in RPMI (Gibco-Life Technologies), and human prostate carcinoma PC3-M cells (American Type Culture Collection) were maintained in DMEM (Gibco) media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were maintained at 37°C.
in 5% CO2 humidified atmosphere. Taxol was purchase from Sigma-Aldrich Chemical, paclitaxel (catalog #T7402). Aurora kinase inhibitors were purchased from Selleckchem.com, VX-680 (Tozasertib, cat. #S1048) and MLN 8054 (cat. #S1100).

siRNA transfection
siRNA was transfected into different prostate cancer cell line (LNCaP, C4-2, and 22RV1) cells using RNAi-MAX (Invitrogen) as per the manufacturer’s protocol. Briefly, 5 × 10^5 cells were seeded in 10-cm dish in growth medium. Next day, transfection complex (siRNA and RNAiMax) was added to the cells when cells are in OptiMEM without serum, the cells were incubated for 4 to 6 hours, transfection complex was removed and washed with PBS, and growth medium was added. Drugs were added, and cells were harvested after 24 hours unless otherwise mentioned. The siRNA sequences are given in Supplementary Methods.

Stable cell lines
Prostate cancer cell lines (LNCaP, C4-2b, and PC3) were stably depleted of CHIP using MISSION TRC shRNA lentiviral particles from Sigma according to the manufacturer’s protocol. Briefly, cells were seeded in 12-well plates, infected with 30 μL of virus particle solution in 1 mL of complete growth medium containing polybrene (4 μg/mL). Cells were selected by puromycin treatment, and CHIP depletion was confirmed by Western blot analysis.

Western blotting and antibodies
Cells were lysed in modified RIPA lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.5% NP-40, 0.1% SDS, 50 mmol/L NaF, 2 mmol/L sodium orthovanadate) supplemented with a protease inhibitor mix (Thermo Scientific). Unless otherwise described, 30 μg of protein was resolved by SDS–polyacrylamide gel electrophoresis (PAGE), transferred, and immunoblotted with various antibodies. The antibodies used were anti-GAPDH (sc-32233), anti-RACK1 (sc-7754), anti-TPX-2(sc-32863) from Santa Cruz Biotechnology (Santa Cruz); anti-CHIP (C3B6), anti-VHL(68437), anti-P-AuroraA/B(D13A11), anti-AuroraA(83092), anti AuroraB(3094), anti-H3(9715), anti-p-H3(ser10;3377), anti-HIF1α(5537), and anti-cyclinB1(4138) from Cell Signaling Technology; and anti-HIF1α (610958) from BD Transduction Laboratories.

Results
CHIP knockdown promotes prostate cancer in xenograft model
The E3 ligase CHIP (C-terminus of Hsp70-interacting protein) is implicated in support of tumorigenesis and invasion in several malignancies (20–23). The human protein atlas shows an inverse relationship between the level of CHIP and stage of prostate cancer (24) but to date the role of CHIP in prostate cancer has not been explored. To experimentally test whether CHIP affects prostate cancer growth, we isolated C42-b cells expressing shRNA targeted for CHIP and cells control with a scrambled sequence shRNA. Both CHIP knockdown and control cells were injected subcutaneously in the flanks of SCID mice and we measured the tumors over 8 weeks. The results (Fig. 1A) clearly showed a statistically significant increase in tumor growth of human prostate cancer cells with knockdown of CHIP. There are several reports supporting hypoxia in prostate tumors and high levels of HIF1α in prostate cancer as compared with benign prostate hyperplasia and normal tissue (13, 25). IHC revealed dramatically higher levels of HIF1α protein in the nucleus of CHIP knockdown tumor cells (Fig. 1B) compared with controls. In addition, the CHIP knockdown tumors had significantly higher microvascular density versus control tumors, as measured by CD31-positive cells. These results showed prostate tumor growth and HIF1α levels were significantly elevated in tumors as a result of reduction of CHIP.

It is possible that the increase in prostate tumor growth of CHIP knockdown cells was due to elevated AR levels because CHIP is also an E3 ligase for AR (17). To explore this possibility, we used PC3 cells that lack expression of AR. We prepared control and CHIP knockdown PC3 cells, injected them subcutaneously into the flanks of SCID mice, and measured tumors for up to 6 weeks. CHIP knockdown in PC3 cells significantly enhanced tumor size in SCID mice (Supplementary Fig. S1) showing AR was not required for promotion of the growth of prostate cancer cells after knockdown of CHIP.

Taxol treatment reduces HIF1α in a dose- and time-dependent manner in human prostate cancer cells
Prostate tumors tend to be hypoxic and taxol is frequently used in prostate cancer treatment; therefore, we tested for the effects of taxol on HIF1α levels in prostate cancer cells. We treated LNCaP, C42, and 22RV1 human prostate cancer cells with increasing concentrations of taxol (0–5 μmol/L) for 16 hours under hypoxic conditions (<1% oxygen). As expected, HIF1α protein was detected by immunoblotting in prostate cancer cells under hypoxia, relative to normoxia (Fig. 2A–C; lane 1 vs. 2). Under hypoxia, the addition of 1 μmol/L to 5 μmol/L taxol reduced HIF1α levels in a dose-dependent manner in LNCaP (Fig. 2A), C4-2 (Fig. 2B), and 22RV1 cells (Fig. 2C). It is important to point out that HIF1β levels were unchanged under these conditions in all three cell lines, revealing the specificity of taxol treatment for HIF1α. We treated LNCaP (Fig. 2D), C4-2 (Fig. 2E), and 22RV1 cells (Fig. 2F) with 0.1 μmol/L taxol under hypoxic condition and harvested after 6 hours, 12 hours, and 24 hours. By immunoblotting, we observed a time-dependent reduction of HIF1α starting at 6 hours, increasing at 12 hours, and becoming nearly complete at 24 hours. This results revealed a dose- and time-dependent reduction in HIF1α levels in response to adding taxol to human prostate cancer cell lines.

Taxol treatment of cells severely decreased the formation of colonies in soft agar (Fig. 2G). This suggests that reduction of HIF1α levels reduces the fitness of prostate cancer cells in the assay. The response to taxol was not seen with CHIP knockdown cells, supporting the idea that without CHIP the cells are resistant to taxol treatment. We tested both taxol (paclitaxel) and taxotere (docetaxel) and observed degradation of HIF1α.

HIF1α protein is degraded by the proteasome after taxol treatment
What was the mechanism for reduction of HIF1α protein levels in response to taxol in prostate cancer cells? To address this, we treated 22RV1 prostate cancer cells with or without the proteasome inhibitor MG132 in the presence or absence of taxol, under both normoxic and hypoxic conditions (Supplementary Fig. S2A). Under normoxia when HIF1α levels are barely detectable (lanes 1, 3), the addition of MG132 blocked proteasome degradation and HIF1α was accumulated in the cells (lanes 2, 4). Under hypoxia, HIF1α was detected (lane 5), and addition of MG132 further increased the levels (lane 6). Addition of taxol under hypoxia provoked the degradation of HIF1α (lane 7 vs. 5), and this was blocked by the addition of MG132 (lane 8 vs. 7). Based on
the effects of MG132, we concluded that taxol was stimulating the proteasome degradation of HIF1α. We confirmed that the effects of taxol were on HIF1α protein levels, not mRNA. Quantitative reverse-transcription PCR (qRT-PCR) was performed and HIF1α mRNA levels were unchanged in taxol treated versus untreated controls in both LNCaP and C4-2 cells (Supplementary Fig. S2C). Likewise, HIF1α-mRNA levels were unchanged in taxol-treated versus untreated controls in 22RV1 cells based on qRT-PCR (Supplementary Fig. S2B). Apparently, taxol was not interfering with HIF1α transcription, splicing, or RNA stability because the levels of mRNA were unchanged.

**Taxol-induced HIF1α degradation is CHIP dependent**

We suspected that CHIP might be the E3 responsible for HIF1α ubiquitination and degradation under hypoxic conditions. Recently, it has been reported that CHIP is required for degradation of HIF1α through autophagy activated by nutrient deprivation (26). Under normoxic conditions, the von Hippel–Lindau (VHL) factor is considered the primary E3 ligase for HIF1α degradation. To test whether VHL and/or CHIP mediate taxol-induced HIF1α degradation, we knocked down CHIP or VHL individually using siRNA in two human prostate cancer cell lines. After siRNA transfection, cells were exposed to low oxygen in hypoxia chamber for 16 hours, in the presence or absence of taxol. A parallel set of transfected cells were kept at ambient oxygen levels. In both LNCaP (Fig. 3A) and C4-2 cells (Fig. 3B), knockdown of CHIP limited degradation of HIF1α when compared with control siRNA-treated samples, under normoxic conditions (lane 1 vs. lane 3). Surprisingly, knockdown of VHL did not elevate HIF1α levels in prostate cell lines tested (lanes 5 and 6). Knockdown of CHIP but not VHL prevented the taxol-induced HIF1α degradation in either cell line (lane 10 vs. 12). Another protein implicated in degradation of HIF1α is the receptor for activated C-kinase 1 (RACK1; ref. 8), originally identified as an anchoring protein for activated protein kinase C (PKC). However, knockdown of RACK1 failed to prevent taxol-induced HIF1α degradation in C4-2 (Supplementary

Figure 1: Knockdown of CHIP enhances growth of prostate cancer xenograft tumors. A, Growth of subcutaneous tumors of human C4-2b cell xenografts in SCID mice. C42b cells were stably knocked down for CHIP by lentiviral transduction with short hairpin RNA targeting CHIP (shCHIP) and cells expressing non-targeted RNA (NTsh) used as controls. Tumor growth was significantly increased with \( P < 0.0001 \) by two-way ANOVA. Photomicrographs of representative excised tumors are shown on the right. B, Immunostaining of tumors showing significant increase in both nuclear HIF1α and CD31 immunostaining in C4-2b_shCHIP compared with C4-2b_NTsh. Right: top box plots representing the number of nuclear staining of HIF1α cells counted in 4 tumor sections, 6 high power fields/section. Significance at \( P < 0.001 \) by two-tailed Student t test; bottom box plots represent the mean vascular density of tumor xenografts calculated from CD31 immunostaining in 4 tumor sections, 6 high power fields/section. Significance of \( P < 0.001 \) by two-tailed Student t test.* < \( 0.05 \); ** < \( 0.01 \).
Fig. S3A) and 22RV1 (Supplementary Fig. S3B) cells in hypoxic conditions. Together, these results support our conclusion that CHIP is the primary E3 ligase triggering the degradation of HIF1α in response to taxol treatment.

The lack of VHL involvement was reinforced by the observation that a VHL-resistant mutant of HIF1α (P402A/P564A) was degraded similar to the wild-type (WT) HIF1α in response to taxol treatment of cells, under both normoxic and hypoxic conditions (Fig. 3C). We noted that levels of this HIF1α mutant were higher than the WT in cells under normoxia. Both the WT and (P402A/P564A) mutant HIF1α were extensively degraded in response to taxol treatment of cells, indicating that the pathway for taxol stimulation did not depend on VHL, consistent with the results in Fig. 3A and B. Moreover, the degradation of mutant HIF1α (P402A/P564A) in response to taxol (Fig. 3D) was compromised in cells knocked down for CHIP. These results provide further evidence that CHIP is required for the taxol-induced degradation of HIF1α. To address the possibility that the effects of siRNA targeting of CHIP were due to off-target effects, we knocked out CHIP using CRISPR/Cas9 in C4-2 cells and stably knocked down CHIP using a shRNA (with a different sequence from siRNA) in LNCaP cells. Knockout or knock-down of CHIP prevented taxol-induced HIF1α degradation in both C4-2 and LNCaP replicating the response seen with siRNA (Fig. 3E). These experiments all support the conclusion that CHIP is the primary E3 ligase for HIF1α degradation in cells treated with taxol.

Transcription of a number of genes including VEGFA and GLUT1 is dependent on HIF1α (27). We assayed for expression of VEGFA and GLUT1 in cells under normoxic and hypoxic conditions. LNCaP LNCaP Hypoxia Hypoxia Taxol ($\mu$mol/L) Taxol ($\mu$mol/L) Taxol (0.1 $\mu$mol/L) Taxol (0.1 $\mu$mol/L) C42 C42 Hypoxia Hypoxia Taxol (0.1 $\mu$mol/L) Taxol (0.1 $\mu$mol/L) 22RV1 22RV1 Hypoxia Hypoxia Taxol (0.1 $\mu$mol/L) Taxol (0.1 $\mu$mol/L) G, Soft-agar colony formation assay in control and CHIP-depleted cells, treated with or without taxol. The bar graph shows three independent experiments; right, a representative image. No., number.

Figure 2.
Taxol induces dose- and time-dependent loss of HIF1α in hypoxic cells. LNCaP (A), C42 (B), and 22RV1 (C) cells were treated with different doses of taxol for 16 hours under hypoxic conditions. Cells were harvested and extracts were immunoblotted for HIF1α and HIF1β, plus GAPDH, used as loading control. LNCaP (D), C42 (E), and 22RV1 (F) cells were treated with 0.1 $\mu$mol/L taxol and exposed to hypoxia for indicated times. Cell extracts were analyzed for HIF1α by immunoblotting. G, Soft-agar colony formation assay in control and CHIP-depleted cells, treated with or without taxol. The bar graph shows three independent experiments; right, a representative image. No., number.
Expression of both genes was relatively low under normoxia, and not much affected by any of the conditions tested (columns 1–4). Expression of both genes was elevated under hypoxia relative to normoxia (column 5) and knockdown of CHIP enhanced expression compared with cells with control siRNA. More important, knockdown of CHIP prevented the reduced expression of both genes when cells were treated with taxol (Fig. 4). Gene expression conditions, and in the presence or absence of taxol (Fig. 4). Expression of both genes was relatively low under normoxia, and not much affected by any of the conditions tested (columns 1–4). Expression of both genes was elevated under hypoxia relative to
reflected the changes in HIF1α protein levels in response to taxol and CHIP knockdown.

**Taxol-induced HIF1α degradation occurs during mitosis**

Taxol acts as a microtubule stabilizing agent and blocks cells in mitosis. We sought to test whether arrest in mitosis was necessary for the CHIP-dependent degradation of HIF1α. We arrested C4-2 cells in S phase by treatment for 24 hours with aphidicolin, an inhibitor of the replicative DNA polymerase. Cells were then treated with or without taxol for an additional 24 hours. Parallel samples were kept in normoxia, as controls, or exposed to low oxygen in hypoxia chamber for 6 hours. The taxol-induced degradation of HIF1α present in hypoxic cells was completely abrogated when cells were stalled in S phase by aphidicolin (Fig. 5A). This suggested to us that degradation of HIF1α was occurring during mitosis. Cyclin B1 levels were analyzed by immunoblotting as a marker for cells in mitosis. Alternatively, we treated LNCaP, C4-2, and PC3M cells (Fig. 5B) with or without the microtubule disrupting drug nocodazole for 10 hours, performed mitotic shake off, and put the cells in a hypoxic chamber for 6 hours in the continued presence of nocodazole. We observed that nocodazole treatment induced HIF1α degradation, just like taxol (Fig. 5B). We treated LNCaP cells stably expressing nontargeting and shCHIP with nocodazole (Fig. 5C). Both sets of cells were arrested in mitosis, based on the increased phosphorylation of Ser10 in histone H3 (Fig. 5C). However, HIF1α was degraded only in the control cells (NT, lane 1) but not in the CHIP knockdown cells (lane 2). HIF1α was not degraded in asynchronous cells that were not in mitosis (untreated), even though CHIP was present. These results show that cells needed to be in mitosis, either by taxol or nocodazole treatment, in order for CHIP to promote the degradation of HIF1α.

**Aurora kinase B is required for HIF1α degradation in response to taxol**

Aurora kinases are activated during mitosis, so we treated LNCaP (Fig. 6A) and C4-2 (Fig. 6B) cells with Aurora A/B inhibitors to test for effects on HIF1α levels. We included cells under normoxic conditions as additional controls, even though HIF1α was not detected. Under hypoxic conditions, taxol induced degradation of HIF1α in both cell lines (lane 5 vs. 6). Interestingly, the relatively specific Aurora A kinase inhibitor MLN8054 (28) had little effect on taxol-induced HIF1α degradation (Fig. 6A, lane 7; Fig. 5B, lane 8). On the other hand, VX680 (29), which inhibits both Aurora A and Aurora B, prevented taxol-induced HIF1α degradation (Fig. 6A, lane 8; Fig. 6A, lane 7). The effects of the inhibitors could be seen by phosphosite immunoblotting for activation of Aurora A and Aurora B. MLN8054 inhibited Thr288 phosphorylation of Aurora A, but not phosphorylation of Aurora B, while, on the other hand, VX680 inhibited phosphorylation of both Thr288 in Aurora A and Thr232 in Aurora B. These results indicated that Aurora B, but not Aurora A, was required for the taxol-induced degradation of HIF1α.

The relative involvement of Aurora A, B, and C kinases in taxol-induced HIF1α degradation was studied by siRNA knockdown in C4-2 (Fig. 6C) and 22RV1 cells (Fig. 6D). Knockdown of Aurora B increased the basal level of HIF1α in the cells and prevented taxol-induced HIF1α degradation (compare lanes 2 and 6). Knockdown of Aurora B alone or in any combination with Aurora A or C was sufficient to prevent the degradation of HIF1α. In contrast, knockdown of either Aurora A or Aurora C, or both together, reduced or failed to prevent taxol-induced HIF1α degradation. In addition, knockdown of TPX2, an activator of Aurora A, failed to prevent HIF1α degradation (Supplementary Fig. S4). Further confirmation of the role of Aurora B was provided by knockdown of INCENP, a subunit of the active Aurora B complex (30), by either one of two independent siRNA. Knockdown of INCENP prevented taxol-induced HIF1α degradation. Both the results with chemical inhibitors and shRNA knockdown strongly support Aurora B as the
mitotic kinase that is required for the CHIP-mediated degradation of HIF1α.

**Aurora kinase B phosphorylates HIF1α in vitro and enhances polyubiquitination**

Aurora B could exert effects through direct phosphorylation of either HIF1α or CHIP. We performed an *in vitro* kinase assay using bacterial expressed, purified recombinant HIF1α, plus purified recombinant Aurora kinase B kinase and γ[32P]ATP (Fig. 7A). The kinase assay showed time-dependent increase in phosphorylation of HIF1α. The extent of HIF1α radiolabeling was comparable to histone H3, included as a known Aurora B substrate (Fig. 7A). Autophosphorylation of Aurora B was evident in all the reactions, including without added substrates. Analysis by LC/MS-MS revealed Aurora B phosphorylation of HIF1α at multiple sites, in particular S247, S465, and S657. The S247 and S465 sites both have sequences (RXPpS) predicted to be Aurora B substrates. HIF1α S657 was phosphorylated by Aurora B, even though it does not conform to the consensus recognition sequence (TSpSP; ref. 31). This site was one of two in HIF1α previously reported to be phosphorylated by PLK3 and mutation of S657A/S657A greatly extended the half-life of HIF1α (32). We concluded that Aurora B phosphorylates HIF1α, including at sites that affect its proteasomal degradation.

To test for effects of Aurora B phosphorylation of HIF1α on reaction with CHIP we performed an *in vitro* ubiquitination assay. These reactions included purified HIF1α along with E1, E2 components, plus HSP70 and CHIP as the E3 ligase. Products were resolved by SDS-PAGE and immunoblotted for HIF1α, with the polyubiquitinated HIF1α appearing as slower migrating species (Fig. 7B). Polyubiquitination of HIF1α by CHIP required complete reactions and omission of ATP as a control prevented formation of ubiquitinated products. The amount of polyubiquitinated HIF1α formed was 1.3-fold greater in reactions that included Aurora B (Fig. 7B, lane 3) compared with HIF1α incubated under identical conditions, but without added kinase (Fig. 7B, lane 4). The activity of Aurora B under the assay conditions was confirmed by phosphorylation of Ser10 in histone H3 (Fig. 7B, bottom). These results demonstrated enhanced polyubiquitination of HIF1α due to phosphorylation by Aurora B.

**Discussion**

In this study, we discovered that prostate tumors with RNAi knockdown of the E3 ligase CHIP grow significantly larger and express high levels of HIF1α. We show CHIP is the dominant E3 ligase for HIF1α in hypoxic prostate cancer cells and HIF1α degradation is enhanced by the clinically relevant agent paclitaxel and docetaxel, which induces mitotic arrest of cells. Nocodazole also blocks cells in mitosis and induces CHIP-dependent HIF1α degradation. Pharmacologic inhibition or RNAi knockdown of mitotic Aurora B attenuated HIF1α degradation. We used *in vitro* assays with purified proteins to demonstrate Aurora B directly phosphorylates HIF1α, and this
increased CHIP-dependent polyubiquitination. Mass spectrometry showed Aurora B phosphorylates multiple sites in HIF1α, including S657, already known to accelerate protein turnover. Thus, arresting cells in mitosis activates Aurora B to phosphorylate HIF1α, making it a better substrate for the E3 ligase CHIP, thereby enhancing HIF1α degradation and reducing the expression of HIF1α-dependent genes.

These new results expand our knowledge about how both mitotic kinases Aurora A and Aurora B regulate selective protein turnover by the E3 ligase CHIP. We previously reported Aurora A (not Aurora B) phosphorylation of CHIP during mitosis enhances AR degradation in prostate cancer cells (33). On the other hand, our present results establish that Aurora B (not Aurora A) promotes CHIP-dependent degradation of HIF1α by phosphorylation of HIF1α. In one case, Aurora A activates the enzyme (CHIP, an E3 ligase), and in the other, Aurora B predisposes a particular substrate (HIF1α) to ubiquitination by CHIP. Aurora A and Aurora B are activated in different stages of mitosis and in distinct intracellular locations, suggesting that timing and location are critical parameters in determining specificity for substrates and functions (34). Aurora A kinase is known to regulate mitotic entry, spindle formation, and centrosome maturation and is concentrated at centrosomes (35). Experimental overexpression of Aurora A overrides the mitotic spindle checkpoint and induces resistance to paclitaxel (36). In contrast, Aurora B kinase is an integral component of the Chromosome Passenger Complex that is associated with chromatin during mitosis, and is differentially regulated at chromosome arms and kinetochores to orchestrate equal chromosome segregation (35). Agents that disrupt microtubule dynamics can arrest cells at different stages in mitosis may therefore have markedly different effects on degradation of proteins, such as AR and HIF1α, that are involved in prostate cancer progression. CHIP is a common factor, and its actions on different substrates that are critical for prostate cancer is dependent on cells being in mitosis.

Our observations have potential implications for the therapy of prostate cancer patients. TCGA data established the inverse relationship between high HIF1α and low CHIP level in prostate cancer, and correlate with poor outcomes for patients. Thus, HIF1α and CHIP could be useful biomarkers to track prostate cancer progression and segregate patients for different treatments. Docetaxel is the most commonly used cytotoxic agent in castrate-resistant prostate cancer. The mechanism of action is thought to be blocking cell-cycle progression by preventing disassembly the mitotic spindle, causing prolonged mitotic arrest. We suggest that taxanes may also inhibit prostate cancer by promoting HIF1α degradation and downregulating HIF1α-dependent genes. The relationship between CHIP, HIF1α, and the response to docetaxel is something for further study. Aurora B inhibitors are in clinical trials for multiple solid tumors. Our data suggest that these inhibitors should be used with caution because we predict they will increase HIF1α levels in tumors, and this is associated with therapeutic resistance and poor prognosis.

Finally, hypoxia is one of the hallmarks of solid tumors and HIF1α is activated under hypoxic conditions, which promotes angiogenesis, EMT, and survival of tumor cells (37, 38). Hypoxia in prostate tumor is considered an early event (39), and several reports supporting hypoxia itself as an independent risk factor for tumor progression, resistance, and treatment failure (40, 41). New knowledge from this study could be useful in creating new therapies for prostate cancer. Agents that directly activate CHIP, or indirectly support its activation by phosphorylation or limit its inactivation by dephosphorylation, could lead to reduced levels of HIF1α and thereby limit tumor growth and survival and prevent resistance to chemotherapies. Phosphorylation of HIF1α promotes ubiquitination and degradation by CHIP, so activators of Aurora B or inhibitors of the phosphatase and/or
regulatory subunit(s) that target S657 in HIF1α could be effective therapeutic agents. Another possibility is for drug inhibitors of the deubiquitinas that reverse the CHIP-mediated ubiquitin modifications, which would promote prosesome degradation of HIF1α to limit the growth of prostate tumors and render them more susceptible to chemotherapeutic drugs.

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

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Biswas, S. Sarkar, N. Said, J.M. Larner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Biswas, S. Sarkar, N. Said, D.L. Brautigan, J.M. Larner

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