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Heterochromatin protein 1α mediates development and aggressiveness of neuroendocrine prostate cancer

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Neuroendocrine prostate cancer (NEPC) is a lethal subtype of prostate cancer (PCa) arising mostly from adenocarcinoma via NE transdifferentiation following androgen deprivation therapy. Mechanisms contributing to both NEPC development and its aggressiveness remain elusive. In light of the fact that hyperchromatic nuclei are a distinguishing histopathological feature of NEPC, we utilized transcriptomic analyses of our patient-derived xenograft (PDX) models, multiple clinical cohorts, and genetically engineered mouse models to identify 36 heterochromatin-related genes that are significantly enriched in NEPC. Longitudinal analysis using our unique, first-in-field PDX model of adenocarcinoma-to-NEPC transdifferentiation revealed that, among those 36 heterochromatin-related genes, heterochromatin protein 1α (HP1α) expression increased early and steadily during NEPC development and remained elevated in the developed NEPC tumor. Its elevated expression was further confirmed in multiple PDX and clinical NEPC samples. HP1α knockdown in the NCI-H660 NEPC cell line inhibited proliferation, ablated colony formation, and induced apoptotic cell death, ultimately leading to tumor growth arrest. Its ectopic expression significantly promoted NE transdifferentiation in adenocarcinoma cells subjected to androgen deprivation treatment. Mechanistically, HP1α reduced expression of androgen receptor (AR) and RE1 silencing transcription factor (REST) and enriched the repressive trimethylated histone H3 at Lys9 (H3K9me3) mark on their respective gene promoters. These observations indicate a novel mechanism underlying NEPC development mediated by abnormally expressed heterochromatin genes, with HP1α as an early functional mediator and a potential therapeutic target for NEPC prevention and management.
Statement of Significance

Heterochromatin proteins play a fundamental role in neuroendocrine prostate cancer, illuminating new therapeutic targets for this aggressive disease.
INTRODUCTION

Neuroendocrine prostate cancer (NEPC) has become a clinical challenge in the management of castration resistant prostate cancer (CRPC). While de novo cases are rare, NEPC as a special subtype of CRPC (~10-20%) is thought to occur via NE transdifferentiation of prostate adenocarcinomas in response to androgen deprivation treatment (ADT), resisting dependence on AR signaling as an adaptive response. As next-generation AR pathway inhibitors (ARPI) such as enzalutamide and abiraterone have made substantial improvements in managing CRPC adenocarcinomas in recent years (1), it is expected that the incidence of NEPC will further increase (2). Unfortunately, the overall median survival of NEPC with small cell feature is less than one year, primarily due to its aggressiveness and limited available treatment options (3). As such, a better understanding of the mechanisms underlying NEPC development remains much needed in order to develop more effective therapeutics.

One distinct histopathological feature of NEPC cells is the frequent manifestation of hyperchromatic nuclei with finely dispersed chromatin and inconspicuous nucleoli, a phenomenon known as “salt and pepper” chromatin (3,4). This is in contrast to prostatic adenocarcinoma cells, which tend to have enlarged nuclei with prominent nucleoli (3,4). This special hematoxylin-staining characteristic suggests a distinct NEPC heterochromatin pattern (5). Heterochromatin is a condensed and transcriptionally inert chromosome conformation, regulated epigenetically by precise and dedicated machineries (6,7). Multiple studies have demonstrated that epigenetic regulation is one major mechanism underlying
NEPC development (8-11). An NEPC-specific heterochromatin gene signature can thus shed light to help better understand the disease and identify novel therapeutic targets.

Another distinct feature of NEPC is the lineage alteration associated with a decrease or loss of crucial adenocarcinoma lineage-specific transcription factors, AR, FOXA1 and REST (12-15). This leads to the repression of AR-regulated genes (e.g. prostate-specific antigen (PSA)) and gain of neuroendocrine markers (e.g. neural cell adhesion molecule 1 (NCAM1/CD56), neuronal-specific enolase (NSE), chromogranin A (CHGA), and synaptophysin (SYP)) (12-14). While recent studies have reported that EZH2 inhibits AR expression and SRRM4 mediates REST splicing (8,16), the mechanisms underlying the constant transcriptional suppression of AR and REST are still not well understood.

One of the major hurdles in studying NEPC is the lack of clinically relevant models, but substantial progress has been achieved recently in modeling NEPC development. Employing genetically engineered mouse (GEM) models, ectopic gain of N-myc and concomitant loss of Rb1 and Tp53 have both been demonstrated to induce de novo NEPC-like tumors (8,11). Potent ADT using abiraterone in Tp53/Pten double deficient mice also promoted overt NE transdifferentiation of luminal adenocarcinoma cells (17). Employing engineered human primary cells or cell lines, N-myc, SOX2, BRN2, and SRRM4 overexpression have all been shown to promote NE transdifferentiation (16,18-20). Our laboratory has established over 45 high-fidelity patient-derived xenograft (PDX) models of PCa including 5 NEPCs (www.livingtumorlab.ca) (21). Among them, LTL331/331R is the first-in-field and unique PDX model of adenocarcinoma-to-NEPC transdifferentiation. Upon host castration, the primary adenocarcinoma (LTL331) initially regresses but relapses within few
months as typical NEPC (LTL331R) (21). Importantly, the whole transdifferentiation process observed in the LTL331/331R model is predictive of disease progression and is fully recapitulated in the donor patient (22), suggesting a strong clinical relevance. Because an overwhelming majority of expressed genes accounting for the NE phenotype in terminal NEPC may obscure the real drivers of disease progression required at earlier stages, we focused on identifying the early changes induced by ADT before NEPC is fully developed, with an additional secondary criterion that these changes persist into terminal NEPC. To this end, we developed a time series of the LTL331 model, for which samples were harvested at multiple points during the transition period following host castration in order to monitor the entire transdifferentiation process and discover potential drivers with early expression changes (22).

In this study, we identified a heterochromatin molecular signature that is commonly upregulated in NEPC. Among the signature genes, we discovered that the upregulation of HP1α (encoded by CBX5), a gene prominently associated with constitutive heterochromatin and mediating concomitant gene silencing, was an early event in our LTL331/331R NE transdifferentiation model. HP1α expression was increased within weeks following castration but before emergence of NE genes, gradually reaching its highest level in terminally developed NEPC. HP1α is also widely expressed in clinical samples of NEPC. We show that HP1α is essential for NEPC cell proliferation, survival, and tumor growth, and its elevated expression promotes ADT-driven NE-differentiation in prostatic adenocarcinoma cells. HP1α ectopic expression reduces expression of AR and REST, two crucial transcription factors
silenced in NEPC, and enriches the repressive histone mark H3K9me3 on their respective gene promoters.
MATERIAL AND METHODS

Patient-derived xenografts and clinical datasets

All PDX tumor lines were grafted in NSG mice as previously described (21). This study followed the ethical guidelines stated in Declaration of Helsinki, specimens were obtained from patients with their informed written consent form following a protocol (#H09-01628) approved by the Institutional Review Board of the University of British Columbia (UBC). Animal studies under the protocol # A17-0165 were approved by UBC Animal Care and Use Committee. The LTL331 castrated tissues and the NEPC-relapsed LTL331R tissues were harvested at different time points after host castration (22). Transcriptomic analysis for all PDX tumors, with the exception of the LTL331-331R castration time-series samples, was performed using GE 8x60K microarray, as previously described (21). Transcriptomic analysis of the LTL331-331R time-series was done using RNA-sequencing data.

The clinical cohorts used in this study are as follows: RNA-seq data for the Beltran et al. 2011 cohort (30 adenocarcinomas, 6 NEPCs) was from Weill Medical College of Cornell University (23); RNA-Seq data for the Beltran et al. 2016 (34 CRPC-adenocarcinomas, 15 NEPCs) the Grasso et al. 2012 (31 CRPC-adenocarcinomas, 4 NEPCs), and the Kumar et al. 2016 (149 CRPC-adenocarcinomas) cohorts were accessed through the cBioPortal (10,24-26); microarray data for the Varambally et al. 2015 cohort (6 CRPC-adenocarcinomas) was accessed from the Gene Expression Omnibus (GEO) database (GSE3325) (27).
Human prostate cancer specimens

PCa specimens were obtained from the Vancouver Prostate Centre Tissue Bank (103 hormone naive primary prostatic adenocarcinomas, 120 CRPC-adenocarcinomas, 8 NEPCs). This study followed the ethical guidelines stated in Declaration of Helsinki, specimens were obtained from patients with their informed written consent form following a protocol (#H09-01628) approved by the Institutional Review Board of the University of British Columbia (UBC). Tissue microarrays of duplicate 1 mm cores were constructed manually (Beecher Instruments, MD, USA). NEPC specimens are histologically either small cell carcinoma or large cell neuroendocrine carcinoma with low or negative AR expression and positive CHGA expression as determined by IHC staining.

Cell line culture and reagents

NCI-H660 and 293T cells were obtained from ATCC. Cells were authenticated with fingerprinting method at Fred Hutchinson Cancer Research Centre (Seattle, USA). Mycoplasma testing was routinely performed at the Vancouver Prostate Centre. NCI-H660 cells was cultured in RPMI-1640 medium (HyClone) with supplements as follows: 5% FBS (GIBCO), 10 nM beta-estradiol (Sigma), 10 nM Hydrocortisone (Sigma), 1% Insulin-Transferrin-Selenium (Thermo Fisher). V16D (20) cells were maintained in RPMI-1640 containing 10% FBS. 293T was kept in DMEM (Hyclone) with 5% FBS. For in vitro NE
phenotype induction in V16D cells, cells were starved with phenol-red free RPMI-1640 (GIBCO) containing 10% Charcoal-Stripped Serum (CSS) (GIBCO) for 24 hours, then cultured in the same media or with the addition of 10 μM enzalutamide (Haoyuan Chemexpress) for another 14 days (20).

Bioinformatics analysis

As previously described, Gene Set Enrichment Analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) was used in this study to determine whether a defined set of genes show significant, concordant differences between two biological phenotypes (e.g. PCa adenocarcinoma vs. NEPC) or two sample groups (e.g. shC vs. KD) (28). All GSEA analyses in this study used whole transcriptomic data without expression level cut-off as expression datasets. Normalized values were used for profiling data from PDX tissues, H660 and V16D stable cell lines, and the three NEPC mouse models (GSE90891, GSE92721, GSE86532); raw read numbers was used for RNA-seq data from the Beltran et al. 2011 cohort; pre-ranked gene list based on p-value from lowest to highest was used for the Beltran et al. 2016 cohort (8,10,11,17,21,23). Unbiased analysis was performed using the latest MSigDB database for each collection (29). Phenotype permutation was applied when the sample size grouped in one phenotype was more than five. Otherwise, gene set permutation was employed. False discovery rate (FDR) q values were calculated using 1000 permutations, a geneset was considered significantly enriched if its normalized enrichment score (NES) has an FDR q below 0.25.
Ingenuity pathway analysis (IPA) was performed as previously described (20).

Differentially expressed genes were selected based on the criteria that their standard deviation between samples of one group (e.g. three HP1α knockdown lines) is below 0.5 and their student’s t-test p-value between groups (e.g. shC vs. KD) is below 0.05.

For the NE scores and the heterochromatin score, the weight for each gene in the score list was first calculated by taking the log2 of the p-value of all genes and multiplying it by the sign of the fold change. The weight for each gene in Lee et al. 2016 was obtained directly from their study. The p-values for the genes contributing to heterochromatin score was calculated by comparing NEPC PDXs with all other adenocarcinoma PDXs with the Student’s t-test. A score was then assigned to each sample by multiplying the weight with the normalized RNA expression value, adding all values for each gene, log-transforming the absolute value of the sum value, and multiplying the sign of the sum value (10,11,18).

The heatmap was constructed using the Gplots and heatmap.2 packages in R.

Hierachal clustering was used to determine sample similarity.

Statistical analysis and data representation

Statistical analysis was done using the Graphpad Prism software. The Student’s t-test was used to analyze statistical significance between groups in discrete measurements while two-way ANOVA was used for continuous measurements. The Kaplan-Meier method was used to estimate curves for relapse-free survival, and comparisons were made using
the log-rank test. Pearson correlation (with 95% confidence intervals) was used to measure
the linear correlation between two variables. Any differences with \( p \)-values lower than 0.05 is
regarded as statistically significant, with \( *p<0.05, **p<0.01, ***p<0.001 \). Graphs show pooled
data with error bars representing standard error of the mean (SEM) obtained from at least	hree replicates and standard deviation (SD) of values from clinical samples. Lines in scatter
plots represent the median value of measurements from multiple samples and mean with 95%
confidence interval (CI) for scores from multiple samples. Box plot with whiskers showing 5-
95% percentile values was used to represent analysis of IHC scores.

Accession numbers

All PDX microarray profiles are available at www.livingtumorlab.ca, and accessible under the
accession number GSE41193 in the GEO database. RNA-seq profiles for the LTL331/331R
castration time-series have been deposited to the European Nucleotide Archive and are
available under the accession number ENA: PRJEB9660. Microarray profiles for cell lines
are deposited (GSE105033).
RESULTS

NEPC has a distinctive heterochromatin gene signature

To determine if a specific gene expression program contributes to the hyperchromatic histological feature of NEPC cells, we first performed an unbiased gene set enrichment analysis (GSEA) using our latest PDX collection and two NEPC clinical cohorts. Transcriptomic profiles from our high-fidelity PDX models (18 adenocarcinomas vs. 4 NEPCs), the Beltran, et al. 2011 cohort (30 adenocarcinomas vs. 6 NEPCs), and the Beltran, et al. 2016 cohort (34 CRPC-adenocarcinomas vs. 15 NEPCs) (10,21,23) all demonstrate that heterochromatin-associated genes are significantly enriched in NEPC compared to adenocarcinoma (Fig. 1A-1C, S1A). We further analyzed three GEM models mimicking the development of NEPC. Interestingly, heterochromatin-associated genes are also significantly enriched as long as NE differentiation occurs, driven by either Pten/Rb double knockout (DKO) or Pten/Rb/Tp53 triple knockout (TKO), Pten/Tp53 knockout with potent ADT, or N-Myc overexpression (Fig. 1D-1F) (8,11,17).

To further decipher the genes contributing to the heterochromatin feature of NEPC, we analyzed the leading edge genes derived from the GSEA of PDX and clinical NEPC samples (Fig. 1A-1C). Since the NEPCs in our PDX collection are all typical neuroendocrine carcinomas without mixed adenocarcinoma tissues, the upregulated genes identified in the NEPC PDXs and validated in the clinical cohorts can be considered heterochromatin signature genes. In addition, three polycomb-group (PcG) genes, EZH2, RBBP4 and RBBP7, are also included in this gene signature as their upregulated expression in NEPC have been
previously reported (9,23). They have also been reported to play important roles in heterochromatin formation (7), but are omitted from the GSEA geneset (GO:0000792) containing other PcG members. As such, 36 genes were identified and selected to form a heterochromatin gene panel, which could successfully distinguish NEPC from adenocarcinoma through hierarchical clustering similar to two reported NE score gene panels (Fig. 1G, S1B-S1E) (10,18). From this heterochromatin gene panel, a weighted score was calculated for each sample in the PDX and clinical cohorts following a similar strategy utilized in recent studies (10,11,18). Notably, similar to NEPC samples having a distinctly higher NE score compared to adenocarcinomas, NEPC samples also have a significantly higher heterochromatin score compared to adenocarcinoma (Fig. 1H). Importantly, the heterogeneity among PDX samples is much smaller than that observed in any clinical cohorts regardless of the scoring system employed, further demonstrating that our PDX collection both clearly reflects the clinical features of NEPC and adenocarcinoma and provide a distinct system to study PCa subtypes. Together, these analyses indicate that a heterochromatin-related gene expression signature is a unique feature of NEPC tumors.

Expression of heterochromatin protein 1α (HP1α) is upregulated early and steadily during NEPC development

Inspired by the critical functional role of heterochromatin structure in modulating cell behavior and gene expression (6,7), we hypothesized that these NEPC-specific heterochromatin signature genes could contribute to NEPC development. As such, we
attempted to identify a potential NEPC driver gene from our 36 heterochromatin signature
genes. In addition to a final upregulation in terminal NEPC, expression of the candidate
driver gene would also be increased early prior to full NEPC development. We thus analyzed
the RNA-seq profile of the LTL331/331R NE-transdifferentiation model with samples from
castration-induced dormant time points prior to full NEPC relapse (21,22). Ranked by the
gene expression difference between pre- and post-castration samples, elevated expression
of HP1α was found to be an early and dramatic event occurring upon host castration.
Conversely, expression of other genes that have been reported to be associated with NEPC
such as EZH2 and CBX2 (9,23) were only upregulated in the fully developed NEPC sample
(LTL331R) (Fig. 2A). Interestingly, we also noticed that Hp1α expression gradually increased
as the NE phenotype progressed from partial to more dominantly overt in the Pten/Tp53
CRPC mouse model (Fig. S2A), lending further support that elevated HP1α expression is an
early event in NEPC development.

To validate our findings, we performed quantitative RT-PCR (qRT-PCR) to detect
HP1α expression at the mRNA level and IHC staining and Western blotting to detect HP1α
expression at the protein level in our PDX collection. Both the mRNA and protein levels of
HP1α are significantly increased in NEPC PDXs (Fig. 2B-2D, S2B). Furthermore, we
confirmed HP1α expression in the LTL331/331R castration time-series samples. Consistent
with the RNA-seq data, both the mRNA and protein expression of HP1α increased upon host
castration, with increased mRNA observed as early as 1 week post-castration and elevated
protein expression observed 3 weeks post-castration (Fig. 2E-2G). Taken together, these
data demonstrate that elevated expression of HP1α is an early and consistent event throughout NEPC development and could be a potential driver of NEPC.

**HP1α expression is upregulated in clinical NEPC samples and correlates with poor prognosis**

To determine the clinical relevance of elevated HP1α expression in NEPC, we analyzed RNA expression profiles from three individual clinical cohorts containing NEPC samples. In the *Beltran* et al. 2011 cohort, HP1α expression was about 4 times higher in NEPCs (n=6) compared to adenocarcinomas (n=30) (Fig. 3A) (23). In the *Beltran* et al. 2016 cohort, HP1α expression was also significantly upregulated in NEPCs (n=15) compared to CRPC adenocarcinoma samples (n=34) by around two folds (Fig. 3B) (10). In another cohort consisting of 35 metastatic CRPC samples, 4 samples with NEPC features expressed significantly higher HP1α mRNA than other typical adenocarcinomas (Fig. 3C) (24). To better understand the relationship between HP1α expression and the NEPC phenotype at a protein level, we performed IHC for HP1α with the Vancouver Prostate Centre (VPC) clinical PCa tissue microarrays (TMAs) containing 103 primary adenocarcinomas, 120 CRPC adenocarcinomas and 8 typical NEPC samples. Consistent with its mRNA level, HP1α protein was significantly overexpressed in NEPCs (mean=2.45) compared to primary adenocarcinomas (mean=1.66, *p*=0.011) and CRPC adenocarcinomas (mean=1.60, *p*=0.014) (Fig. 3D, 3E). These data together demonstrate that HP1α is highly expressed in NEPC.
While HP1α is dramatically upregulated in NEPC, we also noticed that some adenocarcinomas also expressed higher levels of HP1α than others (Fig. 3D). We then investigated whether the expression of HP1α is associated with poor patient prognosis, thus possibly being a pre-disposing factor to poor outcome. Among the 103 primary adenocarcinoma samples in the VPC cohort, there were 37 samples with clinical follow-up information. Kaplan-Meier analysis showed that patients with high expression of HP1α (IHC score $\geq 2$) had a significantly shorter disease-free survival time (HR=4.627, $p=0.0074$) than low HP1α-expressing patients (IHC score <2) (Fig. 3F). For CRPC patients, the group with high expression of $HP1\alpha$ (top 1/3) had a significantly shorter overall survival time after the first hormonal therapy (HR=2.961, $p=0.021$), implying that HP1α positively correlates with poor prognosis upon hormonal therapy (Fig. 3G). Overall, these analyses indicate that increased expression of HP1α is a poor prognostic factor in advanced PCa.

$HP1\alpha$ knockdown inhibits NEPC cell proliferation and induces apoptosis, leading to tumor growth arrest

Considering that HP1α is significantly upregulated in NEPC, we proceeded to investigate its function in NEPC cells. NCI-H660 is a unique and typical NEPC cell line (30). Consistent with the high HP1α expression observed in clinical NEPC samples, HP1α is also expressed at the highest level in H660 cells compared to seven other PCa cell lines at both RNA and protein levels (Fig. S3A). We thus constructed stable H660 cell lines with $HP1\alpha$ knocked down using lentiviral-delivered shRNAs (Fig. 4A). Upon $HP1\alpha$ knockdown, the
overall proliferation of H660 cells was significantly inhibited, as evaluated by both crystal
violet staining and cell counting (Fig. 4B, S3B). We further performed colony formation
assays to evaluate the reproductive and survival ability of single cells. Remarkably, HP1α
knockdown was able to ablate the colony formation ability of H660 cells (Fig. 4C). We then
proceeded to analyze in greater detail the potential cellular mechanisms underlying the
attenuation of cell growth mediated by HP1α knockdown. An EdU incorporation assay
showed that knockdown of HP1α led to a significant reduction of EdU-positive, DNA
synthesis-active cells (Fig. 4D). Furthermore, an apoptosis assay and FACS analysis
indicated that HP1α knockdown also promoted cell early-apoptosis and final death (Fig. 4E,
4F). Molecularly, the apoptosis markers cleaved-caspase 3 and cleaved-PARP1 were both
upregulated in knockdown cells (Fig. 4G); multiple machineries involved in DNA damage
repair and cell cycle progression were also impaired in HP1α knockdown cells as
determined by GSEA and ingenuity pathway analysis (IPA) from stable cell line
transcriptomic profiles (Fig. 4H, S3C, S3D). Thus, HP1α depletion inhibited NEPC cell
proliferation and induced apoptosis in vitro.

We then assessed the functional impact of HP1α knockdown on tumor growth in vivo.
Compared to control H660 cells, xenograft tumor growth of the KD2 stable cell line with 70%
HP1α knockdown (Fig. 4A, S3E) was observed to be dramatically inhibited and much slower,
as determined by continuous tumor volume measurements and final fresh tumor weights
(Fig. 4I, 4J). The other two stable lines (KD1, KD3) with 80%~95% HP1α knockdown were
not able to generate sufficient cell numbers for in vivo tumor formation assays. Taken
together, the in vitro and in vivo studies establish that HP1α knockdown could both inhibit NEPC cell proliferation and induce apoptosis, leading to NEPC tumor growth arrest.

HP1α promotes NE transdifferentiation of prostatic adenocarcinoma cells following ADT

Since HP1α was identified as a potential early driver of NEPC development following hormone therapy, we further investigated whether HP1α could enhance NE phenotype in ADT-induced NE differentiation of adenocarcinoma cells similar to our LTL331/331R NE-transdifferentiation PDX model. We used V16D cells, a LNCaP-derived CRPC cell line (20), to construct stable cell lines with HP1α ectopic expression (Fig. 5A). Upon ADT using charcoal-stripped serum (CSS) or enzalutamide (EnZ) treatment for 14 days, both control and HP1α-overexpressing V16D cells were assessed for the expression of terminal NE markers. HP1α overexpression significantly promoted the expression of NCAM1 (CD56) and NSE, as shown by both qRT-PCR and Western blotting (Fig. 5B, 5C). HP1α overexpression alone was not able to induce NE transdifferentiation (Fig. 5B, 5C). Further transcriptomic analysis of control and HP1α-overexpressing V16D cells upon EnZ treatment revealed that HP1α overexpression consistently upregulated the expression of a panel of NEPC marker genes and enriched neuronal-associated signaling pathways (Fig. 5D, 5E, S4A). Pearson correlation analyses with transcriptomic data from multiple CRPC clinical cohorts (i.e., the Beltran et al. 2016, the Kumar et al. 2016, the Grasso et al. 2012, and the Varabally et al. 2005) further demonstrated that high expression of HP1α is positively correlated with the
expression of terminal NE markers (i.e., NCAM1, NSE, CHGA, CHGB) in advanced PCa (10,24,25,27) (Fig. 5F, 5G, S4B, S4C). Overall, these analyses suggest that HP1α is a potential functional driver promoting NE transdifferentiation.

**HP1α represses AR and REST expression and enriches H3K9me3 on their promoters**

We further analyzed how HP1α contributed to the NE phenotype. A major function of HP1α in heterochromatin is to repress gene expression using epigenetic machineries (31,32), which is an observation supported by our transcriptomic analyses with HP1α knockdown and overexpressing cells (Fig. 5E, S4D). Decreased or loss of expression of the crucial adenocarcinoma lineage-specific transcription factors AR, FOXA1 and REST is a well-established mechanism leading to NE differentiation (12-14). Notably, when HP1α was overexpressed in V16D cells, AR and REST were downregulated at both the mRNA and protein levels (Fig. 6A, 6B, S5A). Consistently, a panel of AR target genes that are repressed in clinical NEPCs are also downregulated upon HP1α overexpression in V16D cells (Fig. 6C). Reciprocally, HP1α knockdown in H660 cells reactivated AR and REST mRNA expression (Fig. S5B), though their protein levels remained undetectable due to low abundance.

We next investigated potential mechanisms underlying the downregulation of AR and REST upon HP1α overexpression. We found that, rather than affecting global heterochromatin related genes, HP1α overexpression significantly enriched (while knockdown impaired) pericentric heterochromatin machineries as determined by GSEA.
Pericentric heterochromatin is a constitutive heterochromatin structure characterized by the repressive histone mark H3K9me3 (32). We then performed a chromatin immunoprecipitation (ChIP) assay to examine the occupancy of H3K9me3 on the promoter regions of \textit{AR} and \textit{REST} following \textit{HP1\alpha} modulation. Interestingly, in V16D cells, \textit{HP1\alpha} overexpression increased the occupancy of H3K9me3 on both the \textit{AR} and \textit{REST} promoters, while its knockdown in H660 cells decreased H3K9me3 enrichment on the \textit{AR} promoter (Fig. 6E, S5F). In accordance with our findings in cell lines, the pericentric heterochromatin geneset is consistently and significantly enriched in NEPCs compared to adenocarcinomas in our PDX models, multiple clinical cohorts, and GEM models (Fig. 6F, S5G). Meanwhile, Pearson correlation analyses with multiple clinical cohorts showed that \textit{HP1\alpha} expression is negatively correlated with \textit{AR} and \textit{REST} expression in advanced PCa samples (Fig. 6G, 6H, S5H) (10,24,25,27). Overall, our data indicate that \textit{HP1\alpha} could regulate expression of the two crucial adenocarcinoma lineage-specific transcription factors \textit{AR} and \textit{REST} potentially via modulating the enrichment of H3K9me3 on their promoters.
DISCUSSION

Loss of luminal epithelial cell characteristics together with gain of small cell neuroendocrine features makes NEPC a completely different disease than typical adenocarcinoma CRPC. This transdifferentiation makes all ARPIs inapplicable despite their otherwise remarkable revolution on the treatment of advanced PCas (1,14). More critically, the application of potent ARPIs for the clinical management of CRPC could accelerate the incidence of NEPC in near future (2,3,33). As such, deciphering the biological and molecular mechanisms underlying NEPC development is fundamentally important for developing novel therapeutics.

Gross differences in nuclear hyperchromatic morphology between adenocarcinoma cells and NEPC cells have long been a criterion for NEPC pathological diagnosis. Our study here unmask, for the first time, the precise molecular basis underlying this NEPC-specific nuclear phenotype and identifies a 36-gene NEPC heterochromatin signature using multiple high-fidelity prostatic adenocarcinoma and NEPC PDXs and two prevalent clinical cohorts. Notably, our heterochromatin gene signature can significantly distinguish NEPCs from adenocarcinomas similar to two other NEPC gene signatures derived from whole-genomic differences, suggesting a crucial function of heterochromatin in NEPC and also a potential application for diagnostic purposes. Furthermore, as epigenetic machineries play a major foundational role in heterochromatin formation and function (6,34), this 36-gene heterochromatin signature also includes 29 epigenetic factors (35). Among them, PcG genes such as \textit{EZH2} have already been demonstrated to play critical roles in NEPC (8,9,36). Thus,
these heterochromatin genes also provide a molecular basis for NEPC development and aggressiveness. Considering that the hyperchromatic nuclear pattern in NEPC is also shared by other small cell carcinomas such as small cell lung cancer (SCLC), this heterochromatin signature may be further applicable to small cell carcinomas of other tissue origins (37).

From these 36 heterochromatin genes, HP1α was identified as a potential early driver of NE transdifferentiation. The longitudinal analyses of LTL331 PDX tissues following host castration demonstrated that the upregulated expression of HP1α is an early event. This is in contrast to a number of genes previously reported to be involved in NEPC development, such as EZH2, CBX2 (9,23), which were only upregulated in the fully developed NEPC LTL331R tumor. Notably, our LTL331/331R model is not only clinically relevant (21,22), but also highly reproducible in delivering the same NE transdifferentiation in 18 individual repeats without exception so long as castration is applied. This robust phenomenon indicates that NE transdifferentiation in the LTL331/331R model is lineage determined, and the early changes detected in castrated tumors may thus reflect an inevitable and not stochastic event. More importantly, we indeed demonstrate that HP1α can promote terminal NE marker expression in adenocarcinoma cells following ADT. In our study, we also noticed that while HP1α was able to repress AR expression and AR signaling under AR-driven prostatic epithelial status, it cannot function as a neural factors to directly induce the NE phenotype. Only when AR signaling is diminished by ADT can HP1α ectopic expression promote NE transdifferentiation in adenocarcinoma cells. This process both recapitulates the in vivo NE transdifferentiation phenotype occurring in the LTL331/331R
model, and also mirrors the clinical progression of NEPC where most cases appear after hormonal therapy (3,33). In terminal NEPC cells where HP1α mainly functions as a regulator of aggressiveness, HP1α knockdown cannot alter NE phenotype (Fig. S6A). Therefore, HP1α could potentially serve as an early therapeutic target to interfere with disease progression before NEPC fully develops.

In addition to being significantly overexpressed in clinical NEPC samples, HP1α plays a crucial role in terminal NEPC as demonstrated by thorough functional studies in the bona fide NEPC cell line NCI-H660. While previous studies have reported the function of HP1α in breast cancer, lung cancer, and cholangiocarcinoma, its function in prostate cancer remains elusive (38-42). Our data demonstrates that HP1α knockdown in NEPC cells dramatically inhibited proliferation, completely ablated colony formation, and induced apoptotic cell death. Consequently, HP1α depletion markedly inhibited NEPC tumor growth in vivo. Alternatively, in V16D adenocarcinoma cells where HP1α overexpression promoted NE transdifferentiation, HP1α overexpression did not significantly enhance proliferation (Fig. S6B). These data suggests that HP1α is particularly essential for NEPC malignancy, with one potential mechanism being HP1α depletion impairs mitotic machineries. While reported to drive and maintain heterochromatin structure (31,32,43), HP1α is prominently associated with constitutive heterochromatins (44) as demonstrated through our study. Depletion of HP1α did not affect heterochromatin-related genes universally, but significantly impaired the pericentric constitutive heterochromatin machineries. Pericentric heterochromatin is a key element ensuring proper chromosome segregation in metaphase (6), which is also a major previously reported function of HP1α (38,45). The abnormally enriched pericentric
heterochromatin genes in NEPCs may also explain the highly proliferative feature of NEPC, for which HP1α may play a driver function. Another potential mechanism underlying the essential function of HP1α in NEPC aggressiveness is that HP1α depletion impaired DNA damage response (DDR) machineries, which is in accordance with previous studies (46,47). Most recently, another study also reported that a DDR pathway is enriched in NEPC, contributing to NEPC cell proliferation (48). Overall, HP1α could potentially serve as a therapeutic target for effective management of developed NEPC.

Our findings also suggest a novel, HP1α-mediated mechanism of NEPC development. AR, FOXA1 and REST are the three crucial adenocarcinoma lineage-specific transcription factors maintaining luminal epithelial characteristics (12-14). Our data demonstrates that HP1α can repress AR and REST expression in adenocarcinoma cells, while its depletion in NEPC cell can reactivate their expression. HP1α gene was first identified to be a modulator of position effect variegation where euchromatic genes abnormally juxtaposed with pericentric heterochromatin could be silenced due to compaction into heterochromatin (49). In our study, we also found that HP1α may play an epistatic role in regulating the pericentric heterochromatin apparatus. The repressive histone mark H3K9me3 is a hallmark of pericentric heterochromatin and also a major substrate recognized by HP1α (31,32). Given that the AR and REST genes are natively located in the vicinity of pericentric heterochromatin (Xq12 and 4q12 respectively, Fig. S6C), they could potentially be sensitive to pericentric heterochromatin deregulation. Our data indeed showed that modulation of HP1α regulates the enrichment of H3K9me3 on the promoters of AR and REST. Considering the significant enrichment of H3K9me3-characterized pericentric
heterochromatin genes in multiple NEPC models and clinical cohorts, our study suggests that the HP1α/H3K9me3 axis may partially explain the absence or loss of AR and REST expression in NEPCs. Further investigation on genome-wide occupancy of HP1α and H3K9me3 will provide valuable insights. HP1α mediates gene silencing together with other precise epigenetic machineries (31). Previous studies have shown that AR expression can also be repressed by the EZH2/H3K27me3 axis (8,36). As such, HP1α/H3K9me3 might coordinate with EZH2/H3K27me3, establishing the complete repression of AR in NEPCs.

Alternative splicing has been suggested to suppress REST in NEPCs (16). HP1α was also reported to mediate mRNA alternative splicing and exon recognition (50). As such, HP1α might be involved in mediating REST splicing as well. Our data here suggests HP1α/H3K9me3 as a new mechanism leading to the silencing of REST mRNA in most NEPC samples.

In summary, our data imply a novel mechanism underlying NEPC development:

- HP1α drives the abnormal formation of pericentric heterochromatin, which in turn promotes ADT-induced NE transdifferentiation via repressing AR and REST expression, and confers the malignant NEPC phenotype via promoting aggressive proliferation and cell survival.
- Taken together HP1α can be considered an early and master mediator of NEPC development and aggressiveness, making it an exceptional novel therapeutic target for potentially effective treatment of NEPC.
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FIGURE LEGENDS

Figure 1. NEPC has a distinctive heterochromatin gene signature. (A-F) GSEA show the enrichment of heterochromatin-associated genes in NEPC from (A) PDX models, (B) the Beltran et al. 2011 cohort (23), (C) the Beltran et al. 2016 cohort (10), and in NE-like GEM models derived from (D) Rb/Tp53 DKO or Pten/Rb/Tp53 TKO (11), (E) NPp53 CRPC with NE differentiation (17), (F) and Nmyc overexpression (8). “NES” stands for normalized enrichment score; FDR q values were calculated using 1000 gene permutations except for (C), where Gsea preranked was applied. (G) Heatmap showing the hierarchical clustering among all PDX samples suggests a unique upregulation of heterochromatin signature genes in NEPC PDX tumors. (H) Weighted NE scores and heterochromatin scores of adenocarcinoma and NEPC PDX tumors and two clinical cohorts. NE scores were calculated based on the gene panels from Beltran et al. 2016 (10) and Lee et al. 2016 (18). Heterochromatin scores were calculated based on the weighted gene panel from (G). Scatter plots show the calculated score of each tumor sample, with lines indicating the mean value and 95% CI. The p-values were calculated using the unpaired two-tail Student’s t-test. See also Fig. S1.

Figure 2. HP1α expression is upregulated during NE transdifferentiation and in NEPC PDX models. (A) A heatmap showing the gene expression changes in the LTL331/331R NE transdifferentiation PDX model. Heterochromatin signature genes, AR signaling targets, and NE markers are included. RNA-seq data from two individual samples at each time point was used (pre: LTL331 pre-castration; Cx: 8 weeks post-castration; Rep: LTL331R NEPC.
relapse). Average differences in expression between castrated and pre-castrated tumors (C vs. P) are shown in a separate heatmap. (B-D) HP1α expression in PDX models, as determined by (B) qRT-PCR, (C) IHC, and (D) Western blotting. Scatter plots show relative mRNA expression or IHC score for each sample, with lines indicating median values. (E-G) HP1α expression in the LTL331/331R castration-induced NE transdifferentiation PDX model, as determined by (E) qRT-PCR, (F) Western blotting, and (G) IHC. Data show mean ± SEM from three replicates. The p-values (B, C, E) were calculated with unpaired two-tail Student’s t-tests. See also Fig. S2.

Figure 3. HP1α expression is upregulated in clinical NEPC samples and correlates with poor prognosis in adenocarcinomas. (A-C) HP1α mRNA expression in NEPC vs. adenocarcinoma from (A) the Beltran, et al. 2011 cohort (23), (B) the Beltran, et al. 2016 cohort (10), and (C) the Grasso, et al. 2012 cohort (24). Scatter plots show RNA expression data of each sample, with lines indicating median values. The p-values were calculated using unpaired two-tail Student’s t-test. (D) Staining intensity for the HP1α protein in primary adenocarcinomas (n=103), CRPC-adenocarcinomas (n=120), and NEPC (n=8) as determined by IHC of the VPC TMA. Box plots show the mean with whiskers representing 5-95% percentile values. The p-values were calculated using unpaired two-tail Student’s t-test. (E) Representative IHC images for the various staining intensities (0 to 3) are shown, with the lower panels being magnifications of the selected regions in the upper panels. Scale bars in the upper and lower panels represent 100 μm and 10 μm respectively. (F-G) Kaplan-Meier survival analyses of estimated (F) relapse-free survival time based on the HP1α IHC score of primary adenocarcinomas from the VPC cohort with follow-up information (n=37).
and (G) prostate-cancer specific survival time after first hormonal therapy based on HP1α mRNA from metastatic adenocarcinomas in the Grasso, et al. 2012 cohort (n=33) (24). The p-values were calculated using the log-rank test to determine the difference in outcomes between patients with high (red) and low (black) HP1α expression.

**Figure 4. HP1α is essential for the aggressive growth of NEPC cells and tumors.** (A) Stable knockdown of HP1α in NCI-H660 cells by lentiviral transduction. Changes to HP1α mRNA and protein levels were determined by qRT-PCR and Western blotting respectively. Bar graph shows mean ± SEM. The p-value was calculated by unpaired two-tail Student’s t-test. (B) Cell growth assay of HP1α knockdown H660 cells as determined by crystal violet staining. Stable cells were plated in four replicate wells for each time point and cell numbers were determined based on the absorbance at O.D. 572 nm of crystal violet dissolved in 2% SDS. Data is graphed as mean ± SEM. Representative images demonstrating cell numbers at the final time point are shown. The p-value was calculated by two-way ANOVA. (C) Colony formation assay of HP1α knockdown H660 cells as determined by crystal violet staining. Cells were plated in four replicate wells for each stable line and colony numbers were counted manually. Bar graph shows mean ± SEM. Representative images demonstrating colony numbers are shown. The p-value was calculated by unpaired two-tail Student’s t-test. (D) An EdU incorporation assay was performed by incubating stable HP1α knockdown H660 cells with 10 μM EdU for 4 hours. EdU-labeled cells (red) and total cells counterstained with DAPI (blue) were counted for at least 10 fields. Bar graph shows the mean (EdU-positive ratio) ± SEM. Representative images are shown with the scale bar representing 100μm. The p-values were calculated by unpaired two-tail Student’s t-test. (E)
An apoptosis assay measuring caspase-3 activity using the ApoLive-Glo™ Multiplex Reagent. Relative apoptosis was determined by the ratio of luminescence (caspase-3 activity) to fluorescence (AFC signal for viable cells). Bar graph shows mean ± SEM (normalized to shC). The p-values were calculated by unpaired two-tail Student’s t-test. (F)

Cell death following stable HP1α knockdown in H660 cells was determined by flow cytometry with 7-AAD staining. 10,000 single cells were collected for analysis with dead cells being 7-AAD positive. Bar graph shows mean ± SEM (normalized to shC). The p-values were calculated by unpaired two-tail Student’s t-test. (G) Apoptosis markers cleaved-caspase 3 and cleaved-PARP1 as determined by Western blotting following stable HP1α knockdown in H660 cells. Intact caspase 3 and PARP1 serve as controls. CI stands for “cleaved”. (H) Selected gene sets enriched in HP1α- versus control- knockdown H660 cells as analyzed by GSEA. The x-axis represents normalized enrichment score (NES). FDR of all gene sets is less than 0.05 calculated by 1000 permutations. (I-J) Stable H660 cell lines shC and KD2 were each grafted into four NSG mice (eight tumors total) to assess in vivo xenograft tumor growth. (I) Tumor volume was measured starting from when palpable tumor appears to when mice were euthanized. Line graph shows mean ± SEM, with p-value calculated by two-way ANOVA. Tumor images are shown in the right panel. (J) Fresh tumors were also weighed at sample collection. Bar graph shows mean ± SEM with The p-value was calculated by unpaired two-tail Student’s t-test. See also Fig. S3.

Figure 5. HP1α promotes NE transdifferentiation of prostatic adenocarcinoma cells following ADT. (A) Ectopic expression of HP1α in LNCaP-V16D cells as determined by Western blotting. (B-C) Induction of NE transdifferentiation with ADT in V16D cells stably
overexpressing HP1α. Relative mRNA and protein expression of terminal NE markers (SYP, CHGA, NSE, NCAM1) were detected by (B) qRT-PCR and (C) Western blotting in stable cells cultured in complete medium (FBS), CSS, and CSS with 10 μM EnZ 14 days. Bar graphs show mean ± SEM. The p-values were calculated by unpaired two-tail Student’s t-tests comparing HP1α- to control-overexpressing cells. Relative band intensities as determined by ImageJ are indicated, with actin serving as internal control. (D) A heatmap comparing expression of NEPC marker genes in the indicated V16D cells upon EnZ treatment. The select NEPC marker genes upregulated by HP1α overexpression are similarly upregulated in clinical NEPCs in the Beltran, et al. 2011 cohort (23). (E) Top 10 pathways significantly enriched in HP1α overexpressing V16D cells compared to control cells as analyzed with IPA. (F-G) Pearson correlation analysis of HP1α mRNA expression and the expression of various NE markers in advanced PCa samples. (F) Correlation with NCAM1 and NSE expression in the Beltran, et al. 2016 cohort (10), and (G) correlation with CHGB and CHGA expression in the Kumar et al. 2015 cohort (25). See also Fig. S4.

Figure 6. HP1α represses AR and REST expression and enriches H3K9me3 on their promoters. (A-B) AR and REST expression in stable V16D cells overexpressing HP1α as determined by (A) qRT-PCR and (B) Western blotting. Bar graphs show mean ± SEM. The p-values were calculated by unpaired two-tail Student’s t-test. (C) A heatmap comparing expression of AR signaling genes in the indicated V16D cells. The select AR target genes downregulated by HP1α overexpression are similarly downregulated in clinical NEPCs in the Beltran, et al. 2011 cohort (23). (D) GSEA of V16D cells with stable HP1α overexpression. Expression of pericentric heterochromatin components are upregulated by HP1α.
overexpression. “NES” stands for normalized enrichment score; FDR q values were calculated using 1000 gene permutations. (E) ChIP-PCR shows the enrichment of H3K9me3 on the promoters of AR and REST in V16D cells with HP1α overexpression. NC is a negative control region. Bar graphs show mean ± SEM. The p-values were calculated by unpaired two-tail Student’s t-test. (F) GSEA show the enrichment of pericentric heterochromatin genes in human NEPC samples and mouse NE-like tumors. The y-axis represents normalized enrichment score (NES) and the x-axis denotes clinical cohorts and GEM models. FDR is less than 0.15 calculated by 1000 permutations. (G-H) Pearson correlation analysis of HP1α mRNA expression and AR and REST mRNA levels from (G) the Beltran et al. 2016 (10) and (H) the Kumar et al. 2016 cohorts (25). See also Fig. S5.
A) PDX LTL331/331R Adeno-to-NEPC model

B) HP1α expression in PDX models

C) HC Score

D) Adeno vs. NEPC

E) HP1α expression in LTL331/331R model

F) Pre, Cx, Rep

G) HP1α

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A. NCI-H660 NEPC cell

**H1α mRNA expression**

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B. 572 nm O.D.

- **shC**
- **KD1**
- **KD2**
- **KD3**

C. Colony number

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D. Edu/DAPI

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E. Relative apoptosis (Cas3/ARC)

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F. Relative cell death

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G. Western Blot

- **Cl-Caspase 3**
- **Caspase 3**
- **Cl-PARP1**
- **PARP1**
- **Actin**

19kD, 17kD

H. Enriched shC and enriched KD

- **GO_Nuclear_Chromosome_Segregation**
- **Reactome_Double_Strand_Break_Repair**
- **Hallmark_E2F_Targets**
- **Reactome_Mitotic_Prometaphase**
- **Hallmark_G2M_Checkpoint**
- **Hallmark_UV_Response_Up**
- **Hallmark_TNFα_Signaling_Via_NFκB**

I. NES

- **shC**
- **KD2**

J. Tumor weight (mg)

- **shC**
- **KD2**

**Tumor weight (mg)**

- **shC**
- **KD2**
Research.

Figure 6

A. V16D

- Bar graph showing mRNA expression of AR and REST for Ctrl and HP1α groups.

B. Ctrl HP1α

- Heatmap showing AR, REST, and Actin expression.

C. V16D Beltran, et al. 2011

- Heatmap of Gene expression.

D. V16D HP1α OE

- GO Pericentric Heterochromatin.

E. Ctrl HP1α

- % of input for AR, REST, and NC.

F. GO Pericentric Heterochromatin

- NES values for HP1α OE in PDX models, Beltran, et al. 2011, Pten/Rb/Tp53 mouse model, and NPP3-CRPC mouse model.


- Scatter plots showing correlation between HP1α mRNA expression and AR/mRNA expression.


- Scatter plots showing correlation between HP1α mRNA expression and AR/mRNA expression.
Heterochromatin protein 1α mediates development and aggressiveness of neuroendocrine prostate cancer

Xinpei Ci, Jun Hao, Xin Dong, et al.

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