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(54) Title: NEAT1 AS A PROGNOSTIC MARKER AND THERAPEUTIC TARGET FOR PROSTATE CANCER

(57) Abstract: This invention relates to diagnosis and prognosis of prostate cancer, as well as therapeutic treatment of prostate cancer. More specifically, the invention provides diagnostic and prognostic methods based on detecting nuclear enriched abundant transcript 1 (NEAT1) levels in a sample. Further provided are methods for treating prostate cancer based on targeting NEAT1 via interfering RNA.



NEAT1 as a Prognostic Marker and Therapeutic Target for Prostate Cancer**CROSS REFERENCE TO RELATED APPLICATION**

[0001] This application claims priority from U.S. Provisional Application No. 61/980,825, filed April 17, 2014, the entire contents of which are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with Government Support under NCI R01 CA152057 and NCI U01 CA111275, awarded by the National Institutes of Health. The Government has certain rights in this invention.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0003] The Sequence Listing in an ASCII text file, named 31008_6063_02_SEQ.txt of 44 KB, created on April 16, 2015, and submitted to the United States Patent and Trademark Office via EFS-Web, is incorporated herein by reference.

FIELD OF THE DISCLOSURE

[0004] This invention relates to diagnosis and prognosis of prostate cancer, as well as therapeutic treatment of prostate cancer. More specifically, this invention provides diagnostic and prognostic methods based on detecting NEAT1 levels in a sample. The invention also provide methods for treating prostate cancer based on targeting and inhibiting NEAT1.

BACKGROUND ART

[0005] The androgen receptor (AR) plays a central role in the progression of prostate cancer (Heinlein (2004)). Androgen ablation is highly effective in treating metastatic prostate cancer, though resistance inevitably develops leading to castrate-resistant prostate

cancer (CRPC). Most cases of CRPC remain dependent on AR signaling, which has led to the clinical development and recent approval of potent AR-targeted therapies for CRPC (i.e., abiraterone, enzalutamide) (de Bono (2011); Scher (2012)). However, similar to first-generation anti-androgen therapies, patients develop resistance to these second-generation hormonal therapies. How CRPC tumors bypass AR signaling is emerging as a significant area of investigation.

[0006] In CRPC, crosstalk between estrogen- and androgen-signaling pathways may present an opportunity for clinical intervention. Estrogen receptor (ER) signaling through ER alpha (ER α) increases with prostate cancer progression (Ricke (2008); Clemson (2009); Rhodes (2007)) and can drive important oncogenic events, including TMPRSS2-ERG expression. Although ER α signaling has been extensively studied in breast cancer (Barwisck (2010); Best (2005); Glinsky (2004)), our understanding of the potential impact of this nuclear receptor on prostate physiology is less clear. Nevertheless, the connection is a particularly intriguing concept given that most cases of prostate cancer arise in the sixth decade of life, a time when testosterone levels are decreasing and estrogens are increasing in men. Mouse models suggest that antagonism of ER α may diminish prostate carcinogenesis (Ricke (2008)).

SUMMARY OF THE DISCLOSURE

[0007] The inventors have demonstrated herein that ER α is recruited to both coding and non-coding regions of the prostate genome and orchestrates expression of non-coding regulatory RNAs. Amongst putatively ER α -regulated intergenic long non coding RNAs (lncRNAs), the inventors have identified Nuclear Enriched Abundant Transcript 1 (NEAT1) as the most significantly overexpressed lncRNA in prostate cancer. Analysis of two large clinical cohorts has revealed that NEAT1 expression is associated with prostate cancer progression. Prostate cancer cells expressing high levels of NEAT1 were recalcitrant to androgen or AR antagonists. Further, the inventors provide evidence showing that NEAT1 drives oncogenic growth by altering the epigenetic landscape of target gene promoters to favor transcription, and that knockdown of NEAT1 decreases proliferation and the invasive properties of the cells. Accordingly, this invention provides diagnostic and prognostic methods, as well as therapeutic methods, for care of prostate cancer patients.

[0008] In one aspect, this disclosure provides a method of determining a risk of developing prostate cancer in a subject who does not yet have prostate cancer, by detecting the level of NEAT1 in a biological sample from the subject, comparing the level of NEAT1 relative to control, and determining the risk of developing prostate cancer in the subject based on an elevated level of NEAT1 in the sample as compared to the control.

[0009] In another aspect, this disclosure provides a prognostic method for determining a risk of advancing the prostate cancer in a subject by detecting the level of NEAT1 in a biological sample from the subject, comparing the level of NEAT1 relative to a control level, and determining the risk of advancing the prostate cancer based on an elevated NEAT1 level relative to the control level.

[0010] In still another aspect, this disclosure provides a diagnostic method for determining the stage of cancer in a subject by detecting the level of NEAT1 in a biological sample from a subject, comparing the level of NEAT1 relative to a control level, and determining the cancer stage based on an elevated NEAT1 level as compared to the control level.

[0011] In a further aspect, this disclosure provides therapeutic methods for treating prostate cancer based on providing to a subject with an interfering RNA molecule that targets NEAT1. The methods of this invention are particularly useful for treating a patient having an advanced stage prostate cancer, including patients showing resistance to hormone therapy. In some embodiments, the interfering RNA molecule is an siRNA molecule, for example, an siRNA molecule comprising a strand that is complementary to any one of SEQ ID NOS: 3-11, 43 or 45. Delivery of the interfering RNA molecule can be achieved using a delivery vehicle composed of nanoparticles.

[0012] In another aspect, the disclosure provides therapeutic methods for treating prostate cancer based on providing to a subject with an interfering RNA molecule that targets NEAT1, in combination with another therapy targeting androgen-receptor mediated functions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1a-1f. ER α plays a distinct role in prostate cancer. (1a) ER α is upregulated in prostate cancer compared with matched benign controls. Waterfall plots depict the qPCR expression levels of ER α mRNA in an independent cohort of benign (n=14) and PCa (n=14). (inset A) The expression of ER α in different prostate cancer cell lines was determined by western blotting and compared with MCF7, a breast cancer cell line. (1b) Analysis of ER α expression in Oncomine public datasets of normal vs. prostate cancer and advanced disease. (1c) Invasion of VCaP and VCaP ER α cells analyzed 48 hrs post-treatment with vehicle control or E2 (10nM) in presence of control or AR-siRNA. Results are expressed as the mean $\pm$ s.d. of three independent experiments. Student's *t*-test was performed for comparisons (% Invasion) between -E2 and +E2 conditions for ER α , ER α -Ctrl siRNA and AR-siRNA and *p<0.05 and **p<0.01 was considered statistically significant. Error bars represent the range of data. (1d) Recruitment of endogenous ER α to target gene chromatin was analyzed in VCaP cells with or without E2 treatment. Results are expressed as the means of percentage of input $\pm$ s.d. of two independent experiments. Error bars represent the range of data. (1e) Computational pipeline for identification of ER α -regulated lncRNA supregulated in prostate cancer: A schematic overview of the methodology employed to identify ER α -regulated lncRNAs that are differentially expressed between benign vs. prostate cancer and prostate cancer vs. NEPC. (1f) Box plots show expression levels of the top three ER α -regulated lncRNAs from 26 benign and 40 PCa cases, with ideogram depicting their chromosomal position. Waterfall plots depict the qPCR expression levels on an independent cohort of benign (n=14) and PCa (n=14) of the three nominated lncRNAs: NEAT1, NR_024490, and FR349599.

[0014] Figure 2a-2g. ER α regulated NEAT1 lncRNA is upregulated in prostate cancer. (2a) NEAT1 is overexpressed in various prostate datasets (Oncomine). (2b) Distribution of the median expression of all genes (core transcript clusters) on the Human Exon 1.0 ST array in the pooled Mayo Clinic cohort (n = 594). NEAT1's expression ranks in the 99th percentile of all genes on the array. (2c) Expression of NEAT1 with/without ER α overexpression and E2 treatment (10nM) at different time points in a panel of prostate cancer cell lines. Results are expressed as the mean $\pm$ s.d. of three independent experiments. (2d) View of NEAT1 genomic location indicates presence of two ER α binding sites in the promoter region. Read coverage tracks derived from RNA sequencing data indicates higher

abundance of NEAT1 transcripts in PCa compared to benign tumors in 3 representative cases. The figure also reports the ChIP sequencing coverage tracks for ER α (VCaP ER α , VCaP and input DNA as control). The bottom panel shows the binding sites of ER α , AR (from Yu et al, GEO Accession GSM353651 - tissue AR), Ace-H3, H3K4me3 and H3K36me3 in VCaP cell line (from Yu et al, GEO Accession GSM353629, GSM353620 and GSM353624), respectively. (2e) Chromatin immunoprecipitation followed by quantitative PCR to study ER α recruitment to NEAT1 promoter in VCaP cells with/without E2 treatment (10nM) was performed with primers spanning the binding regions identified by ER α ChIP-seq data. Primers for non-specific region were used as negative control for ChIP studies. Results are expressed as the means of percentage of input $\pm$ s.d. of two independent experiments. Vertical error bars represent the range of data. (2f) Luciferase based promoter reporter assays was utilized to analyze effect of ER α and/or AR on ERE-Luc promoter in VCaP cells. Cells were transiently transfected with the (ERE)3-SV40-luc reporter plasmid and/or ER α and/or AR treated with/without E2 or R1881 (1nM) for 48 h. Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. (2g) Luciferase based promoter reporter assays was utilized to analyze NEAT1 promoter activity following ER α expression -/+E2 (10 nM) for 24 hr. Results are expressed as the means $\pm$ s.d. calculated from three independent experiments. Error bars represent the range of data. Student's *t*-test was performed for comparisons where indicated and **p*<0.05 and ***p*<0.01 was considered statistically significant.

[0015] Figure 3a-3b. NEAT1 ER α signature correlates with prostate cancer. (3a) qRT-PCR analysis of relative mRNA levels of ER α target genes in VCaP cells with knockout of ER α with and without E2 treatment. The target genes selected for validation are the ones that had the highest log2 fold difference in VCaP and VCaP ER α cell lines. Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. Student's *t*-test was performed (as indicated) for comparisons between -E2 and +E2 conditions for Ctrl siRNA and ER α -siRNA transfections and **p*<0.05 and ***p*<0.01 was considered statistically significant. A representative example is shown for ERG target expression. Vertical error bars represent the range of data. (3b) qRT-PCR analysis of ER α target genes in VCaP cells with ER α overexpression and NEAT1 knockout with and without E2 treatment. Results are expressed as the mean $\pm$ s.d. calculated from three independent

experiments. Error bars represent the range of data. Student's *t*-test was performed for comparisons between –E2 and +E2 conditions for scrambled siRNA and NEAT1 siRNA transfections in VCaP and VCaPER α cells and **p*<0.05 and ***p*<0.01 was considered statistically significant. Vertical error bars represent the range of data. A representative example is shown for SPDEF target expression.

[0016] Figure 4a-4b. NEAT1 ER α signature is upregulated in prostate cancer. (4a)

Relative mRNA levels of genes, analyzed using qRT-PCR in parental VCaP cells transfected with scrambled (Sc) and NEAT1siRNA (N1), respectively, with and without E2 (10nM) treatment. Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. Error bars represent the range of data. Student's *t*-test was performed for comparisons (relative mRNA levels of target gene expression) between –E2 and +E2 conditions for scrambled siRNA and NEAT1 siRNA transfections. A representative example is shown for ADRB1 and PSMA target expression. **p*<0.05 and ***p*<0.01 was considered statistically significant. (4b) Validation of expression of the top target NEAT1 ER α signature genes in a small matched patient cohort of 13 benign and 13 PCa, n=26. Results are expressed as the mean $\pm$ s.d. of two independent experiments. Error bars represent the range of data.

[0017] Figure 5a-5d. NEAT1 is a transcriptional regulator. (5a& 5b) Promoter luciferase reporter assay shows that NEAT1 activates PSMA promoter in PC3 and VCaP cells. Cells were co transfected with empty vector or PSMA luc and Renilla-luc reporter genes alone or with NEAT1, NEAT1+ER α and NEAT1+AR. Luciferase activity was measured 48h post treatment with E2 (10nM) or R1881 (1nM). Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. Student's *t*-test was performed for comparisons (relative PSMA-luciferase activity) between –E2 and +E2 conditions for vector control, NEAT1 and NEAT1+ER α transfections in PC3 and VCaP cells. **p*<0.05 and ***p*<0.01 was considered statistically significant. (5c) Quantitative analysis of NEAT1 ChIRP in VCaP cells with or without E2 treatment (10nM). Recruitment profiles of NEAT1 to PSMA are shown. Results are expressed as the means of percentage of input $\pm$ s.d calculated from two independent experiments. Error bars represent the range of data. Results were reproducible between representative experiments. ***p*<0.01 was considered statistically significant. (5d) Analysis of chromatin landscape at PSMA promoter was

performed by ChIP using antibodies IgG, H3K4Me3, H3K9Me3, H3K27Me3, H3AcK9, and ER α in VCaP cells alone or transected with NEAT1, ER α , NEAT1 ER α , NEAT1 ER α NEAT1_1 siRNA, NEAT1 ER α NEAT1_2 siRNA with and without E2 treatment. qPCR was performed with specific primers for the PSMA promoter. Results are expressed as the means of percentage of input $\pm$ s.d. calculated from two independent experiments. Error bars represent the range of data. Results were reproducible between representative experiments.

[0018] Figure 6a-6l. NEAT1 is a driver of oncogenic cascade. (6a) NEAT1 fold induction in VCaP cells overexpressing ER α . (6b) NEAT1 fold induction in VCaP and VCaP ER α cells overexpressing NEAT1. (6c) % knockdown of NEAT1 in VCaP and VCaP ER α cells expressing scrambled siRNA and NEAT1 siRNA. Mean $\pm$ s.d calculated from three independent experiments is shown. Results were reproducible between representative experiments. (6d) Cell proliferation assays were performed in VCaP vector control, NEAT1 overexpressing cells and also in si scrambled and NEAT1 knockout cells with or without E2 treatment (10nM) at 24h and 48h time points. Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. Student's *t*-test was performed for comparisons (relative cell proliferation) between E2 conditions for vector control, NEAT1 Cl-1 and NEAT1 Cl-2 and E2 conditions for si-scrambled, Neat1-siRNA1 and siRNA2 transfections. ***p*<0.01 was considered statistically significant. (6e) Quantitative bar chart for depicting percentage cell invaded at the completion of invasion assay performed in VCaP vector control, NEAT1 overexpressing cells and also in si scrambled and NEAT1 knockout cells with or without E2 treatment (10nM). Results are expressed as the mean $\pm$ s.d. of three independent experiments. **p*<0.05 and ***p*<0.01, Student's *t*-test. (6f) Soft agar assays were performed with VCaP control and NEAT1 expressing cells. Quantitative bar-plot analysis of stained colonies at 21 days is shown. Results are expressed as the mean $\pm$ s.d. of three independent experiments. ****p*<0.001, Student's *t*-test. (6g) Colony forming assay were performed in VCaP vector control, NEAT1 overexpressing cells with or without E2 treatment (10nM). Right panel depicts the number of colonies at 21 days. Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. **p*<0.05 and ***p*<0.01, Student's *t*-test. (6h) Growth curve for the tumors monitored upto 45 days. Results are expressed as the mean $\pm$ s.d. calculated from three independent experiments. **p*<0.05, Student's *t*-test. (6i& 6j) VCaP and NCI-H660 vector control and NEAT1 overexpressing cells were injected s/c into the flank of male NOD-

SCID mouse. Bioluminescence imaging monitored the tumor growth. Growth curve for the tumors monitored upto 45 days is shown, VCaP (6i) and NCI-H660 (6j). (6k) The average tumor weight is shown. Mean $\pm$ s.d is shown, ** $p < 0.01$, Student's *t*-test. (6l)

Representative immunohistochemistry on the formalin fixed paraffin embedded tissues for ER α . The lower panel shows the H&E staining for the tumors and on the right we see qRT-PCR data for expression of NEAT1 in the xenografts. Data is presented as mean $\pm$ s.d from a representative experiment performed in triplicate ($n = 3$), ** $p < 0.01$. (6m) The average weight of tumors from VCaP vector con and NEAT1 expressing group (top panel) and from NCI-H660 vector con and NEAT1 expressing group (bottom panel). Results are expressed as the means $\pm$ s.d. of three independent experiments. Statistical analysis was performed using Student's *t*-test and ** $p \leq 0.01$.

[0019] Figure 7a-f. NEAT1 in therapy resistance. (7a) NEAT1 expression in VCaP cells treated with E2 (10nM) at different time points alone, E2 + ICI (10nM + 10 μ M) or ICI (10 μ M) alone. Results are expressed as the mean $\pm$ s.d. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test. (7b) NEAT1 expression in VCaP cells treated with E2, E2 + 4OHT (10nM + 10nM), and 4OHT (10nM) alone for 48h. (7c) NEAT1 expression in VCaP cells treated with or without E2 (10nM) or E2 + Enzalutamide (10nM + 10 μ M) at different time points. Results are expressed as the mean $\pm$ s.d. of three independent experiments. * $p < 0.05$ and ** $p < 0.01$, Student's *t*-test. (7d) qRT-PCR analysis of NEAT1, GJB1 and TRPM8 in LnCaP and VCaP control cells, with bicalutamide treatment (10 μ M) alone or in combination with E2 (10nM) for 48h. Results are expressed as the means $\pm$ s.d. of three independent experiments. Vertical bars represent range of data. Results were reproducible between representative experiments. (7e) Quantitation for the RNA ISH signals for RNA ISH of NEAT1 in benign, localized PCa and in advanced disease shown in using RNA Spot Studio. (7f) Scatter plot showing the correlation between ER α and NEAT1 expression by qRT PCR in 9 cases of benign prostate, 7 PCa, and 7 CRPC. Pearson's correlation coefficient $R = 0.86$ (p -value = 1.9×10^{-7}).

[0020] Figure 8a-8j. NEAT1 overexpression is associated with aggressive prostate cancer. (8a-8b) Kaplan Meier curves showing (8a) Biochemical recurrence (BCR) free survival and (8b) metastatic recurrence (MET) free survival for NEAT1 low and high expression groups of samples from the Mayo case-cohort dataset (Erho (2013)) ($n = 216$).

The cut points to define high and low NEAT1 expression were selected using patients from the Mayo nested case-control dataset (n = 378) (Blute (2001)) by maximizing the product of the sensitivity and specificity for each endpoint. The number of patients at risk for each group is shown beneath the plot. AUCs and 95% confidence intervals for the expression of NEAT1_1 transcript predicting BCR (8c), MET (8d) GS > 7 patient outcomes (8e) and PCSM (8f), and NEAT1_2 transcript predicting BCR (8g), MET (8h), GS > 7 patient outcomes (8i) and PCSM (8j). p-values calculated using the Wilcoxon signed-ranked test indicate that both the long and short forms of NEAT1 are found to be significant prognosticators for each outcome.

[0021] Figure 9a-9l. NEAT1 is a strong prognosticator of prostate cancer. (9a-9d)

Univariable forest plots comparing the expression of NEAT1's short (NEAT1_1) and long isoform (NEAT1_2) to clinicopathologic variables in the pooled Mayo cohort (n = 594) (9a) BCR, (9b) MET, (9c) prostate cancer specific mortality (PCSM), (9d) Gleason score (GS) > 7. Pathological tumor stage 3 or greater (pT3+), Lymph Node Invasion (LNI), Surgical Margin Status (SMS) positive, Seminal Vesicle Invasion (SVI), Extra Capsular Extension (ECE), preoperative PSA (pPSA), adjuvant hormone therapy, and adjuvant radiation therapy are shown. (9e-9m) Multivariable odds ratio forest plots for expression of NEAT1_1 and NEAT1_2 transcript. Forest plots show (9e) BCR, (9f) MET, (9g) PCSM and (9h) GS > 7 multivariable odds ratios for NEAT1_2 and (9i) BCR, (9j) MET, (9k) PCSM and (9l) GS > 7 multivariable odds ratios for NEAT1_1 adjusted for clinicopathologic factors and adjuvant treatment (n = 594). NEAT1 is found to contribute significant independent information for the prognostication of each endpoint.

DETAILED DESCRIPTION

[0022] The present inventors have identified NEAT1 expression is associated with prostate cancer progression and a high level of NEAT1 is indicative of prostate cancer, particularly prostate cancer at an advanced stage. Further, the inventors have demonstrated that NEAT1 drives oncogenic growth by altering the epigenetic landscape of target gene promoters to favor transcription, and that knockdown of NEAT1 decreases proliferation and invasive properties of cells. Accordingly, this invention provides methods for diagnosis or prognosis of prostate cancer based on detecting the levels of NEAT1, and methods for treating prostate cancer based on inhibition of NEAT1.

[0023] NEAT1

[0024] The term "NEAT1" is the abbreviation for nuclear enriched abundant transcript 1, a molecule also known as multiple endocrine neoplasia 1 (or "MEN1"). The NEAT1 gene is located on chromosome 11q13.1 and produces two long non-coding RNA (lnc RNA) isoforms, a short isoform of about 3.7kb referred to as NEAT1_1, NEAT1 v1, or MEN epsilon, and a long isoform of about 23 kb referred to as NEAT1_2, NEAT1 v2, or MEN beta. The sequences of the two isoforms (SEQ ID NO: 1 and SEQ ID NO: 2, shown below in their DNA counterparts) completely overlap at the 5' end (i.e., the 3729-nucleotides sequence of the short isoform is identical to nucleotides 1-3729 of the long isoform).

Short isoform (3729bp)- Homo sapiens nuclear enriched abundant transcript 1 (NEAT1) mRNA, complete sequence, GenBank # EF177379.1 (SEQ ID NO: 1):

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AGCAGCTTTAGATAAAGTAAGCAGTTCTGCTTTTCATTTTATAATTTATTTCTACTTTTGTTCATTAATC
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GGGCTTGAGCAAAGTGGGGGAGGGGGCAAAGGCAGTTGGCCCAACACATTCCCCACCCCTTTGAGAGGTC

[illegible]

[0025] Methods Of Diagnosis And Prognosis Of Prostate Cancer

[0026] This invention provides diagnostic methods for determining the stage of prostate cancer, for example, the presence of an advanced stage prostate cancer. The invention also provides prognostic methods for determining a risk for developing prostate cancer, and a risk for an existing prostate cancer to progress towards an advanced stage prostate cancer. These diagnostic and prognostic methods are based on detecting the level of NEAT1, i.e., the level of the NEAT1 RNA molecule, in a biological sample in a subject.

[0027] The term "risk", as used herein, means likelihood, i.e., more likely than not.

[0028] Prostate cancer can be evaluated based on well established procedures and techniques, including physical examination (e.g., tissue biopsy, digital rectal exam); imaging studies such as CT scan (which reveals blood flow and anatomy of tissues in and around the prostate), MRI, PET/CT scan (an advanced nuclear imaging technique which combines

positron emission tomography (PET) and computed tomography (CT) into one machine), ProstaScint scan (which may be used to detect if prostate cancer has spread to the lymph nodes, adjacent tissue or bone), ultrasound imaging; blood or genetic tests (testing established biomarkers of prostate cancer such as prostate-specific antigen or "PSA" and prostate cancer antigen 3 gene or "PCA3").

[0029] Prostate cancer can be staged based on the above evaluations. A commonly used staging scheme is the "TNM" system, which evaluates the size of the tumor ((T1/T2/T3/T4), the extent of involved lymph nodes (Nx/N0/N1), any metastasis (M0/M1/M1a/M1b/M1c), and also taking into account cancer grade (determined by the Gleason Grading system). According to the TNM system, prostate cancer is classified as Stages I-IV. Briefly, Stage I disease is cancer that is found incidentally in a small part of the sample, and the cells closely resemble normal cells and the gland feels normal to the examining finger. In Stage II, more of the prostate is involved and a lump can be felt within the gland. In Stage III, the tumor has spread through the prostate capsule and the lump can be felt on the surface of the gland. In Stage IV, the tumor has invaded nearby structures, or has spread to lymph nodes or other organs.

[0030] In one aspect, this disclosure provides a method of determining a risk of developing prostate cancer in a subject who does not yet have prostate cancer, by detecting the level of NEAT1 in a biological sample from the subject, comparing the level of NEAT1 relative to control, and determining the risk of developing prostate cancer in the subject based on an elevated level of NEAT1 in the sample as compared to the control. According to this aspect of the invention, the control level of NEAT1 can be a pre-established value or a range of values, for example, from healthy individuals (such as healthy individuals who do not develop prostate cancers in follow up studies).

[0031] In another aspect, this disclosure provides a prognostic method for determining a risk of advancing the prostate cancer in a subject, for example, a subject who is diagnosed to have prostate cancer based on other clinical or pathological evaluations. According to this aspect, an elevated NEAT1 level in a biological sample of the subject relative to a control level provides a basis for determining a risk of advancing the prostate cancer, i.e., the cancer progressing towards an advanced stage.

[0032] The present inventors have shown that patients with higher NEAT1 expression have significantly worse outcomes, as reflected by at least high Gleason score, biochemical recurrence, and metastasis events. Thus, the NEAT1 expression level provides a prognosis basis independent of common clinical and pathological variables. More specifically, based on the comparison of the NEAT1 level in a prostate cancer patient to a control level, one can determine a risk of the cancer progressing towards an advanced stage.

[0033] By "advanced stage" prostate cancer, it is meant herein to include prostate cancer of at least Stage III or IV, prostate cancer that has significant involvement of lymph nodes, prostate cancer that has metastasized, prostate cancer that recur after surgical removal or other therapy, and/or prostate cancer that is resistant to hormone therapy or other therapy targeting post castration (such as NEPC and CRPC).

[0034] By "cancer progressing towards an advanced stage" it is meant that the cancer advances or worsens based on one or more clinical or pathological evaluations such as those described hereinabove. Progression of cancer can be, but does not require to be, a change from one stage to a more advanced stage as defined by the TNM system - it can be, for example, a change from a less advanced sub-stage to a more advanced sub-stage within the same TNM stage; and it can also be from any of Stages I-IV to a more advanced, aggressive prostate cancer (e.g., NEPC, or cancer resistant to hormone therapy or therapy targeting post castration).

[0035] According to this aspect of the invention, the control level can be a pre-established value or values or a range of values, for example, based on clinical studies and statistical analysis. The control level may correlate with the NEAT expression levels in healthy individuals and/or individuals having localized prostate cancer and good clinical outcome after therapy in follow up studies. To illustrate without intending to be bound, a control value, in this case, a cut off value or cut point of NEAT levels, can be determined as described in the Examples herein-below. As described in the description of Figures 8a-8b, the cut points to define high and low NEAT1 expression were selected using patients from the Mayo nested case-control data set by maximizing the product of the sensitivity and specificity for each endpoint and to distinguish between low and highly aggressive cancer from indolent cancer. More specifically, two cut points were established for each the NEAT1 long and NEAT1 short expression based on log2 expression determined in

microarray expression analysis, with one for the BCR endpoint and one for the MET endpoint:

BCR - Long - 8.77

MET - Long - 9.08

BCR - Short - 10.22

MET - Short - 10.22

[0036] In still another aspect, this disclosure provides a diagnostic method for determining the stage of cancer, including for example, the likelihood that the prostate cancer is an advanced stage cancer. According to this aspect, the NEAT1 level in a biological sample of the subject relative to a control level provides a basis for determining the cancer stage, with an elevated NEAT1 level correlating with a more advanced stage of prostate cancer.

[0037] The present inventors have demonstrated that NEAT1 levels are elevated in localized prostate cancer, and are more significantly elevated in advanced prostate cancer such as CRPC (see, e.g., Figures 7e-7f). Thus, the NEAT expression level can be used as an independent diagnosis parameter, and can also be integrated in a multi-parameter panel for diagnosis.

[0038] Similar to other aspects of the invention, the control level according to this aspect of the invention can be a pre-established value or values or a range of values, for example, based on clinical studies and statistical analysis. The control level may correlate with the NEAT expression levels in healthy individuals in certain embodiments, and with individuals having a less advanced and less aggressive prostate cancer in other embodiments.

[0039] Sample sources suitable for use include any biological specimen that may contain prostate cancer cells, such as tissue, urine, blood, semen, prostatic secretions or prostate cells. Methods of procuring cell and tissue samples are well known to those skilled in the art, including, for example, tissue sections, needle biopsy, surgical biopsy, and the like. For a cancer patient, cells and tissue can be obtained from a tumor. A cell or tissue sample can be processed to extract, purify or partially purify, or enrich or amplify the nucleic acids in the sample for further analysis. In a specific embodiment, a urine sample is collected immediately following a digital rectal examination (DRE), which often causes prostate cells from the prostate gland to shed into the urinary tract. Samples obtained from the above-

identified sources can be further processed, for example, to enrich for prostate cancer cells or extract the nucleic acid or protein molecules from the cells. The processing may include obtaining the serum or plasma portion of blood, obtaining the supernatant or cell pellet portion of urine, homogenization of tissue, lysis of cells, and the like.

[0040] Suitable assays for detecting and determining the levels of NEAT1 include, but are not limited to, Northern blot, RNA ISH (for *in situ* hybridization), RT-PCR (for reverse transcriptase mediated polymerase chain reaction, including quantitative RT-PCR or "qRT-PCR"), and RNA-Seq, all of which have been well known to the skilled artisan and are also illustrated in the Examples section below.

[0041] Performance of any of the assays may involve the use of a nucleic acid oligonucleotide (a probe for hybridization, or a primer or a pair of primers for PCR) that is specific for NEAT1.

[0042] By "oligonucleotide specific for NEAT1", it is meant that the oligonucleotide is sufficiently complementary to NEAT1 in sequence (e.g., 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% complementary or forming base pairs), such that the oligonucleotide hybridizes under stringent conditions to NEAT1 without significant non-specific hybridization to other molecules. The percentage of non-specific hybridization should be not more than 40%, 30%, 25%, 20%, 15%, 10% or 5%, to be considered insignificant. Stringency is dictated by temperature, ionic strength, and the presence of other compounds such as organic solvents. For example, "high stringency conditions" can encompass hybridization at 42°C in a solution consisting of 5x SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5x Denhardt's reagent and 100µg/ml denatured salmon sperm DNA, followed by washing in a solution comprising 0.1 x SSPE, 1.0% SDS at 42°C or higher. "Medium stringency conditions" can encompass hybridization at 42°C in a solution consisting of 5x SSPE with NaOH), 0.5% SDS, 5x Denhardt's reagent and 100µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0 x SSPE, 1.0% SDS at 42°C.

[0043] In some embodiments, an assay uses one or more oligonucleotides that are specific for the 5' portion of NEAT1 that is shared by both isoforms of NEAT1, which permits detection of both isoforms. In other embodiments, an assay uses one or more

oligonucleotides that are specific for the 3' portion of the long isoform of NEAT1, which permits the detection of only the long isoform of NEAT1. In yet other embodiments, two or more oligonucleotides are use, at least one being specific for the long isoform, and at least another one being specific for the 5' portion of NEAT common to both isoforms.

[0044] According to this invention, one can detect and determine the levels of the long or short isoform of NEAT1 individually, or can detect and determine the collective levels of the long and short isoforms of NEAT1 in combination, based on appropriate choice of assays and oligonucleotides. The findings made by the inventors in respect to the relationship between the level of NEAT1 and prostate cancer, as illustrated in the Examples below, are consistent with the two isoforms. Thus, the diagnostic and prognostic methods disclosed above and herein can be practiced by detecting the level of either of the long or short isoform of NEAT1, or by detecting the combined levels of the long and short isoforms of NEAT1, and comparing to the respective controls.

[0045] **Methods of Treatment**

[0046] This disclosure also provides methods for treating prostate cancer.

[0047] The term "treating" as used herein includes inhibiting and retarding the growth and progression of prostate cancer, causing regression of prostate cancer, preventing or reducing the risk of recurrence post operation or treatment, and/or preventing or reducing the risk of prostate cancer progressing into an advanced stage.

[0048] The therapeutic methods of this invention are based on providing to a subject with an interfering RNA molecule that targets NEAT1. The subject include a subject who has been diagnosed with prostate cancer, especially prostate cancer at an advanced stage, including patients who are resistant to hormone therapy or other therapy targeting post castration, as well as subject who has been determined to have high levels of NEAT1.

[0049] **Interfering RNA Molecules Targeting NEAT1**

[0050] By "an interfering RNA molecule targeting NEAT1", it is meant that the interfering RNA molecule specifically interferes with the level or activity of NEAT1 (i.e., the NEAT1 RNA molecules) via RNA interference. RNA interference (RNAi) is a process

by which double-stranded RNA (dsRNA) is used to silence gene expression. RNAi begins with the cleavage of longer dsRNAs into small interfering RNAs (siRNAs) by an RNaseIII-like enzyme, dicer. siRNAs are dsRNAs that are usually about 19 to 28 nucleotides in length and often contain 2-nucleotide 3' overhangs, and 5' phosphate and 3' hydroxyl termini. One strand of the siRNA, also referred to as the antisense strand, is incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC), which uses this siRNA strand to identify mRNA molecules that are at least partially complementary to the incorporated siRNA strand, and then cleaves these target mRNAs or inhibits their translation. Therefore, the antisense strand of an siRNA that is incorporated into RISC is known as the guide strand. The other siRNA strand, known as the passenger strand or the sense strand, is eliminated from the siRNA and is at least partially identical to the target mRNA.

[0051] Typically, an interfering RNA molecule includes a strand that corresponds to, i.e., substantially identical with, a portion of the DNA sequence coding for the NEAT1 RNA. That is, an interfering RNA molecule includes a strand having a sequence substantially complementary to a portion of NEAT1 (RNA). The portion of NEAT1 to which an interfering RNA molecule is substantially complementary to, is also referred to herein as the target sequence or target site.

[0052] In certain embodiments, target sequences within NEAT1 are selected using design tools and techniques available in the art, for example, those by Tuschl, T. et al., "The siRNA User Guide," available on the Rockefeller University web site; by Technical Bulletin #506, "siRNA Design Guidelines," at Ambion Inc. web site. Parameters for selection can include G/C contents between 35% and 55% and siRNA lengths between 19 and 27 nucleotides. Interfering RNAs corresponding to a selected NEAT1 target sequence can be tested in knock down experiments as described herein.

[0053] Examples of suitable target sequences within NEAT1 include those to which the siRNAs of SEQ ID NOS: 3-11, SEQ ID NO: 43 and SEQ ID NO: 45 correspond to. For example,

Sequence of Sense Strand of siRNAs	Start Site
in NEAT1	
UGGUAAUGGUGGAGGAAGAUU (SEQ ID NO: 3)	998

GUGAGAAGUUGCUUAGAAAUU (SEQ ID NO: 4)	2352
GGAGGAGUCAGGAGGAAUAUU (SEQ ID NO: 5)	366
GCCUUUACUACAUGUGUGA[dT][dT] (SEQ ID NO: 43)	3266
CUUAGAACUUUAAGUGCAA[dT][dT] (SEQ ID NO: 45)	2383

[0054] As described, an interfering RNA molecule includes a strand having a sequence substantially complementary to a portion of NEAT1 (RNA). For example, a double stranded siRNA molecule can contain a sense nucleotide strand that includes a region substantially identical in sequence to a target sequence of NEAT1, and an antisense nucleotide strand substantially complementary to the same target sequence of NEAT1. By "substantially identical", it is meant at least 85%, 90%, 95%, 98% or 99% identity in sequence, or that the differences are not more than 5, 4, 3, 2, or 1 nucleotides between the target sequence of NEAT1 and a region of the sense strand of an interfering RNA. Similarly, by "substantially complementary" it is meant at least 85%, 90%, 95%, 98% or 99% complementarity between the target sequence of NEAT1 and a region of the antisense strand of an interfering RNA. "100%" or "perfect" contiguous complementarity is standard Watson-Crick base pairing. The region of the sense or antisense strands that is substantially identical or complementary to the target sequence of NEAT1 can be at least 13, 14, 15, 16, 17, 18, 19, 20 or more contiguous nucleotides. An interfering RNA molecular may also include a region or regions of one or more bases that is (are) not substantially identical or complementary to target sequence within NEAT1, e.g., the 3', 5' or both ends of a substantially identical or complementary region. A region can be one or more bases or at least two bases.

[0055] In some embodiments of the present invention, an interfering RNA molecule provided to a subject having prostate cancer is an siRNA molecule that targets NEAT1.

[0056] In some embodiments, the siRNA of the invention is a double-stranded nucleic acid molecule comprising two nucleotide strands, each strand having 18 to 30 nucleotides (i.e., 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides). One or both of the strands of double-stranded siRNA may have a 3' overhang of from 1 to 6 (i.e., 1, 2, 3, 4, 5 or 6) nucleotides, which may be ribonucleotides or deoxyribonucleotides or a mixture thereof. In one embodiment, the siRNA comprises a 3' overhang of TT or UU. In another embodiment, the siRNA comprises at least one blunt end. Specific examples of suitable

double stranded siRNAs targeting NEAT1 include those whose sense strands are set forth in Table 2 as SEQ ID NOS: 3-11, SEQ ID NO: 43 and SEQ ID NO: 45.

[0057] In other embodiments, the siRNA molecule provided to a subject is a single stranded siRNA molecule of 18 to 30 nucleotides. Examples of suitable single stranded siRNA molecules include those that comprise a sequence that is complementary to any of the sense strands as set forth in SEQ ID NOS: 3-11, SEQ ID NO: 43 and SEQ ID NO: 45.

[0058] In still other embodiments, an interfering RNA molecule provided to a subject is a short hairpin RNA (shRNA) that targets NEAT1. A hairpin interfering RNA is a single molecule (e.g., a single oligonucleotide chain) that comprises both the sense and antisense strands of an interfering RNA in a stem-loop or hairpin. Examples of shRNAs include those having a sense sequence as set forth in Table 2, and an antisense sequence substantially complementary to the sense sequence.

[0059] In other embodiments, an interfering RNA molecule provided to a subject is another interfering RNA molecule and RNA-like molecule that can interact with RISC and silence gene expression. Examples of such other interfering RNA molecules include microRNAs (miRNAs) and dicer-substrate 27-mer duplexes. Examples of RNA-like molecules that can interact with RISC include siRNA, single-stranded siRNA, microRNA, and shRNA molecules containing one or more chemically modified nucleotides, one or more non-nucleotides, one or more deoxyribonucleotides, and/or one or more non-phosphodiester linkages.

[0060] In some embodiments, a single interfering RNA molecule targeting NEAT1 is provided to the patient. In other embodiments, two or more interfering RNA molecules targeting NEAT1 are provided.

[0061] Interfering RNAs may be generated by chemical synthesis, by in vitro transcription, or by cleavage of longer double-stranded RNA with dicer, for example. Interfering RNAs can also be expressed in cells from plasmid or viral expression vectors or units, many available from commercial sources. Examples of commercially available plasmid-based expression vectors for shRNA include members of the pSilencer series (Ambion, Austin, Tex.) and pCpG-siRNA (InvivoGen, San Diego, Calif.). In the Examples

section below, the inventors made four double stranded siRNAs (see Table 2) using RNA interference lentiviral vectors from ABM (Applied Biological Materials Inc., Richmond, BC, Canada). Unlike conventional shRNA vectors, the RNA interference lentiviral vectors from ABM employ dual convergent promoter system- the sense and antisense strands of the siRNA, which are cloned into a lentiviral vector, are expressed by two different promoters in the vector, rather than expressed as a single strand transcript that folds itself into a hairpin loop.

[0062] Nucleotides in interfering RNAs may be modified on their base portion, sugar portion, or the phosphate portion, for improved function, stability, delivery, or targeting.

[0063] *Delivery Systems for Interfering RNAs Targeting NEAT1*

[0064] Interfering RNAs targeting NEAT1 can be administered directly to a patient (e.g., via intratumoral injection), via a viral vector (which upon administration to a patient gains entry into the cells and expresses the desired interfering RNA molecule in the cells), or via non-viral delivery system. See review by Rao (2009). Non-viral delivery systems typically employ polymeric vehicles that are made of synthetic polymers, natural or biodegradable polymers, or lipids. The delivery vehicles may take the form of nanoparticles. A number of nanoparticle delivery systems have been described for successful delivery of interfering RNAs; see, e.g., Hong (2011), Xu (2013), Fredman (2015), and review by Rao (2009), the contents of all of which are incorporated herein by reference. A microfluidic platform for combinatorial synthesis and optimization of targeted nanoparticles is also described by Valencia (2013), incorporated herein by reference.

[0065] In some embodiments, the interfering RNA molecules targeting NEAT1 are conjugated (or form a complex) with PLA and/or PLGA based nanoparticles for delivery, such as nanoparticles described in Xu (2013) or Fredman (2015), or derived or modified therefrom.

[0066] In other embodiments, the interfering RNA molecules targeting NEAT1 are conjugated (or form a complex) with flexible TEA (Triethanolamine) core PAMAM dendrimers, such as those described by Liu (2009), incorporated herein by reference.

[0067] *Pharmaceutical Compositions and Administration*

[0068] In certain embodiments, the invention provides pharmaceutical compositions (also referred to herein as "compositions") containing an interfering RNA molecule of the invention, or containing a conjugate or complex of a delivery system with an interfering RNA molecule of the invention, provided or formulated in a suitable physiologically or pharmaceutically acceptable carrier, such as water, buffer, saline, glycine, hyaluronic acid, mannitol, and the like. The compositions can be formulated as solutions, suspensions, or emulsions, for administration.

[0069] An interfering RNA molecule is administered to a patient at an effective amount, i.e., an amount of interfering RNA that produces a therapeutic response in a recipient mammal. An effective amount of a formulation may depend on factors such as the age, gender, condition of the recipient, the potency and stability of the interfering RNA, and the type of delivery vehicles used, for example. Generally speaking, an interfering RNA can be provided to a recipient at 0.02-0.5 nmoles, 0.03-0.4 nmoles, or 0.04-0.3 nmoles, per administration; i.e., at least 0.02, 0.03 or 0.04, and not more than 0.5, 0.4 or 0.3 nmoles, nmoles per administration. The precise amount of a formulation that is therapeutically effective can be ascertained by one of ordinary skill in the art.

[0070] Suitable routes for administration of an interfering RNA molecule, a delivery system, or a pharmaceutical composition, include but are not limited to aerosol, intradermal, inhaling, intramuscular, intranasal, intravenous, intraperitoneal, intratumoral, for example.

[0071] Combination Therapy

[0072] In another aspect, the NEAT1 targeting therapeutic treatment described hereinabove is combined with one or more other therapeutic treatments conventionally used for prostate cancer. Generally, treatment for subjects diagnosed with prostate cancer includes cytotoxic chemotherapy, radiation, a therapy targeting androgen receptor mediated signaling. Because ER/NEAT1 mediated signaling has been determined herein to be independent of androgen receptor (AR) mediated signaling, a treatment scheme that

combines a therapy targeting NEAT1 with a therapy targeting androgen receptor mediated functions is particularly advantageous.

[0073] Therapy targeting androgen receptor mediated functions includes androgen deprivation therapy, which lowers the levels of hormones associated with prostate cancer progression, such as androgens. See, e.g., de Bono (2011); and Scher (2012). Androgen deprivation therapy includes, for example, treatment with anti-androgen agents, e.g., a gonadotropin-releasing hormone (GnRH) agonist (such as goserelin or leuprolide). Therapy targeting androgen receptor mediated functions also includes treatment with an agent directly targeting AR (i.e., to an agent that inhibits androgen receptor function, activity or expression), which includes small molecules, antibodies, antisense nucleic acid molecules, and interfering RNAs such as siRNA, shRNA, and miRNA. Therapy targeting androgen receptor mediated functions also includes treatment with an agent that suppresses local synthesis of androgenic steroids in the prostate, e.g., CYP17 inhibitors, such as abiraterone acetate (Zytiga), TAK-700 (orteronel), TOK-001 (galeterone) or VT-464. A wide variety of therapeutic agents targeting androgen receptor mediated functions, including agents directly targeting AR, have been described in U.S. Patent Nos. 4,097,578, 5,411,981, and 5,705,654, U.S. Published Applications 2004/0009969 and 2007/0004753, and PCT international applications published as WO 97/00071, WO 00/17163 and WO 06/124118, the entire contents of which are incorporated herein by reference. Androgen deprivation may also include castration in the form of surgical castration (orchiectomy, i.e., surgical removal of testes) or chemical castration.

[0074] The present description is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, and published patent applications as cited throughout this application) are hereby expressly incorporated by reference.

Example 1. Material and Methods

[0075] Cell culture and treatments: LnCaP and PC3 cells were grown in RPMI 1640 (Invitrogen) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. RWPE1 cells were grown in Keratinocyte Serum Free Medium (K-SFM), Kit (Gibco, #17005-042). VCaP and DU145 cells were grown in DMEM (Invitrogen) and

supplemented with 10% fetal bovine serum (FBS) with 1% penicillin streptomycin. NCI-H660 cells were grown in RPMI 1640 supplemented with 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β -estradiol, 5% FBS, 1% penicillin-streptomycin and an extra 2 mM of L-glutamine (for a final concentration of 4 mM). For cell treatments in several experiments, the inventors used 10-100 nM β -estradiol (Sigma Aldrich), 10 μ M Enzalutamide (Astellas), 10 μ M bicalutamide (Sigma Aldrich), 1-10nM R1881 (PE Biosystems), 10-100 nM 4-hydroxy tamoxifen (4OHT) (Sigma Aldrich), 1-10 μ M ICI 182,720 (Tocris Bioscience).

[0076] Plasmids, siRNAs and transfection: pcDNA 3.1, pcDNA3.1-ER α , pcDNA 3.1 AR, piLenti-GFP, piLenti-NEAT1 siRNA-GFP (set of four, sequences provided in **Table 2**), iLenti-si-scrambled, pLenti-bicistronic-luc-NEAT1. siRNAs for ER α , ER β , AR, NEAT1, NEAT1_2 were used and the sequence is provided in **Table 2**. For the mammalian expression vectors Lipofectamine 3000 (Invitrogen) and Lonza nucleofection were used for transfection. The stable clones for NEAT1 overexpression as well as the scrambled and NEAT1 siRNA expressing cells were generated by using the lentiviral vectors and by selection in puromycin.

[0077] Identification of ER α -regulated lncRNA: A set of known lncRNAs was generated from various data sources: RefSeq: GENCODE v7; - ncRNA.org; and lncRNAdb (Amaral (2011)) and those that were at least 200nt long were selected, resulting in 12,483 lncRNAs. These lncRNAs were characterized according to their potential of being regulated by ER α by using ER α binding sites information from ChIP-seq experimental data. Moreover, several histone marks were considered to provide evidence of transcription, including H3K4me3 and H3K36me3.

[0078] Differential expression analysis: In order to prioritize the experimental validation of lncRNAs, a pair-wise differential expression analysis was performed on the expression values determined by paired-end transcriptome sequencing of 73 samples (26 benign prostate, 40 PCa, and 7 NEPC). A pair-wise Wilcoxon test was performed and all p-values were corrected for multiple hypotheses testing using Benjamini-Hochberg (Benjamini (1995)).

[0079] ER α and NEAT1 signature via Oncomine concept analysis: RNA sequencing was done for VCaP and VCaP ER α -expressing cells as well as in vector control and NEAT1 overexpressing VCaP cells. The expression of the genes was computed and those genes with a log₂-fold change greater than 2 were selected. 588 genes were found to be overexpressed in VCaP ER α cells. A custom concept of this gene list was generated in Oncomine. Similarly, genes from the VCaP NEAT1 group with a log₂-fold change greater than 2 were selected and a custom concept was built in Oncomine using the top 1000 genes from NEAT1 signature. The significantly associated tumor vs. normal concepts with odds ratio > 2.0 and $P < 1 \times 10^{-6}$ considering tumor vs. normal analysis was determined. The resulting concepts and associations are represented through a concept network using Cytoscape version 2.8.2. Each node represents a concept to which the signature is associated at a greater than 3-fold odds-ratio for ER α signature and >2 fold odds ratio for NEAT1 signature. Node size reflects the concept size, i.e. the number of genes in each concept; red and green colors represent correlation with over- or under-expressed genes in the concept, respectively; and edge thickness represents the odds- ratio of the association between concepts, ranging from 1.4 to 29.9 and 1.2 to 637 for ER α and NEAT1 signatures, respectively. The border color of each node represents the tumor type. The layout of the network is based on the Edge-weighted spring-embedded algorithm.

[0080] Luciferase reporter assays: For ERE luciferase assays, VCaP cells were transiently transfected with the (ERE)3-SV40-luc reporter plasmid and/or ER α and/or AR as well as an internal control construct pRL harboring the renilla luciferase gene. VCaP cells were also transfected with empty vector or NEAT1 promoter (1+2) luciferase reporter constructs alone or with ER α as well as an internal control construct pRL harboring renilla luciferase gene. In order to determine the PSMA reporter activity, 293T cells and PC3 cells were co transfected with empty vector or PSMA luc and Renilla-luc reporter genes alone or with NEAT1, NEAT1 + ER α , or NEAT1 + AR.

[0081] 24h post transfection the media was changed to 5% charcoal stripped media and the cells indicated were treated with E2 (10nM) or R1881 (1nM) for 14h. At 48h cells were lysed with passive lysis buffer and luciferase activities were measured using the dual luciferase system (#E1910, Promega) and normalized with renilla luciferase activity.

[0082] RNA *in situ* hybridization for NEAT1: RNA ISH for NEAT1 was performed on 5 benign, 5 PCa and 3 CRPC cases using kits and probes designed by Advanced Cell Diagnostics. Briefly, the single-color chromogenic detection assay uses pairs of specially designed oligonucleotide probes that, through sequence-specific hybridization, recognize both the specific target NEAT1 RNA sequence and the signal amplification system. Unique target probe oligonucleotides were designed to hybridize in tandem to the target RNA. Cross-hybridization to other sequences is minimized by screening against the entire human RNA sequence database.

[0083] The signal amplification system consists of the preamplifier, amplifier, and enzyme-conjugated label probe, which assemble into a tree-like complex through sequential hybridization. Signal amplification occurs at target sites bound by probe pairs only. Nonspecific off-target binding by single probes does not result in signal amplification.

[0084] All steps of NEAT1 RNA ISH staining of the slides are performed manually, optimized in tissue microarrays (TMAs). Briefly, formalin-fixed, paraffin-embedded (FFPE) unstained tissue sections (5 µm) were mounted on positively charged microscopic glass slides, deparaffinized in xylene and rehydrated through a series of alcohols. The rehydrated sections were treated with 3% hydrogen peroxide at room temperature for 10 minutes to block endogenous peroxidase. Sections were then boiled in 1× citric buffer (10 nmol/L Nacitrate, pH 6.0) for 15 minutes and incubated with protease (2.5 mg/mL; Sigma Aldrich, St. Louis, MO) at 40°C for 30 minutes. The slides were hybridized sequentially with target probes (20 nmol/L) in hybridization buffer A (6× saline sodium citrate [SSC] buffer [1× SSC is 0.15 mol/L NaCl and 0.015 mol/L Na-citrate], 25% formamide, 0.2% lithium dodecyl sulfate [LDS], and blocking reagents) at 40°C for 2 hours, signal preamplifier in hybridization buffer B (20% formamide, 5×SSC, 0.3% LDS, 10% dextran sulfate, and blocking reagents) at 40°C for 30 minutes, amplifier in hybridization buffer B at 40°C for 30 minutes, and horseradish peroxidase- or alkaline phosphatase-labeled probes in hybridization buffer C (5× SSC, 0.3% LDS, and blocking reagents) at 40°C for 15 minutes.

[0085] Hybridization signals were detected under bright field microscope as red colorimetric staining (using Fast Red chromogen, BioCare Biomedical, Concord, CA) followed by counterstaining with hematoxylin. Signals were granular and discrete red

signals corresponding to individual lncRNA targets. The signals were scored using the RNA Spot Studio software.

[0086] Chromatin immunoprecipitation: All ChIP experiments were carried out using Millipore EZ-Magna ChIP kit (Catalogue # 17-10086). Briefly 5-10 X 10⁶ cells were crosslinked with 1% formaldehyde for 10 min at room temperature. The crosslinking was then quenched with 0.125 M glycine. Chromatin was sonicated in the lysis buffer to 300-500 bp and the extraction of ChIP DNA was done as per the kit protocol. Antibodies used include ER α (AC-066-100, diagenode, 5 μ g), AR (06-680, Millipore, 5 μ g), H3K4me3 (ab8580, Abcam, 5 μ g), H3K9me3 (ab8898, Abcam, 5 μ g), H3K36me3 (ab9050, Abcam, 5 μ g), H3K27me3 (07-449, Millipore, 5 μ g), and Ace-H3 (no. 06-599, Millipore, 5 μ g).

[0087] ER α ChIP was also performed in crosslinked VCaP cells with E2 treatment for 0, 14h and 48h. In VCaP ER α cells E2 treatment was for 6h, 14h and 48h.

[0088] Chromatin Isolation by RNA Purification (ChIRP): Chromatin immunoprecipitation for NEAT1 was done in VCaP control and NEAT1 expressing cells with and without E2 treatment using the ChIRP protocol (Chu (2012)). Briefly, biotin TEG antisense oligos were generated using singlemoleculefish.com for NEAT1, Lac Z and scrambled NT NEAT1 (see also **Table 4**). The NEAT1 probes were divided into 2 pools. Cells cross-linked in 1% glutaraldehyde were lysed and sonicated. The biotinylated probes were hybridized followed by RNA and DNA isolation. qPCR was performed on the DNA samples.

[0089] Preparation of NEAT1 RNA ISH probe: The full length NEAT1 gene was cloned in pCRII-TOPO (Invitrogen) vector. The plasmid was digested with NotI (New England Biolabs) and purified with the Qiaquick PCR Purification Kit (Qiagen) according to manufacturer's protocol. In vitro transcription was accomplished using 500ng of linearized plasmid DNA and the MEGAscript SP6 kit (Ambion) as directed by the manufacturer. The resultant RNA was cleaned using the RNeasy kit (Qiagen) using the manufacturer's protocol. 5ug of RNA was mixed with 10ul of buffer A, 5 μ l of ML DNP reagent (both provided by Ventana-Roche) and water to 50 μ l. The reaction was incubated at 37°C for two hours. The labeled RNA was cleaned with the RNeasy Kit (Qiagen). 50-250ng/ml of probe was mixed in Ribohybe solution (Ventana-Roche) and 100ul of the probe was used for each

slide. RNA in situ hybridization on FFPE slides was performed using an automated protocol developed for the Discovery XT automated staining system (Ventana-Roche).

[0090] RNA-ISH for NEAT1 on cell lines: Cells were grown on a 15mm, poly-L-lysine coated glass coverslip. At ~70% confluence cells were serum starved in 8% charcoal stripped media for 48h, followed by 48h treatment with 10nM E2. At the end of treatment, cells were fixed in 4% formaldehyde, dehydrated by an ethanol gradient (50-100%) and stored at -20°C. For the hybridization assay cells were rehydrated by an ethanol gradient (100-50%) into PBS. Between subsequent steps cells were washed with PBS. The Affymetrix QuantiGene ViewRNA ISH cell assay kit was used for NEAT1 staining. Cells were permeabilized by 5min incubation at RT in Detergent Solution QC, and digested for 10min at RT by Protease QS (1:4000 in PBS). Then the target specific Probe Set (1:100 in Diluent QF) was allowed to hybridize for 3h at 40±1°C. Between subsequent steps cells were washed by soaking in Wash Buffer. Sequential hybridization steps were conducted for signal amplification—PreAmplifier Mix (1:25 in Diluent QF), Amplifier Mix (1:25 in Diluent QF) and Label Probe Mix (1:25 in Diluent QF) each incubated 30min at 40±1°C. After 2 10min washes in Wash Buffer, nuclei were stained with DAPI and cover slips were mounted to slides with Prolong Gold Antifade Reagent (Life Technologies) for visualization.

[0091] Proliferation assay: Cell proliferation was assessed using the CyQUANT NF cell proliferation assay kit (Life Technology). Cells were seeded in 96-well plates at 3-4 X 10⁴ cells per well. Cells were incubated in DMEM media with 10% FBS for 24h. The cells were then serum starved in 8% charcoal stripped DMEM medium for 48h followed by E2 treatment at 10nM concentration for indicated time points. The media was then aspirated and replaced with the dye binding solution followed by incubation for 30-60 minutes. The fluorescence was then measured in a microplate reader using excitation at 485 ± 10 nm and fluorescence detection at 530 ± 15 nm. The assay was performed in triplicates.

[0092] Invasion assay: The CHEMICON cell invasion assay kit (EMD Millipore) was used for determining the cell invasion. Cells were serum-starved for 48 hours and then seeded at a density of 2 × 10⁵ cells/well in the upper well of the invasion chamber. 500 µl of phenol res free DMEM media supplemented with 8% charcoal stripped serum and 10nM E2

was added to the lower chamber. After 48-hour incubation, the invaded cells were stained by dipping the inserts in the staining solution for 20 minutes. The stained cells were then dissolved in 10% acetic acid and transferred to a 96-well plate for colorimetric reading of OD at 560 nm.

[0093] Migration assay: The Cell Biolabs Inc. Radius™ 96-Well Cell Migration Assay was used to determine cell migration. Cells were serum-starved for 48 hours, then seeded to a pretreated (incubated 20 minutes in Radius™ Gel Pretreatment solution and washed with Radius™ Wash Solution) Radius™ 96-Well Plate at a density of 8×10^4 cells per well with or without E2 (10 nM). After 24 hours incubation, the Radius™ Gel Spot was removed via the Radius™ Gel Removal Solution and pre-migration images were captured. After 24 hours incubation, cells were stained with Cell Stain Solution and post-migration images were captured for analysis using the CellProfiler™ Cell Image Analysis Software (Broad Institute).

[0094] Statistical analysis: The Wilcoxon test was employed with Benjamini-Hochberg (Benjamini (1995)) correction for multiple hypotheses for pair-wise comparisons for differential expression analysis. The Chi-square test was used for comparison of proportions and the Pearson's correlation was used to compare the expression of selected genes. For quantitative real time PCR, the Delta CT value was computed according to the ABI qPCR protocol. To compare qPCR data a student's t-test was employed. Median-rank statistics results are reported for analyses with the Oncomine datasets (Rhodes (2004)).

[0095] Analysis of Mayo Clinic cohort: Affymetrix HuEx microarrays were used to analyze NEAT1 expression in two post-radical prostatectomy cohorts from the Mayo Clinic. Details on tissue preparation, RNA extraction, amplification, hybridization, and clinical characteristics for these cohorts have been described previously (Erho (2013); Karnes (2013)). Both cohorts were filtered using the same criteria (patient either exhibiting preoperative prostate-specific antigen >20 ng/mL, Gleason score ≥ 8 , pT3b, or GPSM (Blute (2001)) score ≥ 10) to increase the homogeneity of patient characteristics. The two sets were pooled to improve analytic power, resulting in a dataset of 594 patients. The patient characteristics of the pooled dataset can be found in **Table 1**.

[0096] A representative Probe Selection Region (PSR) for the genomic span of the short and long NEAT1 isoforms was selected by minimizing the technical variance across the pooled dataset. Based on these two PSRs, the prognostic performance of NEAT1 short and long isoforms was evaluated using univariable and multivariable odds ratios and area under the receiver operating characteristics curve (AUC) for BCR, MET, PCSM, and GS > 7 endpoints. Kaplan Meier (KM) curves were used to perform survival analysis on the Mayo case-cohort patients only (Erho (2013)) since the nested case-control cohort (Blute (2001)) was not suitable for KM analysis.

[0097] RNA isolation, cDNA synthesis and PCR experiments: Total RNA was isolated from frozen prostate tissue samples (for qPCR) and cell lines (for transcriptome sequencing and qPCR) using Trizol (Invitrogen) with DNase I digestion according to manufacturer's instructions. RNA integrity was verified on an Agilent Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized from total RNA using Superscript III (Invitrogen) and random primers (Invitrogen). Quantitative RT-PCR was performed using Power SYBR Green Mastermix (Applied Biosystems) on an Applied Biosystems 7900 Fast Real Time PCR machine. All primers were designed using Primer 3 and synthesized by Integrated DNA Technologies.

[0098] RNA sequencing: Standard poly-A selected RNA sequencing was done for VCaP, VCaP ER α expressing cells as well as for VCaP cells overexpressing empty vector and VCaP NEAT1 overexpressing cell lines using Illumina TruSeq RNA-seq protocol. Reads were aligned to the reference genome NCBI36/hg18 without the minor haplotypes and the minor sequences using STAR aligner3. Reads mapped to the mitochondrial genome were removed. The expression of each gene (UCSC knownGenes) was computed using mrfQuantifier, part of RSEQtools4. The resulting expression data was used to identify variation in gene expression between VCaP and VCaP ER α cells and also between the vector control and NEAT1 expressing cells. The inventors computed the ratio between VCaP and VCaP ER α and control cells and VCaP NEAT1, after adding 1, and selected those genes with a log2-fold change greater than 2.

[0099] ChIP sequencing: ER α ChIP followed by sequencing was performed in VCaP, VCaP + E2, VCaP ER α + E2 expressing cells and in NCI-H660 cells with and without E2 treatment. Cells were treated with E2 for 48hrs. ChIP experiments were carried out using

Millipore EZ-Magna ChIP kit (Catalogue # 17-10086). Briefly 5-10 X 10⁶ cells were crosslinked with 1% formaldehyde for 10 min at room temperature. The crosslinking was then quenched with 0.125 M glycine. Chromatin was sonicated in the lysis buffer to 300-500 bp and the extraction of ChIP DNA was done as per the kit protocol. For ChIP sequencing the concentrations of the ChIP DNA were quantified by Qubit Fluorometer (Invitrogen). ChIP DNA was prepared into libraries and direct sequencing of the ChIP libraries was performed using illumine Genome Analyzer according to standard manufacturers procedures.

[00100] ChIP seq data analysis: Peak detection for all ChIP-seq experiments was performed with ChIPseeqer5, using the same parameters for all datasets (i.e., p-value threshold for peaks=10⁻⁵, minimum distance between peaks=100bp). Genomic annotation of ChIP-seq peaks, comparison between ChIP-seq datasets and motifs analysis were performed using the corresponding tools in the ChIPseeqer software. The binding sites were ranked according to their p-value as determined by ChIPSeeqer and considered the expression levels of the potential target genes. In this case, a target gene is defined if it is within 20KB of the peak and considered only genes whose expression is higher than 1 in at least one condition (either VCAP con or VCAP ER α).

[00101] RNA extraction, sample preparation and sequencing: For RNA sequencing analysis frozen tissue was cored (1.5 mm biopsy cores) and RNA extracted using TRIzol Reagent (Invitrogen, CA). Tissues were obtained from radical prostatectomy series at the Weill Cornell Medical College. All samples were collected with informed consent of the patients and under an Institutional Review Board approved protocol. The extracted RNA was subjected to DNase treatment using a DNA-free TM Kit (Applied Biosystems/ Ambion, Austin, TX, USA). RNA quality was measured using the RNA 6000 Nano Kit on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA with RIN (RNA integrity number) ≥ 8 was used for subsequent library preparation. Illumina's sample preparation protocol for paired-end sequencing of mRNA was used. The paired end reads were then aligned to the human genome (hg18) using ELAND/CASAVA.

Example 2. Results

[00102] ER α in transcriptional regulation of prostate cancer

[00103] To elucidate the role of ER α in prostate cancer, the inventors analyzed ER α protein and transcript levels in a panel of prostate cancer cell lines (n=5) and in a cohort of matched benign prostate tissue (n=14) and prostate adenocarcinoma (PCa) (n=14), respectively. The inventors observed that ER α was significantly upregulated (p=0.03) in prostate tumors compared with benign tissues (**Fig. 1a**). To determine the clinical relevance of ER α in prostate cancer, immunohistochemistry was performed using a tissue microarray composed of tissue cores from 64 samples of benign prostate tissue, 16 high-grade prostate intraepithelial neoplasia (HGPIN), 292 PCa, and 42 neuroendocrine prostate cancer (NEPC). While benign prostate had only low expression levels of ER α , ER α was detected in adenocarcinoma and the adjacent HGPIN through focal nuclear and cytoplasmic staining. ER α is overexpressed in a significant number of prostate cancer cohorts. It was also found to be overexpressed in prostate cancers with high Gleason score compared to those with low Gleason score as well as in those with tumor recurrence when analyzed via the Oncomine database (**Fig. 1b**). Analysis of subcellular distribution in prostate cancer cell lines revealed significant nuclear distribution of ER α in all cell lines tested. ER α protein levels were similar in both AR-positive LnCaP and VCaP cells (**Fig. 1a, inset**). The inventors used parental VCaP and the ER α -positive prostate cancer cell line NCI-H660 as model cell lines to further explore and delineate the specific contribution of ER α to prostate cancer. A ligand-dependent modulation of invasive potential was observed in VCaP cells upon estrogen (E2) treatment (**Fig. 1c**). These results suggest that a functionally relevant, ligand-dependent ER α signaling pathway is active in prostate cancer cell lines.

[00104] To further understand the impact of ER α , the inventors generated VCaP cells that overexpress ER α (VCaP ER α). Stable expression of ER α was confirmed by Western blot. VCaP ER α exhibited significantly higher invasive potential than VCaP parental cells or the vector control cells (**Fig. 1c**). Intriguingly, the noted effects of ER α overexpression were independent of AR status, as experimental silencing of AR in VCaP ER α cells did not compromise the increased invasive potential of E2-treated VCaP ER α cells (**Fig. 1c**). These data suggest that prostate cancer cells can utilize alternate nuclear receptor signaling (e.g., ER α signaling) to propagate, and understanding these mechanisms will help discern the complete spectrum of key regulators of prostate cancer progression.

[00105] Studies have established ER α 's dominant role in transcriptional regulation of target genes in breast cancer. Likewise, high nuclear levels of ER α in prostate cancer cells and their direct association with chromatin implicate ER α in the transcriptional regulation of this cancer, as well. The inventors used ER α chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) in VCaP cells, with and without E2 treatment, and also in VCaP ER α and NCI-H660 cells with E2 treatment to investigate the underlying mechanisms by which ER α might drive a transcriptional program in prostate cancer. The majority of ER α -binding sites were cell-specific. Analysis of the ChIP-seq data for ER α in NCI-H660 and VCaP ER α cells revealed that 64.9% of ER α binding occurred within intergenic regions of the prostate genome. This fraction is higher than the expected fraction if peaks were randomly distributed across the genome ($p=3e-05$).

[00106] Using publicly available datasets (Yu (2010)), the inventors found that 28% of the intergenic ER α binding sites in the prostate cancer genome (from VCaP ER α and NCI-H660 cell lines) overlapped with the active histone marks tri-methylated lysine 4 of histone H3 (H3K4me3) and tri-methylated lysine 36 of histone H3 (H3K36me3) ($p<1e-7$). On the other hand, 20.7% of those sites overlapped with histone marks typical of inactive chromatin, such as tri-methylated lysine 9 of histone H3 (H3K9me3) or tri-methylated lysine 27 of histone H3 (H3K27me3). To prioritize experimental validation of ER α targets, the inventors ranked the peaks according to the average p-value determined by the peak-calling algorithm ChIPSeeqer (Giannopoulou (2011)) and selected the highest ranking peaks for further analysis. The inventors analyzed recruitment of endogenous ER α to the top 11 binding sites in parental VCaP cells (**Fig. 1d**) providing an experimental validation of the ChIP-seq data. A significantly higher recruitment of ER α was evident at the binding sites compared to control IgG.

[00107] Given the enhanced recruitment of ER α to intergenic regions in the prostate genome, the inventors evaluated the likelihood that ER α might influence transcriptional output and thereby the repertoire of non-coding RNA in the context of prostate cancer. The inventors thus analyzed the abundance of non-coding transcripts in RNA-seq data derived from a cohort of 73 prostate tissues which included 26 benign prostate samples, 40 PCa, and 7 NEPC, focusing our analysis on 6,850 intergenic lncRNAs out of 12,143 known lncRNAs.

The inventors identified 1,314 and 1,399 intergenic lncRNAs that were differentially expressed between benign and PCa and between PCa and NEPC, respectively (FDR < 0.01). The inventors identified 140 intergenic lncRNAs putatively regulated by ER α (**Fig. 1e**). An analysis of AR binding sites (Yu (2010)) identified 98 lncRNAs that have an AR binding site within the promoter. This supported the view that ER α might significantly influence the non-coding transcriptome in prostate cancer. Using the RNA-seq data on VCaP and VCaP ER α cell lines to validate the expression levels of the top differentially expressed ER α -regulated lncRNAs, the inventors selected six potential candidate lncRNAs that had higher expression in VCaP ER α compared with VCaP. The inventors used quantitative real-time PCR (qRT-PCR) to validate expression for these six ER α regulated lncRNAs in VCaP and VCaP ER α -expressing cell lines. Expression of three of these lncRNAs was further determined in a cohort of 28 matched benign and prostate cancer samples, confirming upregulation of these three nominated lncRNAs in prostate cancer compared with benign prostate (**Fig. 1f**). Taken together, these analyses indicate that ER α is a transcriptional regulator of the non-coding transcriptome in prostate cancer.

[00108] Among the putatively ER α -regulated intergenic lncRNAs, the inventors identified Nuclear Enriched Abundant Transcript 1 (NEAT1) as the most significantly overexpressed lncRNA in prostate cancer versus benign prostate in our patient cohort (73 samples) (**Fig. 1f**). The NEAT1 gene is located on chromosome 11q13.1 and produces two RNA isoforms that overlap completely at the 5' end. The shorter isoform (hereafter abbreviated as NEAT1_1) is 3.7kb in length and more abundant than the longer, 23kb isoform (NEAT1_2) (Bonf (2009)). NEAT1 lncRNA is essential for the formation of subnuclear bodies called paraspeckles (Bond (2009)), and while both isoforms localize to paraspeckles, their physiological role in prostate cancer remains unknown.

[00109] ER α -regulated NEAT1 lncRNA is upregulated in prostate cancer

[00110] The inventors analyzed the expression data of NEAT1 in the Oncomine database, and observed significant overexpression of NEAT1 lncRNA in several prostate cancer datasets (normal vs. cancer) and aggressive prostate cancer (**Fig. 2a**) (Arredouani (2009); Glinsky (2004); Grasso (2012); Lapointe (2004); LaTulippe (2002); Liu (2006); Luo (2001); Singh (2002); Taylor (2010); Tomlins (2007); Vanaja (2003); Varambally (2005); Wallace

(2008); Yu (2004); Holzbeierlein (2004); Magee (2001); Tamura (2007); Welsh (2001)).

The inventors first confirmed that amplification of chromosome 11q (where NEAT1 resides) was not seen across 109 adenocarcinoma cases (Barbieri (2012)), eliminating chromosome 11q13.1 amplification as an explanation for high NEAT1 expression (Cerami (2012); Gao (2013)). The expression of NEAT1 in two radical prostatectomy cohorts with long-term clinical follow-up from the Mayo Clinic (Erho (2013); Karnes (2013)) was measured using Affymetrix HuEx microarrays (see Methods). **Table 1** contains the patient characteristics of the datasets.

[00111] NEAT1's expression ranked in the 99th percentile of all genes on the microarray (**Fig. 2b**). The inventors determined levels of NEAT1 by RNA *in situ* hybridization (ISH) in a tissue microarray that included 16 benign prostate tissues, 21 PCa, 12 PCa with neuroendocrine differentiation, and 7 NEPC cases. NEAT1 was found to be highly expressed in prostate cancer compared with benign tissue.

[00112] The inventors observed that in a panel of prostate cancer cell lines ER α overexpression and E2 treatment upregulated NEAT1 transcript levels in a time-dependent manner (**Fig. 2c**). In DU145, an ERG-negative cell line, E2/ER α signaling was intact (**Fig. 2c**), supporting an ERG-independent phenomenon. Following ER α overexpression, the inventors recorded an increase in expression of the long isoform NEAT1_2, but to a lesser extent than the short form. This was not surprising as both isoforms of NEAT1 are driven by the same promoter (Nakagawa (2011)). Interestingly, knockdown of ER beta (ER β) did not alter NEAT1 levels, suggesting that NEAT1 regulation is specific for ER α .

[00113] NEAT1 was originally identified with subnuclear organelles called paraspeckles that are free of chromatin and function as repositories of edited RNA and a number of nuclear RNA-binding proteins (Clemson (2009)). Loss of NEAT1 dramatically reduces the formation of paraspeckles. The inventors observed that treatment of the VCaP cells with E2 resulted in re-distribution of NEAT1 from paraspeckles to an enhanced distribution throughout the nucleus.

[00114] The inventors inspected our ER α ChIP-seq data in VCaP ER α and NCI-H660 cells and identified two ER α binding sites on the NEAT1 promoter (**Fig. 2d**). Analysis of chromatin marks using ChIP-seq data sets for histone marks (Yu (2010)) revealed the

presence of active histone marks H3 Acetyl K9 and H3K4me3 in the promoter region of NEAT1, while H3K36Me3 marks were abundant in the gene body (**Fig. 2d**). A recent study revealed that bivalent H3K4Me3 and H3K36Me3 marks are indicators of functional transcriptional loci from the non-coding genome (Guttman (2009)). ER α recruitment to specific regions of the NEAT1 promoter was independently validated by ER α ChIP in VCaP, VCaP ER α and NCI-H660 cells (**Figs. 2e**) using specific primers encompassing ER α binding sites in the NEAT1 promoter. The inventors found that a functional estrogen/ER α signaling pathway was active in VCaP cells, as determined by reporter-based ERE luciferase assays in VCaP cells, with ER α and AR overexpression and E2 or R1881 treatment respectively for 48 h (**Fig. 2f**). To further test whether ER α is required for NEAT1 transcriptional activation, the inventors generated luciferase promoter reporter constructs with both ER α binding sites upstream of the luciferase-coding region. Luciferase reporter assays in VCaP cells confirmed that NEAT1 promoter activity was upregulated in an ER α -dependent manner and further enhanced with E2 treatment (**Fig. 2g**).

[00115] ER α and NEAT1 regulate several prostate cancer genes

[00116] The inventors next sought to understand the physiological role of NEAT1 and to determine the downstream targets of the ER α -NEAT1 axis in prostate cancer. Transcriptome sequencing of VCaP and VCaP ER α cells and pairwise comparison revealed 588 genes to be upregulated in VCaP ER α cells (log2 fold change > 2). The inventors performed a comparative analysis of this 588-gene signature using Oncomine concept analysis. The inventors focused on datasets from prostate cancer studies that included both prostate tumor and benign prostate tissues. The analysis revealed that the ER α gene signature was significantly upregulated in a number of prostate cancer datasets, but was downregulated in other non-prostate datasets, indicating that ER α regulates prostate cancer-specific genes.

[00117] To validate if ER α targets identified by *in silico* analysis are dependent on cellular levels of ER α , the inventors experimentally silenced ER α in VCaP cells using an siRNA approach and determined transcript levels of 10 target genes using qRT-PCR. The target genes selected for validation were those genes that demonstrated the highest log2 fold difference in VCaP and VCaP ER α cells. Results indicated that mRNA levels of the target

genes selected were dependent on ER α (**Fig. 3a**), suggesting a distinct contribution of ER α in determining the transcriptional program.

[00118] NEAT1 is a downstream target in the ER α signaling pathway

[00119] After determining an ER α signature, the inventors next investigated the potential role of NEAT1. Interestingly, knockout of NEAT1 compromised the expression of ER α target genes, suggesting that NEAT1 is not only a downstream target but also a mediator of ER α signaling in prostate cancer cells (**Fig. 3b**). To evaluate this further and to determine if a functional synergy between ER α and NEAT1 pathways exists in prostate cancer cells, the inventors performed RNA-seq of vector control and NEAT1-overexpressing VCaP cells to determine a NEAT1 signature. To achieve this, the inventors limited their analysis to genes that were upregulated four-fold in NEAT1-expressing cells. Interestingly NEAT1 signature showed a strong correlation with the ER α signature genes ($q=1.90E-120$). Analysis of the top 1000 genes of the NEAT1 signature revealed that this signature is upregulated in prostate cancer datasets when compared with other cancer datasets. Furthermore, NEAT1 signature was also upregulated in all prostate cancer datasets (comparing benign vs. PCa; odds ratio > 2.0 and $P < 1 \times 10^{-6}$).

[00120] The inventors also queried Oncomine prostate datasets to identify genes whose mRNA levels correlate with those of NEAT1 (correlation coefficient > 0.5). The inventors compared this gene list with the ER α signature genes from their analysis and identified 155 genes in common. These 155-gene were also found to be upregulated in all prostate cancer datasets compared with other cancer datasets (only normal vs. cancer datasets were considered; odds ratio > 3.0 and $P < 1 \times 10^{-6}$).

[00121] To determine if the genes identified by *in silico* analysis are indeed influenced by NEAT1, the inventors silenced NEAT1 in VCaP cells and determined transcript levels of potential target genes using qRT-PCR. The inventors selected the top 10 genes that were significantly correlated to NEAT1 expression across all prostate cancer concepts. As expected, mRNA levels of these selected target genes were indeed dependent on NEAT1, further confirming a definite role of NEAT1 in the transcriptional program (**Fig. 4a**). In addition to cell lines, the inventors also determined transcript levels of these ER α -NEAT1

signature-selected genes in a small patient cohort (n=26) of 13 matched benign and prostate adenocarcinoma, respectively. It was observed that relative mRNA levels of these NEAT1-ER α signature-selected genes revealed significant upregulation in prostate cancer (**Fig. 4b**). The inventors computed the log2 fold change of expression levels using the 13 paired tumor/benign samples for NEAT1 and for these selected genes, then correlated the fold change values, and observed a moderate to strong correlation between NEAT1 and the associated genes in clinical samples. Among these seven genes, prostate-specific membrane antigen (*PSMA*) and alpha-methylacyl-CoA racemase (*AMACR*) are well-known diagnostic and, in the case of *PSMA*, prognostic markers of prostate cancer progression (Burger (2002); Gumulec (2012); Jiang (2013); Ross (2003); Xiao (2001)). Furthermore, knocking down ER β did not alter expression of key signature genes in LnCaP, PC3, VCaP and NCI-H660 cells, suggesting a non-redundant regulatory role for ER α .

[00122] NEAT1 and chromatin regulation

[00123] To study the potential role of NEAT1 in regulation of target genes *in vivo*, the inventors performed luciferase reporter assays using *PSMA*-luc as a candidate NEAT1 target. NEAT1 induced robust activation of the *PSMA* promoter in PC3 cells (**Fig. 5a**) and VCaP cells (**Fig. 5b**). These results prompted us to investigate if NEAT1 is recruited to chromatin of target genes. The inventors used the ChIRP approach to pull down endogenous NEAT1 from VCaP cells. Analysis of the ChIRP data revealed that NEAT1 is recruited to the *PSMA* promoter, but not the downstream exon 1 (**Fig. 5c**). In addition to *PSMA*, the inventors also tested NEAT1 recruitment to other target genes described in **Fig. 3a** and **Fig. 4a** and observed that in addition to *PSMA*, NEAT1 was also recruited to the promoter region of *GJB1*. This suggests that NEAT1 transcriptionally regulates a compendium of genes known to be involved in prostate cancer progression. The inventors hypothesized that NEAT1 might contribute to gene transcription by interacting with chromatin-modifying proteins and/or interacting with histones. The inventors next analyzed the chromatin landscape at the *PSMA* promoter and observed that NEAT1_1, and not NEAT1_2, facilitated gene transcription by promoting an active chromatin state (**Fig. 5d**). Overexpression of NEAT1_1 significantly increased active chromatin marks at the *PSMA*

promoter (i.e., H3K4Me3 and H3AcK9). Of note, ER α was not significantly recruited to the *PSMA* promoter when expressed alone. Overexpression of NEAT1_1 resulted in subsequent recruitment of NEAT1_1 and ER α to the *PSMA* promoter. These studies indicate that while NEAT1_1 may function as a chaperone for ER α and other chromatin-modifying machinery to target promoters, binding of ER α and/or recruitment to NEAT1_1 targets is not necessary for transcriptional activation.

[00124] As the inventors' data suggest that NEAT1 overexpression favors a chromatin landscape for active transcription, the inventors investigated whether NEAT1 could directly interact with nucleosomal histones. Nuclear lysates from VCaP cells were used in an immunoprecipitation experiment with streptavidin-beads coupled with either scrambled, antisense NEAT1, or antisense NR_024490 (another ER α lncRNA target) oligonucleotides. NEAT1 was found to specifically associate with histone H3 and the specificity of this binding is apparent when comparing Streptavidin-IP using scrambled biotinylated oligos and Streptavidin-IP using antisense-NEAT1 oligos and nuclear lysates from NEAT1 siRNA treated cells, respectively. As an additional negative control, the inventors used scrambled and specific antisense oligos for a different lncRNA, NR_024490, another ER α target. The results indicate that NEAT1 can associate with chromatin via specific interaction with histone H3. The inventors also determined association of NEAT1 with active histone H3 modifications, including H3AcK9 and H3K4Me3. Similar association patterns were seen for NEAT1 in NCI-H660 cells.

[00125] To complement this finding, the inventors performed RNA immunoprecipitation from VCaP ER α cells using anti-histone H3 and anti SNRNP70 (positive control) as the immunoprecipitating antibody. qRT-PCR showed robust binding of NEAT1 to histone H3. The positive control U1 snRNA showed high enrichment in the immunoprecipitate with SNRNP70. To further confirm the specificity of NEAT1 binding to histone H3, the inventors performed a streptavidin-biotin pull down assay in VCaP and VCaP ER α cells with and without E2. These data suggest that NEAT1 can directly interact with the histone H3 component of chromatin.

[00126] NEAT1 promotes prostate tumorigenesis

[00127] To better understand the physiological role of NEAT1 in context of ER α in prostate cancer, the inventors first determined levels of NEAT1 in VCaP cells overexpressing ER α (**Fig. 6a**). Further, the inventors generated stable VCaP and VCaP ER α cell lines that overexpress NEAT1 (**Fig. 6b**). The inventors also knocked down NEAT1 in VCaP and VCaP ER α -expressing cells by stably expressing NEAT1 siRNA targeting different regions of NEAT1 and non-targeting siRNA (**Table 2** and **Fig. 6c**). While overexpression of NEAT1 significantly increased proliferation and cell invasion, knockdown of NEAT1 significantly decreased proliferation and the invasive properties of the cells (**Figs. 6d & 6e**).

[00128] Soft agar assays were performed in both VCaP and VCaP NEAT1 cells. Colonies were monitored over a period of 21 days. Overexpression of NEAT1 resulted in a significantly higher number of viable colonies (**Fig. 6f**). Colony-forming assays performed in NEAT1 clones in VCaP cells with and without E2 demonstrated that E2 treatment in NEAT1-overexpressing cells significantly increased the number of colonies (**Fig. 6g**). These *in vitro* assays establish an oncogenic role for NEAT1.

[00129] To further validate the oncogenic role of NEAT1, the inventors extended their studies to an *in vivo* model system. The inventors performed xenograft studies in NOD-SCID mice. The mice were treated with time-release estrogen pellets. They were divided into two groups: one group was implanted subcutaneously with VCaP ER α cells expressing control siRNA and luciferase reporter, and the other group with VCaP ER α cells expressing NEAT1 siRNA luciferase reporter. The tumor growth was monitored weekly for 45 days and was found to be significantly lower in the NEAT1 siRNA-expressing group compared with the control group (**Fig. 6h**). The tumors were excised and weighed and the NEAT1 siRNA group had significantly smaller tumors (**Fig. 6k**). The inventors confirmed the efficacy of the siRNA *in vivo* by measuring the NEAT1 and ER α levels in the tumors (**Fig. 6l**).

[00130] To further substantiate the hypothesis that NEAT1 plays a role in tumorigenesis, the inventors repeated the experiment in athymic nude mice using VCaP control and VCaP NEAT1-overexpressing cells as well as NCI-H660 and NCI-H660 NEAT1-overexpressing cells. In both these experiments, a significantly higher tumor growth was seen in the

NEAT1-overexpressing cells (**Figs. 6i & 6j, Figs. 6m**) further confirming its oncogenic potential. qRT-PCR analysis confirmed an increased expression of the NEAT1 signature genes in VCaP NEAT1 xenografts compared with control VCaP xenograft tissue.

[00131] NEAT1 is associated with therapeutic resistance

[00132] Studies presented so far show that ER α establishes an oncogenic cascade and that NEAT1 functions as a downstream mediator of ER α signaling. The ER α - NEAT1 axis is functional both in AR-positive and -negative cell lines and drives prostate carcinogenesis. The inventors hypothesized that targeting NEAT1 using mechanisms that can constrain ER α might represent a novel therapeutic strategy in prostate tumors that are resistant to anti-androgen therapy. To test this hypothesis *in vitro*, the inventors evaluated the effect of anti-estrogens and anti-androgens on NEAT1 levels in prostate cancer cell lines. As shown in **Fig. 7a & b**, NEAT1 expression was constrained when cells were treated with the ER α antagonists ICI 182,720 (ICI) and 4-hydroxy tamoxifen (4OHT) in combination with E2. Intriguingly, treatment of ICI and 4OHT alone for longer periods can enhance NEAT1 expression (**Fig. 7a & b**). The inventors observed similar results with AR antagonists enzalutamide and bicalutamide (**Fig. 7c & d**). These results provide compelling evidence to evaluate NEAT1 levels in advanced CRPC cases. RNA-FISH analysis of benign and advanced prostate tumors, including CRPC and NEPC tumor tissue samples illustrated significantly upregulated NEAT1 levels in advanced prostate cancer (**Fig. 7e**), with enhanced focal staining throughout the tumor tissue. The inventors also screened 9 cases of benign prostate, 7 PCa, and 7 CRPC for NEAT1 and ER α expression by qPCR (**Fig. 7f**), and both NEAT1 and ER α levels were significantly higher in the CRPCs. The inventors determined the correlation between NEAT1 and ER α expression by estimating the Pearson's correlation coefficient R. The results indicate a strong positive correlation: R = 0.86 (p-value=1.9e-07). Taken together, our results present a novel role for the non-coding transcriptome in cancer-favorable adaptations.

[00133] NEAT1 is associated with aggressive prostate cancer

[00134] Given the importance of NEAT1 in promoting tumorigenesis both *in vitro* and *in vivo*, the inventors sought to determine the relationship between NEAT1 levels and prostate

cancer clinical outcomes in 594 patients from two radical prostatectomy cohorts with long-term clinical follow-up from the Mayo Clinic (Erho (2013); Karnes (2013)). **Table 1** contains the patient characteristics of men who underwent radical prostatectomy at the Mayo Clinic Comprehensive Cancer Center between 1987 and 2001 for clinically localized prostate cancer.

[00135] The inventors assessed the prognostic potential of NEAT1 expression using several statistical measures and correlating it with biochemical recurrence (BCR) and metastasis (MET), prostate cancer-specific mortality (PCSM) and Gleason score (GS) > 7. Biochemical recurrence was typically determined based on evaluating levels (e.g., protein levels in the blood or other body fluid) of biomarkers characteristic of prostate cancer (e.g., such as PSA). In order to evaluate endpoints of disease aggressiveness and progression based on NEAT1 expression, Kaplan-Meier (KM) analysis was performed for the BCR and MET endpoints. The resulting KM curves (**Figs. 8a & b**) demonstrate that patients with higher NEAT1 expression have significantly worse outcomes for both BCR (p-value: 0.028) and MET events (p-value: 0.016).

[00136] Patient risk discrimination based on the expression profile of NEAT1 was assessed by area under the receiver operating characteristic curve (AUC) with 95% confidence intervals (CI) (**Fig. 8c-8j**). NEAT1 significantly segregates patients who exhibited BCR, MET, PCSM and GS > 7.

[00137] To further compare NEAT1's prognostic ability to other clinicopathologic variables, univariable odds ratios were computed for the BCR, MET, PCSM, and GS > 7 endpoints (**Fig. 9a-d**). NEAT1 was significantly prognostic for segregating high-risk from low-risk patients for each of the endpoints ($p < 0.05$). Further multivariate analysis adjusting for adjuvant radiation and hormone treatment, in addition to the other clinicopathological variables assessed, also demonstrates that NEAT1 was significantly prognostic for BCR, MET, and GS>7, supporting NEAT1 as a prognostic biomarker for aggressive prostate cancer independent of common clinical and pathologic variables (**Fig. 9e-9m**). Overall, these results show that NEAT1 is significantly prognostic for several clinically relevant endpoints.

[00138] Summary of the Results

[00139] Analysis of global ER α recruitment in prostate cancer cells using a ChIP-seq approach revealed that ER α is preferentially recruited to intergenic regions of the prostate genome. Comparison of binding profiles with transcriptome sequencing data suggested that ER α drives expression of non-coding transcripts. From a large compendium of ER α -regulated non coding transcripts, NEAT1 was selected for a detailed biochemical and *in vivo* evaluation, based on an *in silico* approach that demonstrated a strong association of NEAT1 with prostate cancer progression. It has been shown here that ER α transcriptionally regulates NEAT1. NEAT1 is recruited to the promoter of several key target genes and induces an active chromatin state favorable for transcription. These studies indicate that ER α does not function as a molecular chaperone to guide NEAT1 to target chromatin; rather, it is believed that a complex proteome of chromatin-interacting proteins interacts with and guides NEAT1 to promoter targets. Interestingly, both ER α and NEAT1 signaling were refractory to AR inhibitors and the lack of AR or ER β , thus indicating a functional specialization of the ER α -NEAT1 axis for prostate cancer progression. Furthermore, introduction of cells overexpressing NEAT1 could clearly induce prostate cancer progression in experimental animal models.

[00140] It has been shown here that NEAT1 regulates expression of prostate cancer genes by altering the epigenetic landscape at target gene promoters to favor transcription. A closer examination of NEAT1 revealed a previously uncharacterized role in recognition of modified histones. NEAT1 expression independently was sufficient to activate prostate cancer genes in an AR independent manner. Further, the results confirmed an oncogenic role for NEAT1 in an experimental animal model of prostate cancer and in cell culture models.

[00141] The identification of an ER α -NEAT1 axis herein illustrates a mechanism whereby prostate cancer cells may develop therapeutic resistance through positive selection of an alternate nuclear receptor signaling pathway in the absence of AR or during androgen ablation therapy. From a clinical perspective, the results herein indicate for the first time that NEAT1 is significantly prognostic for several clinically relevant endpoints. In prostatectomy specimens from two large cohorts, high NEAT1 expression was associated with a significant increase in both biochemical and metastatic recurrence rates compared to those with low NEAT1 expression.

[00142] In summary, the studies herein provide important insights into a unique mechanism of ER α regulation in prostate cancer and identifies NEAT1 as a novel prognostic marker and potential therapeutic target in this disease. While the studies have identified a previously unexplored function of ER α in regulating lncRNAs, it is also the first of its kind to demonstrate transcriptional regulation of lncRNAs by an alternative steroid receptor in prostate cancer. It is believed that NEAT1 is directly involved in modulation of the phenotype of a leading disease. Combinatorial targeting of NEAT1 and AR may represent a unique therapeutic regimen within a subset of patients with advanced prostate cancer.

Table 1**Patient characteristics for the pooled Mayo nested case-control and Mayo case-cohort datasets**

	%
BCR	68
MET	41
PCSM	25
GS > 7	50
pT3+	68
LNI	18
SMS	62
SVI	42
ECE	53
pPSA > 20	29
AdjHTx	33
AdjRTx	12

Biochemical recurrence (BCR), metastatic recurrence (MET), prostate cancer specific mortality (PCSM), Gleason score (GS) > 7, pathological tumor stage 3 or greater (pT3+), Lymph Node Invasion (LNI), Surgical Margin Status (SMS) positive, Seminal Vesicle Invasion (SVI), Extra Capsular Extension (ECE), preoperative PSA (pPSA), adjuvant hormone therapy, and adjuvant radiation therapy are shown.

Table 2: Sequences of siRNAs

Target	Seq.
siNEAT1 Sense	UGGUA AUGGUGGAGGAAGAUU (SEQ ID NO: 3)
siNEAT1 Sense	GUGAGAAGUUGCUUAGAAAUU (SEQ ID NO: 4)
siNEAT1 Sense	GGAGGAGUCAGGAGGAUAUU (SEQ ID NO: 5)
siNEAT1_2	Sense CCAAAUAGGCUUACAGAUUU (SEQ ID NO: 6)
siNEAT1_2	Sense AGAGAGAAGUUGUGGAGAAUU (SEQ ID NO: 7)
	Sense GCCUUUACUACAUGUGUGA[dT][dT] (SEQ ID NO: 43)
	Antisense UCACACAUGUAGUAAAGGC[dT][dT] (SEQ ID NO: 44)
	Sense CUUAGAACUUUAAGUGCAA[dT][dT] (SEQ ID NO: 45)
	Antisense UUGCACUUAAAGUUCUAAG[dT][dT] (SEQ ID NO: 46)

piLenti-NEAT1 siRNA-GFP 4 siRNAs targeting NEAT1:

NEAT1-1140:

Sense TCATGGACCGTGGTTTGTACTATAGTGT (SEQ ID NO: 8)

Antisense 3' AGTACCTGGCACCAACAATGATATCACA 5' (SEQ ID NO: 47)

NEAT1-1732:

Sense GTTCTTAGCCTGATGAAATAACTTGGGGC (SEQ ID NO: 9)

Antisense 3' CAAGAATCGGACTACTTTATTGAACCCCG 5' (SEQ ID NO: 48)

NEAT1-2352

Sense GTGAGAAGTTGCTTAGAACTTTCC (SEQ ID NO: 10)

Antisense 3' CACTCTTCAACGAATCTTTGAAAGG 5' (SEQ ID NO: 49)

NEAT1-3260

Sense CTGGTATGTTGCTCTGTATGGTAAG (SEQ ID NO: 11)

Antisense 3' GACCATACAACGAGACATACCATTC 5' (SEQ ID NO: 50)

iLenti-si-scrambled

CAACCCGCTCCAAGGAATCG (SEQ ID NO: 12)

Table 3: Primer sequences used in the Examples section

Gene	Sequence (5' --> 3')
NEAT1_2 F:	5' – TTTGTGCTTGGAACCTTGCT – 3' (SEQ ID NO: 13)
NEAT1_2 R:	5' – TCAACGCCCAAGTTATTTTC – 3' (SEQ ID NO: 14)
NEAT1v2 F:	5' – TCTCCATTTCCCATCTGAG – 3' (SEQ ID NO: 15)
NEAT1v2 R:	5' – CAGCCACAGAAAAGGGAGAG – 3' (SEQ ID NO: 16)

Primers used in ChIP Assay

NEAT1_P_2 F:	5' – GGCAGTGACCCTGACAAGTT – 3' (SEQ ID NO: 17)
NEAT1_P_2 R:	5' – TCTGAAGGAGCCTTTTCTGC – 3' (SEQ ID NO: 18)
NEAT1_P_3 F:	5' – CCATCTGACCTTGGCACTTT – 3' (SEQ ID NO: 19)
NEAT1_P_3 R:	5' – CAACCCACCTTGGTCTGACT – 3' (SEQ ID NO: 20)
NEAT1_P_4 F:	5' – GGGAACCTCCCTTCCTCAGTC – 3' (SEQ ID NO: 21)
NEAT1_P_4 R:	5' – CAGCCTCCTGACCACAACCTC – 3' (SEQ ID NO: 22)
NEAT1_P_1 F:	5' – AATTTTCCAGATGTCCTGCC – 3' (SEQ ID NO: 23)
NEAT1_P_1 R:	5' – AACAGTGCTTTTGGGATCG – 3' (SEQ ID NO: 24)
ER_P_NT-NEAT1_1 F:	5' – TCATTGGTCATGGCTTAGCA – 3' (SEQ ID NO: 25)
ER_P_NT-NEAT1_1 R:	5' – AGCCATTTTGTCTCCTGCAC – 3' (SEQ ID NO: 26)
ER_P_NT-NEAT1_2 F:	5' – ACTATTTCAACCGCCACGAG – 3' (SEQ ID NO: 27)
ER_P_NT-NEAT1_2 R:	5' – TGCCTGAGGGCTCTAGTCAT – 3' (SEQ ID NO: 28)

Table 4: Biotin TEG antisense probes

Sequence Name	Sequence (5'-3')
NEAT1_scr1	gagaacatcgaattagcgtc (SEQ ID NO: 29)

NEAT1_scr2	gactatcagacgcctaagat (SEQ ID NO: 30)
NEAT1_scr3	ggtacgtaacgaggaagcga (SEQ ID NO: 31)
NEAT1_185	tgcggatattttccatgcag (SEQ ID NO: 32)
NEAT1_488	agcccttggctctggaaaaaa (SEQ ID NO: 33)
NEAT1_800	aagcgttggtaaatgtgtgc (SEQ ID NO: 34)
NEAT1_1161	tcgcatgaggaacactata (SEQ ID NO: 35)
NEAT1_1516	tgcagcatctgaaaaccttt (SEQ ID NO: 36)
NEAT1_1829	cacaacacaatgacaccctt (SEQ ID NO: 37)
NEAT1_2195	actgtatcttaaccaaccc (SEQ ID NO: 38)
NEAT1_2555	ccaggaggaagctggtaaag (SEQ ID NO: 39)
NEAT1_2939	gatgtgttctaaggcacga (SEQ ID NO: 40)
NEAT1_3325	ccattggtattactttacca (SEQ ID NO: 41)
NEAT1_3691	ttattgtgctgtaaagggg (SEQ ID NO: 42)

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WHAT IS CLAIMED IS:

1. A method of determining the presence or risk of developing or advancing prostate cancer in a subject, comprising
detecting the level of NEAT1 in a biological sample from the subject,
comparing the level of said NEAT1 relative to control, and
determining the presence or risk of developing or advancing prostate cancer in the subject based on an elevated level of NEAT1 in the sample as compared to the control.
2. The method of claim 1, wherein said subject has prostate cancer, and an elevated level of NEAT1 as compared to control is indicative of a risk of the cancer progressing into an advanced stage.
3. The method of claim 1, wherein said subject does not have prostate cancer, and an elevated level of NEAT1 as compared to control is indicative of a risk of developing prostate cancer in the subject.
4. The method of claim 1, wherein an elevated level of NEAT1 as compared to control is indicative of the presence of prostate cancer in the subject.
5. The method of claim 1, wherein said sample is selected from the group consisting of prostate tissue, blood, urine, semen, prostatic secretions and prostate cells.
6. The method of claim 1, wherein said detecting comprising performing an assay selected from Northern blot, RNA ISH, RT-PCR, or RNA Seq.
7. The method of claim 6, wherein said assay utilizes a nucleic acid primer or probe that hybridizes to a 5' portion of NEAT1 molecule.
8. The method of claim 6, wherein said assay is RT-PCR which utilizes a pair of primers, both of which hybridize to a 5' portion of NEAT1 molecule.

9. A method of treating prostate cancer in a subject, comprising administering to the subject an interfering RNA molecule targeting NEAT1.
10. The method of claim 9, wherein said molecule is an siRNA.
11. The method of claim 9, wherein said molecule is an shRNA.
12. The method of claim 9, wherein said siRNA is conjugated with nanoparticles.
13. The method of claim 10, wherein said siRNA is conjugated with PAMAM dendrimers.
14. The method of claim 9, wherein prior to said administration, the subject has been determined to have an elevated NEAT1 level as compared to control.
15. The method of claim 9, wherein the subject exhibits resistance to therapy targeting androgen-receptor mediated functions.
16. The method of claim 9, wherein the patient has an advanced stage prostate cancer.
17. The method of claim 9, wherein the interfering RNA is administered to said patient in combination with a therapy targeting androgen-receptor mediated functions.

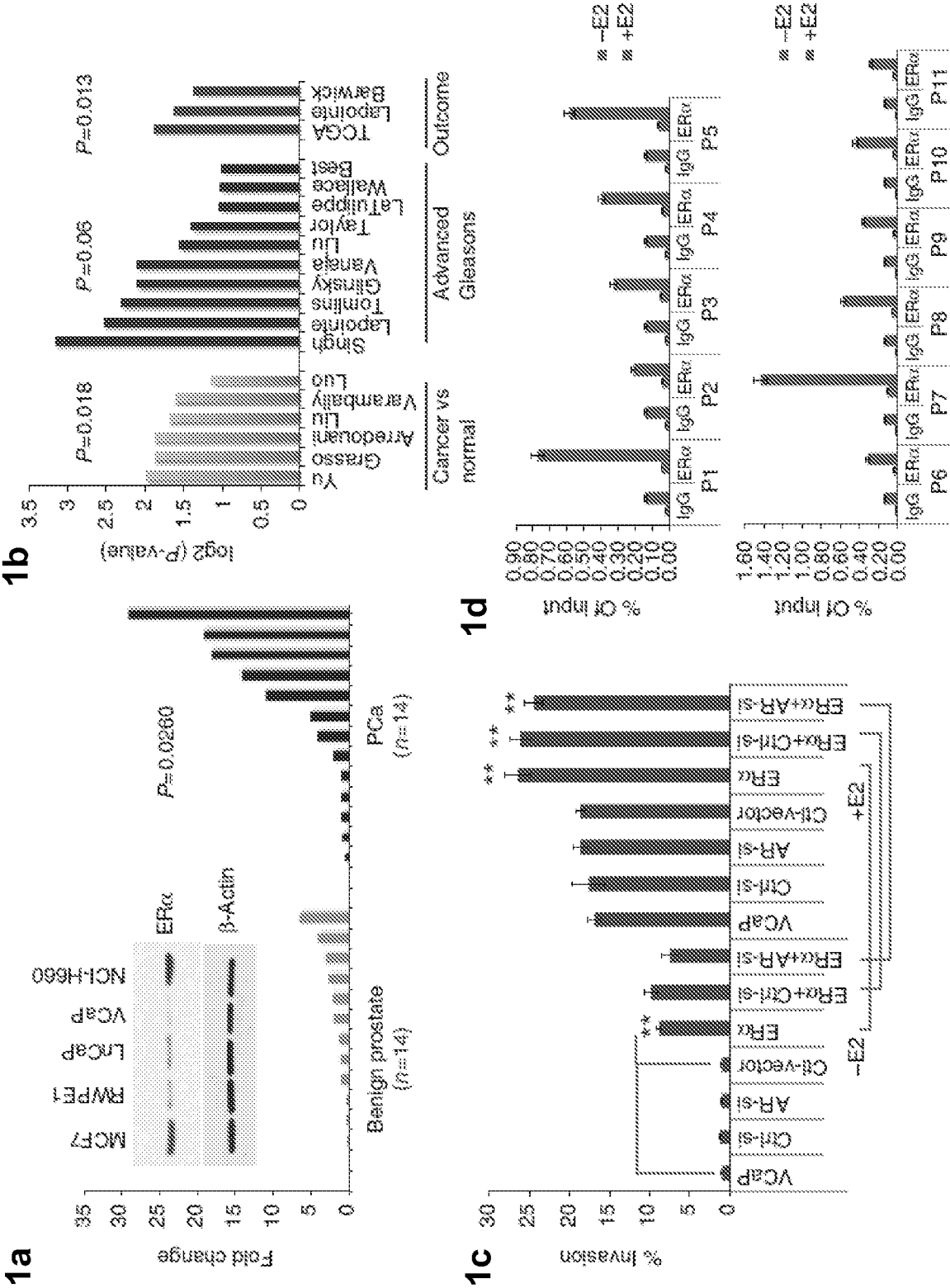


Figure 1a-1d

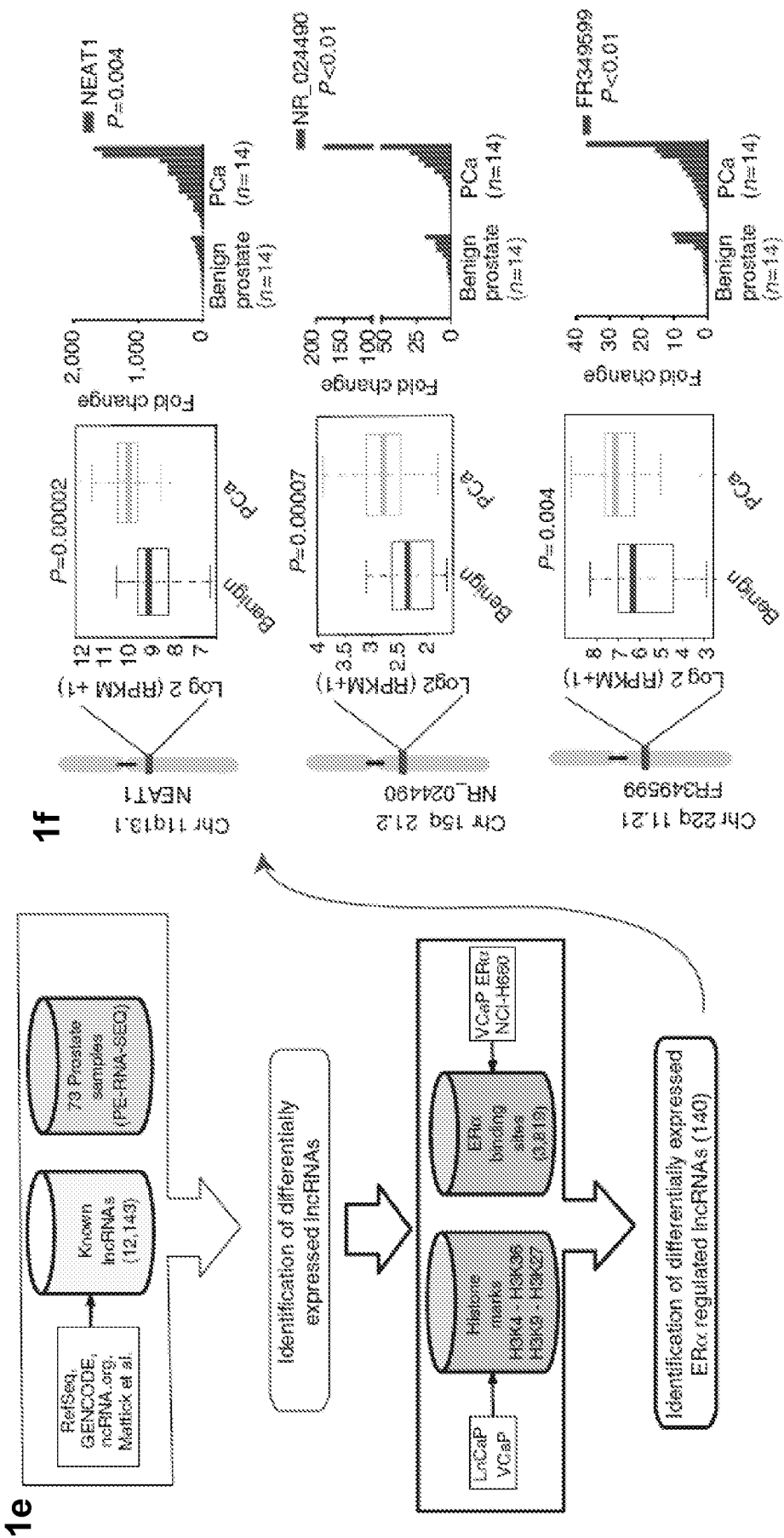


Figure 1e-1f

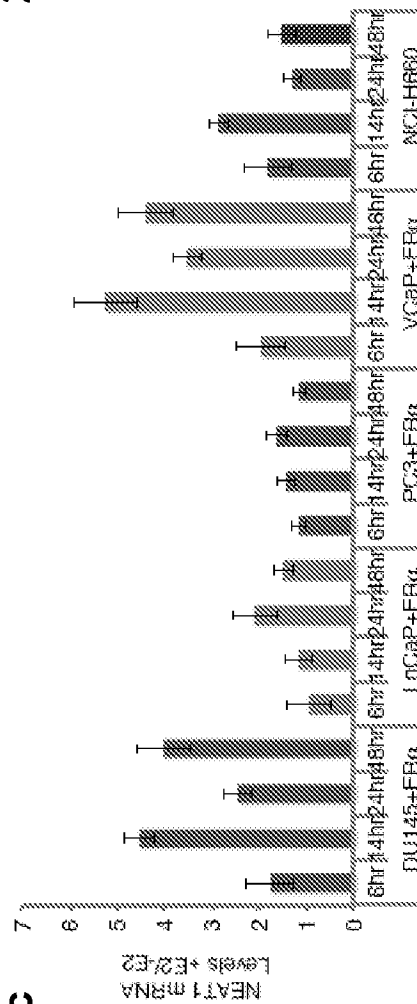
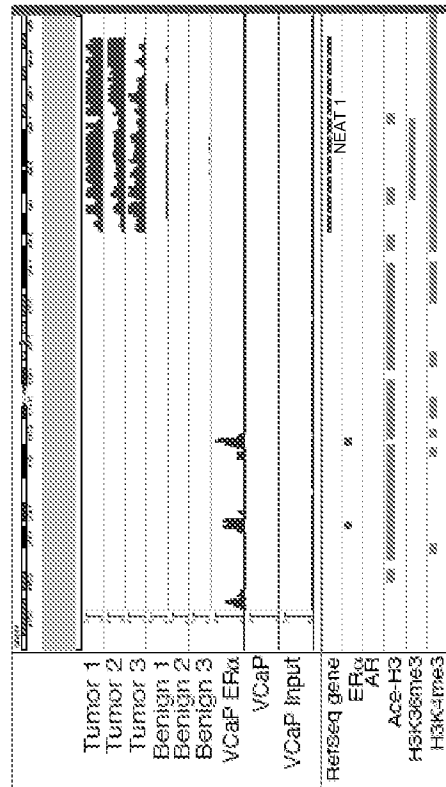
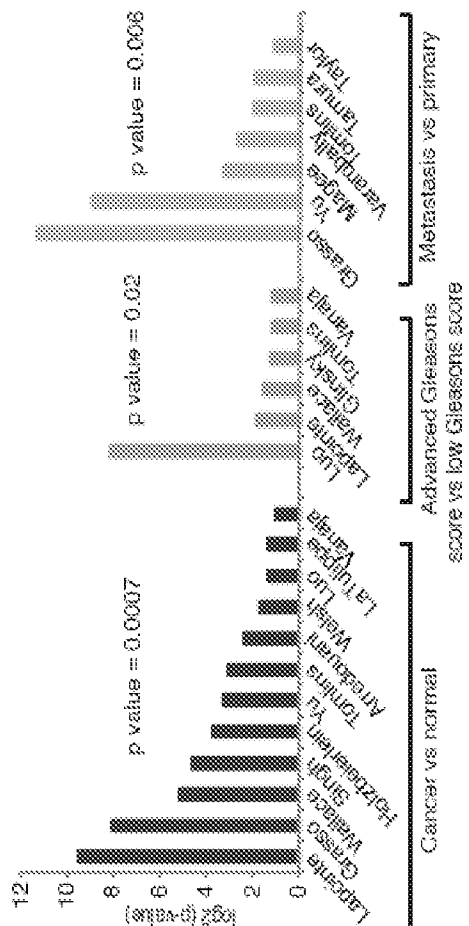
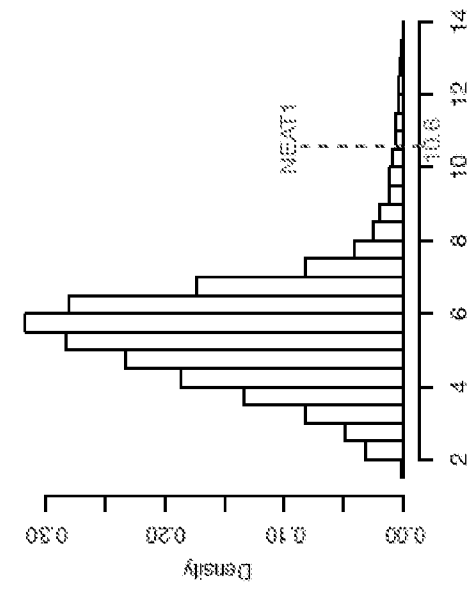


Figure 2a-2d

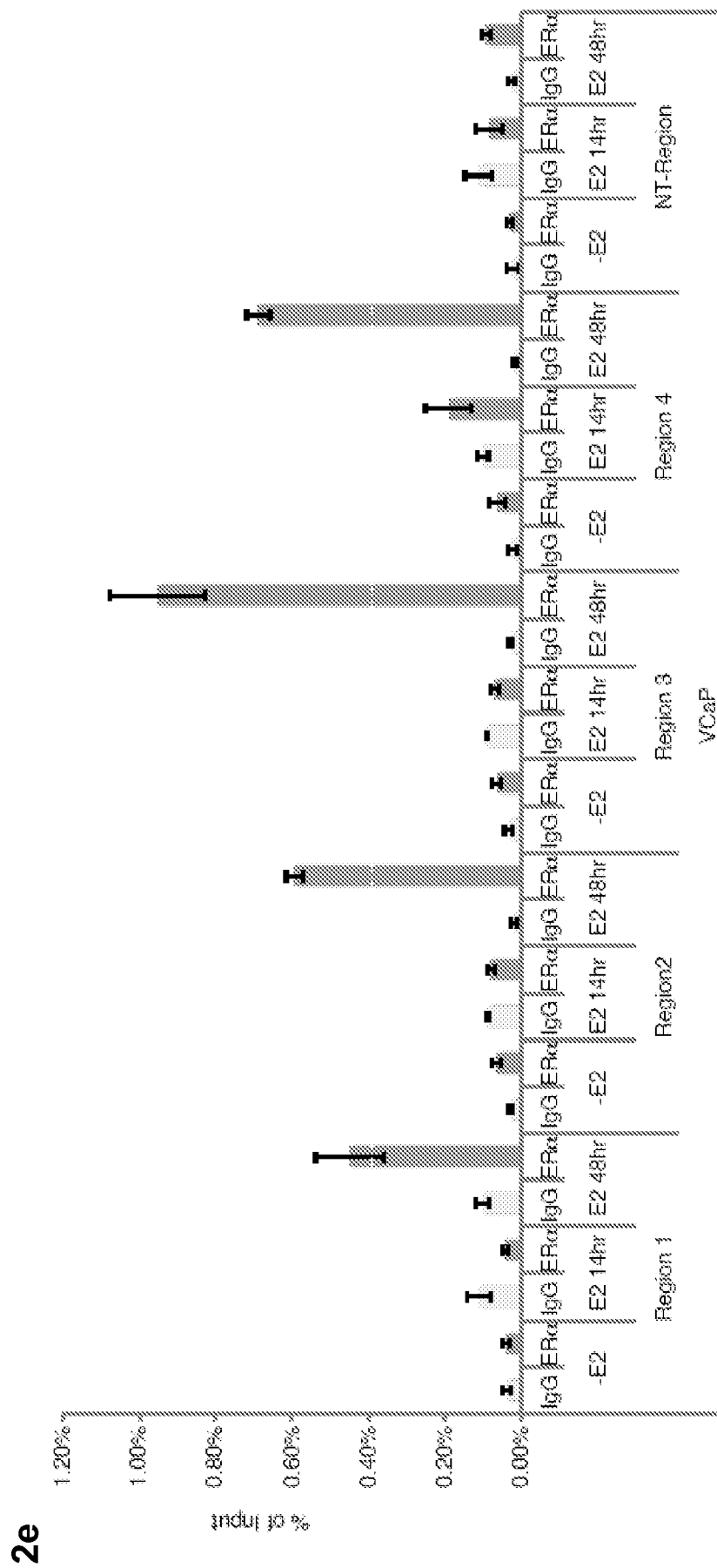


Figure 2e

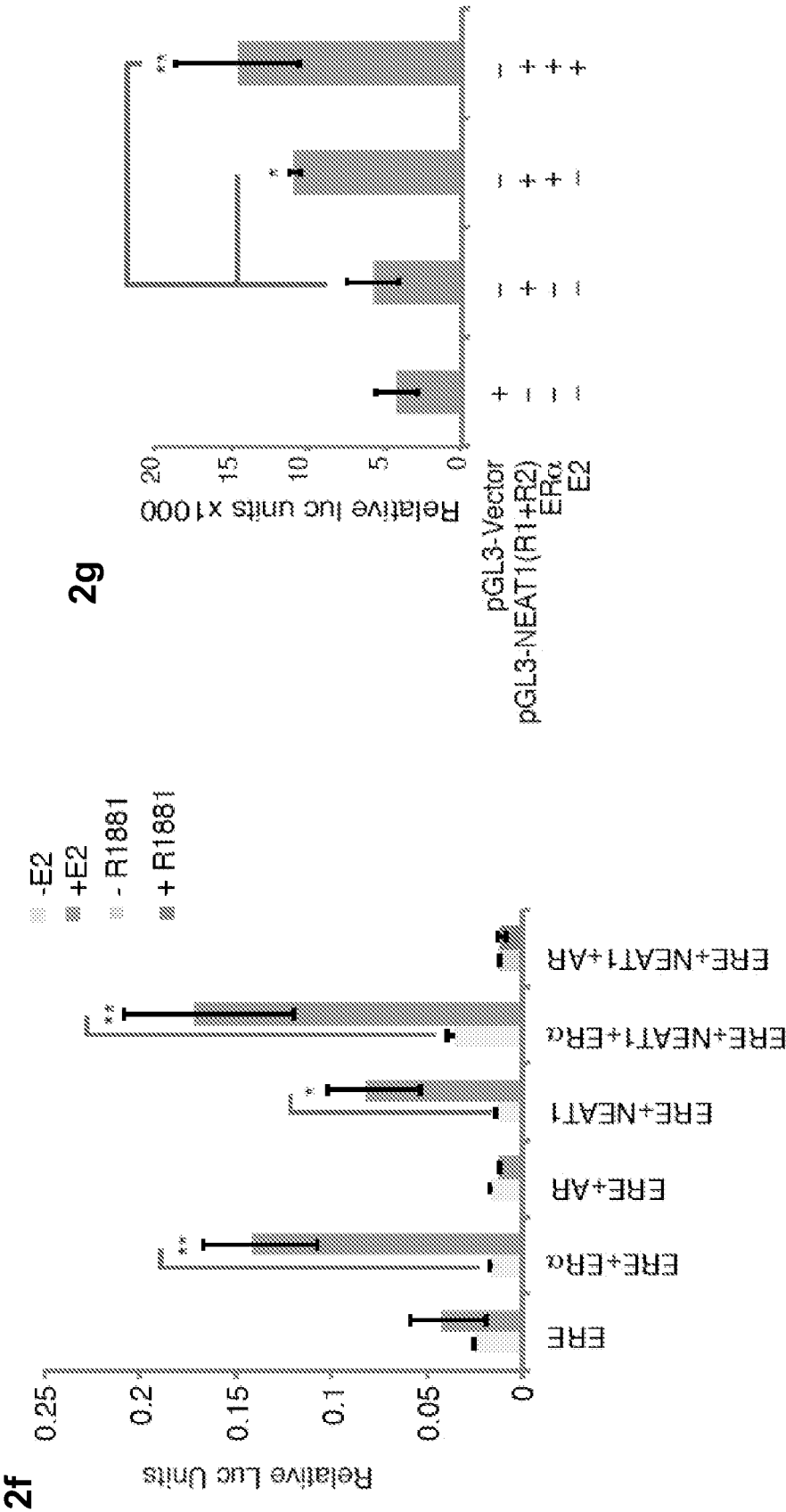


Figure 2f-2g

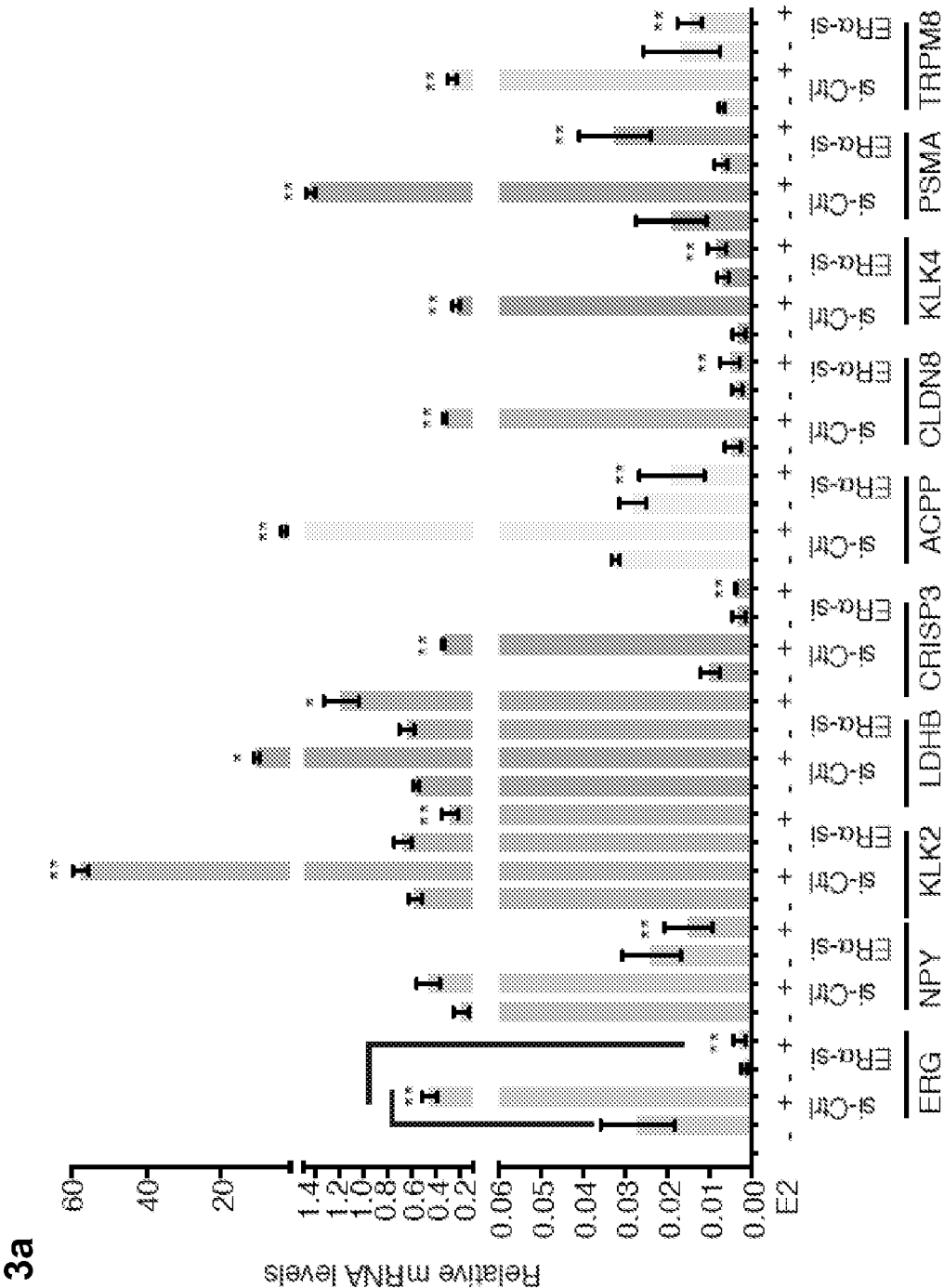


Figure 3a

3b

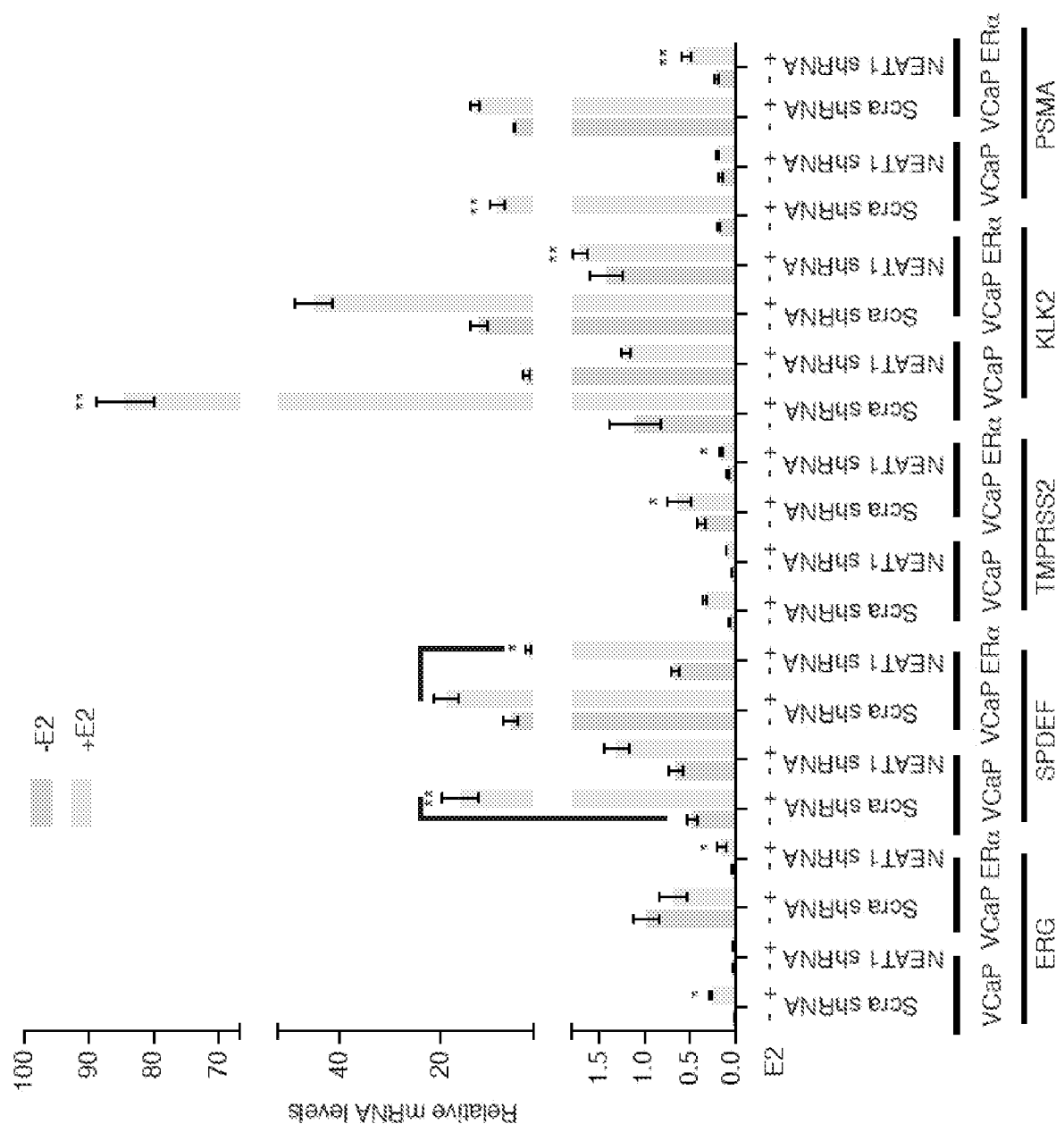


Figure 3b

4a

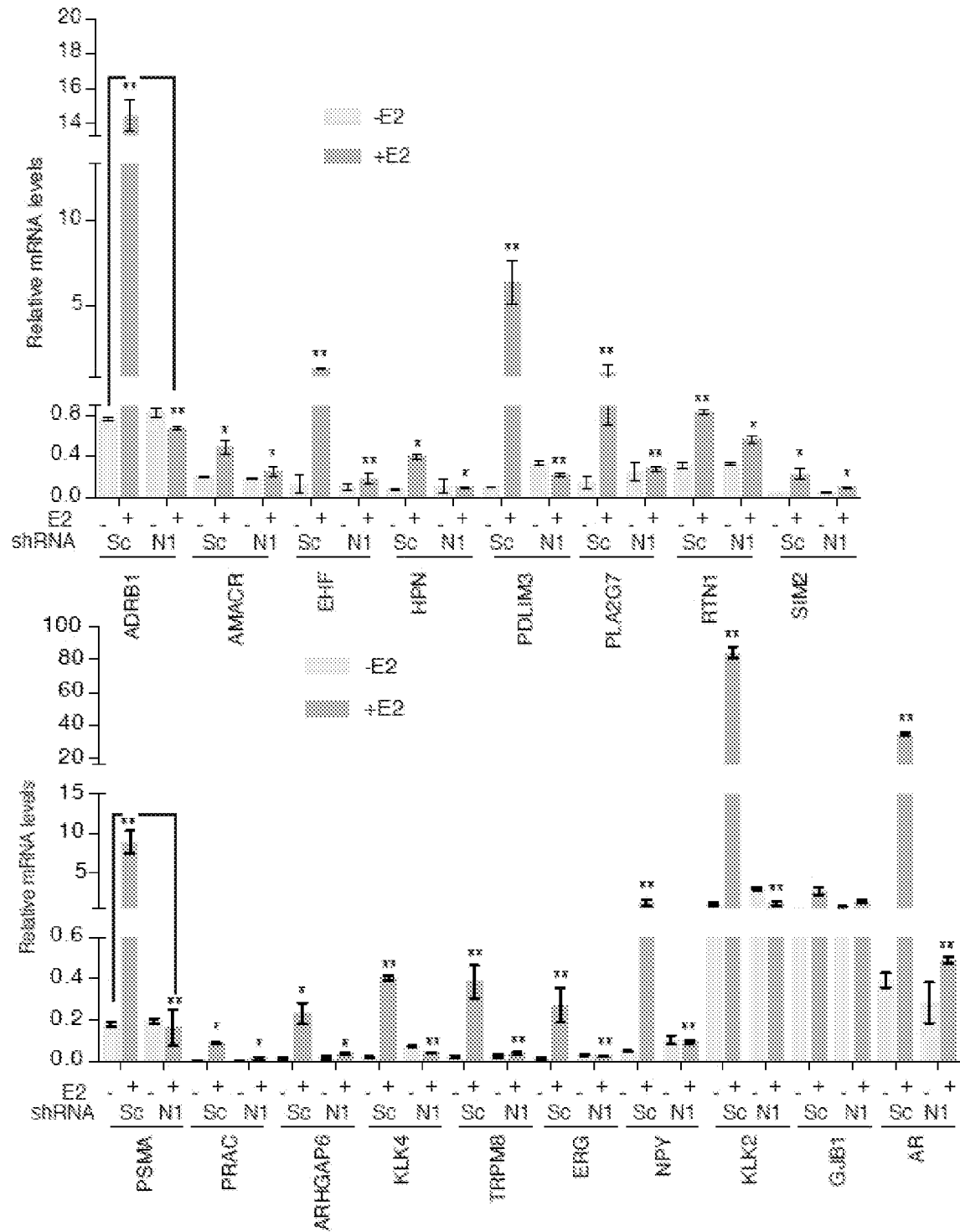


Figure 4a

4b

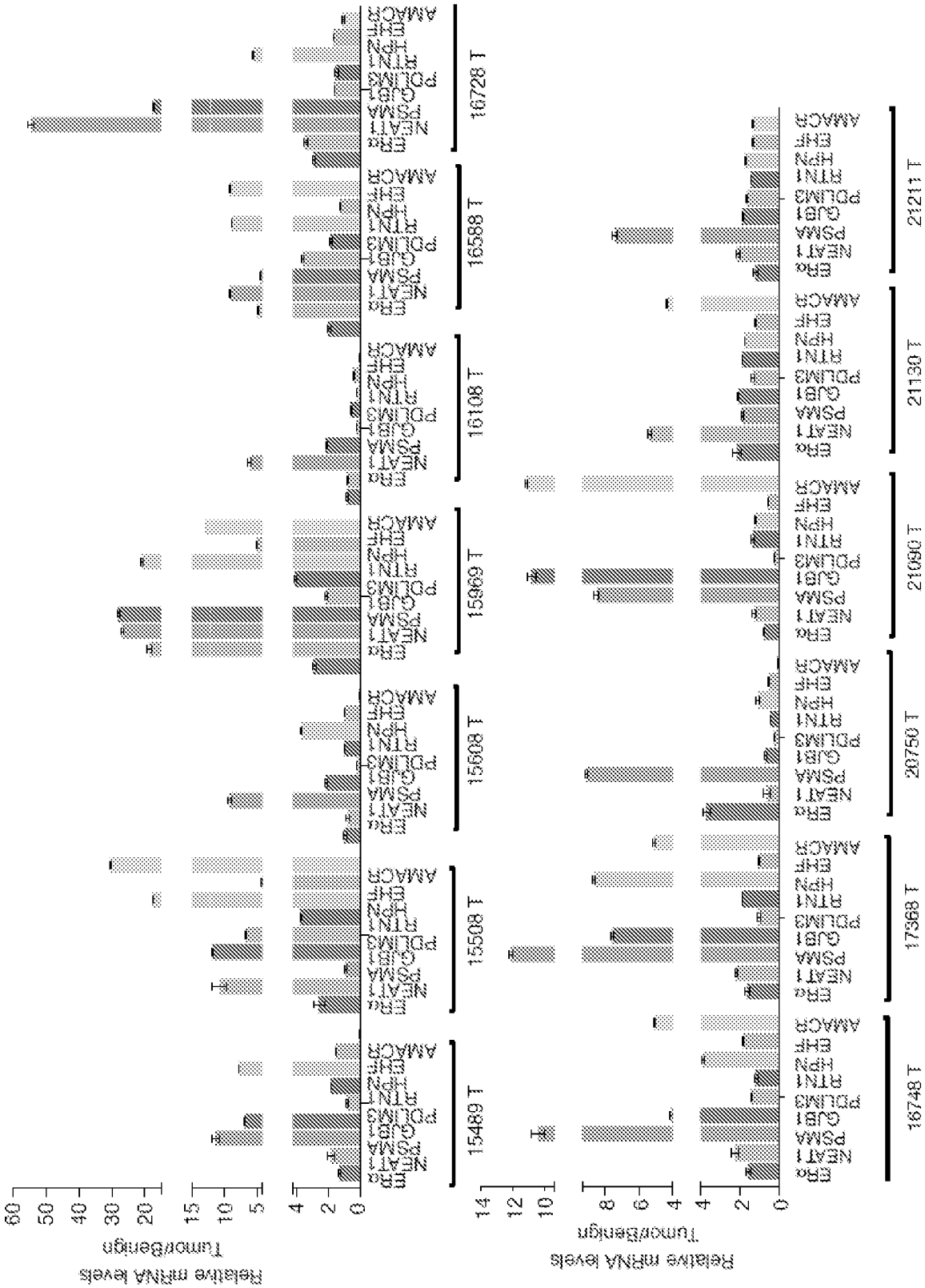


Figure 4b

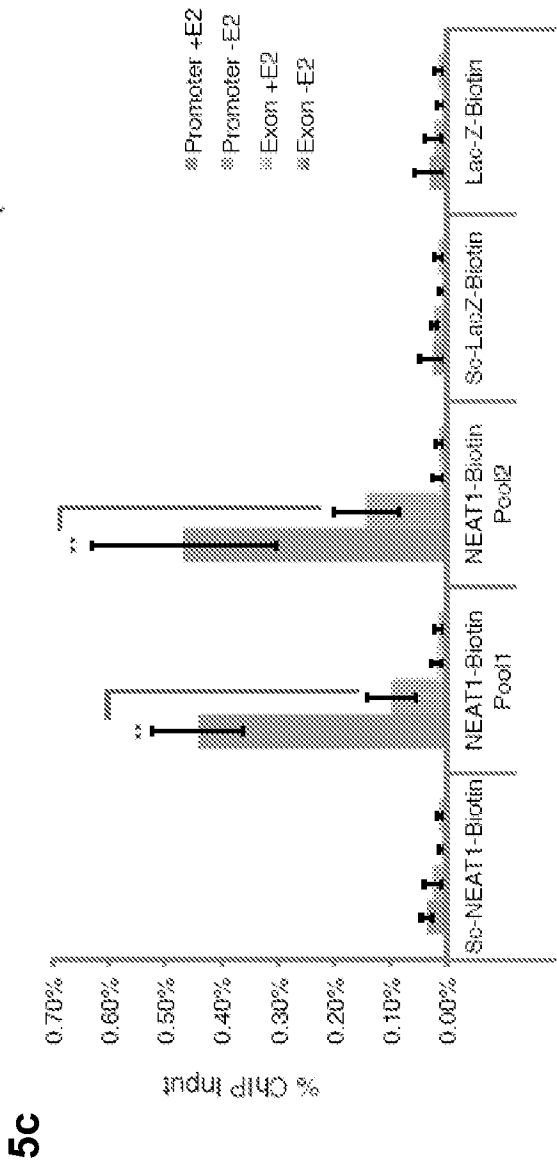
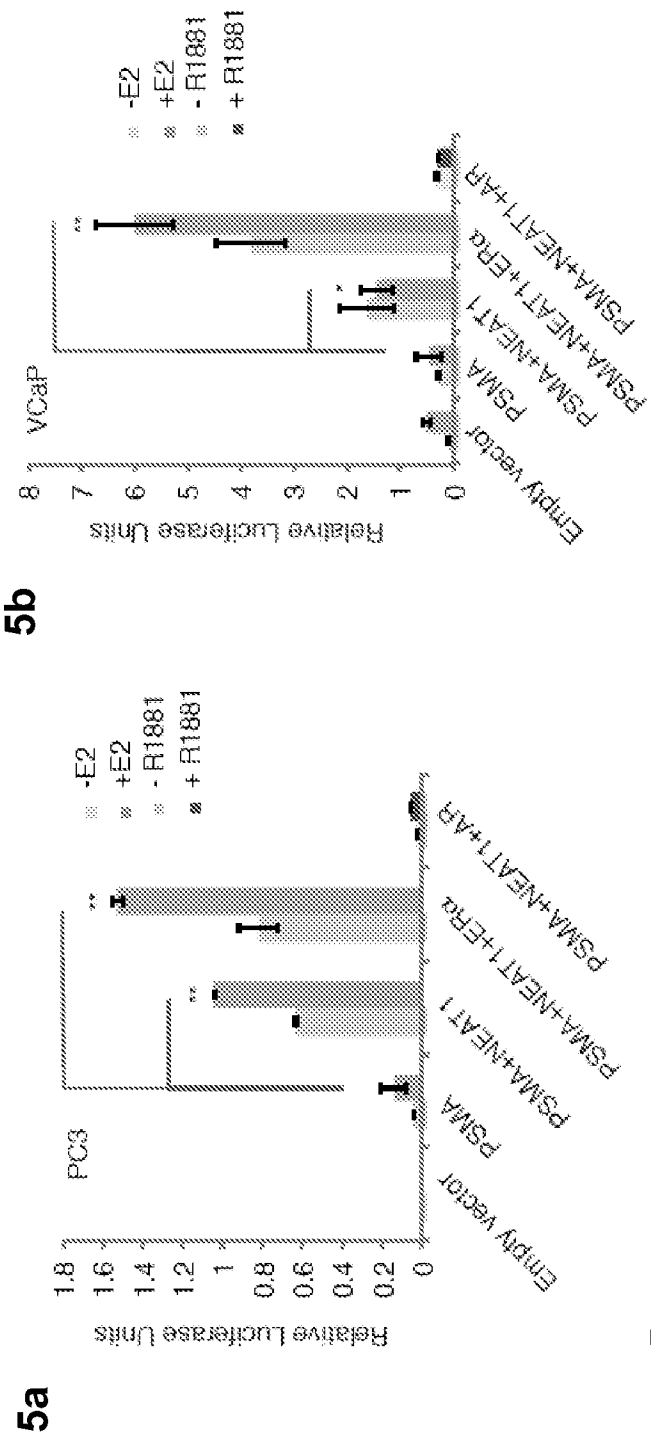


Figure 5a-5c



Figure 5d

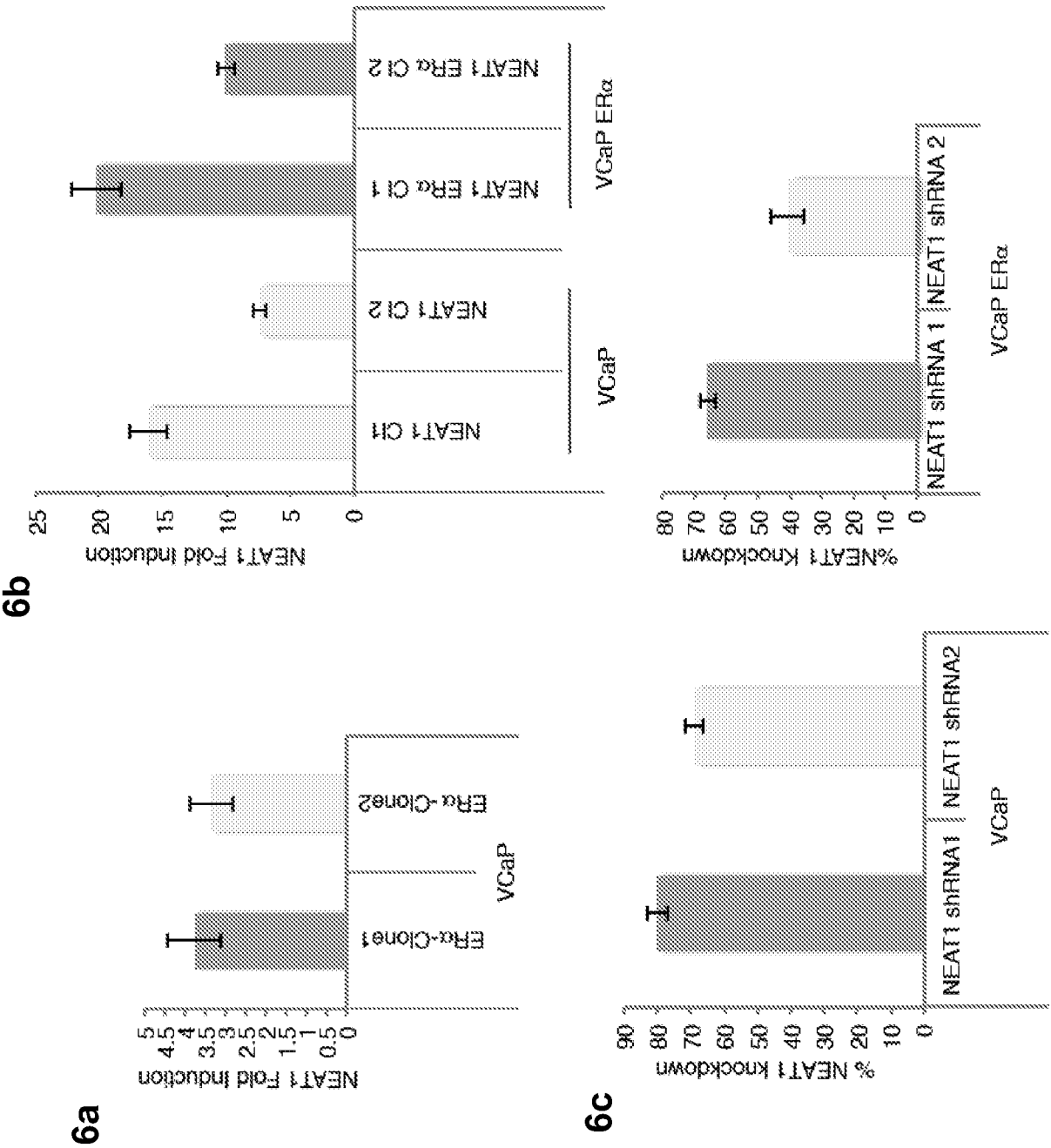


Figure 6a-6c

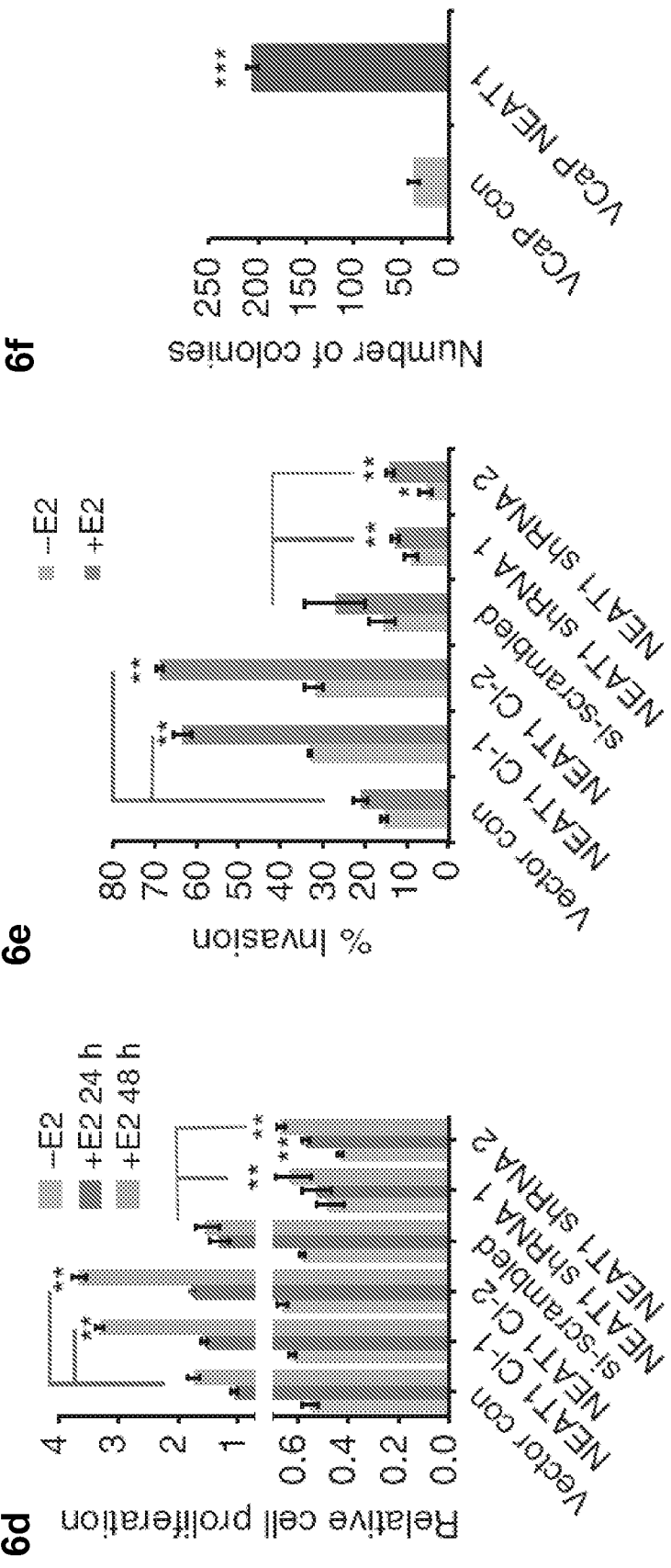


Figure 6d-6f

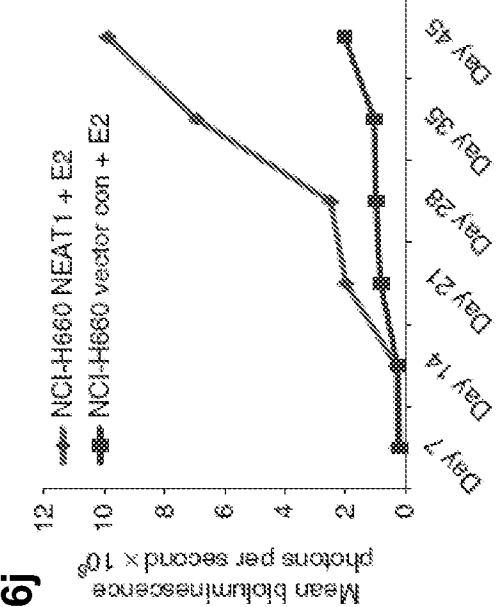
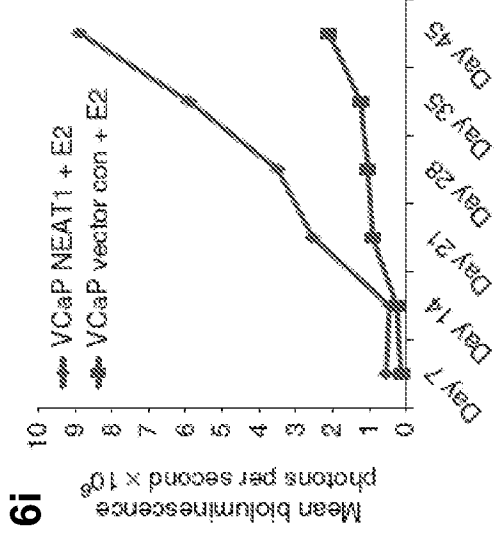
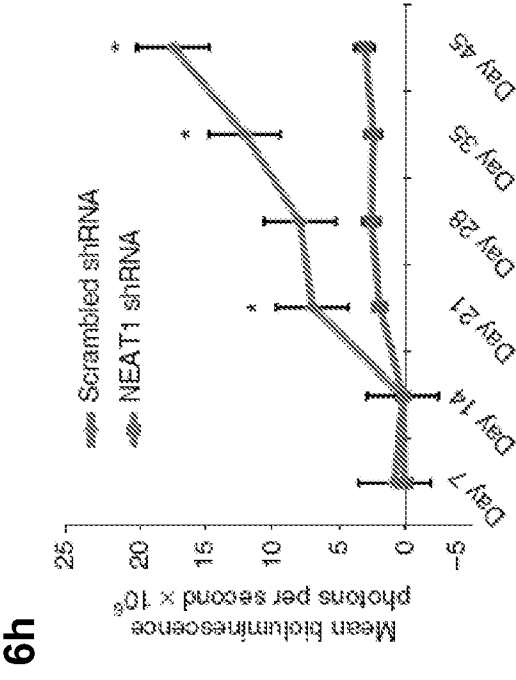
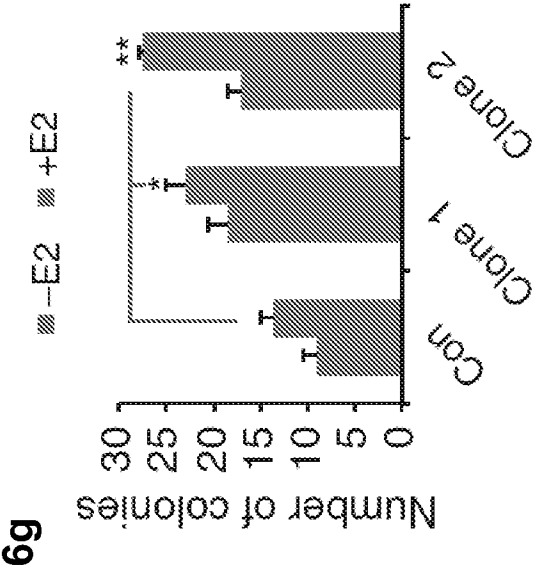


Figure 6g-6j

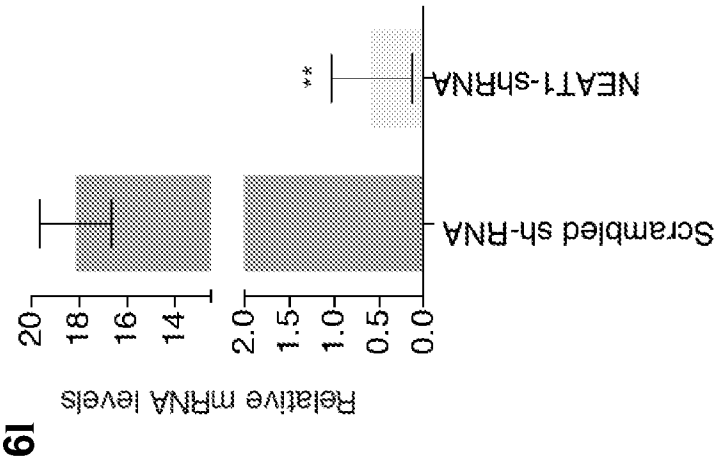
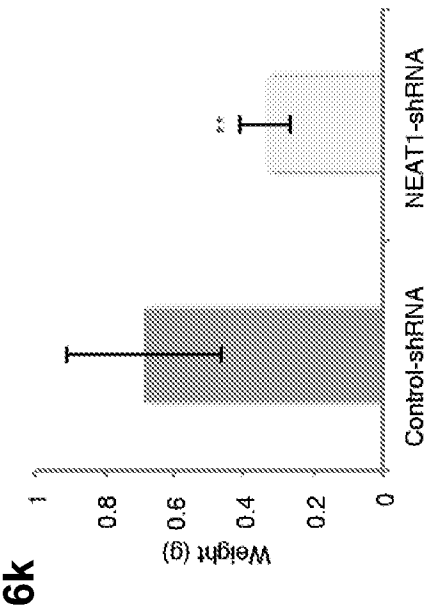
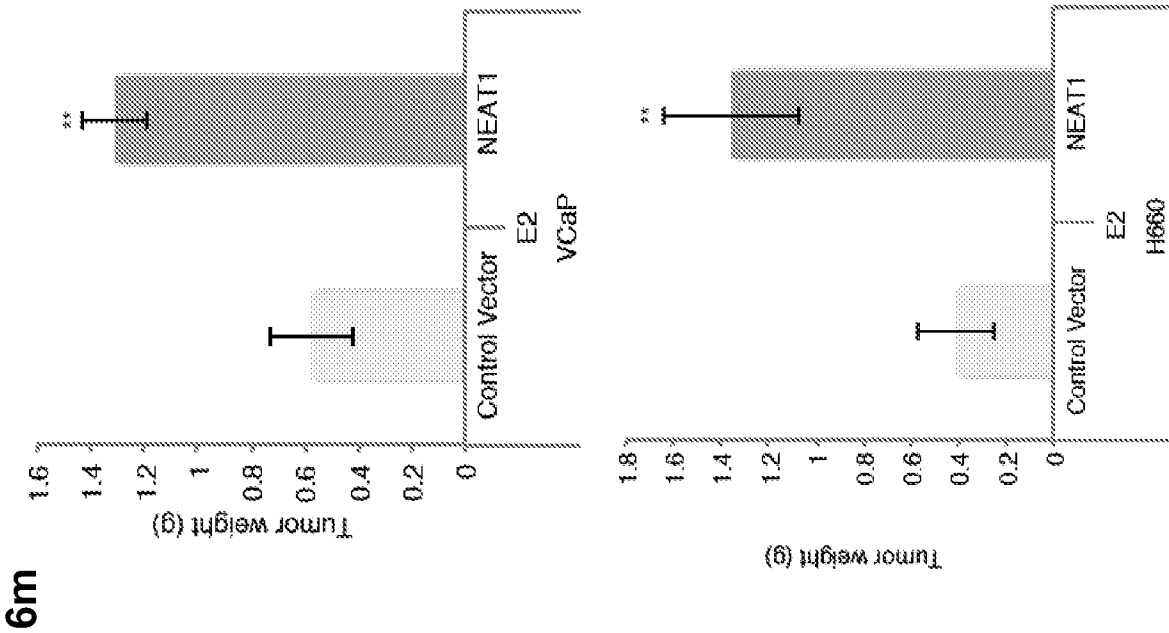


Figure 6k-6m

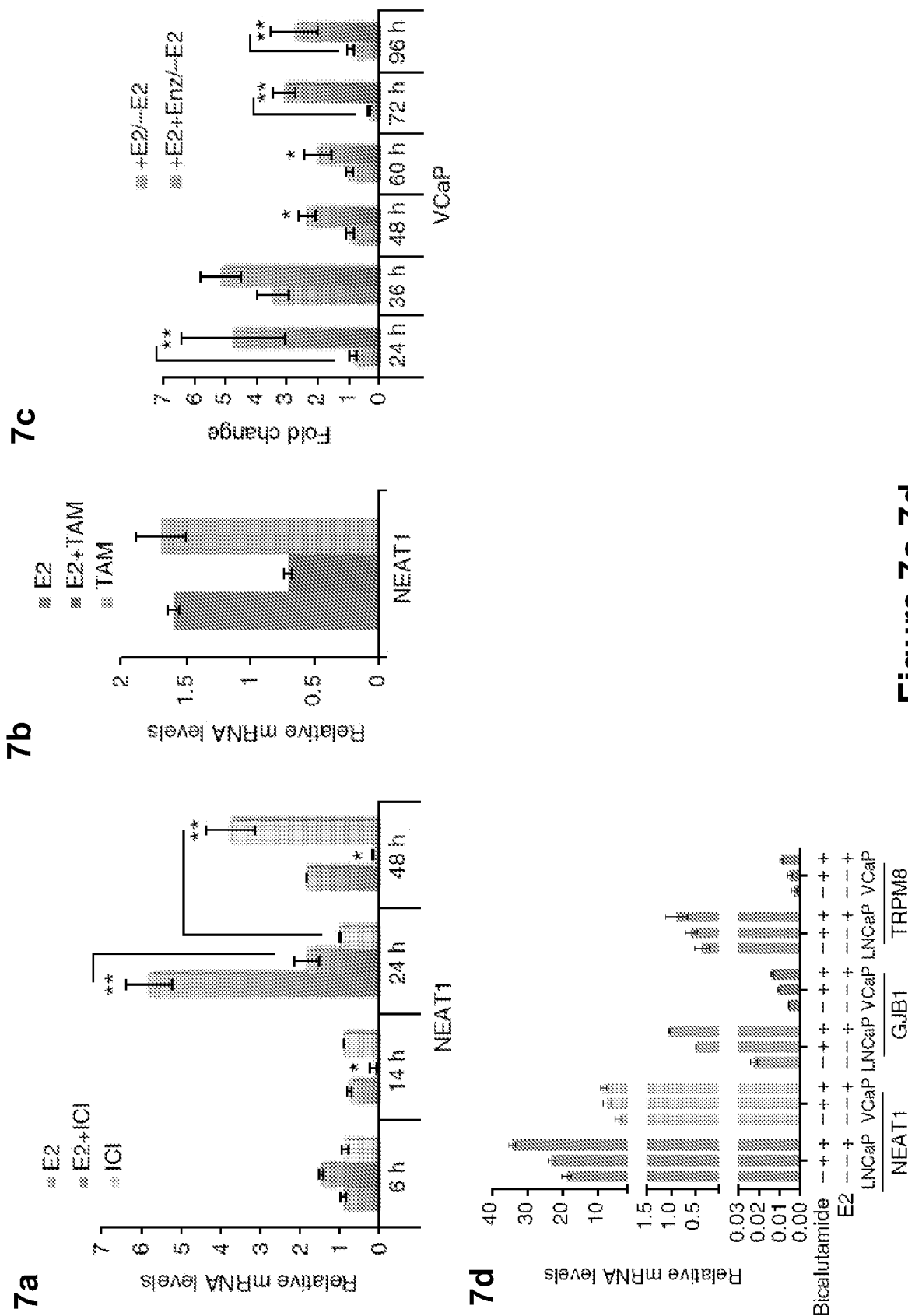


Figure 7a-7d

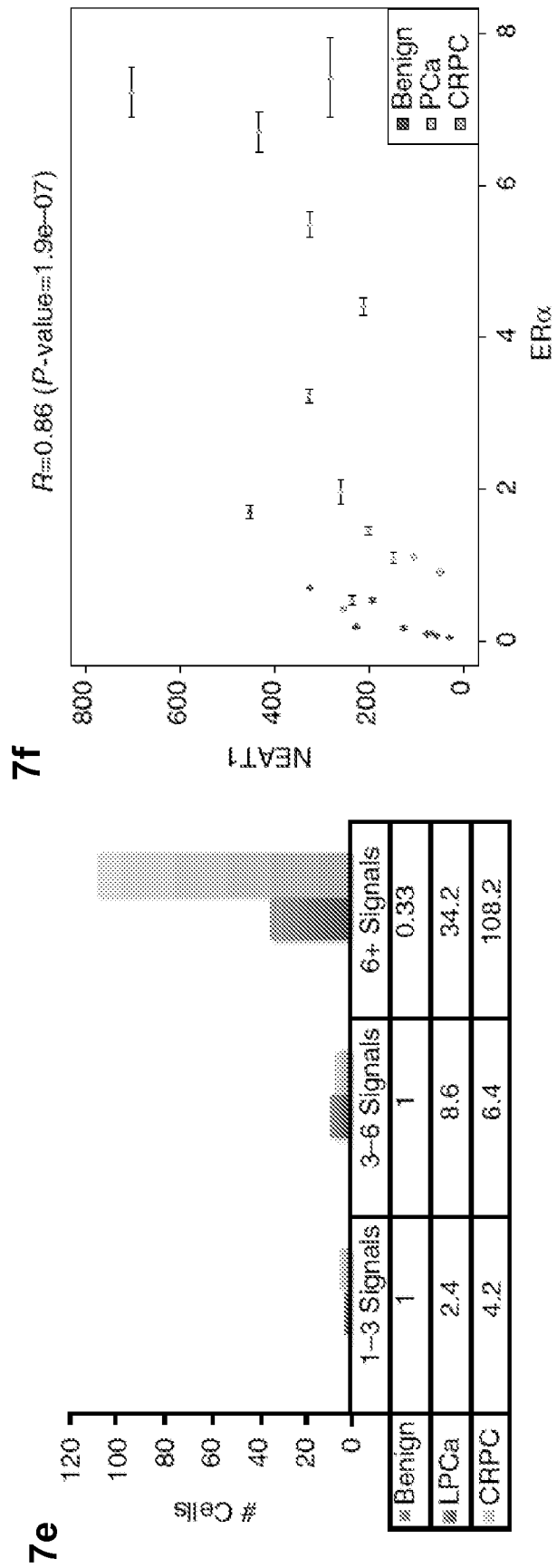


Figure 7e-7f

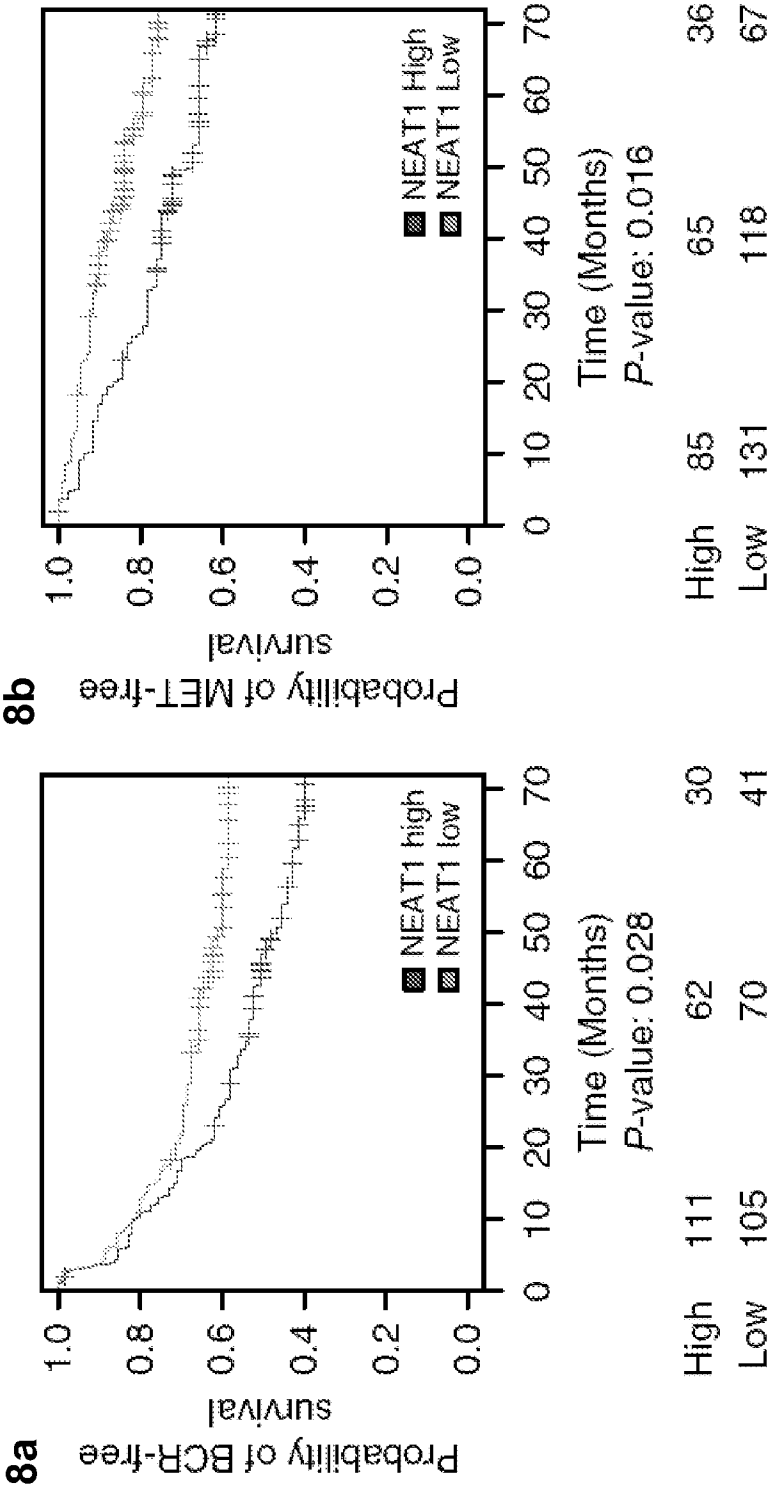
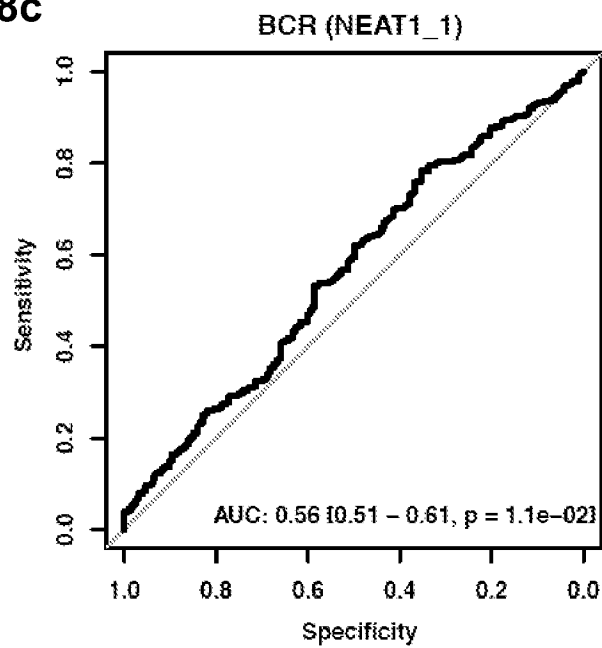
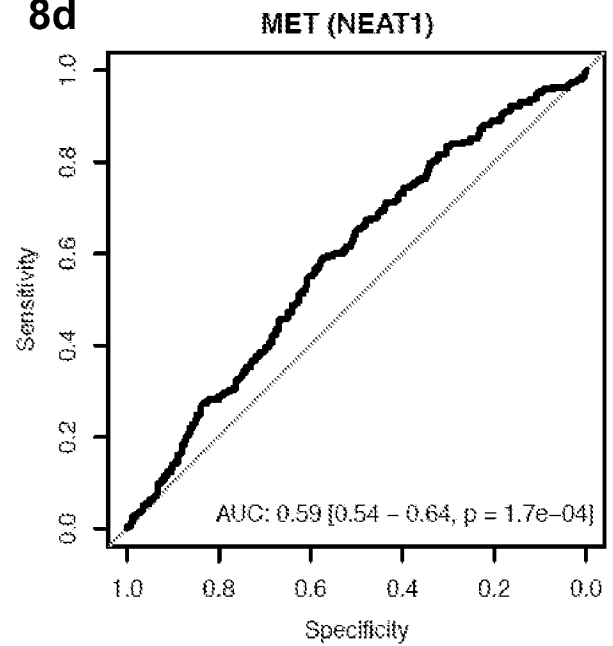
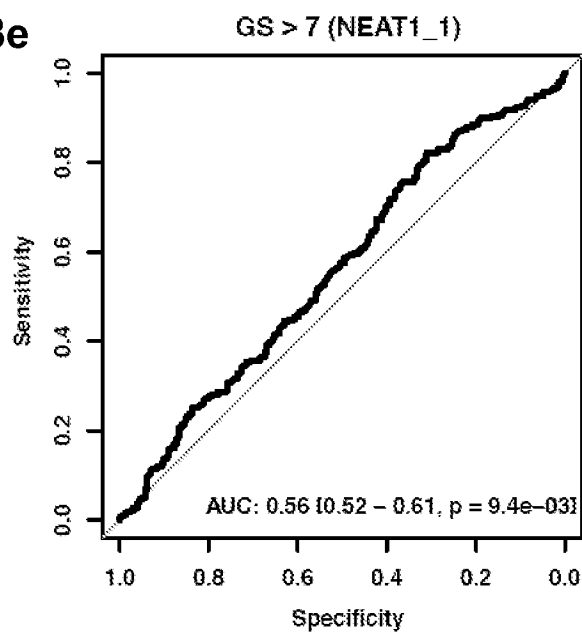
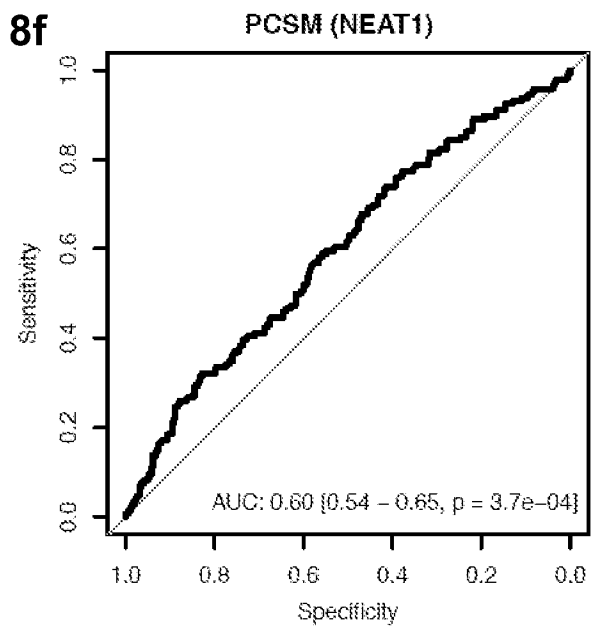


Figure 8a-8b

8c**8d****8e****8f****Figure 8c-8f**

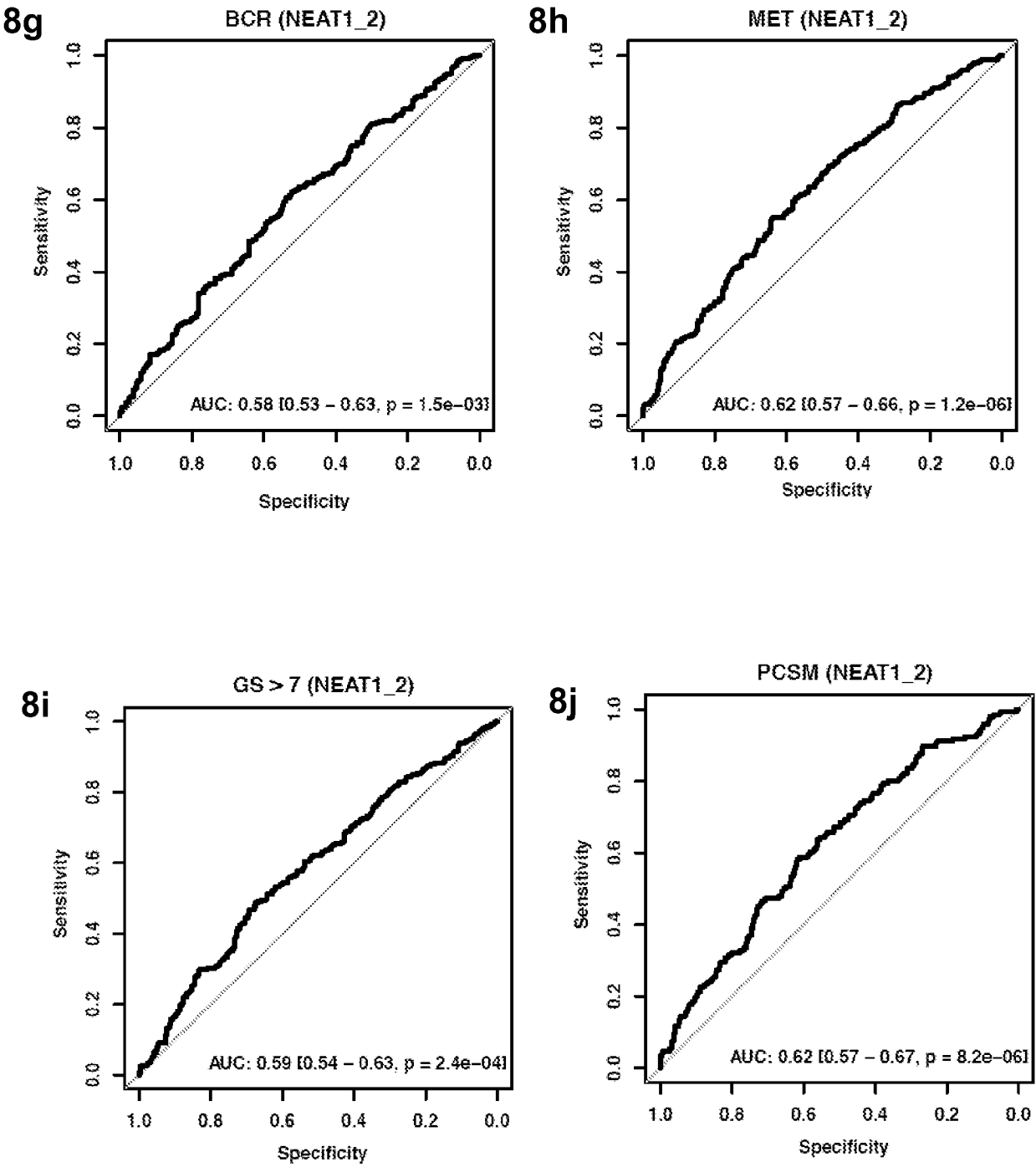
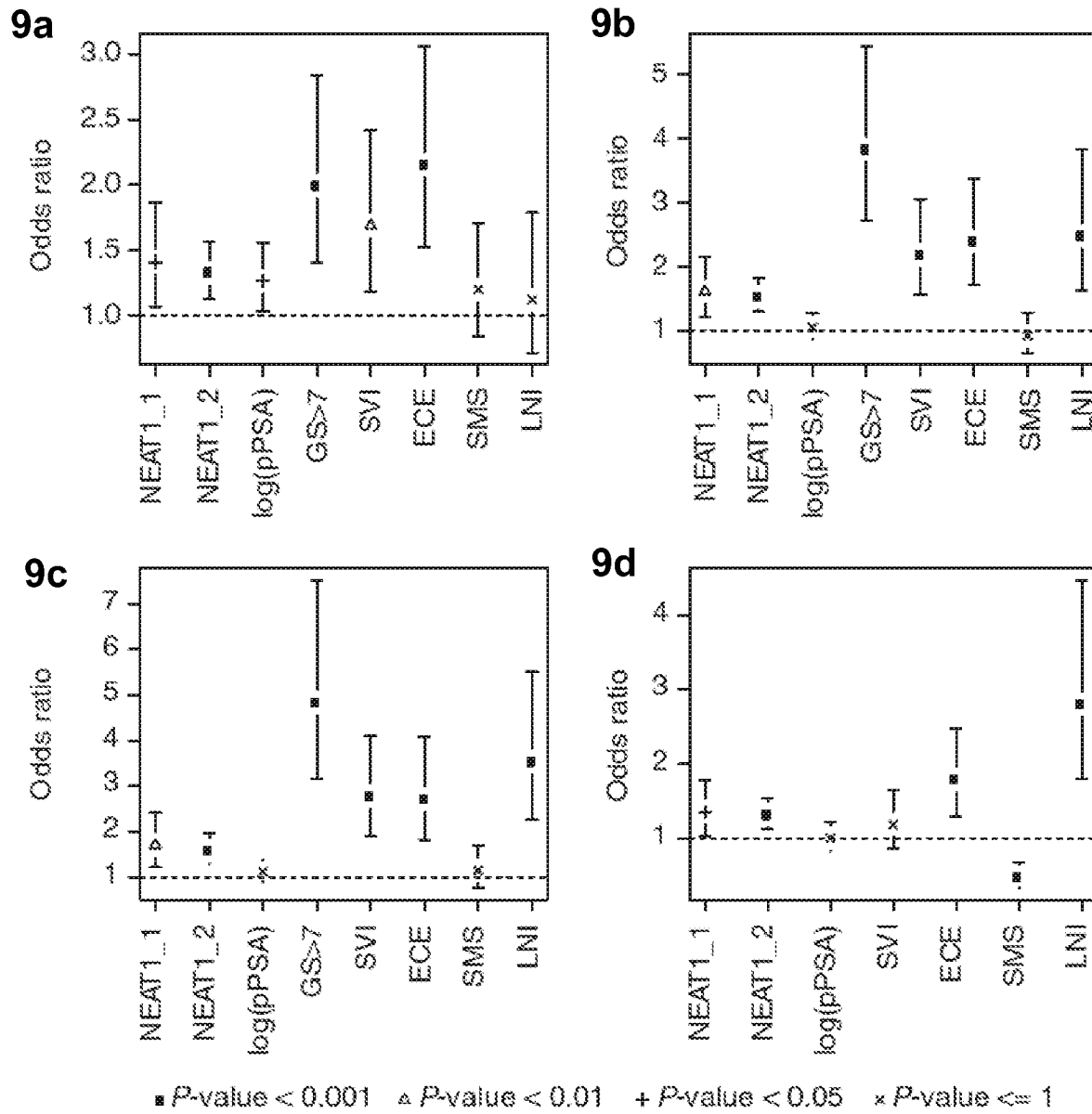


Figure 8g-8j

**Figure 9a-9d**

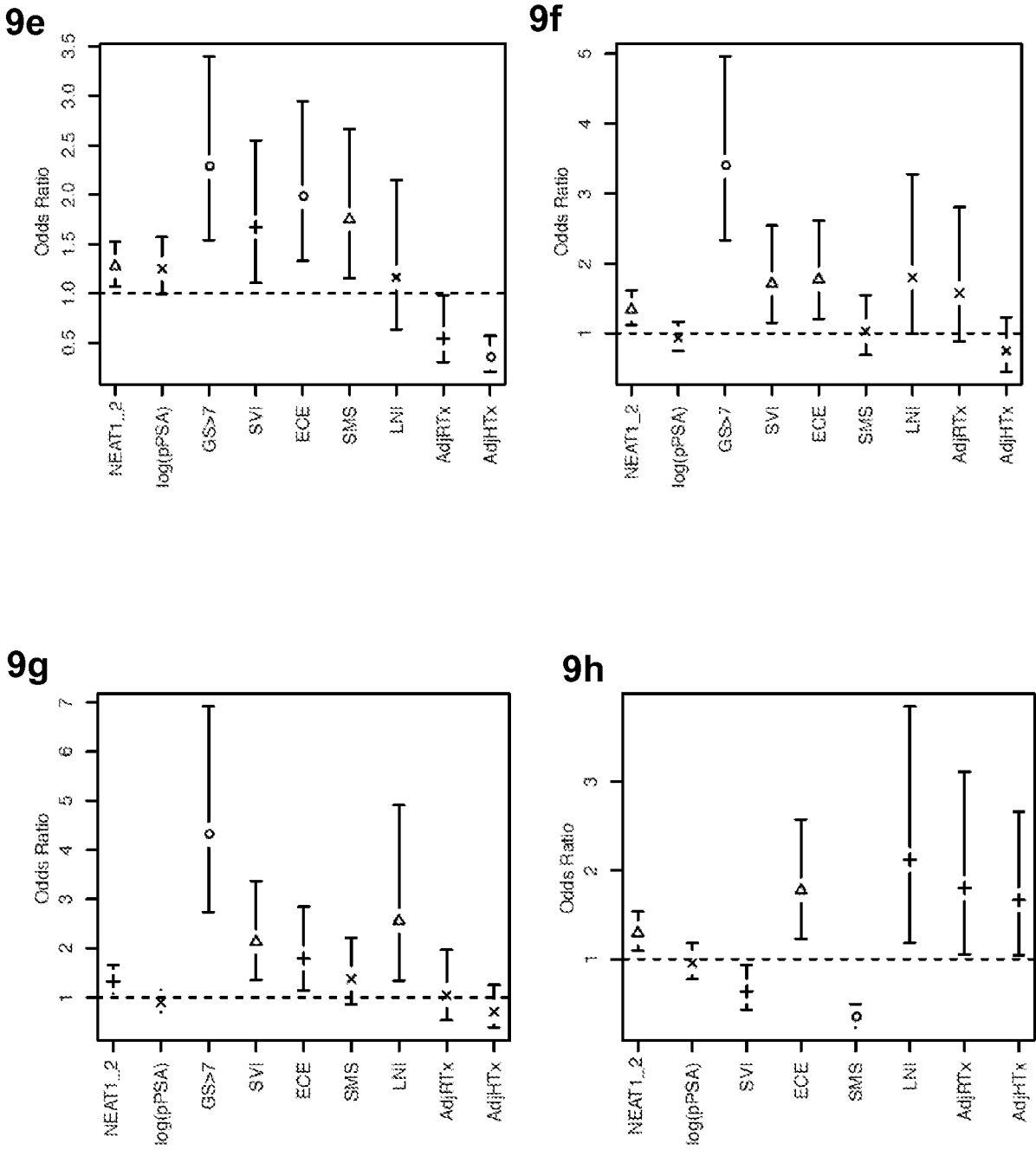
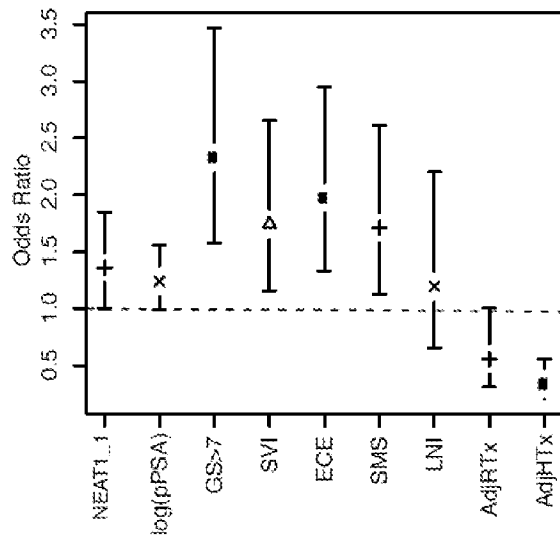
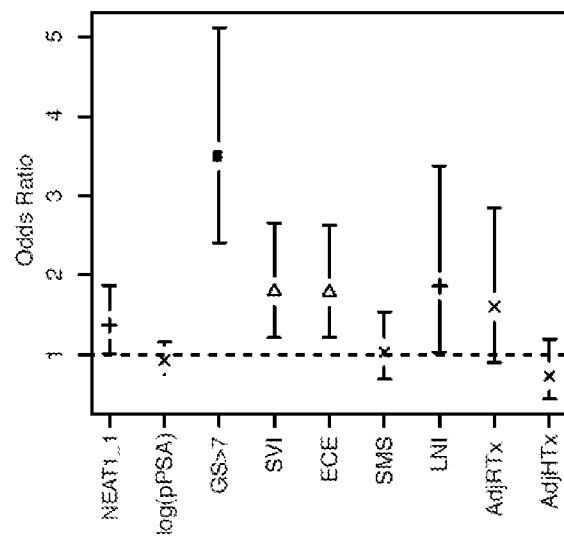


Figure 9e-9h

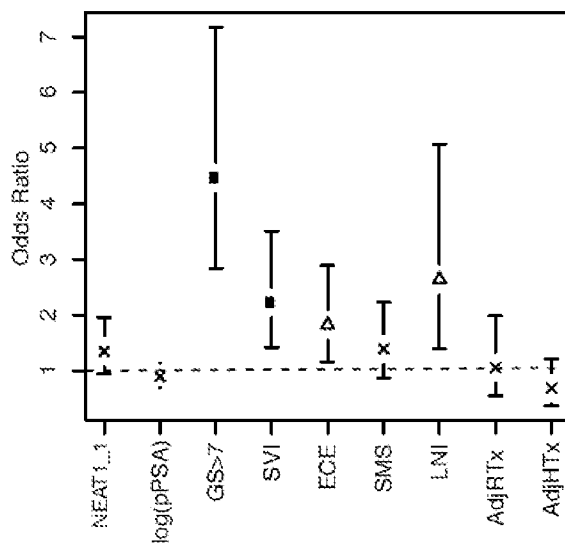
9i



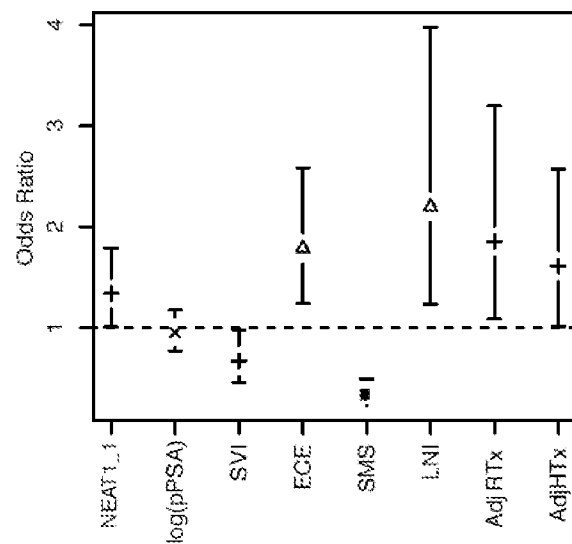
9j



9k



9l



■ p-value < 0.001 △ p-value < 0.01 + p-value < 0.05 × p-value < 0.1

Figure 9i-9l

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/26359

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68; C12N 15/11; A61K 48/00; C07H 21/04 (2015.01)

CPC - C12Q 1/6883; C12Q 1/6844; C12Q 1/6886; A61K 48/00; C12N 15/113; C12Q 1/6876

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12Q 1/68; C12N 15/11; A61K 48/00; C07H 21/04 (2015.01); USPC - 435/6.11,6.12,6.14; 514/44A,44R; 536/24.5,24.33

CPC - C12Q 1/6883; C12Q 1/6844; C12Q 1/6886; A61K 48/00; C12N 15/113; C12Q 1/6876

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

IPC(8) - C12Q 1/68; C12N 15/11; A61K 48/00; C07H 21/04 (2015.01); USPC - 435/6.11,6.12,6.14; 514/44A,44R; 536/24.5,24.33

CPC - C12Q 1/6883; C12Q 1/6844; C12Q 1/6886; A61K 48/00; C12N 15/113; C12Q 1/6876 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline; Google: long non-coding RNAs, lncRNAs, NEAT1, nuclear enriched abundant transcript 1, nuclear paraspeckle assembly transcript 1, MEN1, long non-coding RNA, lncRNA, ncRNA, prostate cancer, risk, advanced, primer, primer pair, probe, RT-PCR, northern blot, RNA ISH, in situ Hybridization, sequencing,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0058130 A1 (Donald) 08 March 2012 (08.03.2012), Abstract, para [0008], [0035], [0036], [0037], [0058], [0071], [0072], [0076], [0107], [0127], [0136], [0216], [0333], [0354], [0358], [0422], [0457], and pg 40, under para [0425], Table 4	1-8
Y	GIBB et al., Human Cancer Long Non-Coding RNA Transcriptomes. PLoS One. 2011, Vol. 6(10):e25915. PDF file: pg 1-10. Abstract; pg 1, col 2, middle para; pg 2, col 1, top para and para 3, col 2, para 2, and Fig 1; pg 5, Fig 4; and pg 6, Fig 5	1-8
Y	SOUQUERE et al., Highly Ordered Spatial Organization of the Structural Long Noncoding NEAT1 RNAs within Paraspeckle Nuclear Bodies. Mol Biol Cell. 2010, Vol. 21(22), p. 4020-7. Abstract; pg 4020, col 1, Footnote, and col 2, middle and last para; pg 4021, col 1, para 4 and 6; pg 4022, col 1, lower para; and pg 4023, Fig 2	7-8
A	US 2013/0225659 A1 (Bennett) 29 August 2013 (29.08.2013), para [0060], [0104], [0153], [0165], and [0169]	1-8
A	US 2013/0303400 A1 (ZEILLINGER et al.) 14 November 2013 (14.11.2013), Abstract, para [0017], [0027], [0041]-[0043], [0047], [0048], and [0063]	1-8
A	MARIN-AGUILERA et al., Identification of Docetaxel Resistance Genes in Castration-Resistant Prostate Cancer. Mol Cancer Ther. 2012, Vol. 11(2), p. 329-39. Abstract; pg 331, col 1, top para; and pg 332, Table 1	1-8



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 June 2015 (26.06.2015)

Date of mailing of the international search report

21 AUG 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/26359

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P.A ✓	NCBI_283131, NEAT1 nuclear paraspeckle assembly transcript 1 (non-protein coding) [Homo sapiens (human)] Gene ID: 283131, updated: 31 May 2015, [online]. [Retrieved on 2015.06.09]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/gene/283131 > PDF file: pg 1-6. pg 4, Other Names	1-8
P.A ✓	CHAKRAVARTY et al., The oestrogen receptor alpha-regulated lncRNA NEAT1 is a critical modulator of prostate cancer. Nat Commun. 2014 Nov, Vol. 5:5383. Entire documentation, especially Abstract	1-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/26359

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-8, directed to a method of determining the presence or risk of developing or advancing prostate cancer in a subject, comprising detecting the level of Nuclear Enriched Abundant Transcript 1 (NEAT1) in a biological sample from the subject.

Group II: Claims 9-17, directed to a method of treating prostate cancer in a subject, comprising administering to the subject an interfering RNA molecule targeting NEAT1.

*****Continued in the extra sheet*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-8

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/26359

Continuation of:
Box No III (unity of invention is lacking)

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature

Group I includes the special technical feature of detecting the level of NEAT1 in a biological sample, not required by Group II.

Group II includes the special technical feature of administering to a subject an interfering RNA molecule targeting NEAT1, not required by Group I.

Common Technical Features

The inventions of Groups I-II share the technical feature of the association of NEAT1 with prostate cancer in a subject [Group I: detecting the level of NEAT1 in a biological sample from the subject; Group II: administering to the subject an interfering RNA molecule targeting NEAT1]

However, this shared technical feature does not represent a contribution over prior art as being anticipated by an article entitled 'Human Cancer Long Non-Coding RNA Transcriptomes' by GIBB et al. (hereinafter 'Gibb'; PLoS One. 2011, Vol. 6(10):e25915. PDF file: pg 1-10) as follows:

Gibb discloses an elevated expression level of NEAT1 is associated with prostate cancer in a subject (pg 6, Fig 5. Legend - 'Expression patterns of lncRNAs in human cancers. (A) lncRNAs with the highest overall expression'; Fig 5A, wherein NEAT1 has an elevated expression level of '1375' in prostate cancer in comparison with the highest expression level of '248' in the normal prostate tissue shown in Fig 4; pg 5, Fig 4, Legend - 'Expression patterns of lncRNAs in normal human tissues. (A) lncRNAs with the highest overall expression'; Fig 4A, wherein NEAT1 showing an expression level of '248' in normal prostate tissue; pg 2, col 2, para 2 - 'nuclear paraspeckle assembly transcript 1 (NEAT1)', which is 'Nuclear Enriched Abundant Transcript 1 (NEAT1)'; Abstract; Please see NCBI_283131, NEAT1 nuclear paraspeckle assembly transcript 1 (non-protein coding) [Homo sapiens (human)] Gene ID: 283131, updated: 31 May 2015, Retrieved from the Internet: <URL: <http://www.ncbi.nlm.nih.gov/gene/283131>> PDF file: pg 1-6. pg 4, Other Names - 'nuclear enriched abundant transcript 1'; Please also see Specification: para [00108] - 'Nuclear Enriched Abundant Transcript 1 (NEAT1)...NEAT1 lncRNA is essential for the formation of subnuclear bodies called paraspeckles').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note:

Claim 1 is objected as lacking a definition for the first appeared abbreviation "NEAT1" limitation in claims. For the purposes of this ISR, "NEAT1" in claim 1 is rewritten as "Nuclear Enriched Abundant Transcript 1 (NEAT1)" according to the Specification (para [00108]).