The mechanism of DAB2IP in chemo-resistance of prostate cancer cells

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Translational Relevance

Docetaxel is the first-line chemotherapy for men with metastatic castration resistant prostate cancer (CRPC) and prolongs survival of most patients; however, the acquisition of chemo-resistance is a significant barrier to cure this disease. It is clear that the DOC-2/DAB2 interactive protein (DAB2IP) has distinct cellular functions, modulating different signal cascades associated with cell proliferation, survival, apoptosis and metastasis. In this study, we demonstrate loss of DAB2IP in prostate cancer cells becomes chemo-resistance and unveil the underlying mechanism of DAB2IP in modulating Clusterin (CLU) gene expression via crosstalk between Wnt/β-catenin and IGF1/IGFR signaling. These findings are of clinical relevance because this is a new mechanism for CLU gene regulation by DAB2IP, which can lead to develop a targeted therapy by aiming CLU along with DAB2IP as a biomarker for patient selection.
Abstract

Purpose: The docetaxel-based chemotherapy is the standard of care for castration resistant prostate cancer (CRPC), inevitably, patients develop resistance and decease. Until now, the mechanism and predictive marker for chemo-resistance are poorly understood.

Experimental Design: Immortalized normal prostate and cancer cell lines stably manipulated with different DAB2IP expression levels were used and treated with chemotherapeutic drugs commonly used in PCa therapy. Cell proliferation was measured using MTT assay; Western blot, quantitative PCR and luciferase reporter assays were used to analyze Clusterin gene regulation by DAB2IP. Immunohistochemistry analysis was performed for evaluating DAB2IP, Clusterin and Egr-1 expression in human PCa tissue.

Results: DAB2IP Knockdown (KD) cells exhibited resistance to several chemotherapeutic drugs, while increased DAB2IP in C4-2 cells restored the drug sensitivity. Parallel, DAB2IP KD cells exhibited higher expression of Clusterin, an anti-apoptotic factor, while elevated DAB2IP in C4-2 cells decreased Clusterin expression. Functionally, knocking down Clusterin by shRNA or antisense oligonucleotide OGX-011 decreased drug resistance, while overexpressing Clusterin in C4-2 D2 enhanced drug resistance. Mechanistically, DAB2IP blocked the crosstalk between Wnt/β-catenin and IGF-1 signaling leading to the suppression of Egr-1 that is responsible for Clusterin expression. Similar result was observed in the prostate of DAB2IP knockout animal. In addition, we observed a significantly inverse correlation between DAB2IP and Egr-1 or Clusterin expression from clinical tissue microarray.
Conclusions: This study unveils a new regulation of Egr-1-Clusterin signaling network by DAB2IP. Loss of DAB2IP expression in CRPC cells signifies their chemo-resistance. Clusterin is a key target for developing more effective CRPC therapy.

Running Title: DAB2IP loss enhances chemo-resistance in CRPC.

Keywords: castration resistant prostate cancer; DAB2IP; clusterin, early growth response-1; chemo-resistance
Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related death among men in the USA (1). Although early PCa is generally treatable, most cases eventually progress to an advanced stage, castration resistant prostate cancer (CRPC) (2). Docetaxel-based chemotherapy is the standard of care regimen for CRPC, however, this regimen only prolongs survival and many patients develop resistance and eventually succumb to their disease (3, 4). The molecular mechanisms underlying acquisition of chemo-resistance by advanced PCa are not well defined.

DAB2IP/AIP1 (DOC-2/DAB2 interactive protein or ASK1 interacting protein) was previously identified as a member of the RAS-GTPase activating protein family (5, 6), and functions as a tumor suppressor in cancer development (7-11). A recent study using genome-wide association analyses revealed a single nucleotide polymorphism of the DAB2IP gene associated with risk of aggressive PCa (12). Indeed, down regulation of DAB2IP in PCa is mainly due to epigenetic regulation, which inversely correlates with tumor-grade and predicts PCa progression (7-11, 13, 14). DAB2IP functions as a scaffold protein to modulate a variety of biologic activities: cell growth, apoptosis, survival and epithelial-mesenchymal transition (EMT) leading to PCa metastasis (15-17). Mechanistically, DAB2IP can inhibit the Wnt-elicited EMT pathway by recruiting PP2A to active GSK-3β. In addition, DAB2IP is able to regulate Ras-MAPK, ASK-JNK, PI3K-Akt and nuclear factor-κB (NF-κB) pathways (5, 16-18). Recently, we reported that DAB2IP loss resulted in resistance to ionizing radiation (IR) due to enhanced DSB...
repair, robust G2-M checkpoint control, and apoptosis resistance (19). However, its potential role and possible mechanism in chemo-resistance of PCa remains largely unknown.

Here, we demonstrate that DAB2IP up-regulation sensitizes PCa cells to chemotherapeutic drugs (i.e., Docetaxel, Epothilones B, Gemcitabine and Istodax) that are commonly used in the clinic. In contrast, DAB2IP loss resulted in increased expression of the secretory form of clusterin (sCLU), a crucial anti-apoptotic protein in CRPC, associated with PCa cell chemo-resistance. Mechanistically, DAB2IP blocked crosstalk between Wnt-β-catenin and IGF1-IGF1R pathways, leading to early growth response-1 (Egr1) gene transcription that in turn regulates sCLU expression. We validated an inverse relationship between DAB2IP and Egr1 or sCLU expression from both DAB2IP−/− mouse and clinical specimens from CRPC patients. Thus, DAB2IP loss confers chemo-resistance in CRPC.
Materials and Methods

Cell Lines
Stable DAB2IP-shRNA-knockdown (KD) and control (Con) shRNA-scrambled cells were derived from PZ-HPV-7, RWPE-1 and LAPC-4 cell lines. Stable DAB2IP-transfected C4-2 sublines (D1 and D2) and its control (Neo) cells were generated as described (16).

Prostate Tumor Specimen
This study was done on the total of 194 PCa specimens obtained from Vancouver Prostate Centre Tissue Bank. 76 of those cases were subjected to neoadjuvant hormone therapy (NHT). The H&E slides were reviewed and the desired areas were marked. Three TMAs were manually constructed (Beecher Instruments, MD, USA) by punching duplicate cores of 1 mm for each sample. All the specimens were from radical prostatectomy except 12 CRPC samples that obtained from transurethral resection of Prostate (TURP). Tissue samples were arrayed according to Gleason score, primary or CRPC status, and with or without NHT, respectively. The Institutional Review Board of UT Southwestern approved the tissue procurement protocol for this study, and appropriate informed consent was obtained from all patients.

Chemotherapeutic Drugs, Antibodies, Chemicals and Conditioned Medium
Chemotherapeutic drugs used for PCa therapy, included Docetaxel (Aventis Pharmaceuticals, Ridgewater, NJ); Epothilone B (Sigma-Aldrich, St. Louis, MO); Gemcitabine (Gemzar, Eli Lilly, Indianapolis, IN); and Istodax (FK228, Fujisawa
Pharmaceutical, Osaka, Japan). Primary antibodies used were: anti-sCLU-α (B-5, Santa Cruz Biotechnology, Santa Cruz, CA); anti-Egr1 (C-19, Santa Cruz) for Western blot; anti-Egr1 (BD, Franklin Lakes, NJ) for immunohistochemical (IHC) staining; anti-β-catenin (BD); anti-p-IGF1Rβ (Tyr1131, Cell Signaling Technology, Danvers, MA); anti-Actin (Sigma); and anti-DAB2IP, generated and used as described (5).

Chemical inhibitors, including Tyrphostin AG1024 (IGF1R kinase inhibitor) and lithium chloride (LiCl, GSK-3β inhibitor), were purchased from Sigma and Calbiochem (Darmstadt, Germany), respectively. Second-generation antisense oligonucleotides (ASO) specific for sCLU (OGX-011) and comparable scrambled oligonucleotides (MM) were provided by OncoGenex Pharmaceuticals (Vancouver, British Columbia, Canada). PCa cells were treated with siRNA or oligonucleotides using previously described protocols (20). Recombinant human IGF1 was purchased from R&D (Minneapolis, MN). Wnt3A- and control-conditioned media (i.e., Wnt-CM and L-CM, respectively) were previously described (16) and used to treat cells for 48 hrs.

**Plasmid Constructs**

Various expression plasmids for DAB2IP, sCLU, Egr1 and β-catenin were previously described (15, 21). The 4250 bp human CLU promoter (pCLU-luc) was previously described (21). An Egr1 mutant (Egr1-mut) at −89 bp of pCLU-luc was made using site-directed mutagenesis and primers: Egr1-mut sense: 5’-GATGCGCCCCCCCCGAATTCCCGAGGGTGCTGCAC-3’; Egr1-mut antisense: 5’-GTGCAGCACCGGGCTGGGAATTCTGGGGGGCCGTCATC-3’. pEgr1-luc and its deleted constructs were obtained from Dr. Eileen D. Adamson (The Burnham Institute for
Medical Research, La Jolla, CA) (22). The pIGF1-luc reporter construct was obtained from Dr. Peter S. Rotwein (Oregon Health & Science University, Portland, Oregon) as described (23, 24).

**Cell Culture, siRNA Oligonucleotides and Transfection, Luciferase Reporter Gene Assay, qRT-PCR Assay, Western Blot Assay, MTT Assay and IHC Staining.** See SI Materials and Methods.

**Statistical Analyses**

Data are presented as means ± SEM of at least three independent experiments performed at least in triplicate. All data analyses were performed by software of SPSS13.0 for Windows. p values ≤ 0.05 were regarded as the threshold value for statistical significance.
Results

**DAB2IP modulates the chemo-sensitivity of PCa cells**

To determine the possible role of DAB2IP in modulating chemo-sensitivities of PCa cells, we knocked down the endogenous DAB2IP expression in two different prostatic epithelial cell lines determined by Western blot (Fig. 1A and B, left panel). By employing FDA approved chemotherapeutic agents commonly used in PCa therapy, we selected four drugs (i.e., Docetaxel, Epothilone B, Gemcitabine and Istodax) with different mechanisms of action. In general, DAB2IP-knockdown (KD) cells showed significantly higher resistance to all four drugs, with increases in IC\(_{50}\) values compared to control (i.e., Con) cells expressing DAB2IP protein.

On the other hand, we generated a stable DAB2IP-expressing subline (i.e., D2) from a CRPC cell line (i.e., C4-2) without detectable endogenous DAB2IP and its control subline (i.e., Neo). D2 cells became more sensitive to chemotherapeutic agents compared to Neo cells (Fig. 1C). These data strongly indicated the impact of DAB2IP on the chemo-sensitivities of CRPC cells.

**DAB2IP regulates CLU gene expression in PCa cells**

To understand the underlying mechanism of DAB2IP in this event, we explored possible downstream signaling pathways. We noticed that DAB2IP-deficient cells express elevated levels of clusterin (CLU) protein, including both intracellular full-length pre-secretory clusterin (psCLU, ~60 kDa), and its mature secretory (sCLU, ~40 kDa) proteins in all DAB2IP-KD cells from PZ-HPV-7, RWPE-1 and LAPC-4 compared with their Con cells.
(Fig. 2A and Fig. S1A). The similar changes were also observed in prostatic epithelia derived from DAB2IP knockout (DAB2IP\(^{-/-}\)) mice as well as MDA-MB-468 breast cancer cells (Fig. S1B and C). In addition, ectopic DAB2IP expression dramatically suppressed sCLU protein expression in C4-2 (i.e., D1 and D2) cells (Fig. 2A). Consistently, both CLU mRNA expression and gene promoter activities inversely correlated with DAB2IP expression levels in these cells (Fig. 2B and C, p < 0.05).

**sCLU mediates the chemo-resistant phenotypes of DAB2IP-deficient PCa cells**

sCLU is a cytoprotective protein that, when endogenously or exogenously overexpressed, can afford chemo-resistance to various cancer cells (25). To determine a role for sCLU as a downstream determinant for DAB2IP loss in the chemo-resistance of PZ-HPV-7 and RWPE-1 KD cells, sCLU levels were knocked down using shRNA specific for the exon I/III boundary region of the secreted form of the protein. Silencing sCLU expression significantly restored the chemo-sensitivity of KD cells to docetaxel (Fig. 3A, p < 0.05). In addition, OGX011, an antisense oligonucleotide (ASO) specifically targeting sCLU has been demonstrated as a promising therapeutic agent against several cancers (26). Our data clearly indicated that OGX-011 ASO not MM could specifically inhibit sCLU expression and synergistically enhance the cytotoxic effects of docetaxel in RWPE-1-KD cells in a dose-dependent manner (Fig. 3B, p < 0.05). In contrast, increased expression of sCLU protein using transient cDNA transfection in C4-2-D2 cells can diminish docetaxel cytotoxicity and prolong cell survival (Fig. 3C, p \(\leq\) 0.05).

**DAB2IP suppresses CLU expression by inhibiting Egr1 gene transcription**
Previously, we reported that the insulin-like growth factor 1 (IGF1)–mediated sCLU induction was regulated by IGF-IR/Src/mitogen-activated protein kinase (MAPK)/ERK signaling, in which Egr1 is a key transcription factor controlling CLU gene promoter activity (21, 24). In DAB2IP-KD cells, elevated Egr1 protein expression was detected (Fig. 4A, left panel and Fig. S1). In contrast, reduced Egr1 protein expression was observed in both D1 and D2 sublines of C4-2 cells compared with control C4-2-Neo cells (Fig. 4A, right panel). Data from qRT-PCR (Fig. 4B, left panel) and CLU gene promoter reporter assays (Fig. 4C, left panel) clearly indicated the inhibitory effect of DAB2IP on Egr1 gene transcription.

To elucidate the role of Egr1 in sCLU gene transcription in the presence of DAB2IP, the activity of wild-type (wt) CLU gene promoter vs. the Egr1 mutant form (i.e., Egr1mut) containing a single mutation at −89 bp for Egr1 binding in CLU gene promoter was determined in PZ-HPV-7 sublines (i.e., Con and KD). A dramatic loss of the reporter gene activity of Egr1mut compared to that of wt CLU was seen in KD cells (Fig. 4D, left panel). On the other hand, the wt CLU promoter activity increased in C4-2-D2 cells in a dose-dependent manner with an incrementally increasing Egr1 protein level by transient cDNA transfection (Fig. 4D, right panel). Thus, DAB2IP-inhibited sCLU gene expression appears to be mediated by Egr1 transcription factor.

**DAB2IP inhibits Egr1 and CLU expression via suppressing Wnt/β-catenin and IGF1/IGF1R signaling**
To dissect the signaling cascade mediated by DAB2IP in regulating Egr1-sCLU expression, we explored the involvement of the Wnt/β-catenin pathway in sCLU expression. Wnt is able to induce sCLU protein accumulation in several prostate cell lines (Fig. 5A and C, and Fig. S2A). Also, using LiCl (a GSK-3β kinase inhibitor) or ectopic expression of β-catenin restored the expression of Egr1 and sCLU in a dose-dependent manner in C4-2-D2 cells (Fig. 5B and D). DAB2IP is able to recruit PP2A to active GSK-3β leading to the inhibition of Wnt/β-catenin signaling (16), in this study, we further demonstrated that DAB2IP could block Wnt-elicited Egr1-sCLU signaling axis in C4-2 and PZ-HPV-7 cells (Fig. 5A and C).

In addition to known regulatory mechanism for sCLU gene expression (27-29), we unveiled that IGF1-induced sCLU gene expression is mediated by β-catenin/T-cell factor (TCF). Here, we examined the potential effect of DAB2IP on the crosstalk between Wnt and IGF1 signaling pathways via Egr1-mediated sCLU gene expression. Significant decreases in IGF1 mRNA and gene promoter activity were noted in the presence of DAB2IP (Fig. 5E, p < 0.05), and this inhibition could be diminished by Wnt (Fig. S2B, p < 0.05). Similar results were observed in A549 lung carcinoma cells (Fig. S3). Consistent activation of IGF1R phosphorylation was observed in DAB2IP-deficient cells, which is correlated with both Egr1 and sCLU protein expression in C4-2-Neo or D2 cells (Fig. 5F). NF-κB is known as a potent inducer for Egr1 promoter via binding of p65/RelA in normal human keratinocytes cells upon UVB irradiation and also DAB2IP has been shown to be able to inhibit NF-κB-elicited pathway (17, 22). However, using several constructs containing deleted NF-κB binding sites in Egr1 gene promoter, the fold of inhibition of Egr1 gene promoter activities by the presence of DAB2IP remained the
same (Fig. S4), implying that the inhibitory effect of DAB2IP on Egr1 gene transcription is not mediated through NF-κB. Taken together, DAB2IP acts as an upstream inhibitor for Wnt–elicited IGF1-Egr1-signaling cascade leading to sCLU gene transcription.

**Inverse correlation between DAB2IP and Egr1 or sCLU in PCa progression**

Loss of DAB2IP expression is often detected in human PCa cell lines and tissues, for example, 70% high-grade PCa tissues were noted with significantly decreased DAB2IP expression compared to associated normal prostate tissue (7, 16, 17). The correlation of DAB2IP with Egr1 or CLU expression in PCa specimens remained undetermined, thus, the expression profile of DAB2IP, Egr1 and sCLU levels using TMAs was performed. Consistent with prior studies that showed positive but weak DAB2IP staining in naive PCa tissues (14, 17), the majority of CRPC tissues showed no expression of DAB2IP (representative staining shown in Fig. 5A, left panel). After quantification, DAB2IP loss became more evident in CRPC than naive specimens (Fig. 5B left panel, p < 0.05). Importantly, stronger nuclear Egr1 and cytosolic sCLU staining were detected in the same CRPC specimens that correlated well with low DAB2IP staining (representative staining shown in Fig. 5A, middle and right panel). Quantitative analyses showed that nuclear Egr1 and cytosolic sCLU expression levels were significantly elevated in specimens obtained from castration recurrence or different months of NHT treatment (Fig. 5B, middle and right panel; Fig. S5, p < 0.05). Indeed, Egr1 and DAB2IP expression levels were inversely correlated (Pearson’s correlation coefficient = -0.14, p = 0.01). Furthermore, sCLU and DAB2IP expression levels were also inversely correlated (Pearson’s correlation coefficient = -0.37, p = 0.02), while Egr1 expression was
positively correlated with sCLU (Pearson’s correlation coefficient = 0.18, p = 0.003). Thus, these data strengthen the notion that loss of DAB2IP in PCa could unleash Egr1-sCLU gene expression, which is associated with chemo-resistance.

Discussion

Understanding the mechanisms for chemo-resistance of cancer is pivotal, because cancer cells eventually develop chemo-resistance (3). For PCa, chemotherapy is commonly used in late stages of disease, such as patients with CRPC that has metastasized to multiple organs. In general, these cancer cells are highly heterogeneous and likely have acquired a resistant phenotype or develop chemo-resistance very quickly. Thus, current chemotherapeutic regimens only prolong patient survival and are not curative. By identifying early molecular mechanisms that promote survival and metastasis, we may develop new therapeutic strategies to enhance the efficacy of chemotherapy to achieve ultimate curative therapies. We and other groups identified DAB2IP as a unique metastatic suppressor that acts as a signalosome to modulate multiple signaling pathways leading to cell growth, survival and apoptosis (15-17). Also, we have demonstrated that DAB2IP-deficient PCa cells exhibit radio-resistance (19). In this study, we have extended these observations and shown that that DAB2IP-deficient cells show chemo-resistance to a wide-spectrum of chemotherapeutic agents, a resistance mechanism that appears to be mediated through sCLU. So one significance of this study has provided another novel mechanism underlying the failure of docetaxel commonly used in CRPC chemotherapy, because two recent publications discovered the effect of this microtubule-targeting chemotherapy in androgen receptor (AR) cellular translocation/trafficking as
critical new insight into mechanisms of resistance of CRPC to taxane (30, 31), meanwhile our very recent study reported that DAB2IP loss facilitated AR activation in CRPC cells (14).

Indeed, sCLU is known as a key contributor to chemo-resistance of many cancer cells (26). The CLU gene codes two isoforms (CLU-1, CLU-2), which gives rise to at least two different functions mediated by two separate protein species: (i) a secreted heterodimeric isoform (i.e., sCLU) and (ii) a prenuclear cytoplasmic (pnCLU) splice variant that lacks the endoplasmic reticulum targeting domain that can be activated to a pro-apoptotic form that can localize to the nucleus (i.e., nCLU) (32, 33). sCLU, a stress-activated cytoprotective chaperone and up-regulated by chemotherapeutic agents, can protect cells from apoptosis (34, 35). However, the regulatory processes that control sCLU expression are not fully elucidated.

Some studies have unveiled several transcriptional regulators for CLU gene promoter including AP1, heat shock factor 1/2, b-MYB, c-MYC, signal transducer and activator of transcription 1 (STAT1) (33, 36, 37). Recently, YB-1 was shown to regulate stress-induced CLU transcription and expression, with CLU playing a dominant downstream role in YB-1-induced cytoprotection and Paclitaxel resistance in prostate cancer cells (38). In addition, we demonstrated that IGF1R-Src-MEK/Erk signaling is involved in the regulation of sCLU expression via the transactivation of Egr1, a known stress-inducible transcription factor (21). In this study, we demonstrate evidence indicating that Wnt-β-catenin signaling is upstream of IGF-1 gene expression. Our data indicate that DAB2IP
is a potent inhibitor that modulates multiple signaling pathways, such as Wnt-β-catenin and IGF1, leading to CLU gene expression. These data are consistent with our previous study (16) in which DAB2IP is critical for regulating EMT by recruiting PP2A and blocking Wnt/β-catenin pathway. Although DAB2IP is known to inactivate NF-κB signaling (17, 22) and NF-κB can be a transactivator of Egr1 promoter (17, 22), our data indicated that NF-κB was not involved in Egr1 regulation and downstream sCLU expression.

The paradox of Egr1 function is commonly seen in cancer. Egr1 mediates apoptosis in response to stress and DNA damage by regulating a tumor suppressor network, but it also promotes proliferation of PCa cells by an unknown mechanism (39). Nevertheless, levels of Egr1 mRNA and protein expression correlate with Gleason scores and inversely correlated with PCa grade (40). However, the status of Egr1 protein in CRPC remained unexplored. Our study provides the first evidence for elevated nuclear Egr1 staining in recurrent PCa or from NHT treatment. An inverse correlation between DAB2IP and Egr1 or sCLU expression was noted not only in PCa but also in cells of DAB2IP−/− mice (Fig. S1C). Most importantly, such inverse correlation was especially evident in human CRPC samples (Fig. 6). DAB2IP is frequently lost in advanced PCa, which underlies the oncogenic function of Egr1 in this disease.

Knowing the pro-survival and anti-apoptotic activities of sCLU, we report the involvement of sCLU in the chemo-resistance of PCa cells to various clinically relevant therapeutic agents. Inhibiting the pro-survival function of sCLU using specific ASO such
as OGX-011 under current clinical trials for prostate and lung cancers can be combined with various cytotoxic agents in order to enhance the therapeutic efficacy (25, 26, 41). Based on the data presented in this work, DAB2IP appears to be another potential factor for boosting the therapeutic efficacy of these agents as well.

In summary, this study at least has shown sCLU and its complex signaling interaction with DAB2IP as a mechanistic basis driving therapeutic resistance in prostate cancer (Fig. 6C). For its significance, understanding such a cross-talk could not only provide a new strategy to improve early prediction of chemo-resistance in CRPC patients through combined detection of multiple molecular markers (i.e., DAB2IP, Egr1 and sCLU), but also indicate DAB2IP as a potential target for developing more effective therapy based on epigenetic modification or peptide strategy in future.
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References

23. Mittanck DW, Kim SW, Rotwein P. Essential promoter elements are located within the 5' untranslated region of human insulin-like growth factor-I exon I. Mol Cell Endocrinol 1997;126:153-63.


Figure legends

Fig 1. DAB2IP enhances the chemo-sensitivities of PCa cells.

The status of DAB2IP protein expression in PZ-HPV-7 (A), RWPE-1 (B), and C4-2 (C) sublines was analyzed by Western blot. Cells were exposed to different concentrations of indicated chemotherapeutic drugs for 48 hrs, and survival assessing relative cell numbers was determined by MTT assays. Results (means, ± SEM) were obtained from three independent experiments, each in triplicate. Asterisks indicate statistical significance between Con and KD cells or between Neo and D2 cells, *p ≤ 0.05. Note: Docetaxel (5 ng/ml = 6.19 nM) and Istodax (5 ng/ml = 9.25 nM).

Fig 2. DAB2IP regulates CLU gene expression in PCa cells.

A, Western blot analyses of DAB2IP and CLU expression were performed in PZ-HPV-7, RWPE-1, and C4-2 sublines. Actin was used as an internal loading control.

B, Levels of DAB2IP and CLU mRNA expression in PZ-HPV-7, RWPE-1, and C4-2 sublines were determined using qRT-PCR. Relative mRNA levels of each gene were determined by normalizing to 18S rRNA. Results (means, ± SEM) were obtained from three independent experiments. *p ≤ 0.05 vs. Con or Neo cells.

C, Cells were transiently co-transfected with the human pCLU-luc reporter, DAB2IP siRNA or a DAB2IP expression vector, along with RSV-β-gal as indicated for 36 hrs as described. Transfectants were then subjected to luciferase and β-gal assays. After normalizing with the β-gal activity, relative CLU promoter activities were calculated. All experiments were performed in triplicate. *p < 0.05 vs. vector control.
Fig 3. *sCLU mediates the chemo-resistance of DAB2IP-deficient PCa cells.*

A, PZ-HPV-7 KD and RWEP-1 KD cells were transiently transfected with shRNA-sCLU and shRNA-Scr control for 24 hrs, and then exposed to 10 ng/ml docetaxel treatment for 48 hrs. sCLU expression levels were determined by Western blot analyses and the relative cell numbers were determined by MTT assays. Results (means, ± SEM) were obtained from three independent experiments. *p < 0.05 vs. control shRNA.

B, RWEP-1 KD cells were treated with sCLU ASO (i.e., OGX-011) or mismatched control (MM) at the indicated concentrations, followed by docetaxel (10 ng/ml) treatment for 48 hrs, and relative cell numbers were determined by MTT assays. *p < 0.05 vs. MM control.

C, C4-2-D2 cells were transiently transfected with various doses of sCLU expression vector or control vector for 24 hrs, followed by docetaxel (10 ng/ml) treatment for 48 hrs, and relative cell numbers were determined by MTT assays. *p < 0.05 vs. vector control.

Fig 4. *DAB2IP targets sCLU expression by blocking Egr1 transcription.*

A, Western blot analyses of DAB2IP and Egr1 expression were performed in the PZ-HPV-7 (i.e., Con and KD) and C4-2 (i.e., Neo and D2) cells. Actin was used an internal control.

B, Levels of Egr1 mRNA expression in PZ-HPV-7 (i.e., Con and KD) and C4-2 (i.e., Neo, D1, and D2) cells were determined using qRT-PCR. Relative mRNA levels of each gene were determined by normalizing to 18S rRNA. Results (means, ± SEM) were obtained from three independent experiments. *p < 0.05 vs. Con or Neo cells.
C, Cells were transiently co-transfected with pEgr1-luc, DAB2IP siRNA or DAB2IP expression vector as indicated for 36 hrs as described in Fig. 2, followed by luciferase and β-gal assays. After normalizing for β-gal activities, the relative Egr1 promoter activities were calculated. Each experiment was performed in triplicate. *p ≤ 0.05 vs. vector control.

D, PZ-HPV-7 sublines were transiently transfected with pCLU-luc (wild-type or Egr1-mut) for 36 hrs, and C4-2 sublines were transiently co-transfected with pCLU-luc and different concentrations of Egr1 expression vector for 36 hrs as described in Fig. 2, followed by luciferase and β-gal assays. After normalizing for β-gal activities, relative CLU promoter activities were calculated, and each result was performed in triplicate. Ectopic Egr1 expression in C4-2 cells was detected by Western blot analysis. Left panel, *p ≤ 0.05 vs. wild-type pCLU-luc activity in KD cells; right panel, *p ≤ 0.05 vs. Neo cells, #p ≤ 0.05 vs. vector control.

Fig 5. DAB2IP inhibits Egr1 and CLU expression via Wnt-β-catenin and IGF1-IGF1R signaling pathways.

A and B, C4-2 sublines were pretreated with LiCl (20 mM, 6 hrs) or conditioned medium (L- or Wnt-CM, 48 hrs), and cell lysates were subjected to Western blot analyses for DAB2IP, CLU and Egr1 levels. Actin was used as an internal loading control.

C, PZ-HPV-7 sublines were treated with L- or Wnt-CM for 48 hrs, and cell lysates were subjected to Western blot analyses of DAB2IP, CLU and Egr1. Actin was used as an internal loading control.
D, C4-2 sublines were transiently transfected with different concentrations of β-catenin expression vector cDNAs for 48 hrs, and cell lysates were subjected to Western blotting to assess DAB2IP, sCLU and Egr1 levels. Actin was used as an internal loading control.

E, Levels of IGF-1 mRNA expression in C4-2 (i.e., Neo and D2) cells were determined using qRT-PCR (left panel). Relative mRNA levels of each gene were determined by normalizing to 18S rRNA. Results (means, ± SEM) were obtained from three independent experiments, each repeated in triplicate. *p ≤ 0.05 vs. Neo cells. C4-2 cells were transiently co-transfected with pIGF1-luc and different doses of DAB2IP expression vector for 36 hrs as described in Fig. 2. Cells were then subjected to luciferase and β-gal assays (right panel). After normalizing with β-gal activities, relative IGF-1 promoter activities were calculated. Each result was performed in triplicate. *p ≤ 0.05 vs. vector control.

F, C4-2-Neo cells were pretreated with the IGF-1R kinase inhibitor, AG1024 (4 μM), or DMSO for 6 hrs, and C4-2-D2 cells were exposed to IGF-1 (100 nM) for 48 hrs. Cell lysates were then subjected to Western blot analyses for p-IGF-1Rβ (Tyr1131), sCLU and Egr1 levels. Actin was used as a loading control.

Fig 6. The relationship of DAB2IP, Egr1 and sCLU expression in PCa tissues.

A, Representative IHC staining of DAB2IP, Egr1 and sCLU levels in clinical specimens. Case-1, detected DAB2IP expression, but lower expression of Egr1 and sCLU in naive PCa specimen; Case-2, no DAB2IP expression with concomitant higher expression of Egr1 and sCLU in CRPC specimen.
B, Histogram of DAB2IP, Egr1 and sCLU protein expression in CRPC compared to naive tissues (CRPC vs. naive tissues, *p < 0.05).

Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5

A

C4-2

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B

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E

IGF1 mRNA level

F

C4-2 Neo

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C4-2 D2

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Fig. 6
The mechanism of DAB2IP in chemo-resistance of prostate cancer cells

Kaijie Wu, Daxing Xie, Yonglong Zou, et al.

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