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The long non-coding RNA HORAS5 mediates castration-resistant prostate cancer survival by activating the androgen receptor transcriptional program

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**Keywords:** lncRNAs, HORAS, HORAS5, prostate cancer, androgen-independence.

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ABBREVIATIONS

AR – Androgen Receptor
CRPC – Castration-Resistant Prostate Cancer
DHT - Dihydrotestosterone
HORAS – Hormone-Resistance Associated non-coding Sequences
HOTAIR - Homeobox Transcript Antisense RNA
IFIT2 – Interferon Induced Protein with Tetratricopeptide Repeats 2
KIAA0101 - PCNA clamp associated factor
LincRNA – Long intergenic non-coding RNA
LncRNA – Long non-coding RNA
LOC283070 – Uncharacterized long non-coding RNA
LTL – Living Tumor Laboratory
MIAT – Myocardial Infarction Associated Transcript
MiRNA – MicroRNA
Mir-645 – MicroRNA 645
NOD/SCID – Non-obese diabetic/severe combined immunodeficiency
ORF – Open Reading Frame
PCa – Prostate Cancer
PCAT4 – Prostate Cancer Associated Transcript 4
PCAT18 – Prostate Cancer Associated Transcript 18
PCGEM1 – Prostate Cancer Associated Transcript 9
PDX – Patient-Derived Xenograft
PSA – Prostate Specific Antigen
qPCR – Quantitative Polymerase Chain Reaction
ABSTRACT

Prostate Cancer (PCa) is driven by the androgen receptor (AR)-signaling axis. Hormonal therapy often mitigates PCa progression, but a notable number of cases progress to castration-resistant PCa (CRPC). CRPC retains AR-activity and is incurable. Long non-coding RNAs (lncRNAs) represent an uncharted region of the transcriptome. Several lncRNAs have been recently described to mediate oncogenic functions, suggesting that these molecules can be potential therapeutic targets. Here, we identified CRPC-associated lncRNAs by analyzing patient-derived xenografts (PDXs) and clinical data. Subsequently, we characterized one of the CRPC-promoting lncRNAs, HORAS5, in vitro and in vivo. We demonstrated that HORAS5 is a stable, cytoplasmic lncRNA that promotes CRPC proliferation and survival by maintaining AR activity under androgen-depleted conditions. Most strikingly, knockdown of HORAS5 causes a significant reduction in the expression of AR itself and oncogenic AR targets such as KIAA0101. Elevated expression of HORAS5 is also associated with worse clinical outcomes in patients. Our results from HORAS5 inhibition in in vivo models further confirm that HORAS5 is a viable therapeutic target for CRPC. Thus, we posit that HORAS5 is a novel, targetable mediator of CRPC through its essential role in the maintenance of oncogenic AR activity. Overall, this study adds to our mechanistic understanding of how lncRNAs function in cancer progression.
INTRODUCTION

Prostate cancer (PCa) proliferation is fueled by activation of the androgen receptor (AR)-signaling pathway (Culig and Santer 2014). Androgens directly bind to the AR and trigger this process. Thus, hormone-deprivation therapy (a.k.a. surgical or medical "castration") is an effective therapeutic strategy for localized and metastatic PCa. Unfortunately, a substantial fraction of prostatic neoplasms (~25%) develop resistance to castration, mainly via genetic and/or epigenetic alterations that enable aberrant ligand-independent activation of AR-signaling (Feldman and Feldman 2001, Scher and Sawyers 2005) and cell survival pathways (Gao, Schwartzman et al. 2013, Karantanos, Corn et al. 2013). Despite the development of new therapies that delay disease progression, castration-resistant prostate cancer (CRPC) is still an incurable disease (Chandrasekar, Yang et al. 2015). Therefore, identification of alternative therapeutic targets is of paramount importance.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides. The human genome encodes for more than 50,000 unique lncRNAs, most of which are uncharacterized (Iyer, Niknafs et al. 2015). In addition to their physiological roles (Li and Chang 2014), lncRNAs are involved in pathological states including cancer (Huarte 2015, Jariwala and Sarkar 2016). For these reasons, lncRNAs have been proposed as a “gold mine” for the discovery of therapeutic targets in oncology. We recently identified PCAT18, a lncRNA that drives androgen-dependent prostate cancer (PCa) proliferation and metastasis (Crea, Watahiki et al. 2014) and MIAT, a neuroendocrine PCa-specific lncRNA (Crea, Venalainen et al. 2016). LncRNAs have been also implicated in PCa drug sensitivity (Malek, Jagannathan et al. 2014), metastatic progression (Shen, Qi et al. 2015), and prognosis (Lee, Mazar et al. 2014). Despite these developments, the role of lncRNAs in hormonal therapy resistance has not been investigated systematically.
Patient-derived PCa tissue xenografts (PDXs) have been very useful for translational cancer research (Hidalgo, Amant et al. 2014). Unlike oligoclonal cell line xenografts, PDXs more closely resemble the complex cellular heterogeneity of human PCa (Siolas and Hannon 2013). We have developed a unique procedure for grafting and serially transplanting primary human cancer tissues in NOD/SCID mice, using the sub-renal capsule graft site (Lin, Xue et al. 2014). These transplantable PDXs accurately recapitulate donor patient’s tissue histology, genetic/epigenetic features, and drug sensitivity (Lin, Wyatt et al. 2014). We have successfully applied PDXs for drug efficacy studies, discovery and validation of therapeutic targets, and personalized cancer therapy (Lin, Wyatt et al. 2014).

Using this technique, we have recently developed pairs of PDXs with opposite sensitivity to castration (Lin, Wyatt et al. 2014). Here, we profiled 3 of these pairs to identify IncRNAs specifically up-regulated in metastatic CRPC. Our results indicate that at least one of these previously uncharacterized transcripts activates pro-survival pathways and is functionally relevant in the progression of PCa to a castration-resistant state.

MATERIALS AND METHODS

Cell Culture

Human prostate cancer cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 media (Gibco, Cat# 11875-093) supplemented with 10% Fetal Bovine Serum (FBS, Gibco Cat# 10099-141) and following ATCC protocols for culture passage and storage of cells (ATCC, Cryogenic storage of animal cells protocol). A humidified 37°C 5% CO₂ incubator was employed for all culturing. Genetic fingerprinting and monthly Mycoplasma tests were conducted at the Vancouver Prostate Centre. Unless otherwise stated, all cells were counted for experiments using a TC20 automated cell counter (BioRad, Cat#
following manufacturer’s protocol (TC20 automated cell counter quick guide) and were maintained until a passage of 20 or lower.

**Patient-derived PCa xenograft**

For generation of PDX models, primary tumor biopsies were collected at the BC Cancer Agency or the Vancouver Prostate Centre with patients’ written consent. All surgical procedures and protocols for the acquisition and research-wise handling of tumor biopsies were approved by The University of British Columbia (UBC) – Research Ethics Board (protocol#: H04-60131). PDX were maintained by transplantation into sub-renal capsules of male NOD-SCID mice obtained from the British Columbia Cancer Research Centre – Animal Resource Centre (Vancouver, Canada), as previously described (Lin, Wyatt et al. 2014). All animal experiments were performed following the ethical guidelines and standards set by the Declaration of Helsinki and in accordance with the established animal care and use protocols approved by the UBC Animal Care Committee (protocol #: A10-0100).

**RNA extraction, reverse-transcription & quantitative PCR (qPCR)**

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) from cultured cells or tissues following manufacturer’s protocol. Upon extraction, 1,000ng of total RNA was reverse-transcribed using the QuantiTect kit (Qiagen) following manufacturer’s protocol. Reverse-transcription, genomic DNA digestion was performed prior to cDNA synthesis following manufacturer’s instructions. Pre-designed or custom TaqMan primers (Life Technologies, Assay IDs for all probes listed in Supp. Table 6) were used for qPCR to assess gene expression as per manufacturer’s protocol. Sub-cellular RNA fractionation was performed using the Paris kit (Ambion, Life Technologies) following the manufacturer’s protocol and performing the optional nuclear pellet washing step to obtain purer fractions.
siRNA-mediated gene knockdown

Gene knockdown were performed using the reverse transfection method (Hattori, Yoshiike et al. 2017). Cells were seeded in a 6-well or 96-well plate along with the lipid:siRNA mixtures prepared using the RNAiMAX (Invitrogen) reagent as per the manufacturer’s protocol. Final siRNA treatment dosages were 2nM and all duplexes were purchased from IDT: *HORAS5* (aka *Linc00161*, anti-*HORAS5* DsiRNA 1: 5’-GUGAUAAUAAUAUAAACUACAGUCA-3’, anti-*HORAS5* DsiRNA 2: 5’-CUAUGACUGUGGUAACAUUUCCAA-3’), *KIAA0101* (anti-*KIAA0101* DsiRNA 1: 5’-GUUUACCUGUAAUCAGAUGTA-3’, anti-*KIAA0101* DsiRNA 2: 5’-AGUGUCAGUUCUUCUAAAUC-3’), and non-targeting negative control, Cat#: 123762010. After 48 or 72hrs post-transfection, treated cells were harvested for extracting total RNA and/or total protein.

Cell proliferation & caspase activity assays

For both LNCaP and C4-2, 2000 viable cells were seeded in a 96-well plate and reverse transfected with either 2nM of control siRNA or siRNAs targeting the *HORAS5* transcript with 6 replicates for each treatment. At days 1, 3, 5 & 7 post-transfection, cell viability was assessed using the colorimetric CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Cat# G3582) according to the manufacturer’s protocol. Wells were incubated at 37°C in a 5% CO₂ incubator for 1.5hrs prior to absorbance measurements using a spectrophotometer set to 490nm. Values were plotted for each treatment after normalizing to the NC Day 1 average reading.

*DHT-rescue experiment:* A total of 2,000 viable LNCaP cells/well were plated in a 96-well plate and reverse transfected with *HORAS5*-targeted or control siRNAs (2nM dosage) as described above. Cells were then daily supplemented with either 10nM DHT or ethanol (vehicle control) and viability was calculated using the MTS assay and described above.

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Caspase activity assays: Cells were plated in a white, flat-bottom 96-well plate and treated with siRNAs as described with 6 replicates for each treatment. At day 3 post-transfection, Caspase-Glo reagent (Promega) was added to cells and total luminescence was quantified following manufacturer's protocol. These luminescence values were normalized to time-match and treatment-matched cell viabilities for each group and were plotted relative to the control siRNA-treated samples.

RNA half-life measurement

A total of 100,000 cell were plated in a 6-well and treated with 50μg/mL of alpha-amanitin (Sigma, A2263), as described (Khalil, Faghihi et al. 2008). Total RNA was extracted at time-points 0, 2, 5, 6, and 10hrs post-incubation and gene expression was analyzed by qPCR as previously described.

Cell cycle analysis

Cell cycle phase estimates were obtained using fluorescence-activated cell sorting flow cytometry. First, LNCaP and C4-2 cells were treated with a scramble control duplex or targeted anti-HORAS5 DsiRNA for 72hrs. A total of $4.0 \times 10^5$ cells were then harvested post-treatment, including untreated and lipofectamine RNAiMAX controls, and resuspended in 100μL of ice-cold DPBS (Gibco, Cat# 14190144). Next, 900μL of ice-cold 70% ethanol (EtOH) was added dropwise while vortexing cells to prevent aggregates from forming. Cells were left in 15mL falcon tubes at 4°C for one week for fixation. The following week, the EtOH was removed by centrifugation and cells were washed with DPBS once. Cell cycle phase was determined using propidium iodide (PI) staining buffer of composition 0.1% Triton X-100, 100μg/mL RNase (Invitrogen, Cat# 12091039), and 10μg/mL of PI (Sigma, Cat# P4170). After 30 minutes in the dark with the PI stain, cells were analyzed by FACScanlibur (Becton Dickenson) and were gated first by side-
scatter vs. forward-scatter, and then by FL2-Area vs. FL2-Width. Counts were then plotted against FL2-Area for selection of G1, S, and G2/M phases. Phasing estimation was obtained using FlowJo v10 software. A total of 20,000 cells were analyzed to get representative counts per phase. Significance was determined by comparing the NC to treated cells in each phase (Student’s T-test). "Cell cycle phase" represents either a single (G1) or double (S/G2-M) complement of DNA.

Western blotting analysis

Cell lysates were created using 100μL of RIPA buffer unless otherwise stated. Proteins (50μg) were resolved via gel electrophoresis on reducing SDS-polyacrylamide gels run at 100V for 1hr. Transfer to nitrocellulose membranes was done at 100V for 1hr as well. The membranes were blocked in 5% skim milk dissolved in TBS-T wash buffer (Tris-Buffered Saline containing 0.1% Tween) at room temperature for 1hr. Following this, blots were incubated overnight at 4°C with protein-specific primary antibodies containing 5% BSA to β-actin at 1:5,000 (Sigma, Cat# A5441), KIAA0101 at 1:1,000 (Abnova, Cat# H00009768-M01), and Androgen Receptor (AR) Primary Ab (Santa Cruz 441, Cat# sc-7305) 1:500 in TBS-T + 5% BSA. After incubation, blots were washed 4 times in TBS-T for 5 minutes each. Lastly, blots were incubated with HRP-conjugated anti-mouse secondary antibody diluted in 10mL of TBS-T at room temperature for 1hr (β-actin 1:5,000, KIAA0101 1:1,000, and AR 1:2,000). After washing as above, chemiluminescence was determined in a GelDoc system using an ECL western blotting substrate kit (ThermoFisher, Cat# 32016).

In vivo gene knockdown

Gene knockdown in vivo was achieved using the AteloGene in vivo siRNA/miRNA Transfection kit (REPROCELL) following manufacturer’s protocol. Castrate-resistant LNCaP cells were treated with either control siRNA or target siRNA and cultured for 24 hours. Cell lysates were then prepared for Western blotting analysis as described above.
xenografted in both dorsal flanks of intact male, immunocompromised NOD/SCID mice as previously described (Kuruma, Matsumoto et al. 2013) and were randomly assigned to each treatment group (n=3, i.e. 6 tumors/treatment). 2nM total dosage of HORAS5 siRNA was locally injected to surround the tumor as per manufacturer’s protocol for two consecutive days. Tumors were harvested 3 days after the first injection to assess tumor volume and target knockdown. All animal experiments were performed following the ethical guidelines and standards set by the Declaration of Helsinki and in accordance with the established animal care and use protocols approved by the UBC Animal Care Committee (protocol #: A10-0100). Total RNA was extracted as described above and genes were quantified using RT-qPCR to assess knockdown efficiency.

Wound-healing (migration) assays

For LNCaP and C4-2 cell lines, 18hrs post-transfection with either NC duplex or anti-HORAS5 siRNA. 2.0*10^6 cells per 6-well for LNCaP cells, and 3.5*10^6 cells per 60mm dish for C4-2 cells were seeded. Only 6-wells were pre-coated with poly-L-lysine for 3hrs (Sigma, Cat# P4707) and rinsed with dH2O. Both LNCaP and C4-2 cells were left to adhere for 4-5hrs before scratching the wells using a sterile P10 micropipette tip. Assays used serum-free media to prevent cell growth. Twenty-four hours post-scratch, cells were visualized and captured using an AxioCam MRc real-time camera (Zeiss). Wound closure was quantified using Adobe Photoshop as described (Chiang, Wang et al. 2014).

Boyden chamber (invasion) assays

LNCaP and C4-2 cells were first either left untreated, or were transfected with NC duplex or anti-HORAS5 siRNA for 18hrs in 60mm dishes. Next, cell invasive potential was examined using polyethylene terephthalate-coated 24-well transwell chambers with a pore size of 8μm.
Prior to the assay, top and bottom chambers were re-hydrated using 500μL of pre-warmed serum-free media for 2hrs at 37°C. Following this, 1.0×10^5 cells were seeded per well in serum-free media, and the assay was carried out according to manufacturer’s protocol. Media supplemented with 10% FBS was used as a chemoattractant in the bottom of each well. Cells were incubated for 48hrs prior to quantification using cell dissociation solution (Trevigen, Cat# 3455-096-05) diluted in MQH₂O and Calcein AM (Trevigen, Cat# 4892-010-01). Once mixed, 300μL of complete dissociation solution was added to the bottom chambers for 1hr. 100μL of dissociated cells were used for 96-well plate quantification using an excitation and emission of 485nm and 520nm respectively. All readings were done in duplicate.

**Immunohistochemical staining**

_In vivo_ treated tumors from all groups were used to generate tissue microarray and stained for cleaved Caspase 3 using a rabbit monoclonal antibody at 1:50 dilution (Cell Signaling, Cat# 9664). Tissue sections were divided into 9 quadrants prior to counting. Five of the total nine quadrants were used to take positively stained cell counts, which were then compared to total cells in the visual field. All five field counts were averaged and converted to a percentage value for control and treatment.

**RNA-Sequencing and differential expression analysis**

Total RNA was used to generate the sequencing library as previously described (Lin, Wyatt et al. 2014). Briefly, RNA sequencing fastq files were processed to read counts using the STAR short read aligner (https://github.com/alexdobin/STAR) with an index built with the hg19 genome GENCODE v19 transcriptome reference, and the -quantMode option. Genes with 0 counts in both libraries were eliminated. Twenty-five (25) was added to the read count for each gene to handle genes with 0 reads in 1 library, and normalized per million mapped reads (RPKM) in Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
each library. Genes were then filtered for 'lincRNA' type, and sorted in descending order of up-regulation in the CRPC (LTL-313BR) sample. To identify the up- and down-regulated lincRNAs, fold-change (FC) cutoff of 3 was used and lincRNAs were ranked in order of FC for HORAS annotation.

**Proteomics analysis**

A total of 10 million viable LNCaP cells were plated in a T150 flask and treated with either control or anti-HORAS5 as described earlier. At 3 days after treatment, cells were washed once with ice cold 1X PBS and snap-frozen to perform the proteomics analysis at the UBC Proteomics Core Facility at the Centre for High-Throughput Biology (Vancouver, Canada). Briefly, protein extracts from treated cells were suspended in SDS sample buffer and run on a short 10% SDS-PAGE gel. Proteins were visualized by colloidal coomassie (Candiano, Bruschi et al. 2004) and digested out of the gel as described (Chan, Howes et al. 2006). Peptide samples were purified by solid phase extraction on C-18 STop And Go Extraction (STAGE) Tips (Ishihama, Rappsilber et al. 2002), and each treatment was labeled by reductive dimethylation using formaldehyde isotopologues (Parker, Guarna et al. 2012). The final product was purified again by C18 STAGE tips as previously done and analyzed by LC-MSMS (Kang, Ge et al. 2014). Protein identification and quantification were performed with MaxQuant v1.5.1.0 as described (Cox, Matic et al. 2009).

**In silico microRNA binding prediction analyses**

In order to predict microRNAs that could be interacting with HORAS5, we used an online transcriptome-wide microRNA binding prediction tool (http://www.mircode.org/index.php) from the Larsson Lekholm Lab from the Institute of Biomedicine, University of Gothenburg (http://larssonlab.org/). The tool was run using default parameters, and the entire output data...
has been summarized in a table in Supp. Fig. 12. Additional information about this prediction software is available on the lab's website.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

RESULTS

Identification of hormone therapy resistance-associated lincRNAs

To identify lncRNAs that are up-regulated upon progression of primary PCa to CRPC, we analyzed our unique collection of PCa PDX models (Lin, Wyatt et al. 2014). These PDXs were grown in the subrenal capsule of immuno-compromised male mice. PDX-bearing mice were surgically castrated at about 10-12 weeks post-engraftment (black arrowheads, Fig. 1A). As expected, castration resulted in a dramatic reduction of plasma PSA levels, which is a canonical AR-target gene and strongly correlated with the total tumor burden (Fig. 1A and Supp. Fig. 1A). Analogous to the clinical progression of some PCas, in three PDX models the tumors eventually relapsed after about 20 weeks to produce a castration-resistant sub-line. The relapsed, castration-resistant tumors displayed signs of AR-signaling re-activation, as evidenced by the parallel increase in plasma PSA levels (Fig. 1A and Supp. Fig. 1A). Of these PDX models, we chose the most well-established and previously described LTL-313B/BR pair for a comprehensive RNA-sequencing analysis (Luk, Shrestha et al. 2017). Notably, the relapsed castration-resistant LTL-313BR sub-line retains AR expression and is markedly resistant to clinical AR-antagonistic drugs bicalutamide and enzalutamide relative to the parental sub-line (Luk, Shrestha et al. 2017). This implies that a broad spectrum of molecular mechanisms that regulate CRPC growth
underlie the survival and proliferation of LTL-313BR under castrate conditions. To investigate this further, we decided to focus on the intergenic sub-class of lncRNAs (hereinafter referred to as lincRNAs) that are coded on distinct chromosomal loci and thus are easier to functionally characterize. Differential gene expression analysis between LTL-313BR versus 313B revealed 57 lincRNAs that were up-regulated (FC≥3), and 329 lincRNAs that were down-regulated (FC≤-3) in the hormone therapy-resistant sub-line (Supp. Tables 1 & 2, respectively). Consistent with previous studies, we found PCa-progression associated PCAT4 (Prensner, Iyer et al. 2011) among the up-regulated transcripts, and in vivo androgen-regulated PCGEM1 (Parolia, Crea et al. 2015) among the down-regulated lincRNAs. Since a relevant portion of these lincRNAs were previously unannotated, we termed them as Hormone Resistance Associated non-coding Sequences (HORAS). To confirm the RNA-Sequencing results and to profile the expression of other differentially expressed lincRNAs in other PDX pairs, we performed qPCR gene expression analyses. Our results confirmed up-regulation of the selected HORAS transcripts in LTL313BR versus LTL-313B, and in two other PDX pairs (Fig. 1B-F). HORAS5 (aka linc00161, NCRNA00161, C21orf100, linc-USP16) was significantly up-regulated in all the three castration-resistant PDXs (4-130-fold; Fig 1B). Therefore, we pursued HORAS5 for further functional characterization in CRPC-derived PCa cells.

The Ensembl database categorizes HORAS5 as a lincRNA gene and annotates it as linc00161 (aka C21orf100, NCRNA00161) with two splice variants. The ORF finder database (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) confirmed that at least 88% of this transcript does not contain an open reading frame. Test code software (Fickett 1982) confirmed that the transcript is non-coding (P<0.01). Interestingly, HORAS5 was first described in a study published over a decade ago which attempted to profile novel genes encoded on chromosome 21 and could be implicated in Down Syndrome (Reymond, Camargo et al. 2002). At the time, the authors confirmed HORAS5 transcript structure via 5’- and 3’-end RACE. Additionally, the same
study showed via qPCR that \textit{HORAS5} was robustly expressed only in normal prostate tissue compared to 20 other normal tissue types (Reymond, Camargo et al. 2002). This prostate-specific expression was corroborated by an independent study using an array-based transcriptomic technique in a panel of 12 normal tissues (Supp. Fig. 2A). Concordantly, we found \textit{HORAS5} to show the highest expression in PCa specimens across all TCGA-sequenced cancers (Supp. Fig. 2B). Of note, a recent study reported that \textit{HORAS5} was induced by chemotherapy in osteosarcoma cells (Wang, Zhang et al. 2016). However, no functional knowledge exists on the biological role of \textit{HORAS5} in PCa pathogenesis.

As a first step, we profiled the expression of \textit{HORAS5} in a panel of PCa cell lines. We found that \textit{HORAS5} was considerably expressed only in AR-positive CRPC-derived cell lines (Thalmann, Anezinis et al. 1994, Sedelaar and Isaacs 2009), and was undetectable in non-neoplastic BPH cells (Fig. 2A). Our data also indicated that the longer \textit{HORAS5} transcript variant was much more abundant than the shorter one in most PCa cells and PDX models (Fig. 2B and Supp. Fig. 3A,B), prompting us to functionally pursue the longer variant (hereafter referred to as \textit{HORAS5} alone). This restricted \textit{in vitro} expression of \textit{HORAS5} in AR-positive CRPC cell lines is consistent with the \textit{in vivo} expression profile observed in our AR-positive CRPC-derived PDX models.

Next, sub-cellular RNA fractionation experiments revealed that \textit{HORAS5} was predominantly localized in the cytoplasm of the cell (Fig. 2C and Supp. Fig. 3C). Since lincRNAs, unlike a protein-coding mRNA, are end functional entities, we investigated the stability of the \textit{HORAS5} transcript itself. To this end, we treated LNCaP cells with α-amanitin, a potent inhibitor of RNA polymerase II, and thereafter measured the relative RNA abundance at various time-points (qPCR). Compared to the mRNA of a known oncogenic factor, \textit{c-Myc}, \textit{HORAS5} possessed a longer half-life of about 3 hours (Fig. 2D). This implies that \textit{HORAS5} is relatively stable inside cells for several hours post-transcription thereby allowing it to mediate important cellular functions.

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**Functional characterization of HORAS5**

So far, we have shown that among the differentially expressed lincRNAs, **HORAS5** is the most consistently up-regulated gene in hormone-therapy resistant PCa and is predominantly retained in the cell cytoplasm. To investigate the biological function of **HORAS5** we used two distinct siRNAs to knockdown this gene in LNCaP and C4-2 cells, both capable of growing under castrate-levels of testosterone, and both expressing a mutated AR gene (Sedelaar and Isaacs 2009, Walker, Abeel et al. 2014). With both the siRNA duplexes, we were able to achieve a robust and consistent knockdown with greater than 80% efficacy at a dosage as low as 2nM (Fig. 3A,B and Supp. Fig. 4). Notably, **HORAS5** knockdown significantly attenuated the growth of both LNCaP and C4-2 cells grown in castrate levels of testosterone (Fig. 3C,D respectively). Consistently, cell cycle analyses revealed a modest, yet significant, decrease in the fraction of cells entering the S-phase after **HORAS5** knockdown relative to control siRNA treatment, with a parallel increase in the G0/G1 fraction (Supp. Fig. 5A,B). In accordance with the previous, induction of apoptotic caspase enzymes was observed upon treatment with both anti-**HORAS5** siRNAs (Fig. 4A-C). However, we did not observe a marked attenuation in invasion or migration potential upon siRNA-mediated knockdown of **HORAS5** at non-toxic doses (Supp. Figs. 6 and 7). Together, these data strongly implicate **HORAS5** in the survival and proliferation of CRPC-derived PCa cells.

Since **HORAS5** was up-regulated in CRPC tumors, we questioned if its biological relevance in PCa was restricted only to castrate conditions. LNCaP cells represent an ideal model to test this hypothesis because they were derived from a castration-resistant PCa, but physiologic levels of testosterone further stimulate their proliferation (Alisky, Tang et al. 2010). To this end, we knocked down **HORAS5** in LNCaP cells either in the absence or presence of dihydrotestosterone (DHT), a potent physiological AR agonist. Addition of DHT markedly rescued cell death observed upon **HORAS5**-knockdown using both siRNAs (Fig. 4D). This indicates that **HORAS5**...
plays a pro-survival role primarily under castrate-levels of androgen, suggesting there may be a functional association of HORAS5 with the AR pathway.

**Mechanisms of HORAS5-dependent PCa cell survival**

Our results so far indicate that HORAS5 plays a key role in CRPC survival and growth. We next set out to investigate the molecular correlates of HORAS5-dependent PCa progression. The majority of the functional studies to date have revealed that lncRNAs fold into three-dimensional structures and interact with a wide range of protein partners, thereby regulating their function (Cao, Luo et al. 2015). Thus, to explore the mechanisms underlying the oncogenic functions of HORAS5, we adopted an unbiased proteomics approach (Supp. Fig. 8).

We first knocked-down HORAS5 in LNCaP cells using two distinct siRNAs and a control non-targeting siRNA. We then performed quantitative, tandem mass spectrometry proteomic analyses using treated cell lysates. Differential protein abundance analysis revealed 224 proteins that were significantly increased and 34 that were significantly decreased by greater than 3-fold in HORAS5-knockdown cells relative to the control (Supp. Table 3 & 4, respectively). Next, we conducted an Oncomine-based pathway analysis of the genes that were significantly down-regulated upon HORAS5 silencing. The most significantly associated concept was "RNA polymerase II-transcription factor activity" (Supp. Table 5). Since AR-RNA Polymerase II complexes have been identified as the main drivers of prostate cancer (Wang, Li et al. 2007), we decided to investigate if the anti-proliferative effect of HORAS5 silencing was at least in part mediated by inhibition of the AR-transcriptional program.
Strikingly, upon *HORAS5* knockdown, several canonical AR-targets were markedly down-regulated (>50%, Fig 5A), including PSA (aka KLK3) and NKX3.1. Beyond canonical targets, a literature search revealed that several other genes that were down-regulated upon *HORAS5* knockdown were also activated by AR. For instance, the second most down-regulated protein, KIAA0101 (>5-fold), was recently confirmed as an AR-activated gene in a study using patient specimens (Shaw, Whitaker et al. 2016). *KIAA0101* has been described as a potent oncogene in many cancers (Hosokawa, Takehara et al. 2007, Zhu, Diao et al. 2013), and has been categorized as an anaphase promoting protein (Emanuele, Ciccia et al. 2011). No study to date has described the biological functions of *KIAA0101* in PCa. Thus, we set out to further explore any cellular interplay between *HORAS5* and *KIAA0101*. In CRPC C4-2 cells, *HORAS5* knockdown led to a significant reduction in the *KIAA0101* mRNA levels by greater than 60% (Fig. 5B). This reduction in *KIAA0101* mRNA translated to a significant reduction of *KIAA0101* protein (Supp. Fig. 9D), also verifying our initial proteomics analyses. To explore this further, we knocked-down *KIAA0101* in CRPC-derived C4-2 cells using two distinct siRNAs (Supp. Fig. 9A,B). *KIAA0101* silencing alone significantly inhibited the growth of C4-2 cells (Fig. 5D), suggesting that the anti-proliferative effect of *HORAS5* inhibition can be partly explained by downstream reduction in oncogenic AR targets like *KIAA0101*. Our proteomic data were further validated by qPCR analysis of another down-regulated gene in *HORAS5* silenced samples, called *STMN1* (Supp. Fig. 10A). Notably, *STMN1* is also an AR-regulated gene (Yan, Xing et al. 2013), and has been previously associated with oncogenic features (Hemdan, Linden et al. 2014, Nie, Xu et al. 2015). Thus, we found several AR target genes to be markedly down-regulated upon *HORAS5* knockdown, which prompted us to query the expression of AR itself. Remarkably, *HORAS5* knockdown significantly reduced AR expression at the transcript and protein level in both LNCaP and C4-2 cells (Fig 5C; Supp. Fig 10B); however, *HORAS5* itself was not regulated by AR-activity (Supp. Fig 10C). From a mechanistic standpoint, we assessed the stability of the *AR* mRNA in presence and absence of *HORAS5*. Here, we found that *HORAS5* knockdown reduced the stability of the *AR* mRNA (Fig. 5E), but found no change in the stability of the control *MYC*.
and HPRT1 mRNAs (Supp. Fig. 11A,B). This strongly suggests that HORAS5 may be involved in the post-transcriptional regulation of the AR. Consistently, upon HORAS5 knockdown we found a significant decrease in the mature AR mRNA, but found no change in the abundance of the pre-mature, intron-containing AR transcript (Supp. Fig. 11C). Together, this evidence, and the experimental data shown in Figure 4D, position HORAS5 as an androgen-independent transcript that is required for activation of the oncogenic AR transcriptional program, through post-transcriptional maintenance of AR mRNA stability.

Next, to clinically interpret the HORAS5-associated protein signature, we individually uploaded the differential expressed genes from the proteomics analyses into the Oncomine database. Proteins that were markedly reduced in response to HORAS5-knockdown strongly correlated with increased odds of disease recurrence at 3 and 5 years after primary treatment (Supp. Fig 8). Direct evaluation of HORAS5 expression in PCa patient samples also confirmed that higher expression of this lincRNA predicted poorer clinical outcome in terms of overall survival (Fig. 5F). These clinical correlations are consistent with HORAS5 mediating major proliferative functions in PCa under hormone-depleted conditions as shown above. The proteins that were up-regulated upon HORAS5-knockdown returned no significant association (p<0.001) with any clinical variables. Next, we queried the TCGA PCa transcriptomic data to identify genes that have significant positive correlation with HORAS5 expression. Using these genes, we then performed a computational analyses, called BART (Amaral, Erikson et al. 2018), to predict the common regulatory transcription factors. Consistent with the involvement of HORAS5 in maintenance of AR-signaling, BART analyses predicted AR to be one of top regulatory factors for the HORAS5-associated genes in patient tumors (Supp. Fig. 11D). As expected, similar analyses for negatively correlated genes did not return the AR as a significant transcription factor (Supp. Fig. 11D).
Therapeutic potential of \textit{HORAS5} inactivation

Thus far, we have established that \textit{HORAS5} drives CRPC growth by supporting oncogenic AR-transcriptional programs and cellular availability of several oncogenic proteins. We then sought to validate the oncogenic roles of \textit{HORAS5} \textit{in vivo}. For this, we generated \textit{in vivo} androgen-insensitive sub-lines of LNCaP as previously described (Kuruma, Matsumoto et al. 2013). Using the AteloGene delivery system (Takeshita, Minakuchi et al. 2005), after 2 consecutive siRNA injections, we were able to achieve a significant knock-down of \textit{HORAS5} by about 50% (Fig. 6A). This inhibition of \textit{HORAS5} expression ensued in a significant reduction in tumor volume relative to the control (Fig. 6B). To confirm if this reduction was due to apoptotic killing of tumor cells, we generated tissue microarrays from the treated tumors and stained for active/cleaved Caspase 3. Complementing the reduction in tumor volume, inhibition of \textit{HORAS5} led to significantly higher staining for cleaved Caspase 3 in tumor cells (Fig. 6C,D). These results indicate that \textit{HORAS5} modulates CRPC cell proliferation and survival \textit{in vivo} and that it could be used as a novel therapeutic target for CRPC.

DISCUSSION

In this study, we have identified \textit{HORAS5} as a mediator of CRPC cell proliferation and as a potential therapeutic and prognostic target for PCa. Pre-clinical data obtained using different cell lines indicate that \textit{HORAS5} is required for CRPC cell survival in castrate levels of testosterone. In keeping with this hypothesis, we showed that \textit{HORAS5} is highly expressed in early clinical PCa specimens and in CRPC patient-derived xenografts. Additionally, higher \textit{HORAS5} expression in primary PCa samples predicts poorer clinical outcome.

Our results also indicate that \textit{HORAS5} is predominantly contained in the cytoplasm of PCa cells where it regulates the stability of the AR mRNA. In osteosarcoma cells, this IncRNA has been
recently shown to be associated with the AGO complex, where it buffers mir-645 and indirectly increases the levels of the pro-apoptotic IFIT2 protein (Wang, Zhang et al. 2016). However, our proteomic (Supp. Tab. 3&4) and miRNA binding prediction data (Supp. Fig. 12A) did not show the existence of this pathway in PCa cells, where the lncRNA seems to play an oncogenic role. Moreover, mir-645 was not expressed in AR-positive PCa cells (Supp. Fig. 12B). Instead, our results are more in line with recent evidence showing that HORAS5 overexpression is associated with worse prognosis in hepatocellular carcinoma patients (Xu, Chen et al. 2017). The previous study also showed that HORAS5 drives the metastatic potential of liver cancer cells. Taken together, this evidence indicates that the effects of HORAS5 expression are tissue- and disease-specific, as described for other lncRNAs (Schmitt and Chang 2016, Huarte 2015).

In this study, we have shown using different techniques that HORAS5 silencing leads to a marked inhibition of the KIAA0101 oncogene. KIAA0101 has been described as a pro-survival oncogene in several cancer types (Hosokawa, Takehara et al. 2007, Zhu, Diao et al. 2013) and as a driver of cell cycle progression (Emanuele, Giccia et al. 2011). Our data show that HORAS5 silencing attenuates cell cycle progression and induces caspase-dependent apoptosis in CRPC-derived cells. Interestingly, HORAS5 silencing significantly attenuates the AR-transcriptional program, with marked reduction in oncogenic AR targets such as KIAA0101. This can be explained by the decrease in the expression of AR mRNA itself likely due to reduced cytoplasmic stability in absence of HORAS5. Several other oncogenic proteins are positively associated with HORAS5 expression as well (Supp. Table 3). This suggests that targeting HORAS5 could simultaneously block multiple key oncogenic pathways in CRPCs.

Other lncRNAs have been previously identified as potential drivers of CRPC. In particular, LOC283070 has been shown to induce the proliferation of LNCaP cells in androgen-depleted media (Wang, Lin et al. 2016). Since this lncRNA had not been described before, we decided to...
analyze its expression in our models. Unfortunately, we were unable to do so, since we found that the primers used to measure LOC283070 expression perfectly overlap with the CAMK1D protein, which has been identified as a potential interactor of LOC283070. In addition, the siRNA sequences used to silence LOC283070 show a substantial overlap with the CAMK1D protein. Based on these findings, we question the assumption that LOC283070 is a true lncRNA. On the contrary, HOTAIR is a well-characterized oncogenic lncRNA, which is mainly expressed in the nucleus and binds directly to the AR. A recent study identified HOTAIR as a putative driver of CRPC progression (Zhang, Zhao et al. 2015). Notably, this study did not test the therapeutic potential of HOTAIR knockdown in vivo. Hence, this manuscript reports the functional characterization of a lncRNA that drives CRPC in vitro and in vivo.

CONCLUSION

Our study indicates that the cytoplasmic lncRNA, HORAS5, mediates CRPC progression in an AR-dependent manner by regulating AR mRNA stability, and is associated with poorer clinical outcome in human PCa samples. In addition, our in vivo data further implicate lncRNAs as putative therapeutic targets for CRPC (Fig. 6). Thus, our study adds to the mounting evidence that centrally implicates lncRNAs in cancer biology, and warrants the development of innovative therapies that target oncogenic non-coding RNA molecules in advanced, lethal PCa.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.
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Authors’ contributions

AP and EV conducted most of the experiments and wrote the manuscript. HX, DL, and RW conducted the in vivo experiments. RM and PP conducted in silico analyses. JR conducted proteomics analyses. JRE and FF analyzed RNA sequencing data. CC critically revised the paper. YW and FC ideated and supervised the study, and critically revised the paper.

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Not applicable.

REFERENCES


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Figure Legends

Figure 1 | **HORAS5 is up-regulated in CRPC.** (A) PSA levels in three independent hormone-sensitive patient-derived xenografts in NOD-SCID mice. Arrows represent androgen-deprivation (via surgical castration) followed by relapse around 20–30 weeks. (B-F) qPCR expression of \( \text{HORAS5} \) (B, aka \( \text{linc00161} \)), \( \text{HORAS1} \) (C, aka \( \text{RP11-945A11.1} \)), \( \text{HORAS2} \) (D, aka \( \text{AF131217.1} \)), \( \text{HORAS27} \) (E, aka \( \text{PCAT4} \)), \( \text{HORAS20} \) (F, aka \( \text{AP001604.3} \)) identified from differential expression analyses between hormone-sensitive (LTL313B) and relapsed (LTL313BR) PCa PDXs. Results expressed as means ± S.D. from two independent replicates. Student's \( t \)-test was performed for statistical comparisons. *\( P<0.05 \) and **\( P<0.01 \). \( \text{HORAS1} \) was undetectable in the remaining samples.

Figure 2 | **HORAS5 is a stable cytoplasmic lncRNA in AR-positive PCa cells.** (A) Basal expression (qPCR) of \( \text{HORAS5} \) in a panel of PCa cell lines. Gene expression was normalized to average of \( \text{GAPDH} \) and \( \text{HPRT1} \) and shown relative to BPH1. (B) Taqman qPCR expression of \( \text{HORAS5} \) long and short variants (as identified in the Ensembl database) in two CRPC-derived cell lines, namely \( \text{LNCaP} \) and \( \text{C4-2} \). (C) qPCR expression of \( \text{HORAS5} \) in the nuclear and cytoplasmic sub-cellular fractions of AR-positive \( \text{LNCaP} \) cells. \( \text{GAPDH} \) and \( \text{snoRNA55} \) are used as controls for cytoplasmic and nuclear fractions, respectively. Results from two independent experiments are plotted. (D) qPCR-based transcript expression of \( \text{HORAS5} \) and c-Myc in \( \text{LNCaP} \) cells treated with alpha-amanitin for various time points (0, 2, 5, 6, 10hrs post-treatment). Relatively stable \( \text{GAPDH} \) expression was used for normalization and data is plotted relative expression at 0h. Results from two independent experiments are plotted as means ± S.D..

Figure 3 | **HORAS5 knockdown attenuates cell proliferation in vitro.** (A,B) qPCR expression levels of \( \text{HORAS5} \) in \( \text{LNCaP} \) (A) and \( \text{C4-2} \) (B) cells upon treatment with non-targeting control siRNA (NC) or two distinct anti-\( \text{HORAS5} \) siRNAs (2nM dosage). Data shown is a single representative trial. (C,D) MTS cell proliferation assays for \( \text{LNCaP} \) (C) and \( \text{C4-2} \) (D) upon treatment with anti-\( \text{HORAS5} \) siRNAs or controls siRNAs. Two-way ANOVA with multiple comparisons (Tukey's post \( t \)-test) performed to measure significance, ****\( P<0.0001 \). Results are from three independent experiments.
Figure 4 | \textit{HORAS5} knockdown induces caspase-mediated apoptosis \textit{in vitro}. (A-C) Caspase 3/7 (A), Caspase8 (B), and Caspase9 (C) activities normalized to relative LNCaP cell number three days post-transfection of \textit{HORAS5}-siRNAs versus non-targeting control siRNA. One-way ANOVA with Tukey’s Post Test, $$^{****}P<0.0001$$. Results are expressed as means ± S.D. from two independent replicates. (D) Relative viable cell counts of LNCaP cells upon knockdown of \textit{HORAS5} (48hrs) with or without DHT supplementation. Results are expressed as means ± S.D. from two independent replicates. Student’s $t$-test performed for statistical comparisons with $$^{****}P<0.0001$.

Figure 5 | \textit{HORAS5} regulates the expression of AR target genes and has prognostic value in PCa. (A) mRNA expression of the labeled genes after siRNA-mediated knockdown of \textit{HORAS5} in LNCaP cells. (B) Relative mRNA expression (qPCR) of \textit{KIAA0101} post \textit{HORAS5}-knockdown in LNCaP and C4-2 cells. (C) Relative mRNA expression (qPCR) of \textit{AR} post \textit{HORAS5}-knockdown in LNCaP and C4-2 cells. (D) MTS cell proliferation assay in C4-2 cells upon treatment with anti-\textit{KIAA0101} siRNAs (2nM dose) or a control siRNA (5nM dose). (E) Relative expression (qPCR) of \textit{AR} mRNA in LNCaP treated with either \textit{HORAS5}-targeting or control siRNA (5nM for 24h), followed by treatment with the transcriptional inhibitor Actinomycin-D (20μM) for increasing durations. (F) post-prostatectomy disease-free survival based on \textit{HORAS5} expression (cBioportal, TCGA prostate cancer database, 456 vs. 35 (Elevated \textit{HORAS5}) patients). All qPCR results shown as data from at least two independent replicates, statistical analyses using One-way ANOVA with Tukey’s Post Test, $$^{****}P<0.0001$.

Figure 6 | \textit{HORAS5} knockdown attenuates tumor growth \textit{in vivo}. (A) Relative expression of \textit{HORAS5} (qPCR) 72 hours following AteloGene:siRNA injections (n=3 in each arm). Raw values were normalized to \textit{GAPDH} and \textit{HPRT1} and shown relative to the control group ($^{***}P<0.001$). (B) Quantitative measurements of tumor volume post-knockdown of \textit{HORAS5} (72hrs). Statistical significance from Student’s $t$-test with $^*P<0.05$. (C) Representative IHC images for cleaved Caspase 3 from control siRNA (NC) and anti-\textit{HORAS5} siRNA treatment groups. The scale bar at the bottom marks a 20μm width. (D) Overall IHC staining quantification from C. Results are expressed as percent of positive cells to total cells in the visual field. Data are shown as means ± S.D. and a Student’s $t$-test was used to calculate significance, $^*P<0.05$. 

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Supporting Information

Supplementary Figure 1 | Tumor volume and serum PSA levels for LTL313B/BR paired PDX-pair in NOD/SCID mice. Tumor volume (blue line) and serum PSA (red line) dramatically increase after LTL313B tumor engraftment. When tumors reach ~150mm$^3$ volumes, mice were surgically castrated. In response to castration, tumor volume and PSA both sharply decline. However, tumor eventually relapses around week 20 with concurrent increase in serum PSA levels. The castration-resistant tumor is referred to as LTL313BR.

Supplementary Figure 2 | Basal HORAS5 expression in a panel of normal and cancerous tissues. (A) HORAS5 expression is highest in healthy prostate tissue relative to 11 other tissue types. This microarray data was downloaded from GEO Profiles (ID: 2921923) and replotted using GraphPad. (B) CBioPortal analysis of HORAS5 abundance in multiple cancerous tissues.

Supplementary Figure 3: Expression of Long and short HORAS5 splice variants in PCa cell lines and LTL PDXs. (A) Schematic showing the two HORAS5 splice variants and the exon-spanning TaqMan probes used for qPCR to distinguish the two variants. (B) Basal expression (TaqMan qPCR) of both HORAS5 transcript variants in 5 different paired hormone-sensitive/-resistant LTL PDXs. Data is plotted as mean ± S.D. from a representative sample and is normalized to the HORAS5 long transcript variant expression in the 313B sample for clarity. (C) qPCR expression of HORAS5 in the nuclear and cytoplasmic sub-cellular fractions of AR-positive C4-2 cells. GAPDH and snoRNA5 are used as controls for cytoplasmic and nuclear fractions, respectively. Results from two independent experiments are plotted.

Supplementary Figure 4 | HORAS5 short transcript knockdown in two prostate cancer cell lines. LNCaP and C4-2 cells were treated with either a NC scramble or anti-HORAS5 siRNA for 48hrs prior to qPCR analysis. Data is depicted as means ± S.D. from duplicate independent experiments.

Supplementary Figure 5 | HORAS5 knockdown affects cell cycle progression in vitro. (A,B) Fraction of LNCaP (A) and C4-2 (B) cells in different phases of the cell cycle 72hrs post-
knockdown of $HORAS5$. Student $t$-test were used to measure significance, *$P<0.05$. Cell cycle data shown as means ± S.D. from three independent experiments.

**Supplementary Figure 6 | $HORAS5$ silencing does not alter cell migration potential.** (A,B) Wound-healing (scratch) assays following 18hr knockdown of $HORAS5$ in LNCaP (A) and C4-2 (B) cells. Images show wound size when produced (0 hrs) and 24 hrs following. Results are shown as means ± S.D. from three independent experiments. Significance tested using a Student’s $t$-test. The scale bars mark the 20$\mu$m width.

**Supplementary Figure 7 | $HORAS5$ silencing does not mediate cellular invasion.** LNCaP (A) and C4-2 (B) cell invasive potential examined after 18hr knockdown of $HORAS5$. Boyden chamber 48-well invasion assays were utilized and results are shown as means ± S.D. from three independent trials. Significance tested using a Student’s $t$-test.

**Supplementary Figure 8 | Proteins down-regulated in response to $HORAS5$ knockdown are associated with clinical PCa recurrence.** Differentially expressed protein genes (Supplemental tables 3 and 4) identified from in vitro knockdown of $HORAS5$ in LNCaP cells followed by MS/MS proteomics analysis were investigated for clinical associations using the Oncomine web portal. Proteins up and down-regulated (≥ 3-fold) in LTL313BR versus LTL313B were uploaded into Oncomine separately, and only significant clinical associations are reported above.

**Supplementary Figure 9 | $KIAA0101$ knockdown in CRPC-derived PCa cells.** (A,B) Immunoblots showing B-Actin (control) and $KIAA0101$ protein expression 72 hours after knockdown of $HORAS5$ in LNCaP (A) and C4-2 (B) cells. (C) Relative mRNA expression (qPCR) of $KIAA0101$ post knockdown in LNCaP and C4-2 cells. Results are from a representative sample. (D) Immunoblot showing B-Actin (control) and $KIAA0101$ protein expression 72 hours after knockdown of $KIAA0101$ by control and targeted DsiRNAs in LNCaP and C4-2 cells. All blot were cut for primary antibody incubation, and have been boxed to indicate results which are from the same run.
Supplementary Figure 10 | Several key oncogenic proteins are down-regulated upon HORAS5 knockdown. (A) STMN1 mRNA expression (qPCR) in LNCaP and C4-2 cells 72 hours post-knockdown of HORAS5. One-way ANOVA with Tukey’s Post Test. qPCR data are shown as means of three independent replicates ± S.D.. (B) Immunoblot of the AR and control B-actin 72h after the knockdown of HORAS5 using two distinct siRNAs (2nM dosage) in LNCaP and C4-2 cells. Immunoblot data shown as a representative sample. (C) mRNA expression of labeled genes in LNCaP cells following DHT addition (10nM) for various durations. A single representative experiment is shown here. Student’s t-test performed for statistical comparisons with **P<0.005, ***P<0.001, and ****P<0.0001.

Supplementary Figure 11 | Functional regulation of AR activity by HORAS5. (A,B) Relative expression (qPCR) of (A) MYC or (B) HPRT1 mRNA in LNCaP treated with either HORAS5-targeting or control siRNA (5nM for 24h), followed by treatment with the transcriptional inhibitor Actinomycin-D (20uM) for increasing durations. (C) Relative expression (qPCR) of mature (exonic) or premature (intronic) AR mRNA in LNCaP cells treated with either control or HORAS5-targeting siRNA (5nM) for 72h. *p<0.05; ****p<0.0001 (two-way ANOVA and Tukey’s test). (D) BART transcription factor prediction results of genes either negatively or positively correlated with HORAS5 expression in the TCGA PCa tumors (n=499; data accessed through cBioportal). AR is highlighted in red.

Supplementary Figure 12 | HORAS5-miRNA interaction predictions. (A) Table adapted from miRcode.org representing potential transcriptome-wide HORAS5-miRNA interactions based on UCSC GRCh37/hg19 genome assembly. (B) Relative expression (qPCR) of U6 snRNA and miR-645 in LNCaP cells treated with either HORAS5-targeting or control siRNAs for 72hrs. Data shown as a representative replicate.
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Figure 2 | NORASS is a stable cytoplasmic lncRNA in AR-positive PCA cells. (A) Basal expression (qPCR) of NORASS in a panel of PCA cell lines. Gene expression was normalized to average of GAPDH and HPRT1 and shown relative to BPH1. (B) Taqman qPCR expression of NORASS long and short variants (as identified in the Ensembl database) in two CRPC-derived cell lines, namely LNCaP and C4-2. (C) qPCR expression of NORASS in the nuclear and cytoplasmic sub-cellular fractions of AR-positive LNCaP cells. GAPDH and snoRNA5 are used as controls for cytoplasmic and nuclear fractions, respectively. Results from two independent experiments are plotted. (D) qPCR-based transcript expression of NORASS and c-Myc in LNCaP cells treated with alpha-amanitin for various time points (0, 2, 5, 6, 50hrs post-treatment). Relatively stable GAPDH expression was used for normalization and data is plotted relative expression at 0h. Results from two independent experiments are plotted as means ± S.D.
Figure 3 | HOPRASS knockdown attenuates cell proliferation in vitro. (A,B) qPCR expression levels of HOPRASS in LNCaP (A) and C4-2 (B) cells upon treatment with non-targeting control siRNA (NC) or two distinct anti-HOPRASS siRNAs (2'OMe design). Data shown is a single representative trial. (C,D) MTS cell proliferation assays for LNCaP (C) and C4-2 (D) upon treatment with anti-HOPRASS siRNAs or controls siRNAs. Two-way ANOVA with multiple comparisons (Tukey's post test) performed to measure significance. ****P<0.0001. Results from three independent experiments.
Figure 4 | HORAS5 knockdown induces caspase-mediated apoptosis in vitro. (A-C) Caspase 3/7 (A), Caspase8 (B), and Caspase9 (C) activities normalized to relative UNCAP cell number three days post-transfection of HORAS5-siRNAs versus non-targeting control siRNA. One-way ANOVA with Tukey’s Post Test, ****p<0.0001. Results are expressed as means ± S.D. from two independent replicates. (D) Relative viable cell counts of UNCAP cells upon knockdown of HORAS5 (48hrs) with or without DXT supplementation. Results are expressed as means ± S.D. from two independent replicates. Student’s t-test performed for statistical comparisons with ****p<0.0001.
Figure 5 | HOMASS regulates the expression of AR target genes and has prognostic value in PCs. (A) mRNA expression of the labeled genes after siRNA-mediated knockdown of HOMASS in LNCaP cells. (B) Relative mRNA expression (qPCR) of KISAA0101 post HOMASS knockdown in LNCaP and C4-2 cells. (C) Relative mRNA expression (qPCR) of AR post HOMASS knockdown in LNCaP and C4-2 cells. (D) MTS cell proliferation assay in C4-2 cells upon treatment with anti-KISAA0101 siRNAs or a control siRNA (20μM dosage). (E) Relative expression (qPCR) of AR mRNA in LNCaP treated with either HOMASS-targeting or control siRNA (20μM for 24h), followed by treatment with the transcriptional inhibitor Actinomycin-D (20μM) for increasing durations. (F) post-prostatectomy disease-free survival based on HOMASS expression (cBioportal, TCGA prostate cancer database, 456 vs. 35 elevated HOMASS patients). All qPCR results shown as data from at least two independent replicates, statistical analyses using One-way ANOVA with Tukey’s Post Test. ****p=0.0001.
Figure 6 | HCRASS knockdown attenuates tumor growth in vivo. [A] Relative expression of HCRASS (qPCR) 72 hours following AteloScience siRNA injections (n=3 in each arm). Raw values were normalized to GAPDH and MRP17 and shown relative to the control group (**P<0.001). [B] Quantitative measurements of tumor volume post-knockdown of HCRASS (72hrs). Statistical significance from Student’s t-test with *P<0.05. [C] Representative IHC images for cleaved Caspase 3 from control siRNA (NC) and anti-HCRASS siRNA treatment groups. The scale bar at the bottom marks a 20μm width. [D] Overall IHC staining quantification from C. Results are expressed as percent of positive cells to total cells in the visual field. Data are shown as means ± S.D. and a Student’s t-test was used to calculate significance, *P<0.05.