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NOVEL MECHANISMS OF β-ADRENERGIC SIGNALING IN PROSTATE CANCER PROGRESSION

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A

DISSERTATION

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DOCTOR OF PHILOSOPHY

by

MOHIT HULSURKAR, MS

Houston, Texas

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Dedication

To my parents, Manjusha and Madan Hulsurkar;

My sister Rashmi and my wife Shweta…

This journey would be incomplete without your love…

Acknowledgements

I am fortunate to have received help and support when I stumbled, guidance when I was lost and inspiration when I failed. I am grateful to everyone in my life for this, and I can only make a humble attempt to acknowledge all of you here.

I would like to begin by thanking my guide and advisor Dr. Wenliang Li. You showed faith in me and offered an opportunity to be your student. You always challenged me to perform better, to aim higher and to strive for perfection. At the same time, you supported me when I failed. Your guidance helped me overcome my failures and achieve my goals. You trained me to be able to think on my own from very early days of my tenure, and encouraged me to be an independent thinker. In spite of being a student, you gave me an opportunity to lead teams and coordinate the lab function. It has not only prepared me for future responsibilities but helped me to grow as a better person. I am immensely grateful to you for that. It is with these qualities that I am confident of succeeding in future, however, it is going to be challenging without the security of your support.

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I am thankful to my parents for allowing me to follow my dreams. Mom and dad, you taught me to give my best in everything I did. Your trust in me has kept me going even during tough times. Thank you, Rashmi for making me smile and keeping me grounded. During the course of my graduate studies, my wife Shweta has shared every moment with me. Thank you, for always being by my side and holding me in my joy and depression, love and frustration. Your belief in me means the world to me and will keep me motivated. Without all of your love, this entire struggle would have been futile, thank you for being with me.

NOVEL MECHANISMS OF β-ADRENERGIC SIGNLING IN PROSTATE CANCER PROGRESSION

By

Mohit Hulsurkar, MS

Advisory Professor: Wenliang Li, Ph.D.

Prostate cancer is the second leading cause of cancer death among American men. The American Cancer Society estimates that 180,890 men will be will be diagnosed with prostate cancer in 2016 in the USA. (http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-key-statistics). Androgen deprivation therapy (ADT) is the standard treatment for early stage prostate cancer. But most patients relapse with aggressive variants of prostate cancer, with survival time between 1-3 years. In order to develop cure for such aggressive variants of prostate cancer, our present understanding of the mechanisms underlying its progression needs to be advanced.

Recently, it has been found that activation of β-adrenergic signaling pathway leads to aggressive variants of prostate cancer. β-adrenergic signaling involves the activation of β-adrenergic receptors (ADRBs), eventually leading to increased activation of cAMP response element-binding protein (CREB). Downstream targets of CREB activation in neuroendocrine differentiation as well as in neoangiogenesis are largely unknown, indicating that the underlying mechanisms of β-adrenergic signaling in prostate cancer progression are far from completely understood. For instance, while the epigenetic regulation by histone deacetylases 2 (HDAC2) is

necessary for stress to induce cardiac hypertrophy, its mechanism are unknown in cancer progression. Similarly, another regulator of β-adrenergic signaling, GRK3 was recently shown to be a new critical regulator of prostate cancer progression and tumor angiogenesis. However, mechanisms of GRK3 in prostate cancer progression and its regulation by ADRB2 signaling remain unknown.

Our **hypothesis** is that GRK3 and HDAC2 are critical downstream effectors of β-adrenergic signaling-activated CREB in promoting prostate cancer progression. Here, we show that CREB directly activates GRK3 transcription by binding to its promoter and this up-regulation of GRK3 expression by ADRB2/CREB pathway is sufficient as well as necessary to induce the neuroendocrine differentiation of prostate cancer cells. We also show that downstream of chronic stress and ADRB2, CREB binds to HDAC2 promoter and activates its expression. HDAC2 further suppresses the expression of thrombospondin 1 (TSP1) in order to induce angiogenesis, thus acting as a mediator for the β-adrenergic signaling pathway.

Here, we have introduced two new pathways acting downstream of the ADRB2/CREB axis. We show that the CREB/GRK3 axis leads to neuroendocrine prostate cancer progression. We have introduced a new paradigm that β-adrenergic signaling and epigenetic gene expression regulation may be working synergistically resulting in cancer progression.

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Chapter One:

Introduction

Prostate Cancer Progression

Prostate cancer (PCa) is the second most common cancer and second leading cause of cancer related death in American men (http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-key-statistics). The American Cancer Society estimates in 2016, about 180,890 new patients will be detected and about 26,120 deaths from prostate cancer will occur in the US (http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-key-statistics). Worldwide, around 300,000 men died of prostate cancer in 2012 and this number is estimated to be 630,000 in 2035[1]. Prostate-specific antigen (PSA) screening is evolving constantly and is helping in the early detection of prostate cancers. According to a recent study, more than 80% of the patients detected via PSA screening showed non-metastatic, localized prostate cancers[2]. Early detection aids in the treatment of prostate cancer with radical prostactomy, which controls the localized cancer in most men[3]. However, cancer recurs in most of the patients (**Fig. 1.1**) and is associated with rising PSA levels. At this stage of disease, androgen deprivation therapy (ADT) is prescribed with medical or surgical castration being the standard practice. Ever since the dependence of prostate cancer on androgen was demonstrated by Huggins *et al.* in 1941[4-6], ADT by castration is being used to treat prostate cancers. Castration results into low blood testosterone levels (< 50 ng/dL) and leads to cell apoptosis as well as inhibition of prostate cancer progression. However, the disease becomes resistant to ADT and evolves into castration resistant prostate cancer (CRPC).

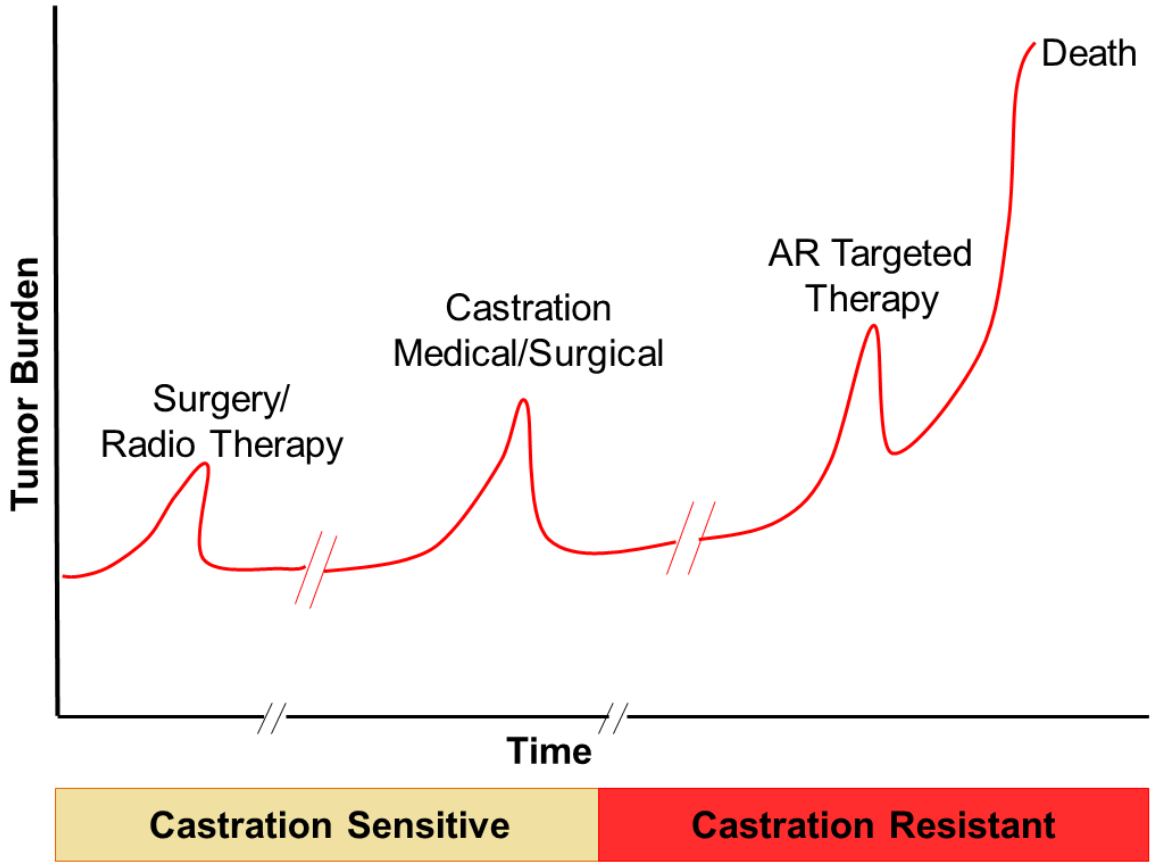

Figure 1.1 Prostate cancer progression with time. A model depicting different stages of prostate cancer in patients with time and available therapy. Developed based on the model from Ramalingam S, Pollak KI, Zullig LL, Harrison MR. What Should We Tell Patients About Physical Activity After a Prostate Cancer Diagnosis? **Oncology (Williston Park).** 2015 Sep;29(9):680-5, 687-8, 694.

Targeting the androgen receptor (AR) is a standard course of action at early stages of CRPC, when the disease is still non-metastatic. However, most patients relapse with aggressive variants of prostate cancer, resistant to currently available therapies and show metastasis into bones, lymph nodes, lungs and liver[7]. At this stage, the prognosis of disease is poor, with survival time between 1-3 years[8-16].

In order to develop cure for such aggressive variants of prostate cancer, our present understanding of the mechanisms underlying its progression needs to be advanced.

Prostate cancer progression and β-adrenergic signaling

It has been found recently that in addition to androgen deprivation therapy and exposure to ionizing radiation, activation of the β-adrenergic signaling pathway also leads to aggressive variants of prostate cancer[17,18]. Interestingly, β-adrenergic signaling has been found to be responsible for the progression of breast[19], colon and pancreatic[20], lung[21], skin[22], ovarian[23] cancers. Furthermore, inhibition of β-adrenergic signaling by β-blockers, a group of drugs used to treat multiple conditions such as hypertension and cardiac arrhythmia, is reported to have better cancer prognosis[24-34]. According to epidemiological studies, cancer patients using β-blockers showed reduced cancer related mortality in prostate[26,33,34], ovarian, and non-small cell lung cancers[25]. In breast cancer patients, it was associated with reduced metastasis, disease free survival, cancer recurrence and mortality[30-32]. Based on these epidemiological studies, phase II clinical trials are underway to study the effects of a β-blocker, propranolol, on the progression of ovarian, cervix, colorectal, and breast cancers (*ClinicalTrials.gov identifiers: NCT01504126, NCT01308944, NCT01902966, NCT00888797, and NCT01847001*). However, no clinical trials are being conducted to study the effects of propranolol in prostate cancer, indicating that studying the mechanisms of β-adrenergic signaling could lead to development of a novel therapy to inhibit prostate cancer progression.

β-adrenergic signaling involves the activation of β-adrenergic receptors (ADRBs) by epinephrine and norepinephrine, catecholamines that mediate body's fight or flight response[35]. Expression of ADRBs is high in the normal prostate, especially ADRB2, which is highly expressed in the luminal cells[36-39]. ADRB2 is predominantly

expressed in the epithelial cells in normal as well as malignant tissues[40,41] and its expression is up-regulated in prostate cancer at mRNA level[40,42] as well as at protein level[40,41,43]. However, expression of ADRB2 was reported to be down-regulated following castration and androgen deprivation[40,44], indicating that the mechanisms involved in ADRB2 signaling need to be studied further to better understand its role in prostate cancer progression.

β-adrenergic signaling and neuroendocrine prostate cancer

Because of its role in transducing the signals of sympathetic nervous system, activation of β-adrenergic receptors is widely considered to be responsible for the onset of neuroendocrine differentiation of prostate adenocarcinoma cells[45-48]. Neuroendocrine prostate cancer (NEPC) is an aggressive variant that causes around 25% of the prostate cancer deaths[49-52]. It is resistant to currently available therapies and the survival period is less than a year[8-16]. NEPC onset is considered as a mechanism by which cancer cells resist the androgen deprivation therapy[53-55] and with the use of more potent androgen deprivation drugs, NEPC incidence is expected to rise[56-59].

β-adrenergic signaling and neoangiogenesis

Dr. Judah Folkman reported his 'tumor angiogenesis hypothesis' in 1971 stating that tumor growth is correlated with neovascularization and without angiogenesis, tumor growth would be restricted to microscopic size[60,61] (**Fig. 1.2**). His pioneering research created interest in studying tumor angiogenesis, lead the eventual discovery of VEGF, originally hypothesized as tumor angiogenesis factor (TAF), and has resulted into development of antiangiogenic drugs[62,63]. Hanahan and Weinberg have reported induction of angiogenesis to be one the six hallmarks of cancer[64,65] (**Fig. 1.3**). It is now well established that the solid tumors need supply of nutrients and oxygen as well as removal of metabolite waste, which is provided by formation of new capillaries.

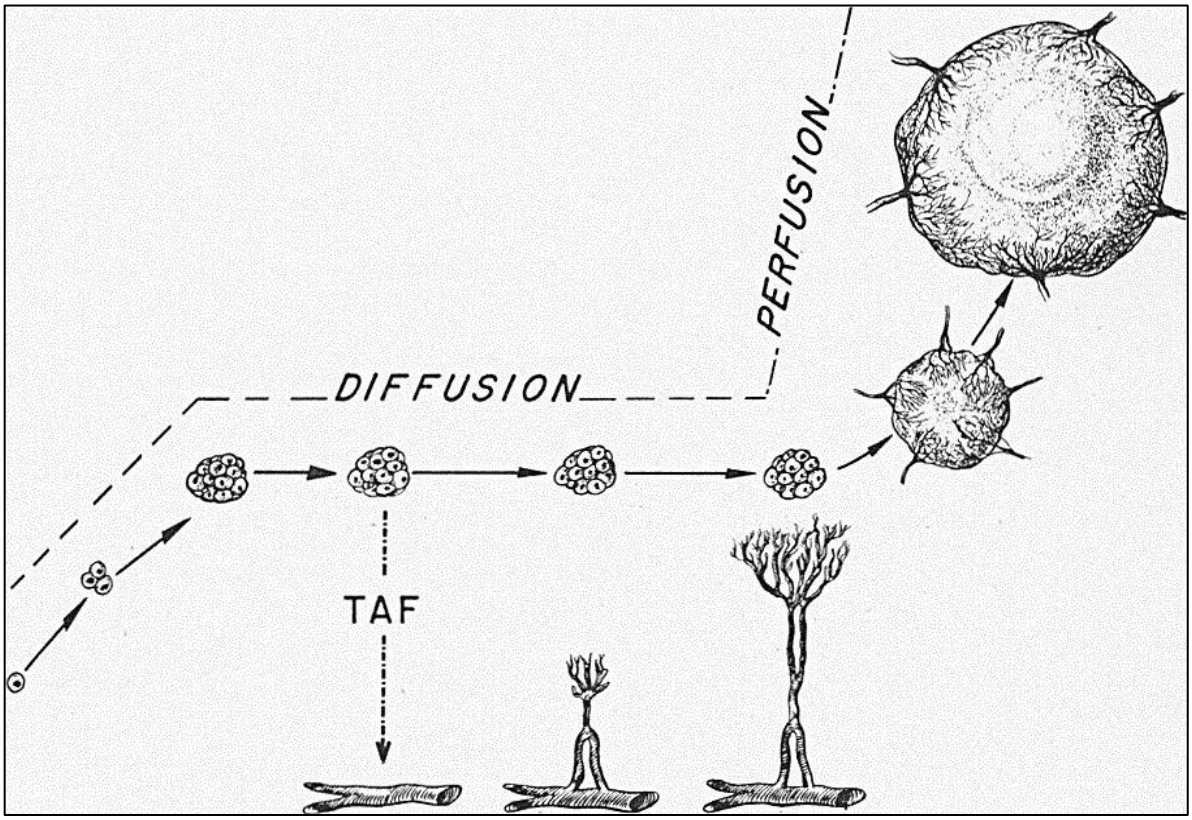

Figure 1.2 Folkman model of tumor angiogenesis. Illustration of the concept that most solid tumors may exist early as tiny cell populations living by simple diffusion in the extracellular space (further growth requires vascularization, and the tumor then maintains itself by perfusion). Tumor-angiogenesis factor (TAF) may be the mediator of neovascularization.

Reproduced with permission from Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971 Nov 18;285(21):1182-6., Copyright Massachusetts Medical Society.

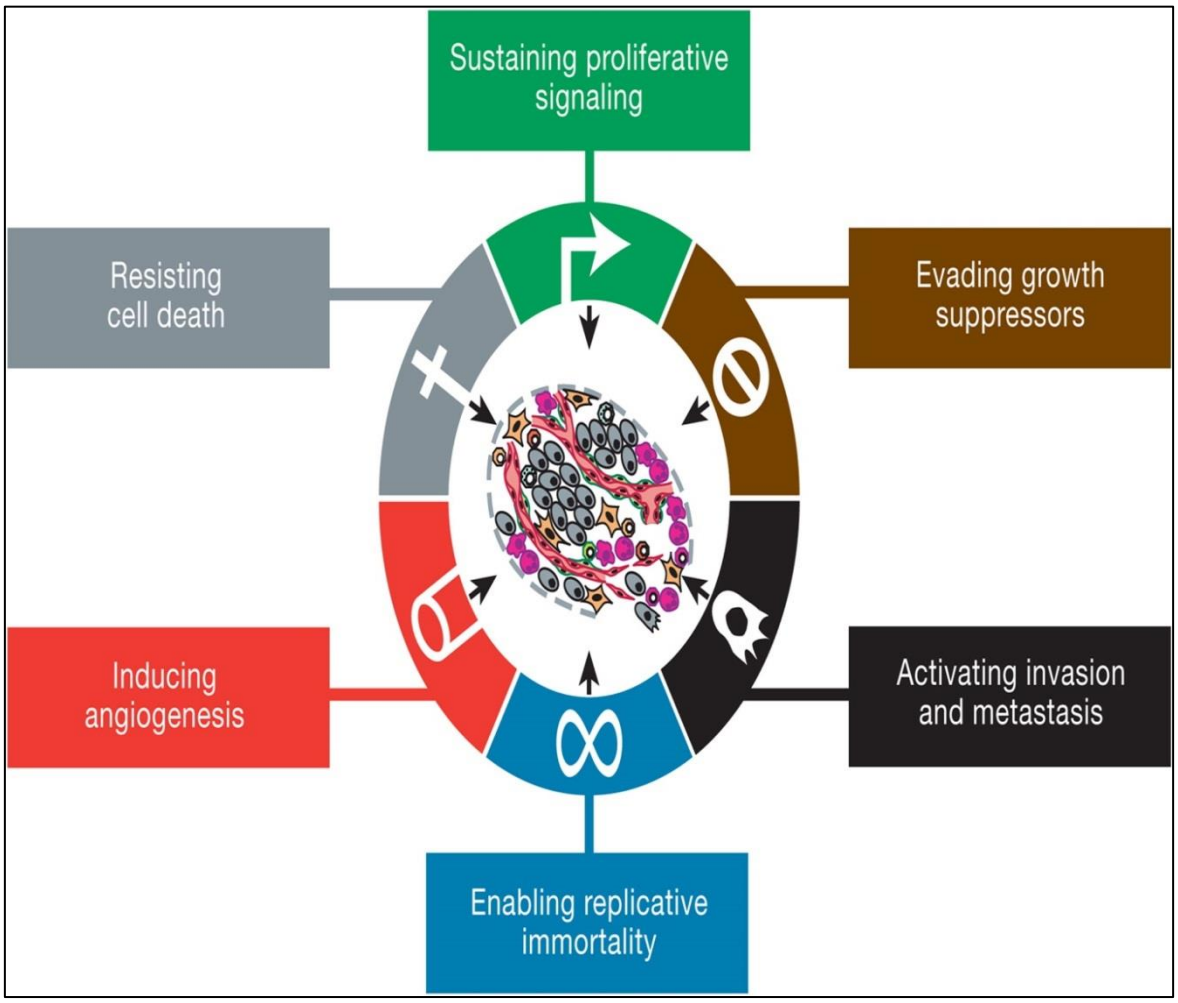

Figure 1.3 The Hallmarks of Cancer Acquired capabilities of cancer. We suggest that most if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies.

Reproduced with permission from Douglas Hanahan, Robert A Weinberg. **The Hallmarks of Cancer: The Next Generation.** Cell, Volume 144, Issue 5, 2011, 646-674. http://dx.doi.org/10.1016/j.cell.2011.02.013

In normal physiological conditions in adults, angiogenesis is turned on only transiently. However, in tumors, it is usually on and results into sprouting of new capillaries accompanied by tumor growth.

Induction of angiogenesis is another mechanism through which β-adrenergic signaling is considered to promote cancer progression. Its activation by chronic behavioral stress results into up-regulation of VEGF expression and increased angiogenesis in ovarian carcinoma[23]. Furthermore, in LNCaP and PC3 cells, activation of ADRB2 by epinephrine and norepinephrine respectively resulted into increased VEGF expression[66,67]. Interestingly, inhibition of ADRB2 by β-blocker propranolol resulted into reduced blood vessel volume in rats[68], supporting the observation that activation of β-adrenergic receptors results into increased tumor angiogenesis in the prostate. However, another study reported no up-regulation in VEGF expression upon activation of β-adrenergic signaling by chronic stress[18], indicating that β-adrenergic signaling might induce angiogenesis through other mechanisms as well.

Interestingly, neuroendocrine cells are found to be the primary source of VEGF in the prostate[69]. Also, the number of neuroendocrine cells is shown to correlate positively with neo-angiogenesis in prostate cancers[70,71]. Furthermore, multiple factors that promote the neuroendocrine differentiation are also known to promote angiogenesis[17], indicating that these two processes are correlated, with ADRB2 being the putative upstream regulator.

Downstream mechanisms of β-adrenergic signaling in prostate cancer

Activation of ADRBs leads to increased adenylyl cyclase activity and elevated cAMP levels (**Fig. 1.4**). cAMP then binds to the cAMP binding domain (CBD) of protein kinase A (PKA) and exchange proteins directly activated by cAMP (EPAC1 and EPAC2)[72-75]. Epac is known to activate Rap1, independent of PKA. Depending upon their relative abundance, distribution and localization, as well as the specific cellular environments, Epac and PKA may act independently, converge synergistically, or oppose each other in regulating a specific cellular function[76]. Upon activation of PKA by cAMP, its catalytic subunit is released and is considered to primarily bind to cAMP response element-binding protein (CREB) and phosphorylate it at S133. CREB is a transcription factor, which leads to activation of transcription of multiple genes. Recently, the cAMP/PKA pathway was shown to phosphorylate BCL2-associated agonist of cell death (BAD) leading to stress mediated resistance to apoptosis in prostate cancer cells[18,77]. Activated PKA also inhibits the RhoA-ROCK pathway, leading to cytoskeletal remodeling and neurite outgrowth[78]. Another signaling cascade targeted by activated PKA is the PI3K/AKT pathway, which leads to up-regulation of VEGF in HIF-1α dependent manner[66].

Activation of cAMP/PKA pathway upon stimulation of ADRB2 by its agonists is known to induce neuroendocrine differentiation[46,47]. Increased cAMP activity in prostate cancer cells LNCaP, PC3 and PC3M leads to neuroendocrine differentiation[45,47,79-81] and overexpression of constitutively active PKA is sufficient to promote the neuroendocrine differentiation of LNCaP cells[46]. Conversely, elevated cAMP activity was unable to induce the neuroendocrine differentiation upon

overexpression of inactive mutant of PKA[47]. Activated PKA phosphorylates cAMP response element-binding protein (CREB) at S133 and activates it[46,47,82,83].

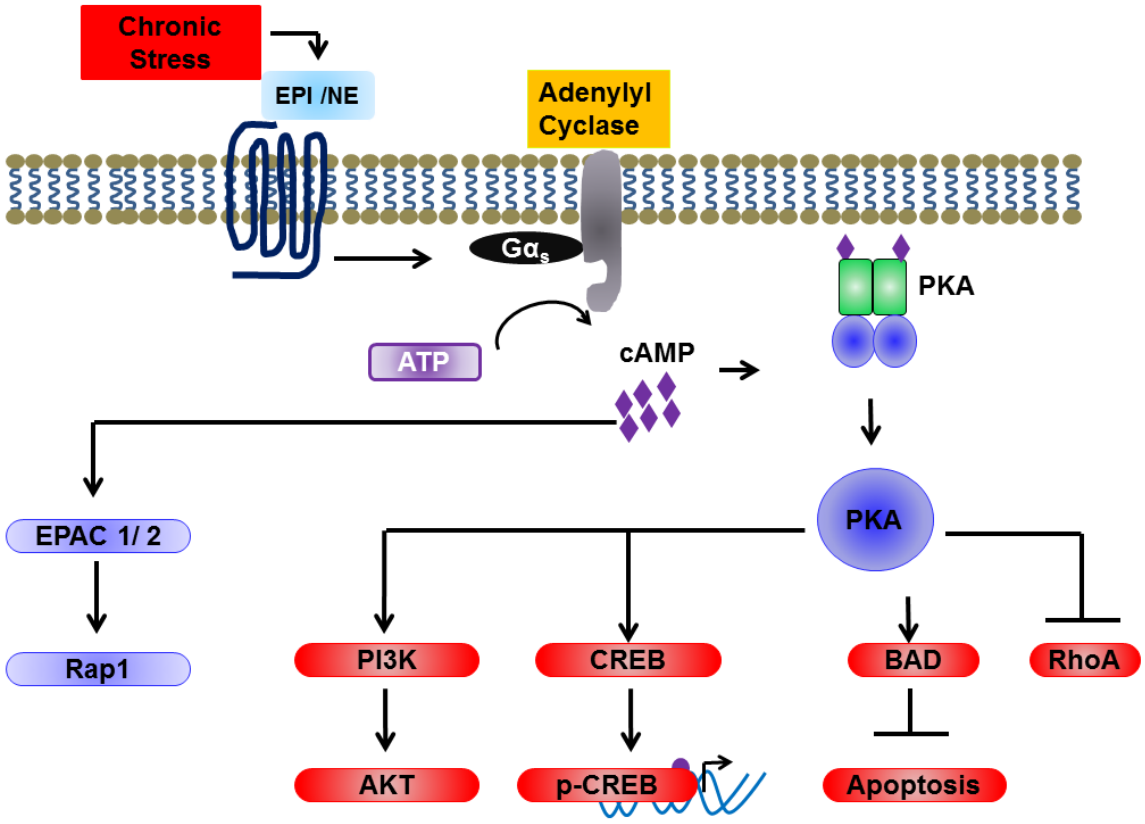

Figure 1.4 Downstream mechanisms of β-adrenergic signaling. Activation of ADRB results into increased cAMP levels, which activates EPAC and PKA that further regulate multiple signaling pathways.

Suppressing this cAMP/PKA induced activation of CREB inhibits the induction of neuroendocrine differentiation[46,47,82-86], suggesting that downstream of ADRB2, the cAMP/PKA/CREB pathway leads to neuroendocrine differentiation. Interestingly, activation of this pathway by chronic behavioral stress is found to induce tumor angiogenesis in mouse models of ovarian cancer[23]. Thaker *et al.* found that VEGF expression was up-regulated upon activation of the ADRB2/cAMP/PKA/CREB pathway. However, it was not clear whether CREB, a transcription factor, induces VEGF expression directly.

Objective and hypothesis of dissertation

Downstream targets of CREB activation in neuroendocrine differentiation as well as in neoangiogenesis are largely unknown, indicating that the underlying mechanisms of ADRB2 signaling in prostate cancer progression are far from completely understood. For instance, epigenetic regulation by histone deacetylases (HDACs) is necessary for stress to induce cardiac hypertrophy[87-89]. Specifically, HDAC2 has been implicated as a key mediator in this process[90]. However, it is unknown if and how ADRB2 signaling directly regulates HDAC2 activity. Furthermore, the involvement of HDAC2-mediated epigenetic regulation in ADRB2-promoted cancer progression also remains unclear. Another regulator of ADRB2, GRK3, a kinase that phosphorylates ADRB2, was recently shown to be a new critical regulator of prostate cancer progression and tumor angiogenesis[91]. It is necessary for the survival and proliferation of metastatic cancer cells, and sufficient to promote primary tumor growth in prostate. Notably, GRK3 is overexpressed in human prostate tumors, especially in soft tissue metastases[91]. However, role of GRK3 in NEPC progression and its regulation by ADRB2 signaling remain unknown.

We have studied the role of GRK3 and HDAC2 in promoting prostate cancer progression, downstream of ADRB2 signaling. We have investigated whether stress activated β-adrenergic signaling modulates HDAC2-mediated epigenetic regulation to promote prostate cancer progression. To understand how GRK3 and NEPC progression contribute to poor prognosis in prostate cancer, we have investigated the mechanisms of GRK3 overexpression in prostate cancer and its connections to ADT, CREB activation and NEPC development. We have also studied whether and

how the ADRB2/cAMP/PKA/CREB pathway regulates the expressions of GRK3 and HDAC2.

Our hypothesis is that GRK3 and HDAC2 are critical down-stream effectors of β-adrenergic signaling-activated CREB in promoting prostate cancer progression. **Fig. 1.5** demonstrates our proposed working model. To test our hypothesis, I have divided my dissertation into two parts: i) to study the regulation of GRK3 expression and its role in NEPC progression downstream of ADRB2 signaling, and ii) to study the regulation of HDAC2 expression and its role in ADRB2 signaling promoted prostate cancer progression and increased tumor angiogenesis.

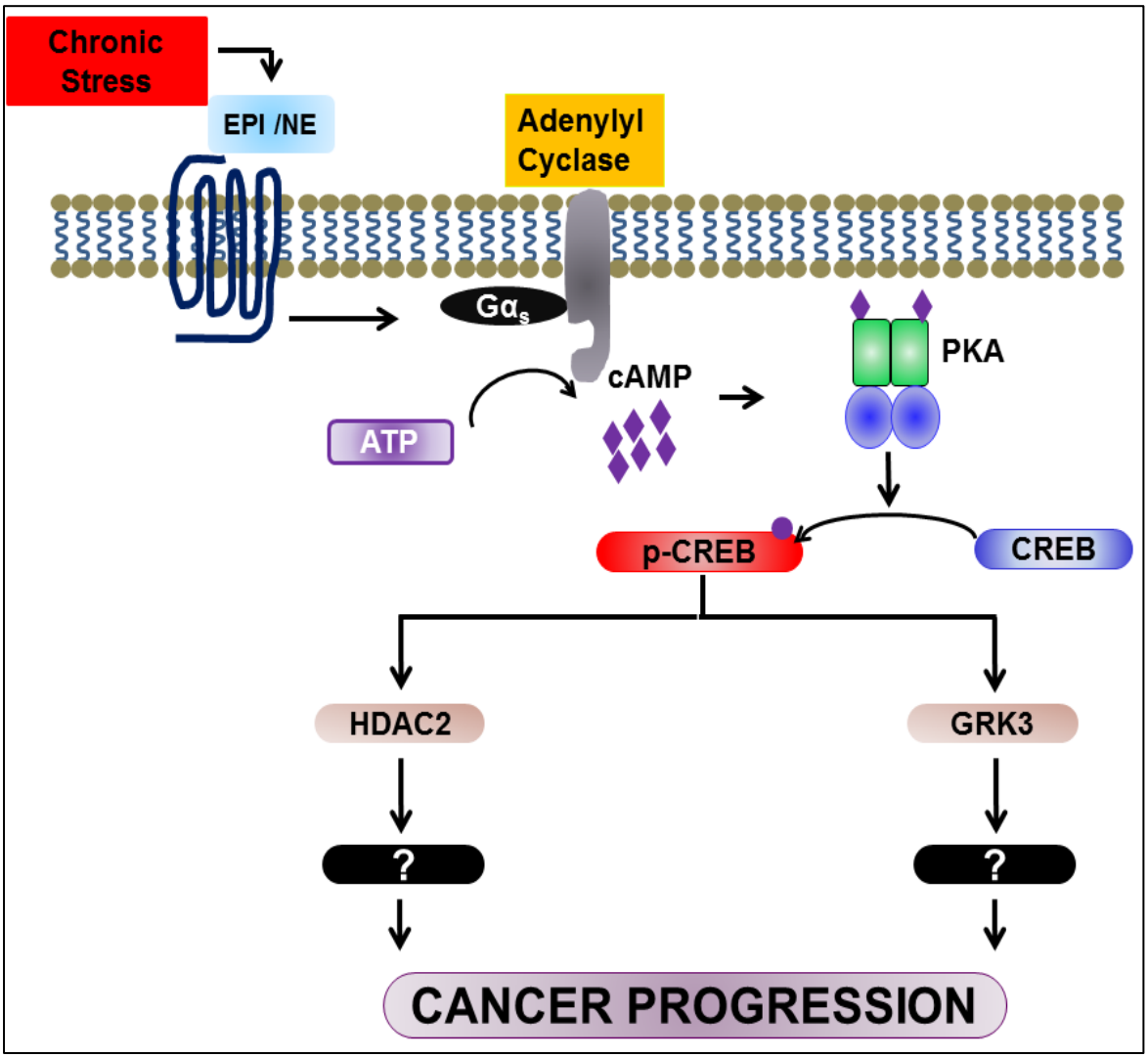

Figure 1.5 Our **hypothesis** is that GRK3 and HDAC2 are critical down-stream effectors of β-adrenergic signaling activated CREB in promoting prostate cancer progression. Activated by cAMP binding, PKA phosphorylates and activates CREB, which eventually binds to GRK3 and HDAC2 promoters and activates their expression. Overexpression of GRK3 and HDAC2 eventually leads to cancer progression.

Here, we show that CREB directly activates GRK3 transcription by binding to its promoter and this up-regulation of GRK3 expression by ADRB2/CREB pathway is sufficient as well as necessary to induce the neuroendocrine differentiation of prostate cancer cells **(Fig. 1.6)**. We also show that downstream of ADRB2, CREB binds to HDAC2 promoter and activates its expression. HDAC2 further suppresses the expression of thrombospondin 1 (TSP1) in order to induce angiogenesis, thus acting as a mediator for the β-adrenergic signaling pathway **(Fig. 1.6)**.

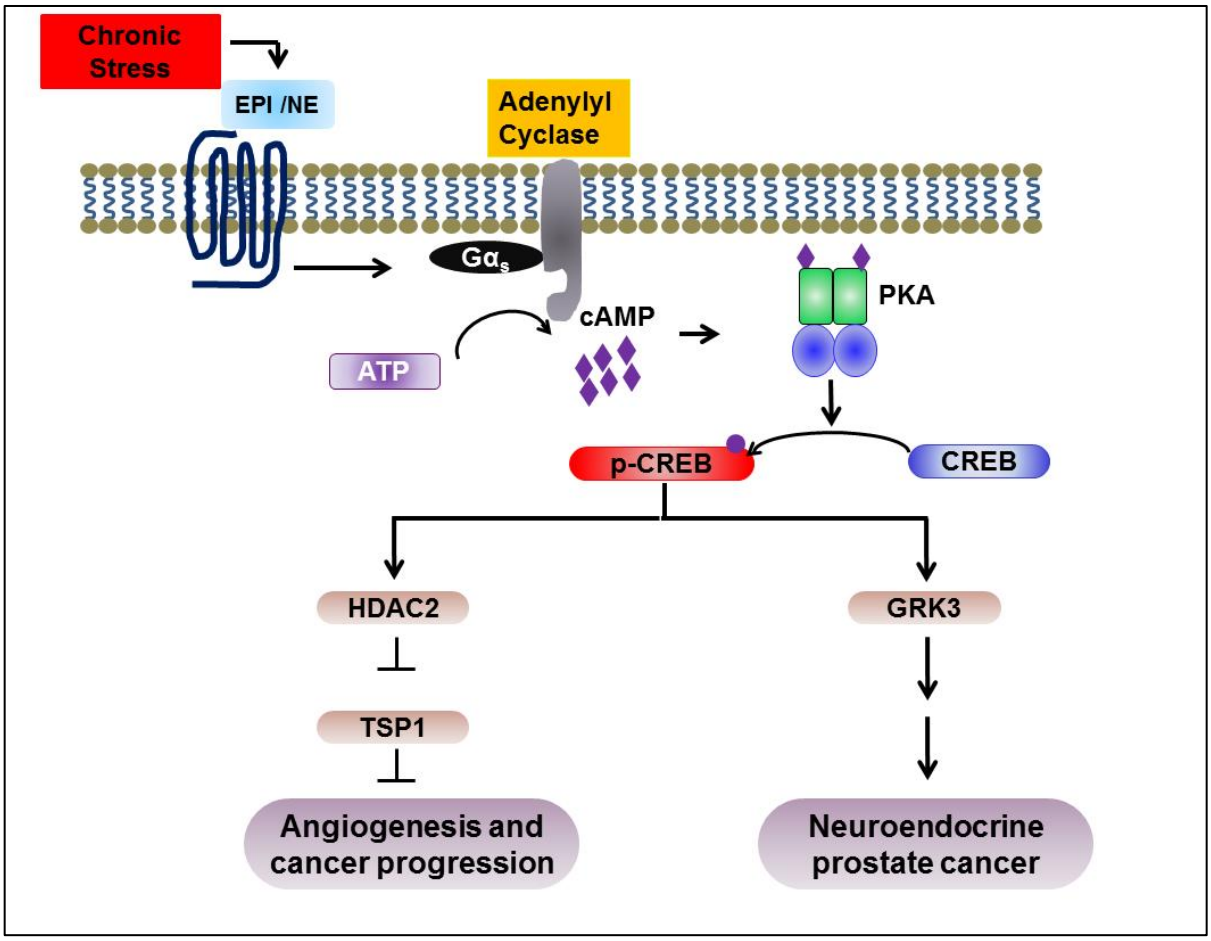

Figure 1.6 Our **hypothesis** is that GRK3 and HDAC2 are critical down-stream effectors of β-adrenergic signaling activated CREB in promoting prostate cancer progression. Activated by cAMP binding, PKA phosphorylates and activates CREB, which eventually binds to GRK3 and HDAC2 promoters and activates their expression. Overexpression of GRK3 leads to neuroendocrine prostate cancer progression, while HDAC2 overexpression eventually leads to increased angiogenesis and cancer progression through TSP1 suppression.

Chapter Two:

Materials and Methods

Cell culture

PC3 prostate cancer cells used in this study are a poorly metastatic variant that was kindly provided by Dr. Isaiah Fidler[91,92] and were confirmed to match with the PC3 cells from ATCC by DNA finger printing. Mouse endothelial cells SVEC4-10, kindly provided by Dr. Vihang Narkar, were originally ordered from ATCC. SVEC4-10 cells were cultured in DMEM (Mediatech), with 10% FBS and 1% penicillin-streptomycin. ADT-induced NEPC cells NE1.3 were maintained in phenol red-free RPMI 1640 medium supplemented with 5% charcoal-striped FBS (Gibco) and 1% penicillin and streptomycin[93]. Prostate neuroendocrine small cell carcinoma cells NCI-H660 were obtained from ATCC and cultured according to ATCC guidance. VCaP cells were maintained in the DMEM medium supplemented with 10% FBS (Gibco), 1% glutamine, 1% penicillin and streptomycin. RWPE-1 cells were grown in keratinocyte serum free medium with 0.05 mg/ml BPE and 5 ng/ml EGF, according to ATCC guidance. Cultures were grown in a 37°C incubator with 5% CO_2. All other prostate cancer cell lines were purchased from ATCC and were cultured in RPMI 1640 media (Mediatech), supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Mediatech).

Animal experiments

NOD/SCID mice were purchased from Charles River Laboratories. All the mouse procedures were approved by the Animal Care and Use Committees of UTHealth and performed in accordance with institutional polices (Protocol # AWC 14-050). One million PC3 or LNCaP cells expressing luciferase were injected subcutaneously

on the flanks and shoulders of NOD/SCID mice. In the stress experiment, the mice were subjected to behavioral stress by restricting the movements for one hour, twice a day. In the chemical treatment experiments, the control group received buffered saline, the 'ISO' group received 10 mg/kg isoproterenol, twice a day. In each experiment, all the mice were sacrificed when mice with the biggest tumors reached the humane endpoint (1 cm diameter). The luciferase signal from the xenograft tumors was measured with IVIS Lumina II platform machine (Caliper Life Sciences).

Vector constructs and virus preparation

All shRNA constructs are in pLKO.1 vector and were purchased from Sigma-Aldrich. TRCN 6321 and TRCN7086 shRNAs were used to downregulate HDAC2 and were packaged into viral particles using 293T cells[94], according to previously described method[91]. PC3 cells were transfected with mammalian expression vector pcDNA3.1, Flag-pcDNA3.1-CREB-WT (wild type), or Flag-pcDNA3.1-CREB-Y134F[95] (kindly provided by Dr. Rebecca Berdeaux) using TransIT-LT1 transfection reagent and selected with 400μg/ml of G418.

cDNA/shRNA transduction and transfection in prostate cancer cells

LNCaP cells expressing GFP, GRK3 (wild type cDNA) or GRK3-KD (kinase dead cDNA) were generated through retroviral transduction as previously described[91]. Preparation and usage of shGRK3 lentivirus have also been described[91]. LNCaP and NE1.3 cells were infected by shGRK3 lentivirus at MOI of 5 in the presence of polybrene (8 μg/ml). shScramble was used as control. PC3 cells were transfected

with the mammalian expression vector pcDNA3.1, Flag-pcDNA3.1-CREB (wild type), Flag-pcDNA3.1-CREB (Y134F)[95] (kindly provided by Dr. Rebecca Berdeaux at UT-Houston) using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA). Transfected PC3 cells were selected with 400μg/ml of G418.

Western blotting analysis

Western blotting analysis was carried out as previously described[91]. Primary antibodies used were as follows:

anti-GRK3 (3460-1 Epitomics, USA), anti-TSP1 (ab1823, Abcam), anti-p-CREB (#9198, Cell Signaling Technology), anti-CREB (#9197, Cell Signaling Technology), anti-HDAC2 (#2545, Cell Signaling Technology), and anti-actin (SC47778, Santa-Cruz Biotechnology).

After washes, the membranes were incubated with HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (Cell Signaling Technology) for 1 h at room temperature. Finally, the immunoreactive bands were developed with Pierce ECL Western Blotting Substrate (Thermo Scientific) on Blue Basic autoradiography Film (Bioexpress).

Immunofluorescence (IF) and immunohistochemistry (IHC) staining

IF staining was carried out as previously described[96]. Primary antibodies used were as follows: anti-TSP1 (ab85762, Abcam) and anti-IB4 (B-1205, Vector Labs). IHC staining procedure was similar to that we previously described[96,97]. Briefly, slides with 5-μm sections of formalin-fixed, paraffin-embedded xenograft tumors were

deparaffinized, rehydrated and subjected to antigen retrieval with 10 mM sodium citrate pH 6.0 for 40 min. Slides were then incubated with hydrogen peroxide as Peroxidase Suppressor (Thermo Scientific Pierce) and 2.5% horse serum as blocking buffer, followed by incubation with CD31 primary antibody (ab28364, Abcam) overnight at 4°C, PBS washes and then HRP conjugated secondary antibody 1 h at room temperature. The immunohistochemistry reaction was developed with a DAB substrate Kit (Vector Labs), slides were counterstained with hematoxylin and mounted in VectaMount permanent mounting medium (Vector Labs).

Immunohistochemistry staining on human prostate tumor tissue microarray

The Universal Elite ABC kit (Vector Labs) was used for immunohistochemistry (IHC) staining, according the manufacturer's instructions. Briefly, slides of five micrometer sections from 78 cases of formalin-fixed and paraffin-embedded prostate cancer and normal tissue blocks were dewaxed in 60°C oven for 2 hours and rehydrated through incubating in xylene and alcohol series. Antigen retrieval was done in 10 mM sodium citrate buffer (pH 6.0) in a food steamer for 30 minutes. After suppressing the endogenous peroxidase activity the sections were incubated in normal horse serum to prevent nonspecific immunoglobulin binding. Upon PBS wash, the sections were then treated with the anti-human p-CREB (Cell Signaling Technology) or anti-human GRK3 antibody (Epitomics) at 4°C overnight. A streptavidin-HRP detection system was used to reveal specific binding. Immunoreactivity was scored as following: staining intensity -/+, <25% positive cells

(weak, score 1); staining intensity ++, 25-50% positive cells (intermediate, score 2); and staining intensity +++, >50% positive cells (strong, score 3). Percent of positive cells and staining intensity were scored independently by two experienced researchers.

Reverse transcription and quantitative PCR analysis

TRIzol (Invitrogen) was used to isolate RNA from cells. cDNA was generated by reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad). Real time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) in Bio-Rad CFX96 Real-Time PCR Detection System using the following primers:
HDAC2-F: cagatcgtgtaatgacggtatca, HDAC2-R: ccttttccagcaccaatatcc; TSP1-F: caatgccacagttcctgatg, TSP1-R: tggagaccagccatcgtc, GRK3-F: gcagtgccgactggttct, GRK3-R: gtctgaaagggctgtgacct, CREB-F: ggagcttgtaccaccggtaa, CREB-R: gcatctccactctgctggtt, CHGA-F: tacaaggagatccggaaagg, CHGA-R: ccatctcctcctcctcctct, CHGB-F: cacgccattctgagaagagc, CHGB-R: tctcctggctcttcaaggtg, ENO2-F: ctgtggtggagcaagagaaa, ENO2-R: acacccaggatggcattg, AR-F: gccttgctctctagcctcaa, AR-R: ggtcgtccacgtgtaagttg, PSA-F: cacagcctgtttcatcctga, PSA-R: atatcgtagagcgggtgtgg, GAPDH-F: agccacatcgctcagacac, and GAPDH-R: gcccaatacgaccaaatcc.

For all RT-PCR analysis, GAPDH was used to normalize RNA input and expression levels were calculated according to the comparative C_T method ($\Delta\Delta C_T$).

Chromatin Immunoprecipitation (ChIP)

Cells treated with ISO or ISO+PRO were crosslinked with formaldehyde and lysed in SDS lysis buffer. Cell lysate was sonicated on ice, for 12 cycles of 20 sec ON and 40 sec OFF at 40% amplitude using Branson Low Power Ultrasonic Systems 2000 LPt/LPe sonicator (Fisher Scientific) and the supernatant was used for ChIP experiments with anti-CREB (# 06-863, Merck Millipore), anti-p-CREB (# 9198, Cell Signaling Technology), anti-HDAC2 (ab51832, Abcam) or negative control IgG. The immunoprecipitates were washed sequentially with low salt buffer, high salt buffer, LiCl buffer and finally TE buffer. DNA was eluted and the reversal of crosslinking was done with proteinase-K and 0.2 M NaCl. The DNA was extracted using phenol chloroform and PCR was conducted using the following primers:

GRK3-CREB-F: GCCTCTAAGATCACCCAGCA, GRK3-CREB-R: AGACCTGACATCTGCCTACA, HDAC2-CREB-F: CATTGCTGCAGAGTGGAACA HDAC2-CREB-R: AGGTGGAGGCAGATTAAGGA, TSP1-HDAC-F: GTCATACAACACTCCCACGC and TSP1-HDAC-R: CCAGGGCATAGGTAGAAGCT.

Endothelial cell migration assay

PC3 cells were treated as indicated overnight in RPMI-1640 with no serum. SVEC4-10 cells were grown till 70% confluence, starved overnight, trypsinized and resuspended in the conditioned media collected from PC3 cells. 50,000 SVEC4-10 cells were seeded per Boyden chamber insert (8 µm, BD Biosciences). Cells were

allowed to migrate for 4 hours and the inserts were fixed and stained with crystal violate dye to observe migrated cells. Cell migration was analyzed qualitatively.

Endothelial cell tube formation assay

PC3 cells were treated as indicated overnight in RPMI-1640 with no serum. SVEC4-10 cells were starved overnight, trypsinized and resuspended in the conditioned medium from PC3 cells. 20,000 SVEC4-10 cells/well in PC3 conditioned medium were seeded on the Matrigel. Pictures were taken under 4X and 10X magnification and the number of branches in SVEC tube formation were quantified.

Microarray data mining

The indicated GSE microarray data sets were downloaded from the GEO database http://www.ncbi.nlm.nih.gov/gds.The TCGA_PCa data on CREB and GRK3 were downloaded from http://www.cbioportal.org[42,98]. The normalized and transformed gene expression values from the sources were used in our analysis and statistical calculation.

Statistical analyses

P-values were obtained through Student's t-test, unless otherwise indicated. Spearman correlation coefficient and associated P-values for gene expression from public datasets were calculated using the statistical tool at

http://www.fon.hum.uva.nl/Service/Statistics/RankCorrelation_coefficient.html, confirmed by additional statistical analysis at http://vassarstats.net/corr_rank.html.

Cell proliferation assay

AlamarBlue® cell viability reagent (Thermo Fisher Scientific) was used to estimate the cell numbers and the cell proliferation assay was performed according to the manufacturer's protocol. Briefly, 1000 cells were seeded in each well of 96 cell well plates and were allowed to proliferate for 72 hours in regular culture media and conditions. AlamarBlue® cell viability reagent was added to the cells and incubated at 37°C for 1-4 hours. Infinite® M1000 spectrophotometer was used (Tecan US, Inc.) to read fluorescence at excitation wavelength 535 nm with emission wavelength at 595 nm. The readings were plotted with Y-axis showing the relative cell number.

MDV3100 treatment

LNCaP-GFP, GRK3 and GRK3-KD cells were seeded in 24 well plates (4000 cells per well, six replicates per cell line, per treatment). Cells were treated with vehicle or 5 μM MDV3100 for 10 days. Fresh media and drugs were replenished after 5 days of treatment. Cell viability was studied with the AlamarBlue® cell viability reagent (Thermo Fisher Scientific) as described above.

Chapter Three:

GRK3 is a direct target of CREB activation and regulates neuroendocrine differentiation of prostate cancer cells

This chapter is based upon

Sang, M*., **Hulsurkar M***., Zhang, X*., Song, H., Zheng, D., Zhang, Y., Li, M., Xu, J., Zhang, S., Ittmann, M.,Li, W.. GRK3 is a direct target of CREB activation and regulates neuroendocrine differentiation of prostate cancer cells. *Oncotarget*

*These authors contributed equally.

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Background

Neuroendocrine prostate cancer

Progression to castration resistant prostate cancers (CRPC) is a major therapeutic challenge for prostate cancer patients. Unfortunately, the mechanisms underlying CRPC development remain largely unclear. Approximately 25% of men who die of prostate cancer have tumors with a neuroendocrine phenotype[56,99-101]. Neuroendocrine prostate cancer (NEPC) is characterized by loss of androgen receptor (AR) expression, resistance to hormonal therapies, and elevated levels of NE-related proteins, such as enolase 2 (neuronal, ENO2) and chromogranin A and B (CHGA/CHGB[56,99-101]. NEPC is associated with aggressive disease, frequent metastases to soft tissues and a short survival time[11-13,15,16]. With the recent introduction of potent ADT drugs, such as enzalutamide and abiraterone acetate, the incidence of NEPC is expected to increase dramatically[56-59,99,102,103]. A better understanding of the molecular events underlying NEPC development is urgently needed to develop a therapeutic solution for CRPC/NEPC.

NEPC can arise *de novo*, but most commonly evolves from preexisting prostate adenocarcinoma (PAC)[93,104-106]. The majority of evidence to date favor a transdifferentiation model of NEPC origin, where PAC treated extensively with androgen deprivation therapy (ADT) or radiation therapy develop into NEPC, as a mechanism of adaptive response and drug resistance[14-16,46-48,54,81-86,93,105-108]. Recently, neuroendocrine differentiation (NED) has been observed in a patient-derived xenograft model of prostate adenocarcinomas that developed NEPC after medical castration[103,109,110].

cAMP response element-binding protein (CREB)

CREB is a transcription factor that binds to DNA at cAMP Response Element (CRE) site. Multiple stimuli induce CREB activation, resulting into myriad of responses like neuronal signaling, cell growth and motility etc[111]. CREB phosphorylation at S133 results into its activation[46,47,82,83] and many upstream kinase regulators, including PKA, are responsible for CREB activation[111] **(Fig. 3.1)**. All the CREB target genes share an upstream CRE sequence of TGANNTCA, the binding site where CREB interacts with DNA[112,113]. More than 100 genes are reported to be targeted by CREB[111]. It is still unclear how this complex network of multiple upstream kinase regulators and hundreds of target genes is regulated with specificity (**Fig. 3.2**), suggesting that the underlying mechanisms of CREB transcription regulation are not completely understood.

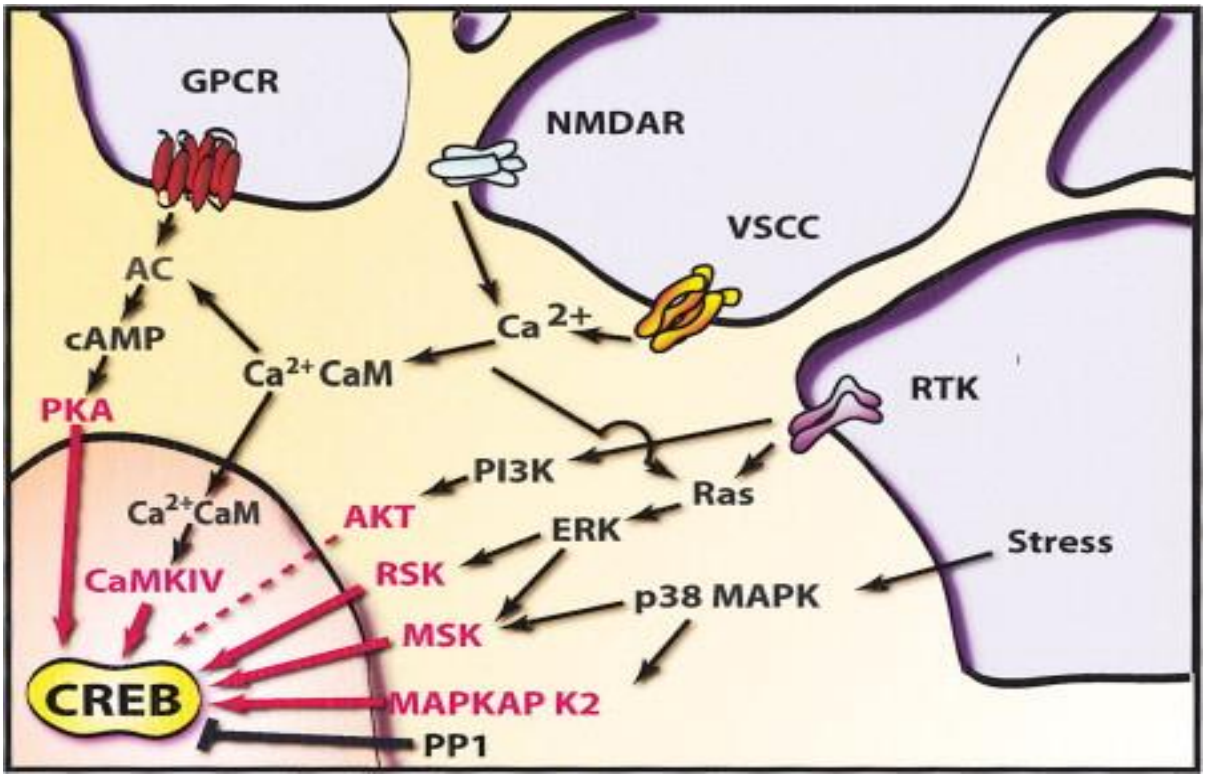

Figure 3.1. An Overview of signaling pathways that converge on CREB

Excitatory neurotransmitters, ligands for GPCRs, neuronal growth factors, and stress inducers are among the stimuli that activate signaling pathways that converge upon CREB. As described in the text, multiple stimulus-dependent protein kinases have been implicated as CREB kinases in neurons, and a high degree of crosstalk exists between these signaling pathways. Stimulus-dependent CREB kinases include PKA, CaMKIV, MAPKAP K2, and members of the pp90RSK (RSK) and MSK families of protein kinases. Protein phosphatase 1 (PP1) has been implicated as the predominant phospho-CREB phosphatase[111,114,115].

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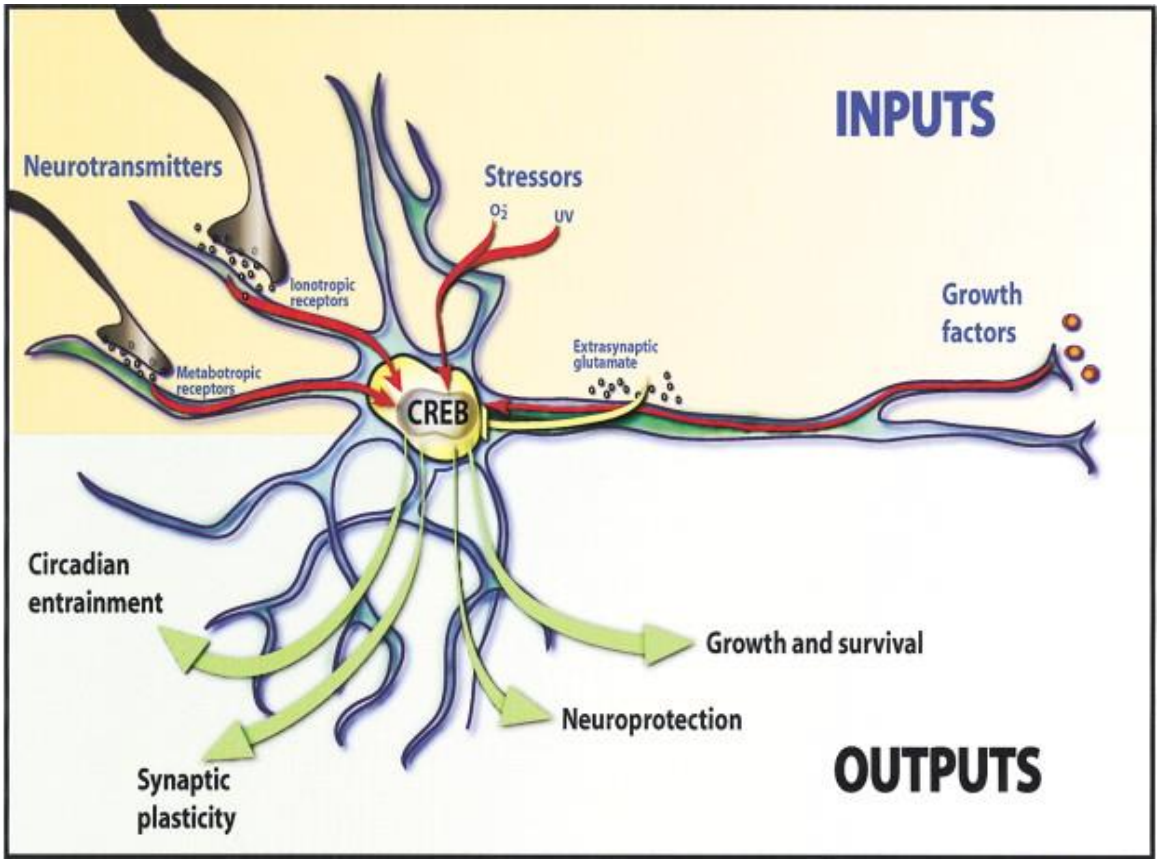

Figure 3.2. CREB-dependent gene expression is critical for a variety of functions.

Shown are some of the processes for which CREB-dependent gene expression has been implicated. Stimuli and conditions that promote CREB phosphorylation and CREB-mediated gene expression are indicated in the upper half of the diagram, while physiological and pathological consequences of CREB activation are depicted in the lower half.

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GRK3

GRK3 belongs to the subfamily of G-protein coupled receptor kinases (GRKs). GRKs are best known for their roles in the phosphorylation and desensitization of agonist-bound GPCRs[116-120], including beta-adrenergic receptors (ADRBs). ADRBs act through the increase of cAMP by adenylyl cyclase (AC) and activation of PKA/CREB pathway[121-123]. Therefore, PKA/CREB can be activated through ADRB stimulation (such as isoproterenol)[121], or by a direct activator of AC, forskolin (FSK) and the inhibitor of phosphodiesterase, IBMX[124].

Through unbiased shRNA and cDNA screening of hundreds of human kinases, our lab has previously shown that G-protein coupled receptor kinase 3 (GRK3) is a new critical activator of prostate cancer progression[91]. Not only is it necessary for the survival and proliferation of metastatic cancer cells *in vitro* and *in vivo*, but it is also sufficient to promote primary tumor growth in the prostate and metastases in soft tissues. Notably, GRK3 is overexpressed in human prostate tumors, especially in soft tissue metastases[91]. However, it is unknown what biological processes are responsible for GRK3 overexpression in prostate cancers and how GRK3 contributes to prostate cancer progression.

Working hypothesis

Our working hypothesis is that activated by ADT, CREB induces GRK3 expression and GRK3 acts as a mediator of CREB to induce neuroendocrine differentiation of prostate cancer cells.

To understand how GRK3 and NEPC progression contribute to poor prognosis in prostate cancer, we investigated the mechanisms of GRK3 overexpression in prostate cancer and its connections to ADT, CREB activation and NEPC development. We show that GRK3 indeed controls NED phenotypes of prostate cancer cells, and is induced by ADT as a direct target and critical mediator of CREB activation. These results elucidate the mechanisms of NED in prostate cancer cells and may facilitate establishment of GRK3 as a new therapeutic target for NEPC.

Results

ADT induces neuroendocrine differentiation of human prostate adenocarcinoma LNCaP cells.

To investigate the signaling pathways and molecular mechanisms of neuroendocrine prostate cancer cells, we compared the classic AR-positive adenocarcinoma (PAC) LNCaP cells with NE1.3 cells, neuroendocrine differentiated NEPC cells derived from LNCaP cells through long term androgen deprivation treatment (ADT)[48,93]. As shown in **Fig. 3.3 A**, LNCaP cells have an epithelial morphology, whereas NE1.3 cells show a neuronal morphology with rounded cell bodies and extended, finely branched processes. NE1.3 cells expressed low levels of AR and AR target PSA, and high levels of NE markers CHGA, CHGB and ENO2 (**Fig. 3.3 B-C**). This is consistent with the literature that long term ADT induces NED in PAC cells, mouse models and patients[14,82,93,100,104,105,125-127]. In addition, we observed that the expression of NE markers was significantly higher in another NEPC cell line NCI-H660 than in PAC cell line LNCaP[128] (**Fig. 3.3 D**).

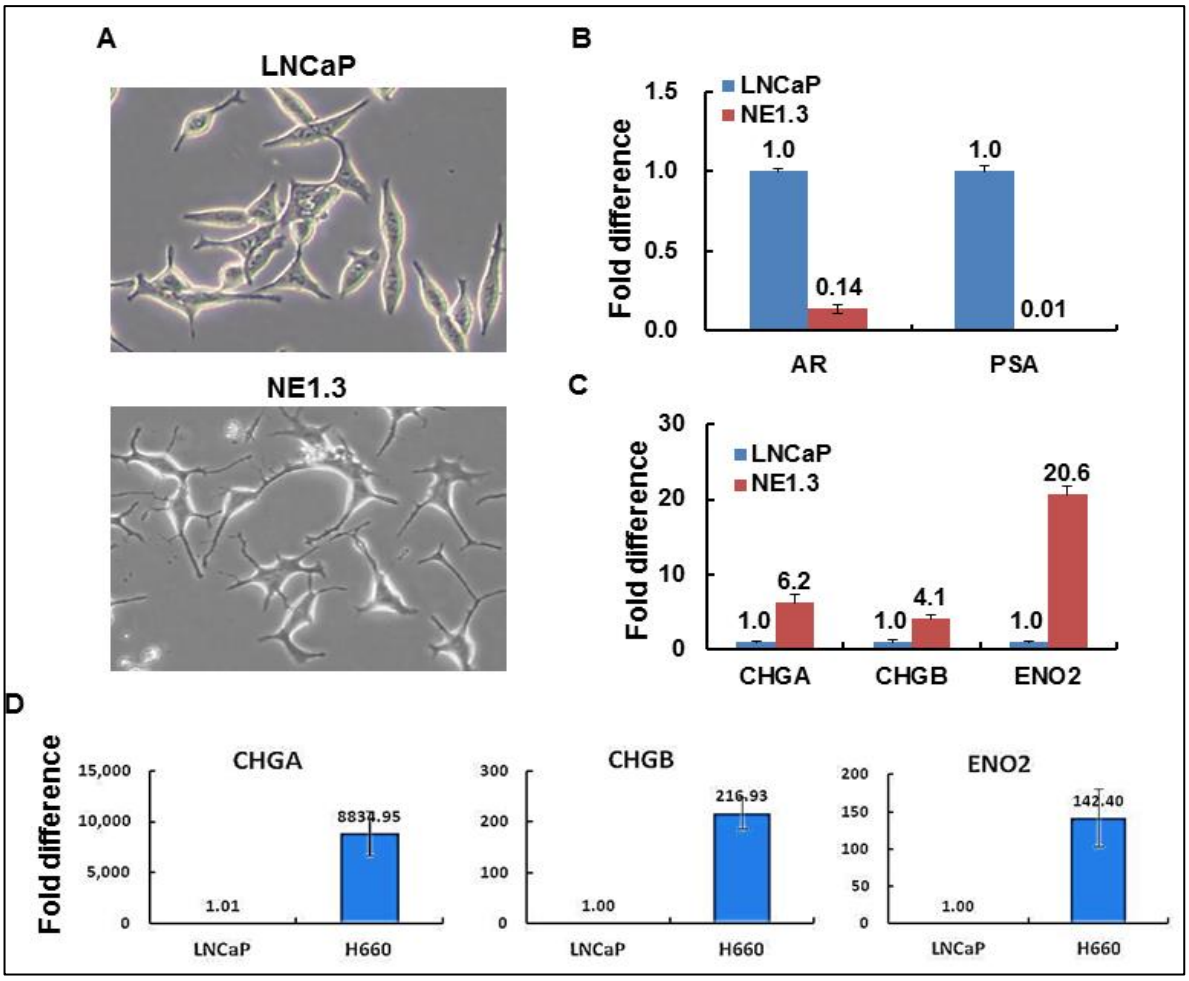

Figure 3.3. Androgen deprivation treatment (ADT) induces neuroendocrine differentiation in prostate cancer cells. (A) Representative images show that prostate adenocarcinoma cells LNCaP have an epithelial morphology, whereas the ADT-induced LNCaP-derived neuroendocrine cancer cells NE1.3 show a neuronal morphology with compact, rounded cell bodies and extended, finely branched processes. **(B)** RT-PCR shows the expression patterns of androgen receptor (AR) and AR target, prostate specific antigen (PSA), in LNCaP and NE 1.3 cells. Y-axis shows the relative fold differences in expression, normalized to GAPDH. **(C, D)** RT-PCR shows the expression patterns of neuroendocrine markers chromogranin A and

B (CHGA, CHGB) and enolase 2 (ENO2) in NE1.3 **(C)** and NCI-H660 **(D**) as compared to LNCaP cells. Y-axis shows the relative fold differences in expression, normalized to GAPDH. *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

GRK3 is up-regulated in ADT-induced NEPC cells.

We hypothesized that GRK3 promotes NEPC development. Indeed, we found that GRK3 was significantly up-regulated in NEPC cells NE1.3 and H660 at both mRNA and protein levels as compared with the PAC cells LNCaP and VCaP (**Fig. 3.4 A-B**). By analyzing data from a time course study of androgen deprivation of LNCaP cells (GSE8702)[129], we found a similar result, i.e. GRK3 and NE marker ENO2 are up-regulated as the LNCaP cells become androgen-independent after long term ADT (**Fig. 3.5**). To mimic castration-induced neuroendocrine differentiation *in vivo*, we compared the expression of GRK3 and NE markers between untreated PAC prostate cancer patient-deprived xenograft (PDX) LTL331 and NEPC PDX LTL331R that derived from LTL331 after relapse from castration[103,109,110,130]. GRK3 and NE markers (ENO2, CHGA and CHGB) are significantly up-regulated in LTL331R (**Fig. 3.4 C**).

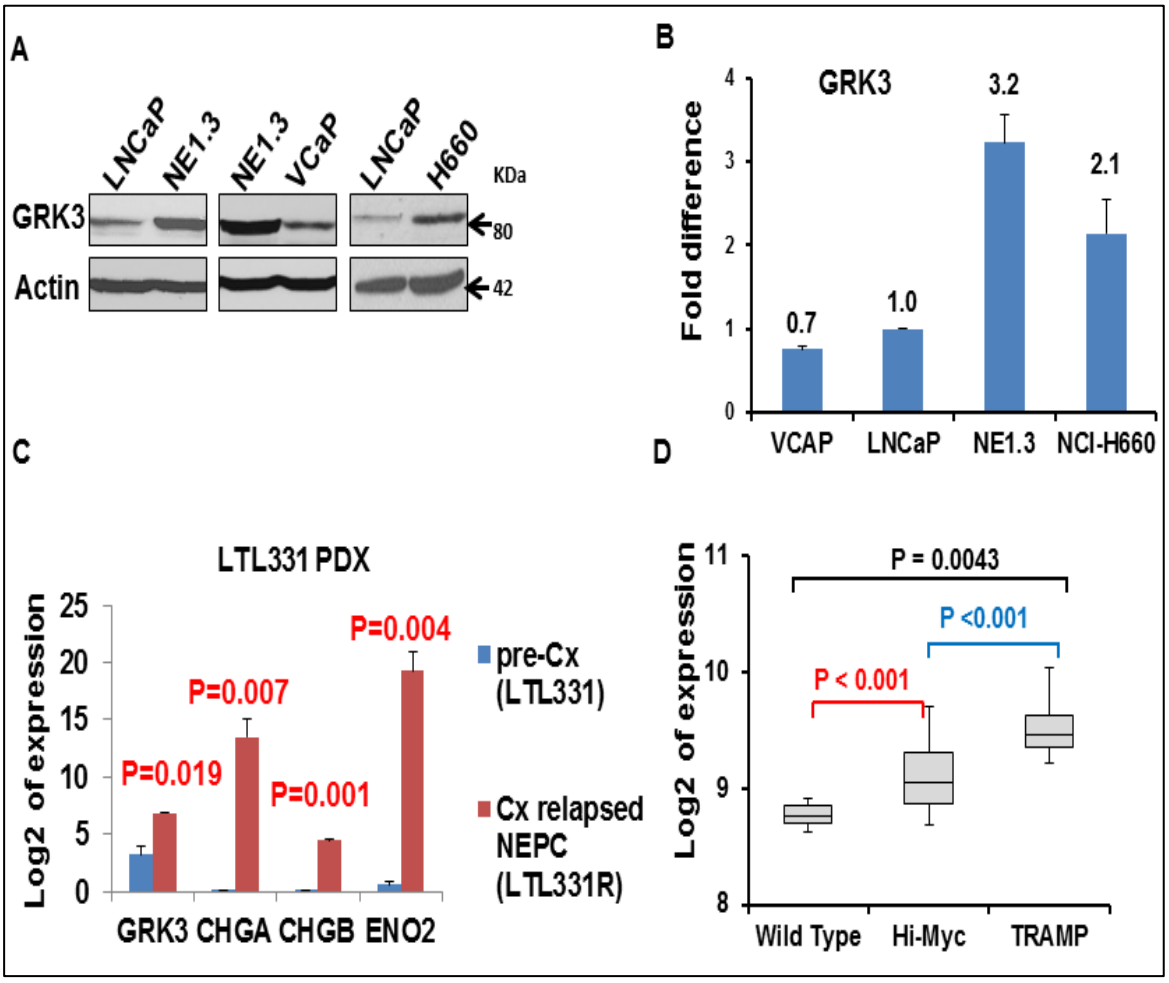

Figure 3.4. GRK3 is up-regulated in neuroendocrine prostate cancer (NEPC). Western blot assays **(A)** and RT-PCR **(B)** comparing the expression of GRK3 in prostate adenocarcinoma (PAC) cells LNCaP and VCaP to NEPC cells NE1.3 and NCI-H660. Y-axis: relative differences in expression normalized to GAPDH. **(C)** GRK3 is significantly up-regulated when the prostate cancer patient derived xenograft (PDX) PAC model LTL331 tumors relapsed after castration and become CRPC/NEPC (LTL331R). RNA-seq data by Akamatsu S et al[130] were obtained and analyzed for GRK3 expression. Y-axis indicates the log2 of the expression levels. **(D)** Expression of GRK3 in different genetically engineered mouse (GEM) models of

prostate cancer (GSE53202). TRAMP mice are a classic GEM model for NEPC while Hi-Myc mouse is a classic model of prostate adenocarcinoma (PAC). Y-axis shows the normalized and transformed expression values in the indicated data sets obtained from the GEO database (**B** and **D**). *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

To further strengthen these results from human prostate cancer cells and PDX models, we investigated GRK3 expression in a classic NEPC genetically engineered mouse (GEM) model, the TRAMP mouse[131,132]. Analysis of microarray data in GSE58822 and GSE53202[133,134] revealed GRK3 levels were significantly higher in prostate tumors of the TRAMP mice than in normal prostate tissues of wild type mice (P=0.0043 in GSE58822 and P=2.17E-16 in GSE53202) (**Fig. 3.4 D**). Interestingly, GRK3 is also expressed more highly in tumors of the TRAMP mice than in a classic GEM model for PAC, the Hi-Myc mice (P=1.02E-6, GSE53202) (**Fig. 3.4 D**). All together, these results show that GRK3 is induced by ADT and up-regulated in NEPC.

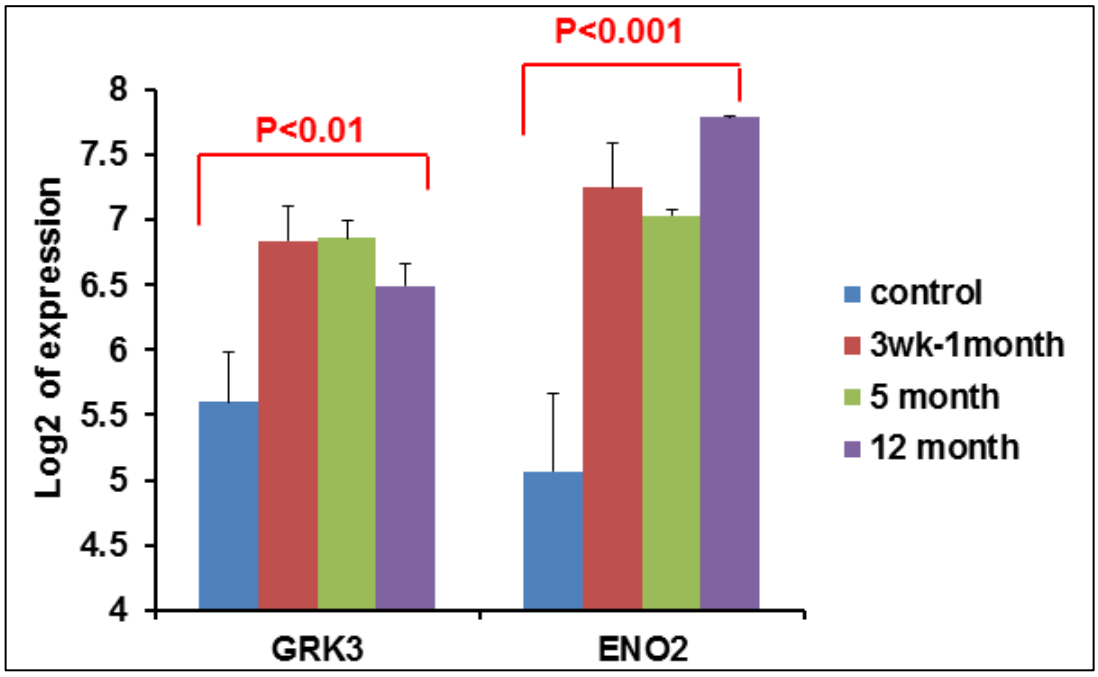

Figure 3.5. GRK3 and ENO2 expressions are up-regulated upon androgen deprivation. Results from data mining of a time course study of androgen deprivation of LNCaP cells (GSE8702). Y-axis shows the log2 transformed expression of GRK3 and NE marker ENO2 at different durations of androgen deprivation of LNCaP cells (untreated; early, 3 week-1 month; midterm, 5 months; late, 12 months). *Results in this figure were obtained in collaboration with Li W.*

GRK3 is a direct target of CREB activation that is induced by ADT.

Consistent with reports in literature that CREB activation promotes NED in prostate cancer cells[85,86,135], we found that CREB was up-regulated and activated (by pS133 level[136-138]) in NEPC cells NE1.3 and H660 (**Fig. 3.6 A-B**). Moreover, overexpression of either the CREB wild type cDNA or constitutively active mutant CREB-Y134F cDNA increased the expression of NE markers and GRK3 in prostate cancer cells (**Fig. 3.6 C-D**).

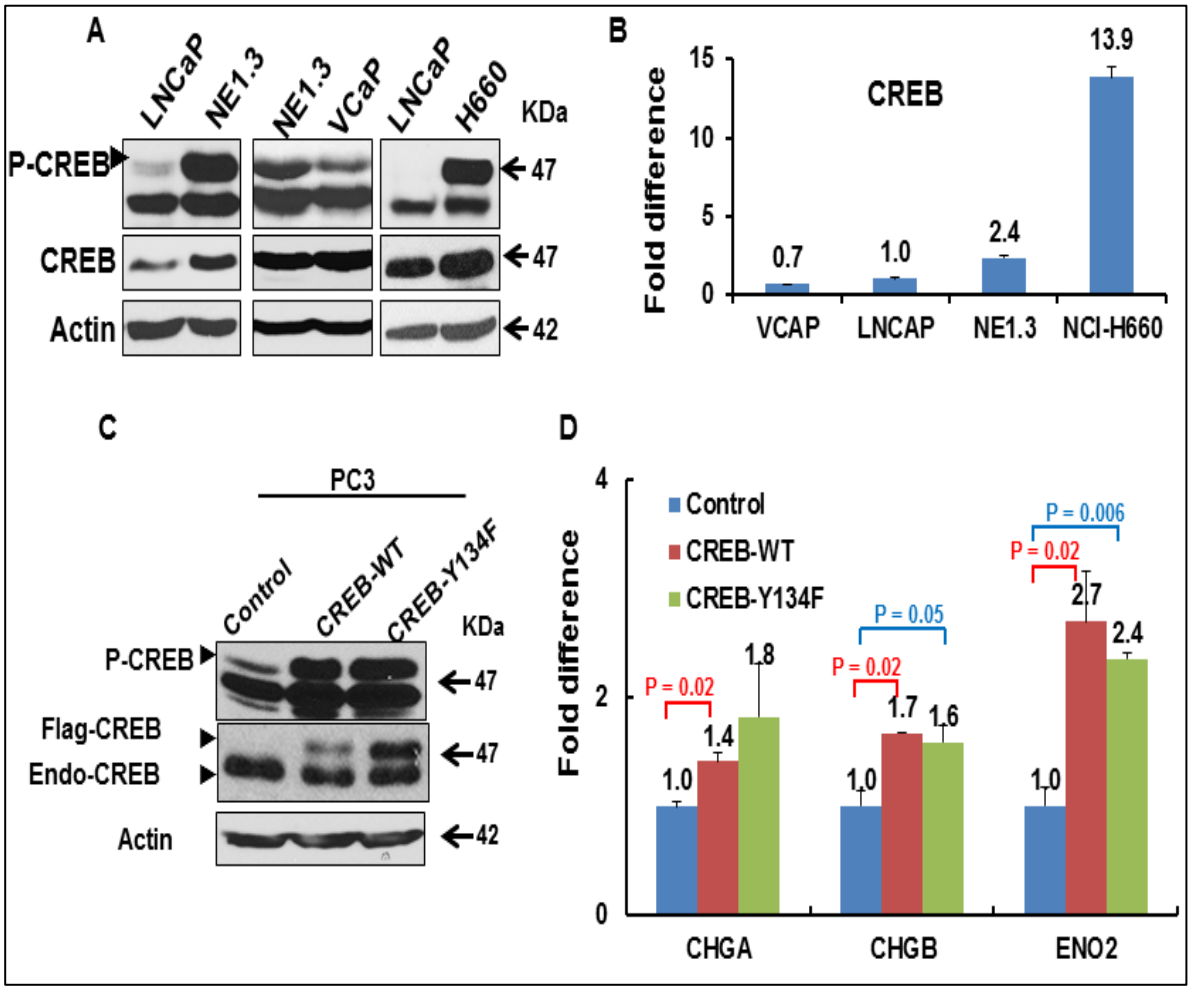

Figure 3.6. CREB activation is induced by androgen deprivation treatment (ADT) and promotes neuroendocrine differentiation of prostate cancer cells. **(A)** Western blots show that CREB is up-regulated and hyper-phosphorylated at S133 (activated) in ADT-induced NE1.3 and in NCI-H660 cells, as compared to LNCaP and VCaP cells. **(B)** RT-PCR shows elevated expression of CREB in NEPC NE1.3 and NCI-H660 cells compared to LNCaP and VCaP cells. Y-axis shows the relative fold differences in expression, normalized to GAPDH. **(C)** A Western blot shows overexpression of flag-tagged wild-type and constitutively active Y134F mutant of CREB. **(D)** RT-PCR shows elevated expression of NE markers CHGA,

CHGB and ENO2 in the prostate cancer cells overexpressing flag-tagged wild-type or constitutively active Y134F mutant of CREB. Y-axis shows the relative fold differences in expression, normalized to GAPDH. *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

CREB-Y134F contains a mutation in which tyrosine 134 is changed to phenylalanine, which increases its affinity to PKA, and thus enhances its phosphorylation and activation by PKA[95]. Induction of GRK3 was also observed when we treated prostate cancer cells with beta-adrenergic receptor agonist isoproterenol (ISO), or adenylyl cyclase activator forskolin (FSK) with phosphodiesterase inhibitor IBMX (FSK+IBMX) (**Fig. 3.7 B, Fig. 3.8 B**). Both treatments are known to activate CREB through PKA phosphorylation at S133[137-141]. These results support our hypothesis that GRK3 is induced by CREB activation.

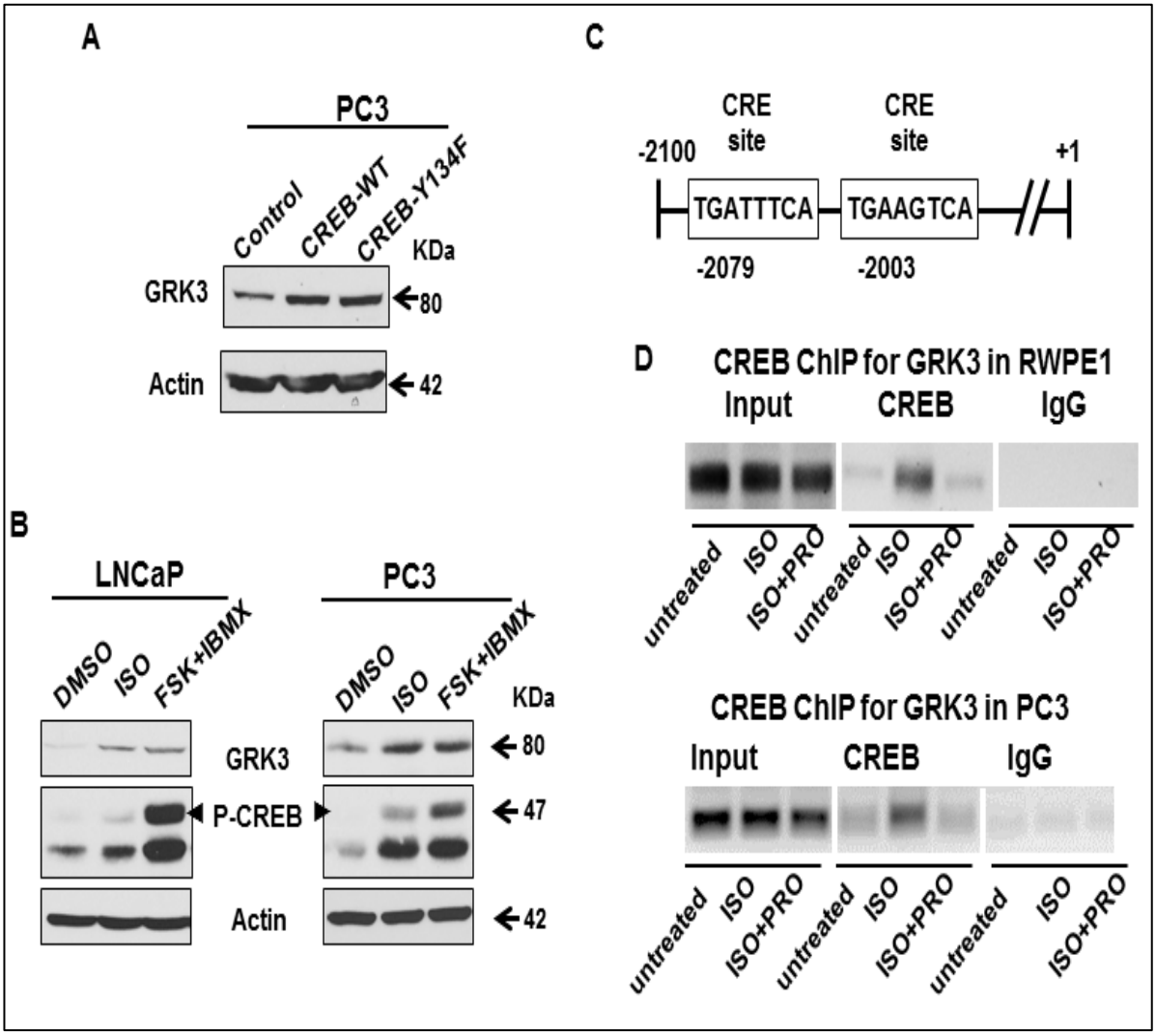

Figure 3.7. GRK3 is a direct transcriptional target of CREB activation. (A) Western blot shows that GRK3 expression is up-regulated in prostate cancer cells overexpressing CREB-WT and CREB-Y134F cDNAs. **(B)** PC3 and LNCaP cells were treated with 10 μM isoproterenol (ISO, beta-adrenergic receptor agonist), or 10 μM forskolin (FSK, adenylyl cyclase activator) + 0.5 mM IBMX (phosphodiesterase inhibitor) for 4 hours. Western blot analysis shows that CREB was hyper-phosphorylated at S133 and GRK3 was significantly up-regulated in both LNCaP and PC3 cells upon treatment with ISO or FSK+IBMX. **(C)** Two consensus cAMP

response element (CRE) sites, TGANNTCA, are located ~2000 bp upstream of the transcription initiation site in GRK3 promoter. **(D)** PC3 and RWPE1 cells were treated with 10 μM ISO or 10 μM ISO+propranolol (PRO, beta-adrenergic receptor antagonist). Chromatin immunoprecipitation (ChIP) was done with anti-CREB and anti-IgG antibodies, followed by PCR using primers designed to recognize the GRK3 promoter sequence around the CRE sites. The ChIP-PCR results were confirmed by DNA gel electrophoresis, using inputs as loading controls. The quantitative measurements of CREB binding to GRK3 promoter are shown in Fig. 3.9. *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

We further found two consensus cAMP response element (CRE) sites on GRK3 promoter (**Fig. 3.7 C**), which suggests that GRK3 is a direct transcriptional target of CREB activation. To confirm that CREB directly binds to GRK3 promoter, we carried out chromatin immunoprecipitation (ChIP) assay in PC3 and RWPE1 cells. The specific binding of CREB to GRK3 promoter was significantly increased after ISO treatment, and inhibited by beta-adrenergic receptor antagonist propranolol (PRO)[142] (**Fig. 3.7 D**, **Fig. 3.9**). These results indicate that GRK3 is a direct target of CREB activation.

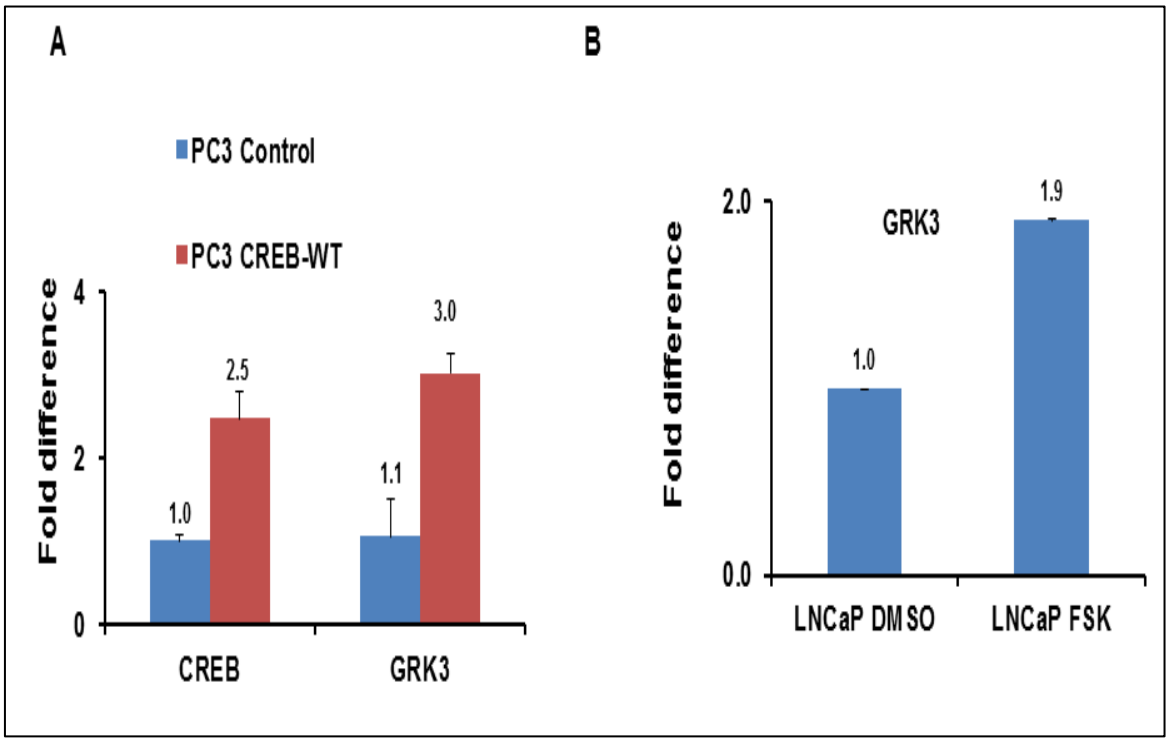

Figure 3.8. GRK3 expression is up-regulated upon CREB activation at mRNA level. (A) RT-PCRs showing the expressions of CREB and GRK3 in PC3 cells overexpressing CREB cDNA. GRK3 expression is up-regulated in cells overexpressing CREB. **(B)** PC3 cells were treated with CREB activator forskolin (FSK, 10 μM, 4 hours). GRK3 expression was up-regulated in cells treated with FSK. Y-axis shows the relative fold differences in expression, normalized to GAPDH.

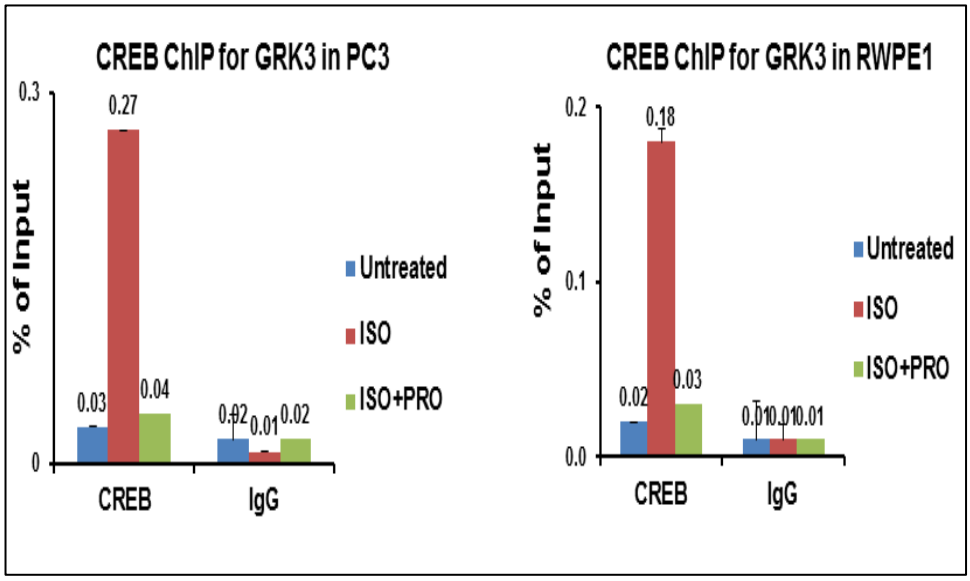

Figure 3.9. CREB binds to GRK3 promoter. The ChIP-PCR results were quantified for Fig. 3.9 as % binding of the input and plotted on the Y-axis. PC3 and RWPE1 cells were treated with 10 μM ISO (isoproterenol, beta-adrenergic activator) or 10 μM ISO + 10 μM PRO (propranolol, beta-adrenergic receptor antagonist). Chromatin immunoprecipitation (ChIP) was done with anti-CREB and anti-IgG antibodies, followed by PCR using primers designed to recognize the GRK3 promoter sequence around CRE sites.

GRK3 expression positively correlates with CREB and NE markers in human cancer tissues and cell lines.

Our results reveal that GRK3 is a direct target of CREB, which suggests a positive correlation between CREB and GRK3 expression in human cancer cells and tissues. Indeed, mRNA expression of CREB and GRK3 are positively correlated in several widely cited prostate cancer datasets, such as Yu_PCa[143] (GSE6919), Taylor_PCa[144] (GSE21034) and TCGA_PCa (obtained from www.cBioPortal.org[42,98]), with Pearson correlation coefficients r = 0.36, 0.44 and 0.52, respectively, and P<0.000001 for all three (**Fig. 3.10 A**). Furthermore, we carried out analysis of the levels of GRK3 protein and CREB activation (by pS133-CREB) in a tissue microarray with 78 cases of human prostate cancer and normal samples. The p-CREB level was found to positively correlate with the expression of GRK3 (Chi Square χ^2 = 22.2, P=0.0002) (**Fig. 3.10 B** and **Table 3.1**). These results support our finding that GRK3 is a target of CREB activation and suggest that the CREB/GRK3 axis is active in human prostate tissues.

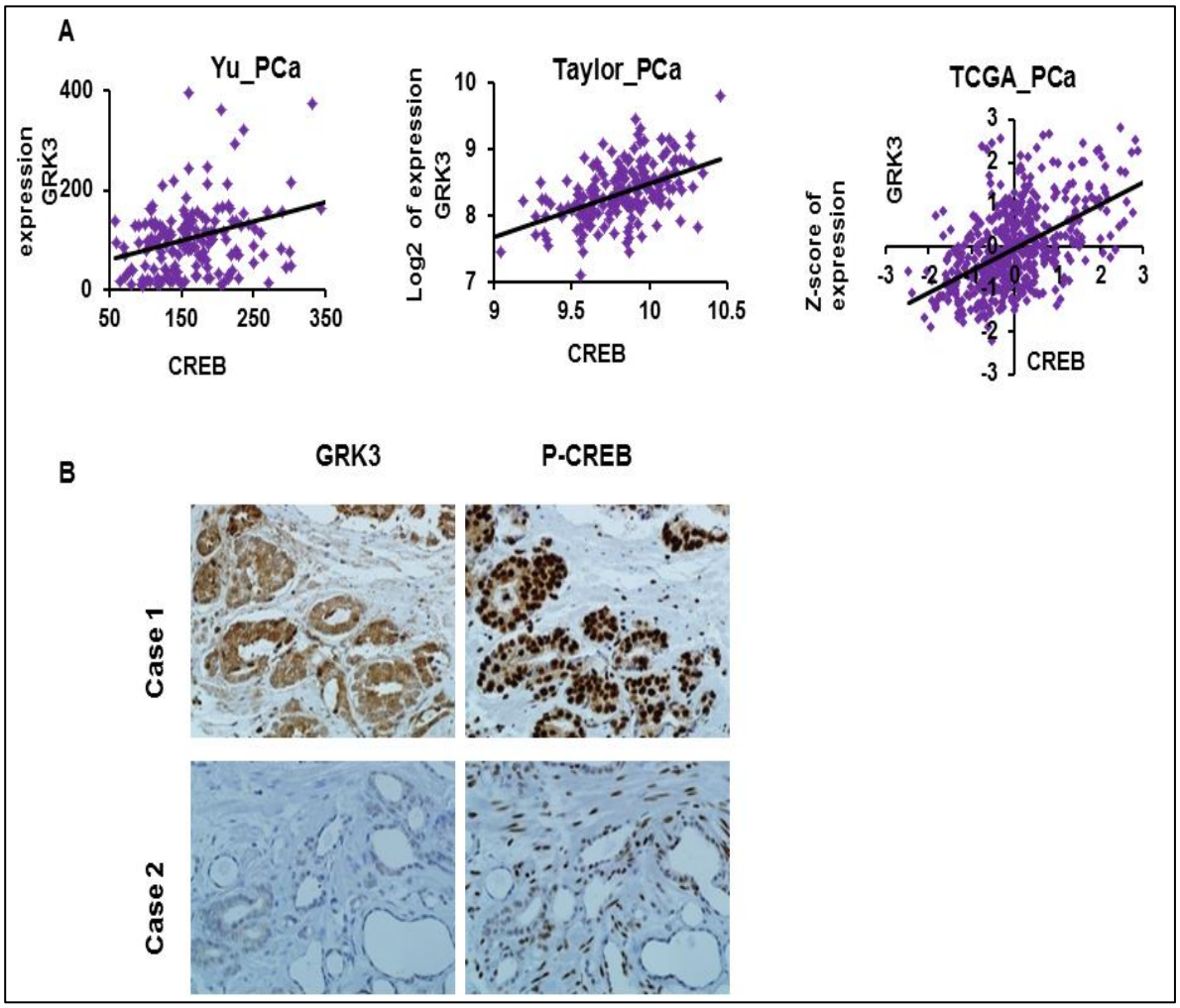

Figure 3.10. GRK3 expression positively correlates with CREB expressions. **(A)** Results from data mining of published prostate cancer datasets Yu_PCa, Taylor_PCa and TCGA_PCa for mRNA expressions of CREB and GRK3. CREB and GRK3 expressions positively correlate with each other, with Pearson correlation coefficients r = 0.36, 0.44 and 0.52, respectively, and P<0.000001 in all three datasets. **(B)** Immunohistochemistry staining was performed on a tissue microarray with 78 cases of human prostate cancer and normal samples using anti-GRK3 and

anti p-CREB (S133) antibodies. Staining results in each sample were scored according to the staining area and staining intensity on a scale of 1 to 3. Two representative tumor cases are shown. *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

Table 3.1. Results of IHC staining of tissue microarrays with 78 cases of human prostate cancer and normal samples. Chi-square test showed that p-CREB levels positively correlate with the expression of GRK3 (Chi Square $\chi^2 = 22.2$, P=0.0002)

p-CREB	GRK3		
	+ (1)	++ (2)	+++ (3)
+ (1)	22	7	1
++ (2)	7	9	5
+++ (3)	4	14	9

To determine if the positive correlation between CREB and GRK3 exists beyond prostate cancer, we analyzed their expression patterns in ~1000 human cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE)[145]. GRK3 expression indeed positively correlates with CREB expression (Pearson correlation coefficient r = 0.43, P<0.000001) (**Fig. 3.11 A**). To test whether GRK3 expression correlates with the expression of NE markers as well, we analyzed the CCLE cancer cell lines with measurable CHGA or CHGB expression (log2 transformed expression >4) and found that GRK3 expression positively correlates with CHGA (r = 0.41, P<0.00001) and CHGB expressions (r = 0.24, P<0.00001) (**Fig. 3.11 B**). These results suggest that positive correlation between GRK3 and NED markers exists broadly in human cancer cell lines.

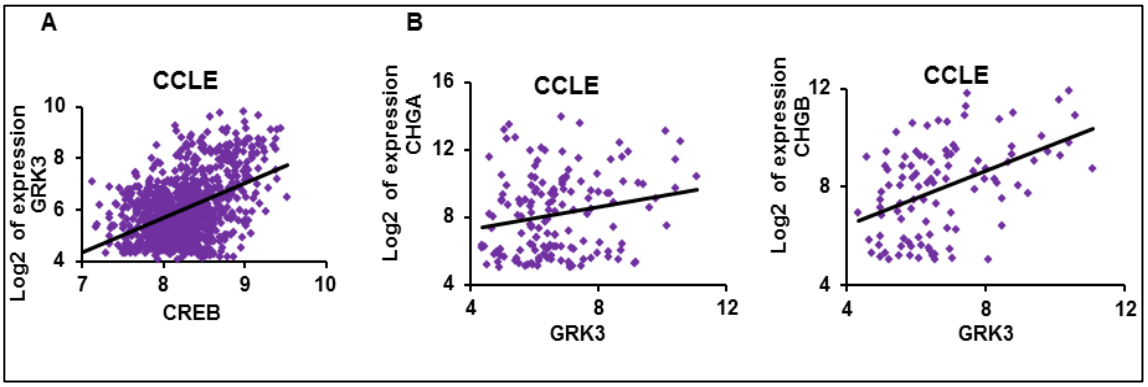

Figure 3.11. GRK3 expression positively correlates with CREB and NE marker expressions. (A) GRK3 expression positively correlates with CREB expression in ~1000 human cancer cell lines from the Cancer Cell Line Encyclopedia (Pearson correlation coefficient r = 0.43, P<0.000001). **(B)** GRK3 expression positively correlates with CHGA and CHGB expression in human cancer cell lines from the Cancer Cell Line Encyclopedia (P<0.00001 in both). Only cell lines with meaningful CHGA or CHGB level (log2 expression >4) were included in this analysis. The normalized and transformed expression values downloaded from GEO database or www.cBioPortal.org were used in our analysis and are plotted on X and Y-axes: normalized expression values for Yu_PCa, Z-scores of expression for TCGA_PCa, log2 transformed expression in all other scatter plots. *Results in this figure were obtained in collaboration with Li W.*

GRK3 is a critical activator for NE phenotypes of prostate cancer cells.

Upon showing that GRK3 is up-regulated in NEPC as a direct target of CREB activation, we next investigated whether GRK3 plays a critical role in promoting NED induced by ADT or CREB activation. Consistent with the literature[47,83], LNCaP cells developed features of neuronal morphology upon CREB activation by FSK+IBMX treatment (**Fig. 3.12 A**). As expected, the treatment also significantly increased expressions of NE markers CHGA, CHGB and ENO2 (**Fig. 3.12 B**, **Fig. 3.13**). We simultaneously carried out the same FSK+IBMX treatment on LNCaP cells expressing GRK3 shRNA# 1 or shRNA# 2 (**Fig. 3.12 C-D**). Notably, FSK+IBMX could no longer induce the expression of NE markers and obvious NE morphology upon GRK3 knockdown (**Fig. 3.12 C-D**). These results indicate that GRK3 is required for NED induction by CREB activation in LNCaP cells.

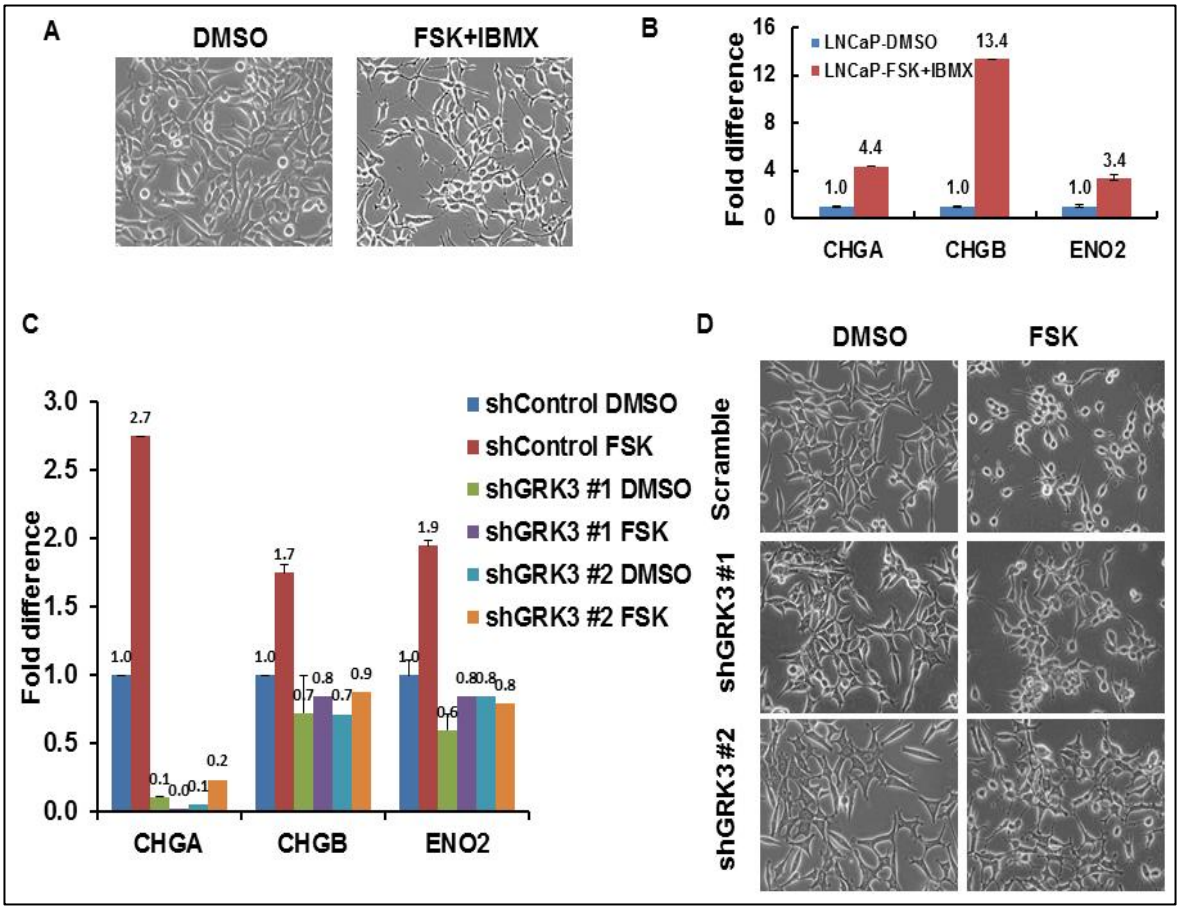

Figure 3.12. GRK3 is critical for CREB induced neuroendocrine differentiation of prostate cancer cells. (A) LNCaP cells exhibited a typical epithelial, fusiform morphology, tapering into unbranched processes typically less than one cell body length, whereas FSK+IBMX treatment (10 μM FSK + 0.5 mM IBMX) treatment resulted in a neuronal morphology with compact, rounded cell bodies and extended numerous long, fine, branched processes with defined growth cones. **(B)** RT-PCR comparing the expressions of NE markers CHGA, CHGB and ENO2 in the mock or FSK+IBMX treated LNCaP cells. Y-axis shows the relative fold differences in expression, normalized to GAPDH. **(C)**RT-PCR results show that the expressions of CHGA, CHGB and ENO2 could not be up-regulated with FSK+IBMX upon GRK3

down-regulation in LNCaP cells. Y-axis shows the relative fold changes in expression, normalized to GAPDH. **(D)** Representative images of LNCaP cells that express either Scramble control shRNA or two specific GRK3 shRNAs after treatment with either DMSO vehicle or forskolin (FSK, 10uM) for 4 ours. The GRK3 knockdown efficiency in LNCaP-shGRK3 cells is shown in Supplemental Fig. S6A. *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

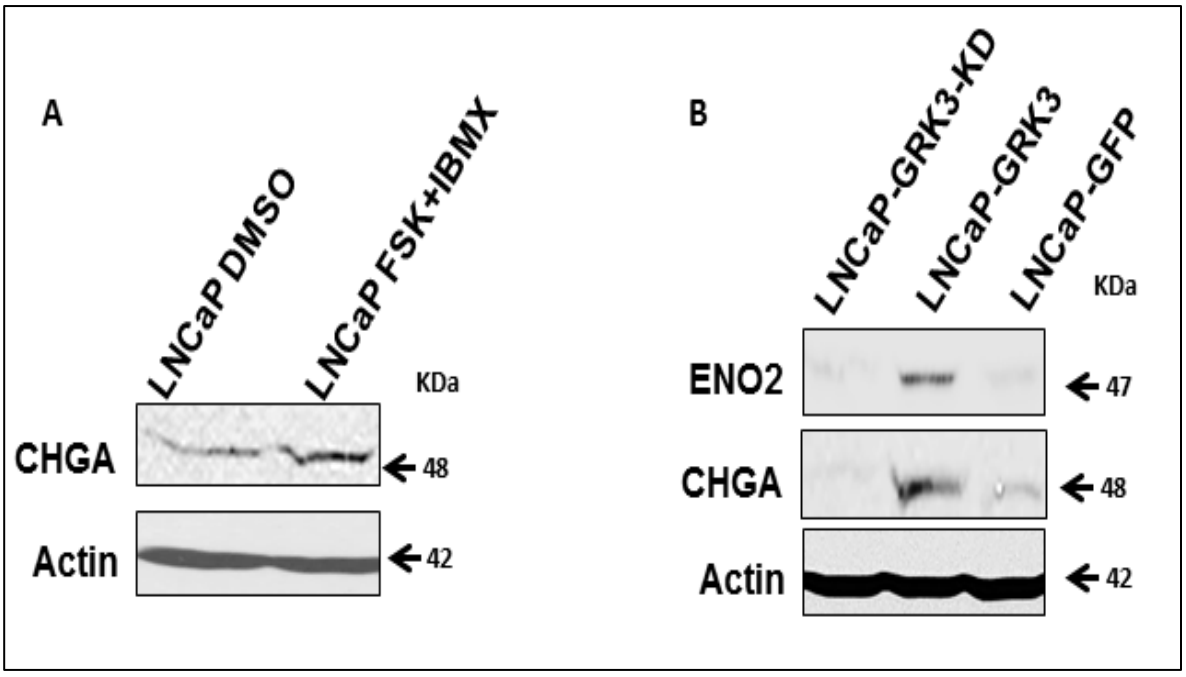

Figure 3.13. NE-marker expressions are up-regulated at protein level. (A) LNCaP cells were treated with CREB activator forskolin (FSK, 10 μM, 4 hours) and 0.5 mM IBMX (phosphodiesterase inhibitor, 4 hours). A western blot shows that treatment with forskolin and IBMX (FSK+IBMX) results in the higher expression of NE marker CHGA. **(B)** Overexpression of GRK3, but not its kinase-dead mutant GRK3-KD, results in the up-regulation of NE markers CHGA and ENO2 in LNCaP cells.

To evaluate whether GRK3 suppression is sufficient to reverse ADT-induced NED, we down-regulated GRK3 expression in ADT-induced NEPC cells NE1.3 using GRK3 specific shRNA[91] (**Fig. 3.14 B**). As shown in **Fig. 3.15 A**, the expression of NE markers CHGA, CHGB and ENO2 were reduced upon GRK3 down-regulation. In addition, the neuronal morphology – small and rounded cell bodies, and extended, fine branches –disappeared (**Fig. 3.15 B**). Notably, GRK3 knockdown inhibited the proliferation of NE1.3 cells (**Fig. 3.15 C**).

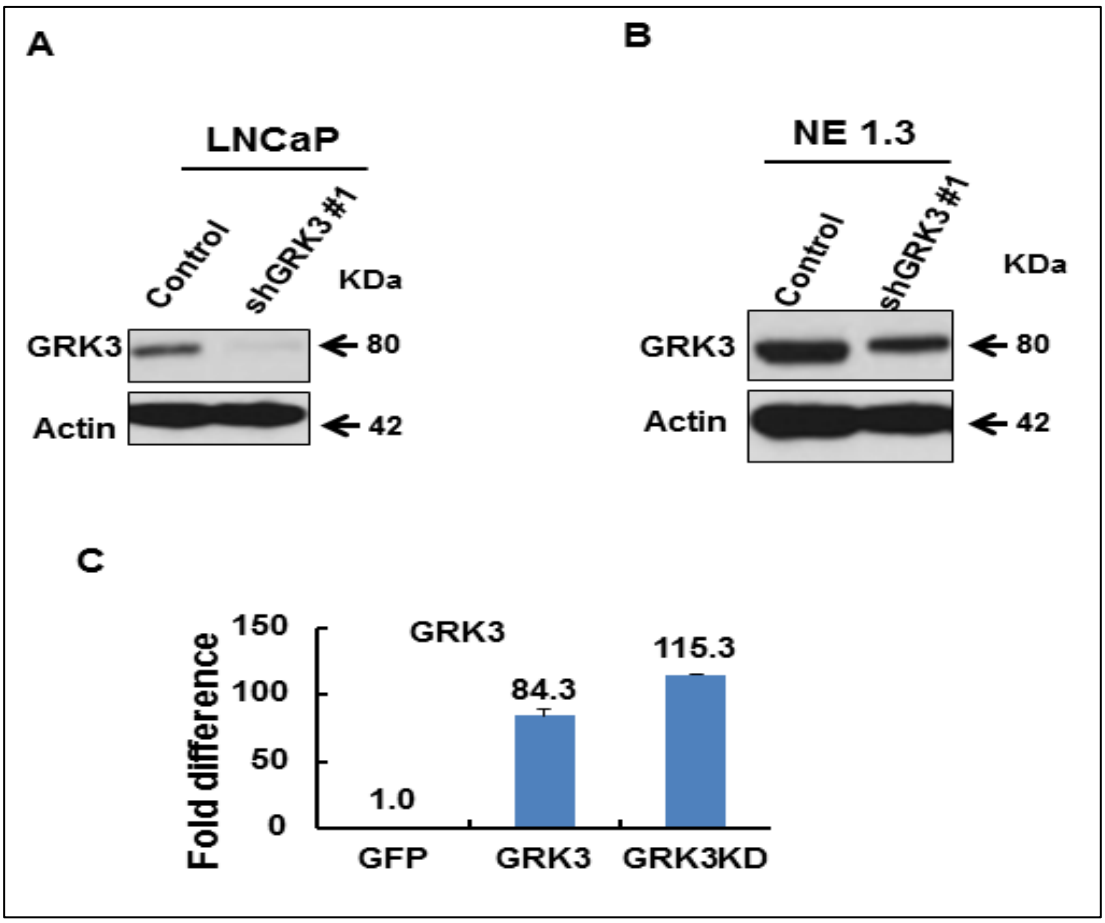

Figure 3.14. GRK3 expression is altered with shRNA and cDNA. (A-B) Western blots confirming the down-regulation of GRK3 in LNCaP-shGRK3 and NE1.3-shGRK3 cells. **(C)** RT-PCR confirming the overexpression of GRK3 in LNCaP-GRK3 and LNCaP-GRK3 KD cells.

To further establish an essential role of the CREB-GRK3 axis in NED of prostate cancer cells, we next tested the hypothesis that upon GRK3 knockdown in NEPC cells, inhibition of CREB cannot further repress the expression of NE markers. Results from propranolol (PROP), an inhibitor of beta-adrenergic signaling and CREB activation, provide evidences supporting this hypothesis (**Fig. 3.15 D**). This result, together with the data in **Fig. 3.12 C-D**, indicates that GRK3 is a key mediator of CREB activation in promoting NED of prostate cancer cells.

Finally, to directly examine a causal role of GRK3 in NED of prostate cancer cells, we overexpressed GRK3 wild type (WT) cDNA and kinase dead (KD) cDNA with a K220R mutation[91,146] in LNCaP cells (**Fig. 3.14 C**). GRK3-WT, but GRK3-KD does not, induced the expression of NE markers CHGA, CHGB, and ENO2, which suggests that the GRK3 kinase activity is required for its induction of NE markers (**Fig. 3.15 E**). Importantly, expressing GRK3-WT cDNA, rendered LNCaP cells more resistant to MDV3100 than expressing GFP control or GRK3-KD cDNA (**Fig. 3.15 F**). In addition, overexpressing GRK3 promoted LNCaP cell-derived primary tumor growth *in vivo* [37]. Collectively, these results indicate that GRK3 is a critical activator of NE phenotypes, ADT resistance, and progression of prostate cancer cells.

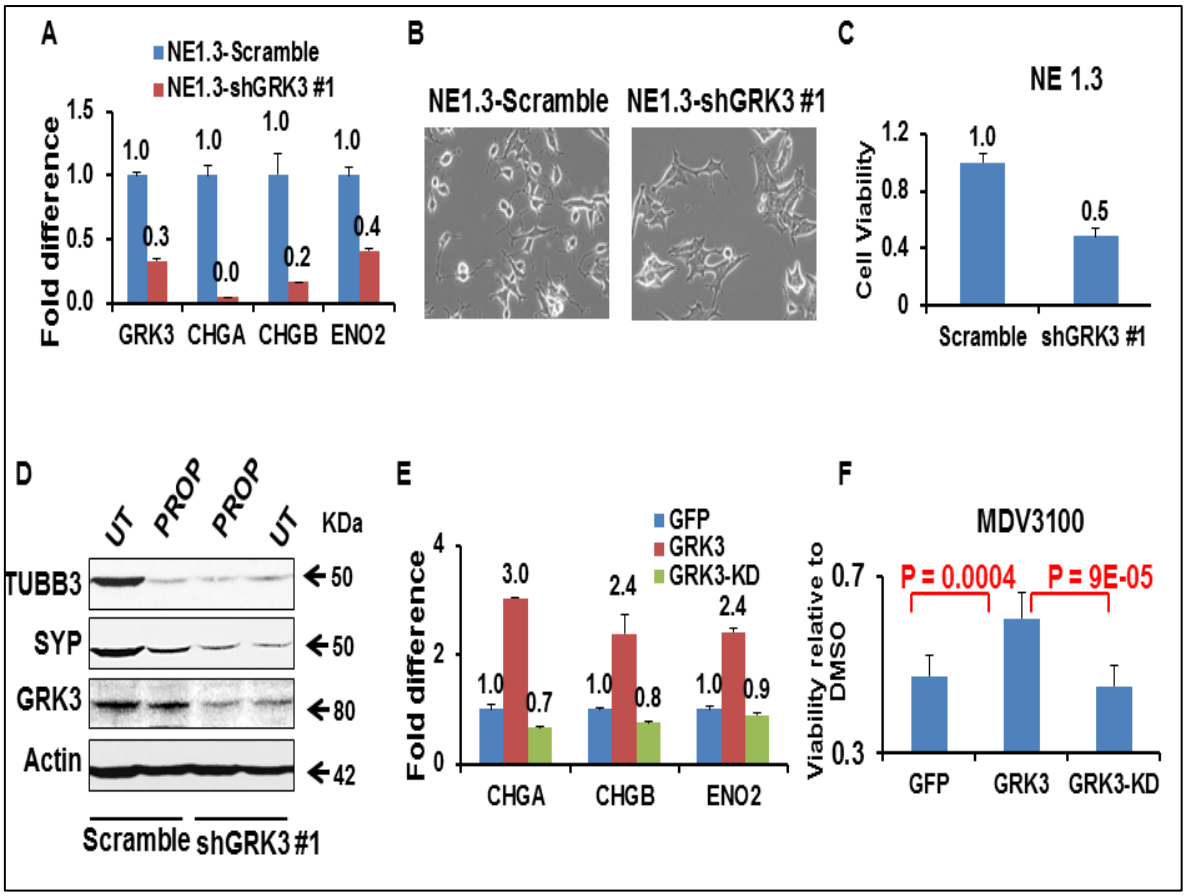

Figure 3.15. GRK3 controls neuroendocrine phenotypes of prostate cancer cells. (A) RT-PCR comparing the expressions of NE markers CHGA, CHGB and ENO2 in NE1.3 cells upon GRK3 down-regulation. Y-axis shows the relative fold differences in expression, normalized to GAPDH. **(B)** Representative pictures of NE1.3 cells expressing either Scramble control shRNA or GRK3 shRNA. Upon GRK3 down-regulation, the neuronal morphology of the NE1.3 cells (such as the compact, rounded cell bodies and extended and branched processes) disappeared. **(C)** NE1.3 cells with and without GRK3 down-regulation were seeded in 96 well plates and were allowed to grow for 72 hours (6 replicates). The cell numbers were measured using Alamar Blue® cell viability assay and the fold difference is plotted on

the Y-axis. **(D)** NE1.3 cells expressing Scramble or shGKR3 were either untreated (UT) or treated with beta-adrenergic receptor antagonist, propranolol (PROP, dissolved in H_2O, 10 μM, 4 hours), followed by western blotting analysis for expression of NE markers synaptophysin (SYP) and tubulin-beta III (TUBB3)[147,148]. **(E)** RT-PCR comparing the expression of NE markers in LNCaP cells upon overexpression of GFP, GRK3 (wild-type) or GRK3-KD (kinase dead) cDNA. Y-axis shows the relative fold differences in expression, normalized to GAPDH. **(F)** LNCaP-GFP, LNCaP-GRK3 and LNCaP-GRK3-KD cells were treated with DMSO vehicle or 5uM MDV3100 (enzalutamide) for 10 days. The cell numbers were measured using Alamar Blue® cell viability assay. The fold difference in viability in MDV3100 relative to DMSO for each cell lines are plotted on the Y-axis. P values were calculated using Student t-test, based on data from eight replicates. The GRK3 knockdown in NE1.3-shGRK3 cells and GRK3 overexpressing. *Results in this figure were obtained in collaboration with Sang M and Zhang XC.*

Summary

In this study, we demonstrated that GRK3 is induced by androgen deprivation treatment (ADT) as a direct target of ADT-activated CREB, and that expression of GRK3 positively correlates with expression and activity of CREB in prostate cancer cells and tissues. Of note, overexpression of GRK3 is sufficient to promote neuroendocrine differentiation (NED) and resistance to MDV3100, while GRK3 silencing blocks CREB-induced NED, reverses NE phenotypes, and inhibits proliferation of NEPC cells. These results suggest that ADT activates a critical signaling pathway, the CREB/GRK3 axis, in promoting NED of prostate cancer cells.

Chapter Four:

Beta-adrenergic signaling promotes tumor angiogenesis and prostate cancer progression through HDAC2-mediated suppression of thrombospondin-1

This work is based on

Beta-adrenergic signaling promotes tumor angiogenesis and prostate cancer progression through HDAC2-mediated suppression of thrombospondin-1.

Hulsurkar M, Li Z, Zhang Y, Li X, Zheng D, Li W. In press in ***Oncogene.***

Background

Chronic behavioral stress and cancer progression

Behavioral stress and β-adrenergic signaling lead to cardiac hypertrophy and congestive heart failure (CHF)[149,150], and have been increasingly associated with cancer progression[18,23,151]. Recently, it was shown that chronic behavioral stress and activated β-adrenergic signaling promote cancer progression in ovarian and prostate cancers[18,23]. The cAMP/PKA signaling pathway was shown to be necessary for behavioral stress mediated cancer progression[18,23], through induction of angiogenesis in ovarian cancer[23] and activation of a PKA/BAD anti-apoptotic signaling pathway in prostate cancer[18]. In addition, epidemiology studies on melanoma, breast, lung and prostate cancers indicated that cancer patients who took β-blockers, the hypertension drugs that interfere with signaling of the stress hormones adrenaline and noradrenaline, had better cancer clinical outcomes[23,32,34,152,153].

Histone deacetylase 2 (HDAC2)

Histone proteins bind to eukaryotic DNA to form nucleosomes, the basic structural unit that allows the DNA to be packaged into chromatins[154,155]. As a part of epigenetic regulation of gene expression, these histones are modified, changing the conformation of chromatin[156,157]. These modifications of histone, including methylation/de-methylation, acetylation/de-acetylation, control the extent to which the DNA is wrapped to histones[156,157]. Acetylation of histones results into more 'relaxed' conformation, resulting into increased transcription of the target genes, whereas deacetylation results into gene silencing[158]. Histone deacetylates (HDACs) are a group of proteins that catalyze this reaction[159]. There are four classes of HDACs [160,161]. Class I (HDACs 1, 2, 3 and 8) Class IIA and IIB (HDACs 4, 5, 6, 7, 9 and 10) and Class IV (HDAC 11) form the classical family of HDACs[160-162]. Epigenetic regulation by HDACs is necessary for chronic behavioral stress to induce cardiac hypertrophy[87-89]. Specifically, HDAC2 has been implicated as a key mediator in this process[90]. However, it is not known whether and how HDAC2 acts as a downstream mediator of stress induced cancer progression.

Thrombospondin 1

Thrombospondin 1 (TSP1) was the first identified endogenous inhibitor of angiogenesis[163-165]. TSP1 is a 140 KDa glycoprotein that was initially identified to be secreted from platelets[166-168]. TSP1 potently inhibits angiogenesis directly by interfering with endothelial cell migration and survival[165,169]. TSP1 also acts as an antagonist for VEGF and inhibits its action through multiple pathways[165,169] (**Fig. 4.1**). Suppression of TSP1 results in increased angiogenesis[165]. Interestingly, TSP1 is overexpressed upon treatment with HDAC inhibitors (HDACi) [170-172], suggesting that it is repressed by HDACs.

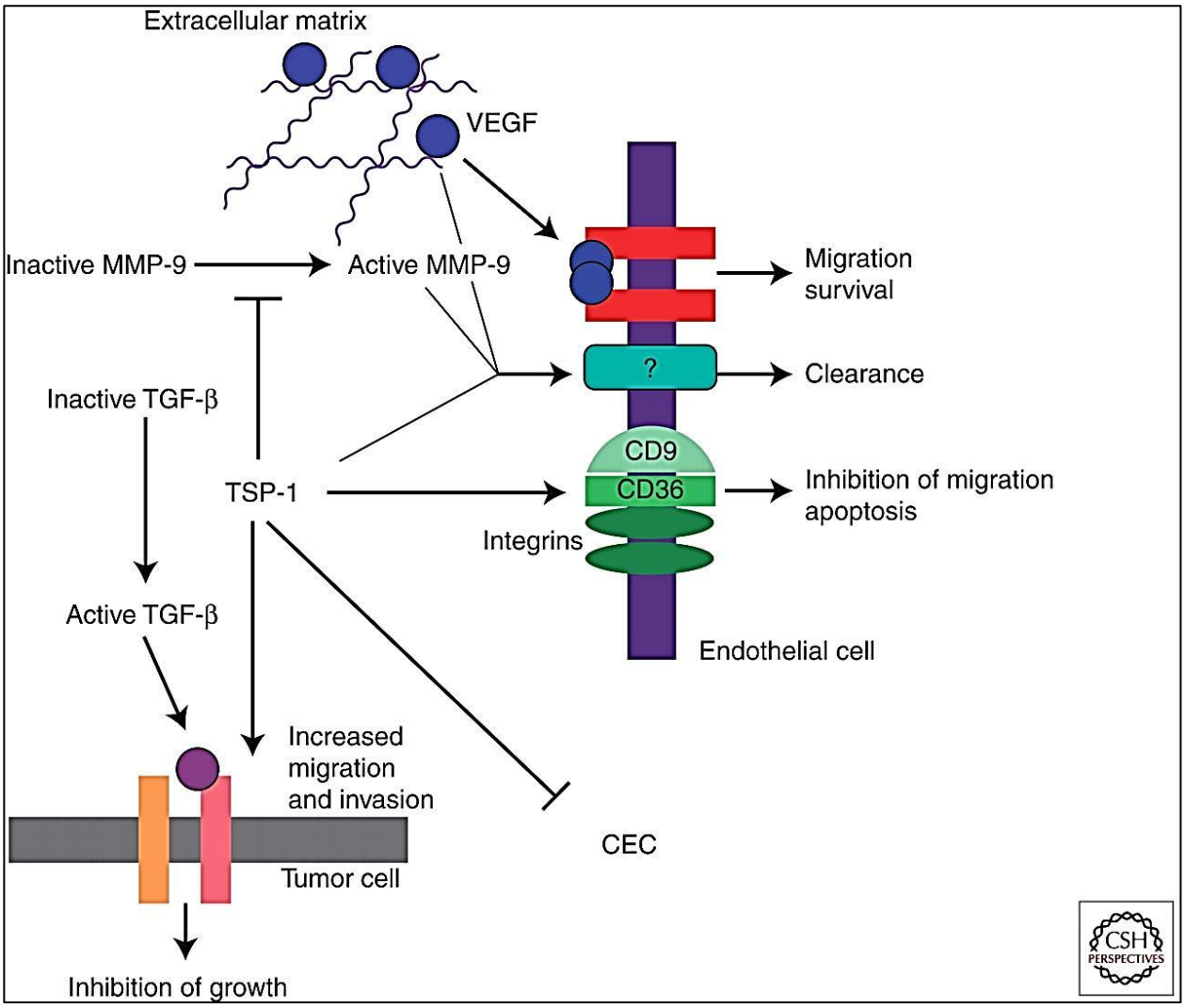

Figure 4.1 Schematic representation of the role of TSP-1 in the tumor microenvironment.

Taken with permission from Patrick R. Lawler, and Jack Lawler Cold Spring Harb Perspect Med 2012;2:a006627. ©2012 by Cold Spring Harbor Laboratory Press.

Working hypothesis

We hypothesize that activated by β-adrenergic signaling, CREB activates HDAC2 transcription and HDAC2 further represses TSP1 expression leading to induction of angiogenesis and cancer progression. In this study, we show that upon activation of the β-adrenergic signaling pathway, its downstream effector molecule, CREB, directly binds to the promoter of HDAC2 and induces its expression. HDAC2 in turn suppresses TSP1 expression epigenetically, thus inducing angiogenesis and ultimately promoting β-adrenergic signaling-mediated prostate cancer progression.

Results

Chronic behavioral stress induces tumor progression *in vivo*

To investigate the mechanisms through which chronic behavioral stress promotes prostate cancer progression, we injected PC3 cells stably expressing luciferase into NOD/SCID mice and subjected the mice to chronic stress. The tumor growth was monitored non-invasively by imaging the luciferase-expressing tumor cells. After establishing the baseline of tumor growth, the mice were randomly divided into two groups – 'calm' and 'stress' groups. The stressed mice were subjected to behavioral stress by physically restraining their movement twice daily, for 1 hour. This periodic physical restraint system has been reported to induce chronic behavioral stress in mice[23]. After 25 days, the mice in the 'stress' group showed a 15.3-fold increase in the tumor size (measured by luminescence signal) compared to the 1.4-fold increase in the control 'calm' group ($P = 0.02$, Student's t-test) (**Fig. 4.2**).

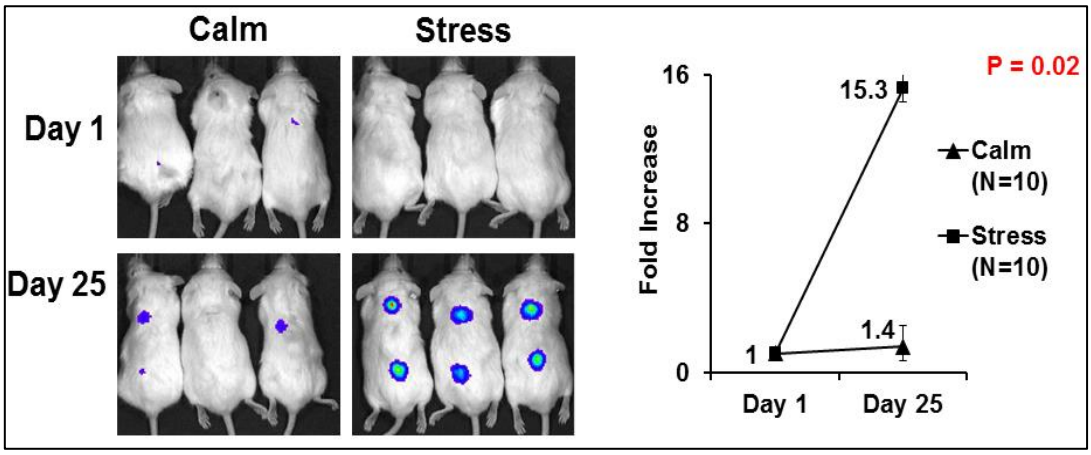

Figure 4.2. Chronic stress promotes the growth of mouse xenografts of prostate cancer cells. Representative mice images showing that chronic behavioral stress promoted PC3 tumor growth in mouse xenografts. Luciferase labelled PC3 cells were injected s.c. into both the flanks and shoulders of NOD/SCID mice. Three days after injection, the mice were randomly assigned to the undisturbed Calm group, or to the Stress group where the mice were subjected to restraint behavioral stress for one hour, twice a day for 25 days (n=12 tumors, 6 mice/group).

Activation of cAMP/PKA pathway downstream of the β-adrenergic receptors has been shown to mediate the effects of chronic behavioral stress in C4-2 prostate cancer xenograft models[18]. We next investigated whether activation of β-adrenergic signaling similarly induces xenograft tumor growth of prostate cancer cells PC3 and LNCaP. We used isoproterenol (ISO), a β-adrenergic receptor agonist, to stimulate β-adrenergic signaling[173,174]. Luciferase-expressing PC3 and LNCaP cells were injected into the mice. After establishing the baseline of tumor growth, the mice were divided into 2 groups. The control group received buffered saline; the 'ISO' group received 10 mg/kg ISO twice a day for 21 days (PC3 tumors) or 56 days (LNCaP tumors). Consistent with the literature[23], the tumor growth was faster in the ISO treated group than in the control group ($P = 0.04$ for PC3 and $P= 0.05$ for LNCaP tumors, Student's t-test) (**Fig. 4.3a-b**). Downstream of chronic stress/β-adrenergic receptor, CERB activation is responsible for ovarian cancer progression[23]. Therefore, we investigated whether the overexpression of constitutively active CREB promotes prostate cancer progression. We injected PC3 cells overexpressing CREB Y134F, a constitutively active CREB mutant, into NOD/SCID mice. We found that the tumor growth was faster in the mice injected with PC3 cells with CREB Y134F ($P=0.02$, Student's t-test) (**Fig. 4.3c**). These results show that both chronic behavioral stress and activation of β-adrenergic signaling pathway promote tumor growth in xenograft mouse models for prostate cancer cells.

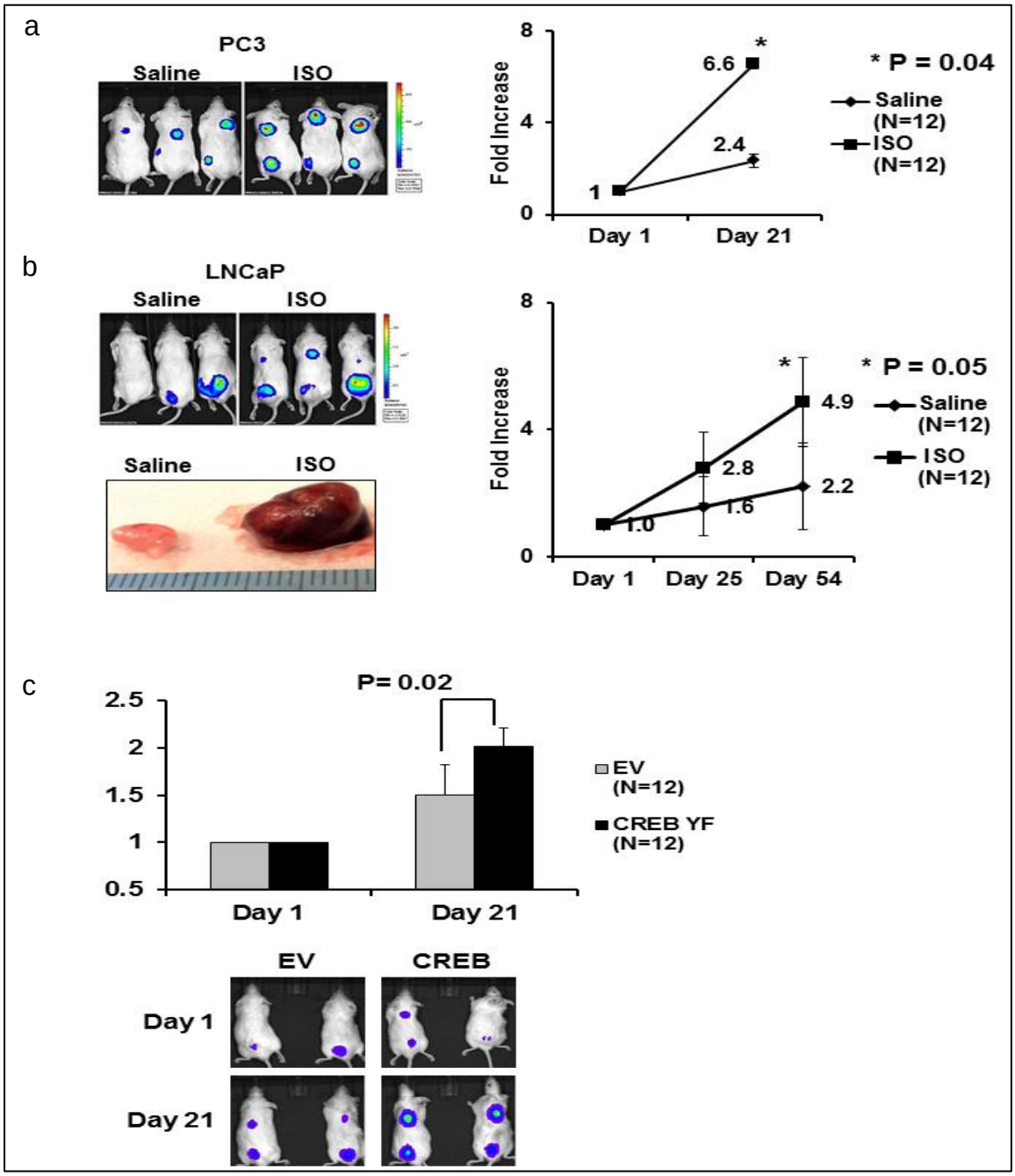

Figure 4.3. Chronic stress and the activation of β-adrenergic signaling promote the growth of mouse xenografts of prostate cancer cells. (a-b) Representative mice images showing that the activation of β-adrenergic signaling in mice promoted the growth of tumor xenografts for PC3 (a) and LNCaP cells (b).

Mice were randomly assigned to receive either saline or 10 mg/kg ISO twice a day for 21 days (PC3, B) or 56 days (LNCaP, C) (n=12 tumors, 6 mice/group). The growth of each tumor was quantified using IVIS Lumina II platform. Fold increases on Y-axes were relative to day one. (c) Representative mice images showing that the overexpression of constitutively active CREB promoted the growth of tumor xenografts for PC3 cells. Luciferase labelled PC3 cells with or without overexpressing constitutively active CREB were injected s.c. into both the flanks and shoulders of NOD/SCID mice (n=12 tumors, 6 mice/group). Tumor growth was monitored for 21 days and fold increase for each tumor was calculated compared to day one.

Activation of β-adrenergic signaling induced angiogenesis

Chronic stress has been shown to promote cancer progression by inducing tumor angiogenesis in ovarian carcinoma, which is mediated by β-adrenergic signaling[23]. We investigated if activation of β-adrenergic signaling by ISO treatment increased tumor angiogenesis in our xenograft prostate cancer models. Tumor tissues from control and ISO-treated mice were stained for Isolectin B4 (IB4), an angiogenic marker[175,176]. As shown in **Fig. 4.4a**, IB4 levels were elevated in tumors from ISO-treated mice, indicating an increase in angiogenesis. In addition to this *in vivo* finding, we evaluated the effects of activation of β-adrenergic signaling in prostate cancer cells on endothelial cell migration and tube formation, two commonly used *in vitro* angiogenesis assays[177,178]. Using conditioned medium from cancer cells to treat endothelial cells for studying their migration and tube formation abilities mimics the *in vivo* angiogenesis processes[177]. Conditioned media from PC3 cells treated with ISO increased the migration of SVEC4-10 cells, an endothelial cell line widely used for angiogenesis assays[179] (**Fig. 4.4b**). In addition, SVEC4-10 cells formed more tubes (capillary-like structures), when incubated with conditioned medium from ISO-treated PC3 cells **(Fig. 4.4c)**. Taken together, these data show that activation of β-adrenergic signaling in prostate cancer cells induces angiogenesis *in vitro* and *in vivo*.

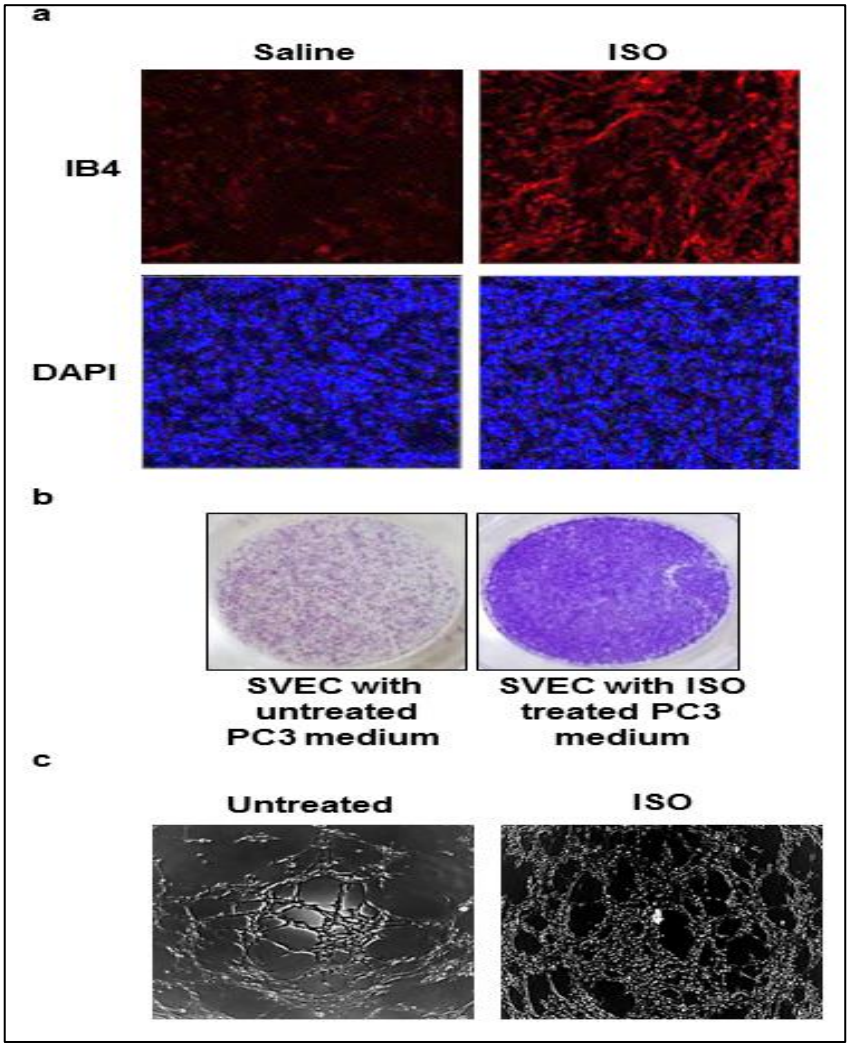

Figure 4.4. Activation of β-adrenergic signaling induces angiogenesis. (A) A representative image for immunofluorescent (IF) staining of PC3 xenograft tumor tissues shows that ISO treatment increased the levels of IB4 (red), an endothelial marker and an indicator of angiogenesis. Tissue sections of multiple tumors from the untreated and ISO groups were stained for IB4. DAPI was used to visualize cell nuclei. **(B)** SVEC endothelial cell migration assay. Conditioned media from 16 hours 10 μM ISO treated or untreated PC3 cells were used to induce migration of serum-

starved SVEC4-10 cells seeded on top of Boyden chambers. The assays were performed 3 times with similar results and the representative images are shown. **(C)** SVEC endothelial cells tube formation assay. Conditioned media from 16 hours 10 μM ISO-treated or untreated PC3 cells was used to culture serum-starved SVEC cells, seeded on growth factor reduced matrigel. The assays have been done at least 3 times with comparable results. All the results were confirmed by reproducing at least once.

HDAC2 is a downstream mediator of the β-adrenergic signaling induced angiogenesis

Trivedi et al. showed that HDAC2 is critical for stress and β-adrenergic activation-induced congestive heart failure (CHF) [90]. Whether β-adrenergic signaling directly regulates HDAC2 in CHF and whether this pathway is activated to promote cancer progression are unclear. Therefore, we next investigated the regulation of HDAC2 expression by β-adrenergic signaling and its role as a downstream mediator of the β-adrenergic signaling pathway in promoting angiogenesis. HDAC2 levels were found to be increased upon ISO activation of β-adrenergic signaling in PC3 and DU145 cells (**Fig. 4.5a**). We then used LNCaP derived neuroendocrine prostate cancer cells NE1.3[93], in which CREB is hyper-phosphorylated (**Fig. 4.5b,** left), to study the effect of inhibition of CREB on HDAC2 expression. We inhibited CREB directly by protein kinase A inhibitor peptide 14-22 (PKI) as well as indirectly by β-adrenergic receptor antagonists ICI118, 551(ICI) and propranolol (PRO). Protein kinase A phosphorylates CREB at S133 and activates it. Therefore, PKI treatment results into inactivation of CREB. On the other hand, ICI and PRO inhibit the β-adrenergic signaling pathway, leading to inactivation of CREB. Upon treatments with these CREB inhibitors, HDAC2 was indeed downregulated (**Fig. 4.5b,** right). Moreover, HDAC2 expression was upregulated upon the overexpression of wild-type CREB cDNA and constitutively active mutant CREB-Y134F cDNA[95] (**Fig. 4.5c**). In addition to these *in vitro* findings, activation of β-adrenergic signaling *in vivo* by ISO treatment in LNCaP xenograft tumors induced HDAC2 expression (**Fig. 4.5d)**.

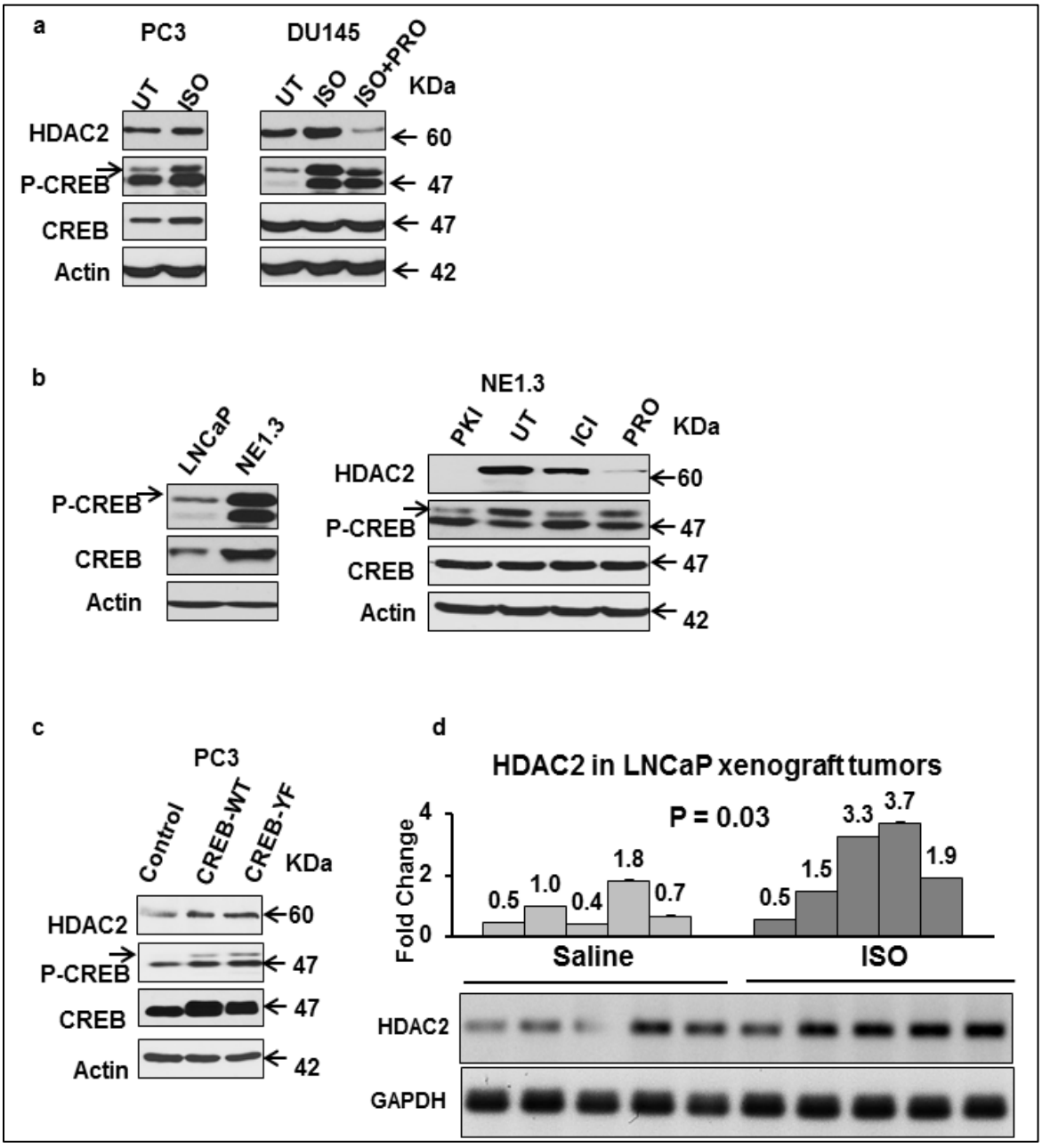

Figure 4.5. HDAC2 expression is upregulated upon activation of β-adrenergic signaling pathway. (A) PC3 and DU145 cells were untreated (UT), or treated with 10μM isoproterenol (ISO, β-adrenergic receptor agonist), or 10 μM ISO + 10 μM propranolol (PRO, β-adrenergic receptor antagonist) for 4 hours. **(B)** Inhibition of PKA/CREB signaling in CREB-high NE1.3 cells led to HDAC2 downregulation.

LNCaP-derived neuroendocrine prostate cancer cells NE1.3 were untreated (UT), or treated with 10 μM PKI (PKA inhibitor), ICI 118 551 (ICI, β-adrenergic receptor antagonist) or PRO for 4 hours. Levels of HDAC2, total CREB, and pS133-CREB were measured by western blotting. **(C)** HDAC2 expression was elevated when wild-type CREB or constitutively active mutant of CREB (CREB Y134F) was overexpressed in PC3 cells. **(D)** Quantitative RT-PCR result (**top**) shows that HDAC2 expression is upregulated in LNCaP xenograft tumors from mice treated with ISO. Y-axis shows the relative fold changes in expression, normalized to GAPDH. P-value was calculated using Student's t-test. The PCR products were also analyzed by DNA gel electrophoresis (**bottom**). **(E-G)** Analyses of ~1000 human cancer cell lines in the CCLE database (**E**), the TCGA prostate cancer dataset (**F**), and metastatic prostate cancer samples by the SU2C/PCF Dream Team (**G**) [42,98,180,181] showed that the expressions of HDAC2 and CREB are positively correlated. All the western blots results were confirmed by reproducing at least once.

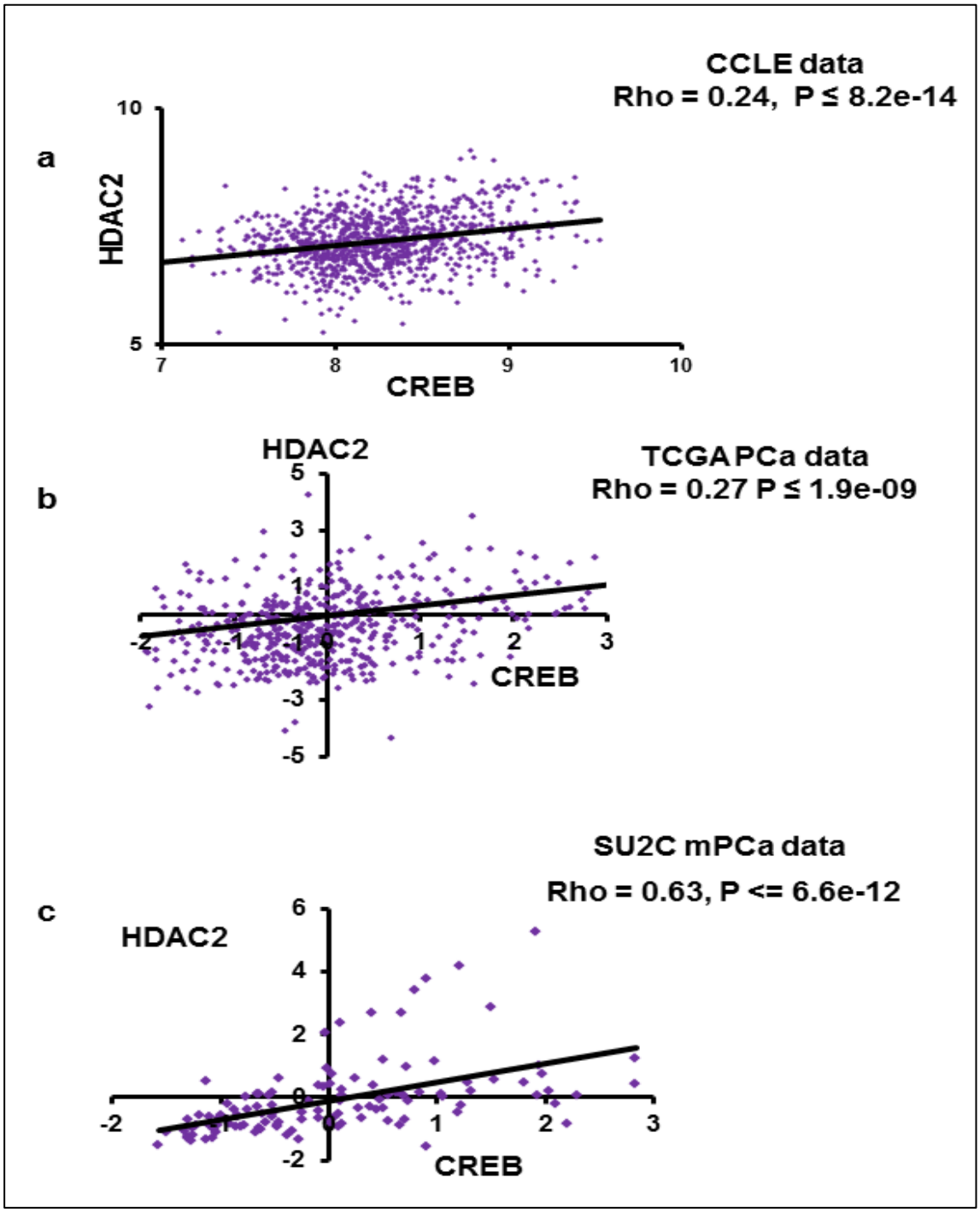

Figure 4.6. Correlation between HDAC, CREB and TSP1 expressions. (a-c) Analyses of ~1000 human cancer cell lines in the CCLE database (**a**), the TCGA prostate cancer dataset (**b**), and metastatic prostate cancer samples by the

SU2C/PCF Dream Team (**c**) [42,98,180,181] showed that the expressions of HDAC2 and CREB are positively correlated. All the western blots results were confirmed by reproducing at least once.

Since CREB is a key downstream effector of β-adrenergic signaling pathway, we postulated that CREB may directly regulate HDAC2 expression. Analysis of The *Cancer Cell Line Encyclopedia* (CCLE) database[145] showed that CREB expressions are positively correlated with those of several HDACs in ~1000 human cancer cell lines, with HDAC2 having the strongest correlation with CREB expression (Spearman's correlation Rho = 0.24 and P <= 8.2e-14) (**Fig. 4.6a,** and **4.7a**). In addition, according to the data obtained from cBioPortal for cancer genomics, HDAC2 and CREB expressions are also positively correlated in TCGA prostate cancers (Rho = 0.27, N = 487, P <= 1.9e-09, **Fig. 4.6b**)[42,98,180,181] and in metastatic prostate cancer samples comprehensively analyzed by the SU2C/PCF Dream Team (Rho = 0.63, N = 118, P <= 6.6e-12, **Fig. 4.6c**)[42,98,180,181]. These correlations may appear relatively modest, with Rho = 0.24, 0.27 and 0.63, respectively. However, considering the large number of samples analyzed by these datasets and the heterogeneity of cancers, these correlations are significant.

Interestingly, there are three full length CREB binding sites (TGANNTCA) at the HDAC2 promoter (**Fig. 4.8a**). To investigate if CREB binds to the HDAC2 promoter at these putative CRE sites, we performed a ChIP-PCR assay by using anti-CREB, anti-pS133-CREB antibodies, and PCR primers designed from the HDAC2 promoter region harboring these CRE sites. CREB indeed bound to the promoter region of HDAC2 in PC3 and DU145 cells (**Fig. 4.8b-c)**. Notably, the binding increased upon ISO treatment, and decreased with additional treatment of propranolol (PRO), an antagonist of β-adrenergic signaling. These results indicate that HDAC2 is a direct target of CREB transcriptional activation.

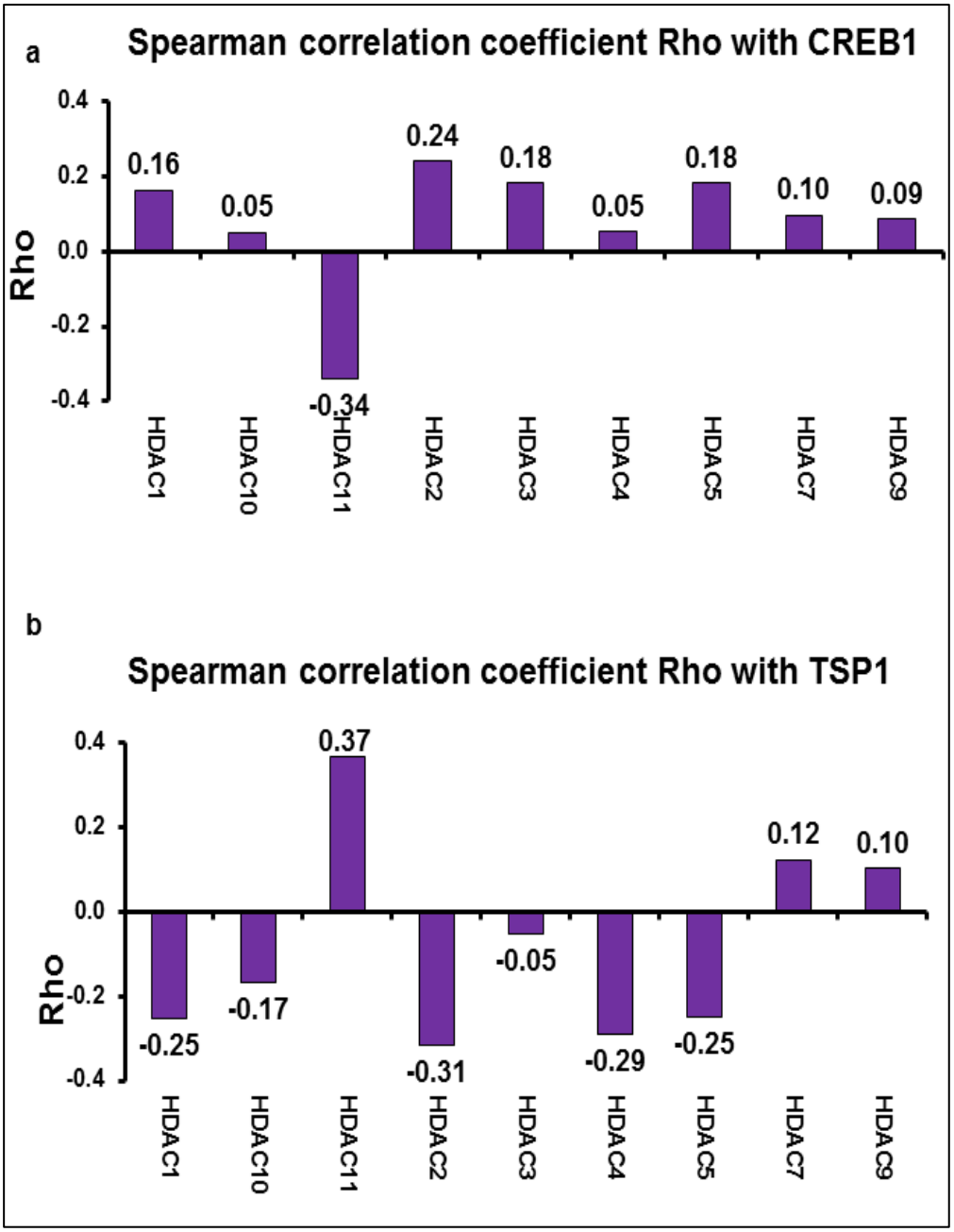

Figure 4.7. Correlation between HDAC, CREB and TSP1 expressions. (a) Analysis of The *Cancer Cell Line Encyclopedia* (CCLE) database[145] showed that CREB expressions are positively correlated with those of several HDACs in ~1000 human cancer cell lines. Correlation between HDAC2 and CREB expressions is the

strongest (Spearman's correlation Rho = 0.24 and P <= 8.2e-14). **(b)** Analysis of the CCLE database[145] showed a strong negative correlation between TSP1 and several HDACs in ~1000 human cancer cell lines. Correlation between HDAC2 and CREB expressions is the strongest (*Spearman's rank correlation* coefficient Rho= -0.31, P <1.38e-22).

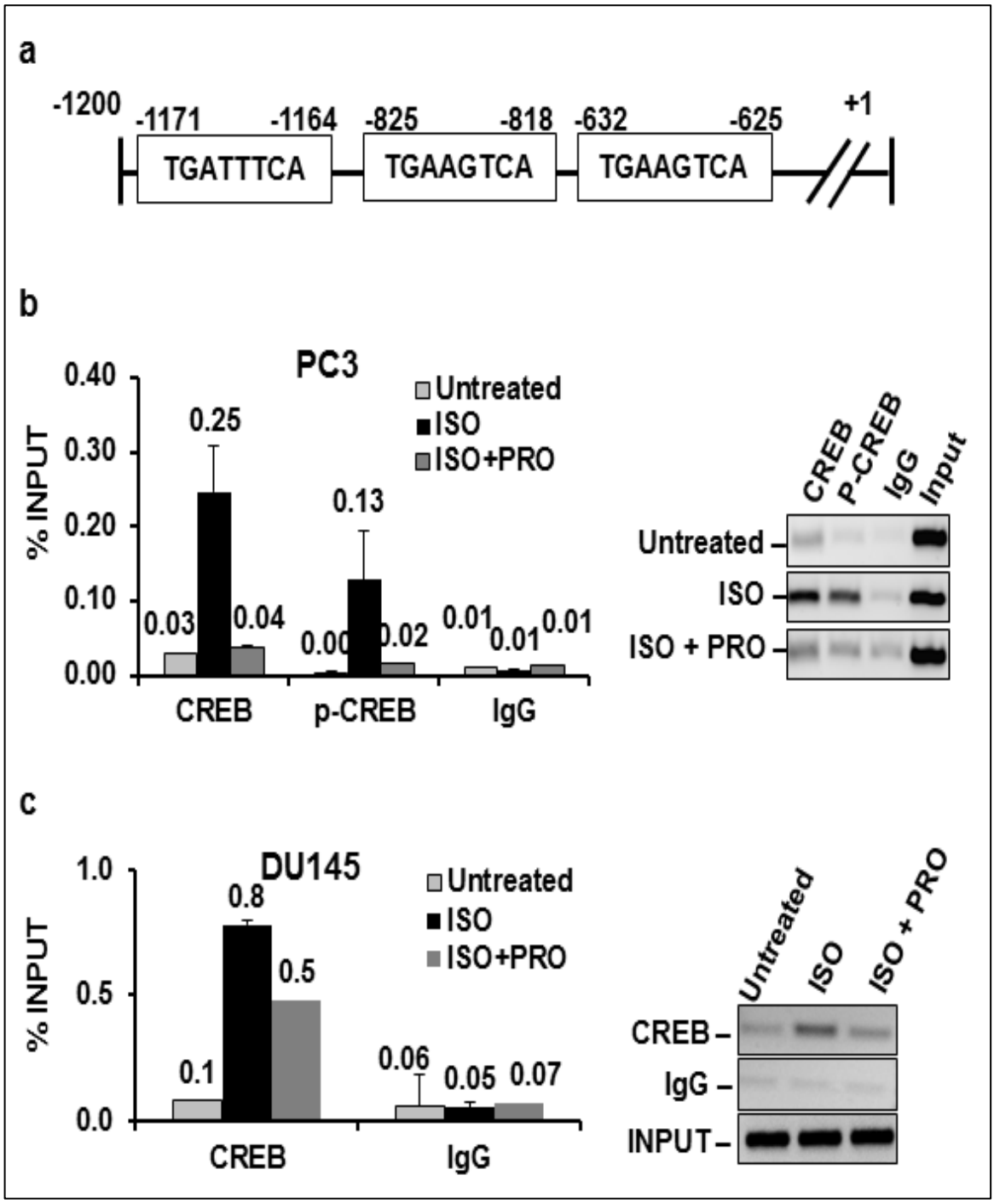

Figure 4.8. CREB induces HDAC2 expression by binding to its promoter. (A) Three putative cAMP-response elements (CRE) in the promoter region of HDAC2. **(B-C)** PC3 and DU145 cells were treated with 10 μM ISO, or 10 μM ISO + 10 μM

PRO for 4 hours. A ChIP assay was conducted using anti-CREB antibodies or IgG control, followed by PCR to amplify the HDAC2 promoter region around the putative CRE sites. The ChIP-PCR result was presented as % of the input, which was confirmed through DNA gel electrophoresis. All the ChIP results were confirmed by reproducing at least once.

We next determined whether HDAC2 acts as a critical downstream mediator for β-adrenergic signaling to promote cancer progression. We injected luciferase-expressing PC3 cells, with/without stable HDAC2 knockdown (**Fig. 4.9a**), into NOD/SCID mice. After establishing the baseline of tumor growth, the mice injected with PC3-shHDAC2 cells were divided into 2 groups. The control group received buffered saline, the 'ISO' group received 10 mg/kg ISO twice a day for 21 days. Consistent with the literature[23], the tumor growth was faster in the ISO treated group than in the control group (P = 0.005, Student's t-test). However, ISO activated β-adrenergic signaling was not able to induce tumor growth upon HDAC2 down-regulation (P = 0.001, Student's t-test) (**Fig. 4.9b)**. To confirm the role of HDAC2 in mediating the effect of activated CREB overexpression *in vivo*, we injected PC3 cells overexpressing CREB Y134F, a constitutively active CREB mutant with stable HDAC2 knockdown, into NOD/SCID mice. We found that the tumor growth was slower in the mice injected with PC3 cells with both, CREB Y134F and HDAC2 knockdown, compared to PC3 cells with only CREB Y134F overexpression. (P=0.02, Student's t-test) (**Fig. 4.9c**).

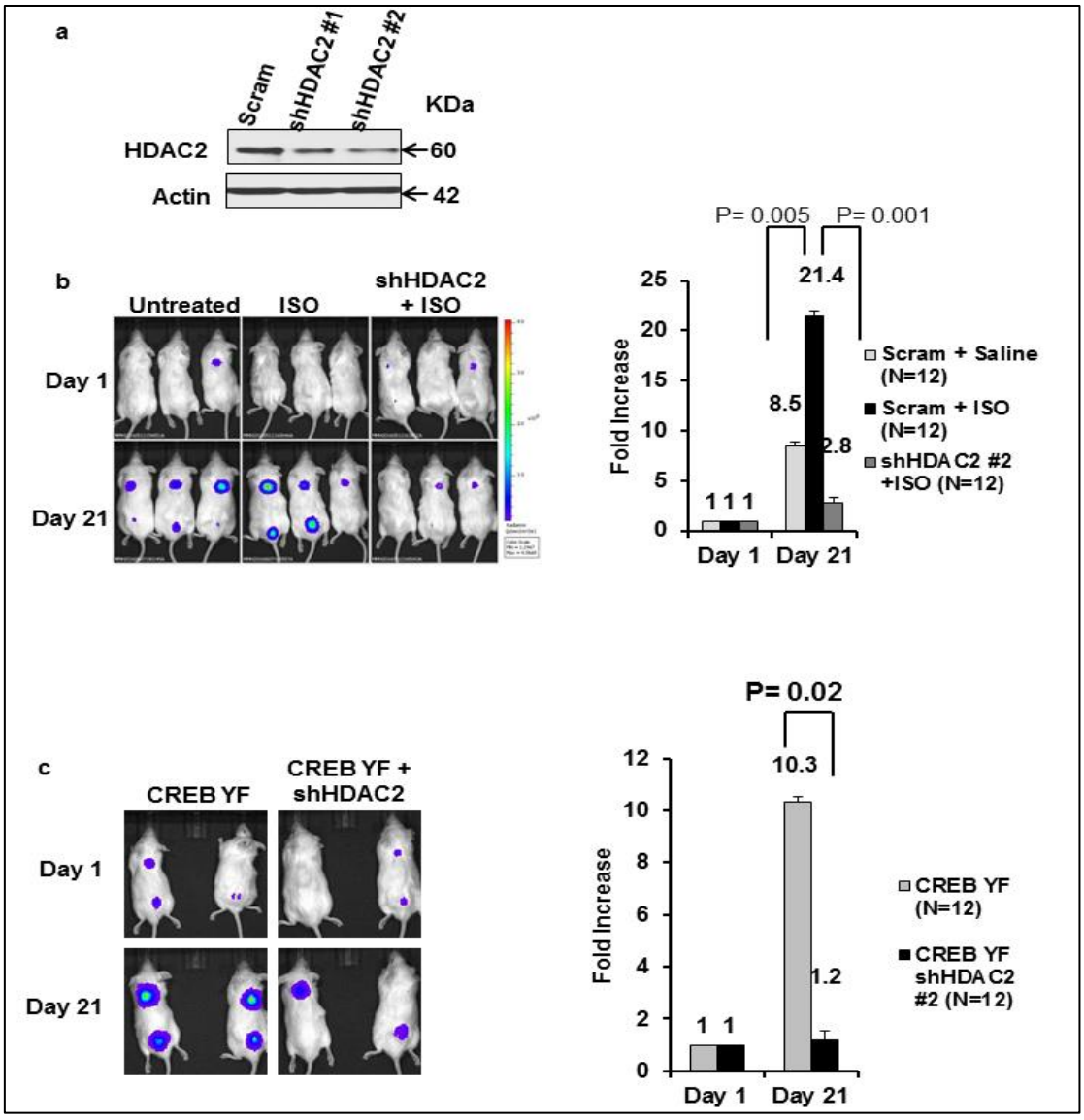

Figure 4.9. HDAC2 is critical for tumor growth induced by β-adrenergic signaling. (A) Western blotting shows that both the HDAC2-shRNAs stably downregulated HDAC2 expression in PC3 cells. **(B)** Representative mice images showing that the activation of β-adrenergic signaling in mice could not promote the growth of tumor xenografts for PC3 cells with HDAC2 down-regulation. Mice were randomly assigned to receive either saline or 10 mg/kg ISO twice a day for 21 days (n=12 tumors, 6 mice/group). The growth of each tumor was quantified using IVIS Lumina II platform. Fold increases on Y-axes were relative to day one. **(C)**

Representative mice images showing that the overexpression of constitutively active CREB could not promote the growth of tumor xenografts for PC3 cells with HDAC2 down-regulation. Luciferase labelled PC3 cells overexpressing constitutively active CREB with or without HDAC2 down-regulation were injected s.c. into both the flanks and shoulders of NOD/SCID mice (n=12 tumors, 6 mice/group). Tumor growth was monitored for 21 days and fold increase for each tumor was calculated compared to day one.

Further, we investigated whether HDAC2 acts as a critical downstream mediator for β-adrenergic signaling to induce *in vitro* angiogenesis. Conditioned media from prostate cancer cells treated with ISO, with and without HDAC inhibitor trichostatin A (TSA), were used in angiogenesis tube formation assay of SVEC4-10 endothelial cells. Indeed, TSA treatment of PC3 cells inhibited the ISO-induced tube formation (**Fig. 4.10a**). Since TSA is a pan-HDAC inhibitor, to confirm the role of HDAC2, we next examined the effect on HDAC2 down-regulation on *in vitro* angiogenesis. Knockdown of HDAC2 expression by two independent HDAC2 shRNAs in prostate cancer cells (**Fig. 4.10b**) abrogated the ISO-induced tube formation of SVEC4-10 cells (**Fig. 4.10c**). Taken together, these results indicate that CREB binds to HDAC2 promoter and induces its expression, and HDAC2 is critical in mediating the effect of β-adrenergic signaling on angiogenesis.

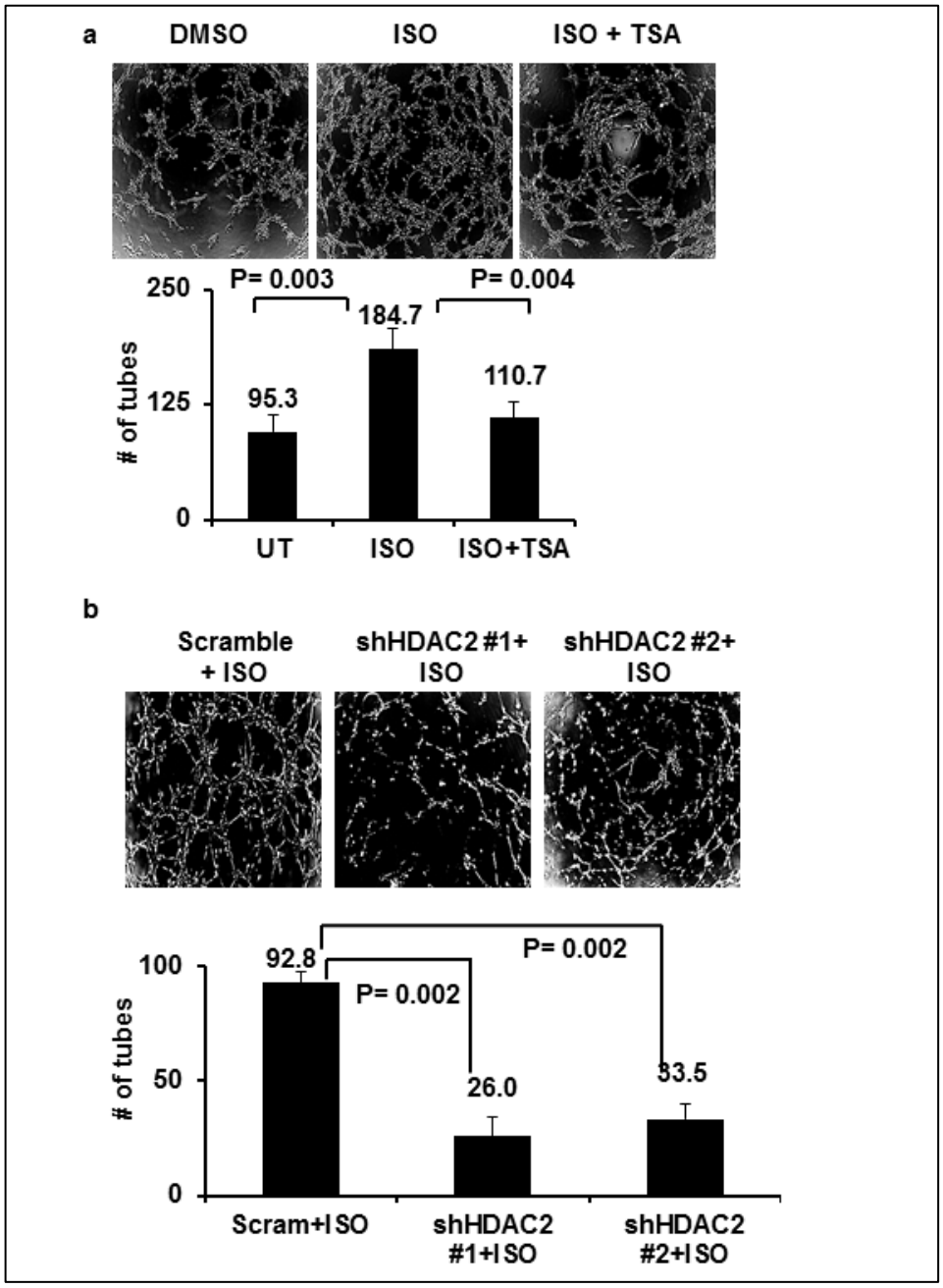

Figure 4.10. HDAC2 is critical for angiogenesis induced by β-adrenergic signaling. (A) PC3 cells were treated with 10 μM ISO with or without 0.3 μM Trichostatin A (TSA, HDAC inhibitor) for 16 hours in serum free media. Conditioned media from the PC3 cells were then used to culture serum starved SVEC cells

seeded on growth factor reduced matrigel for angiogenesis tube formation assay. **(b)** Conditioned media from ISO-treated PC3 cells expressing either scramble control shRNA or HDAC2-shRNA # 1 or #2 were used to culture serum-starved SVEC cells, seeded on growth factor reduced matrigel. Tube formation was quantified as number of nodes/branches per field, 3 fields per well, duplicates per sample. All the P-values were calculated using Student's t-test. The assays have been conducted twice with comparable results.

β-adrenergic signaling suppresses the expression of anti-angiogenic protein TSP1

Knowing that both HDACi treatment and HDAC2 down-regulation inhibit β-adrenergic signaling induced angiogenesis, we hypothesized that HDAC2 represses some anti-angiogenic proteins. In a phase-2 clinical trial of HDACi Vorinostat (suberoylanilide hydroxamic acid, SAHA), it was shown that after 4 weeks of therapy with Vorinostat, reduction in the microvessel density in the patient tumors was correlated with the increased TSP1 expression[171]. TSP1 is also shown to be up-regulated upon HDACi treatment in neuroblastoma, bladder and cervical cancer cells[170-172]. Therefore we were interested in determining if TSP1 is suppressed by the β-adrenergic signaling pathway to induce tumor angiogenesis and whether this suppression is mediated by HDAC2. Indeed, β-adrenergic signaling activators ISO and epinephrine (EPI) suppressed, while its inhibitors PRO and ICI118 551(ICI) induced, TSP1 expression in PC3, DU145 and LNCaP C4-2B prostate cancer cells (**Fig. 4.11a**). Treatment of neuroendocrine prostate cancer cells NE1.3 with CREB inhibitors inhibited CREB activation and induced TSP1 expression (**Fig. 4.11b**). To further determine the role of CREB in TSP1 regulation, we overexpressed wild-type and constitutively active CREB in PC3 cells and observed that TSP1 levels were suppressed (**Fig. 4.11c**).

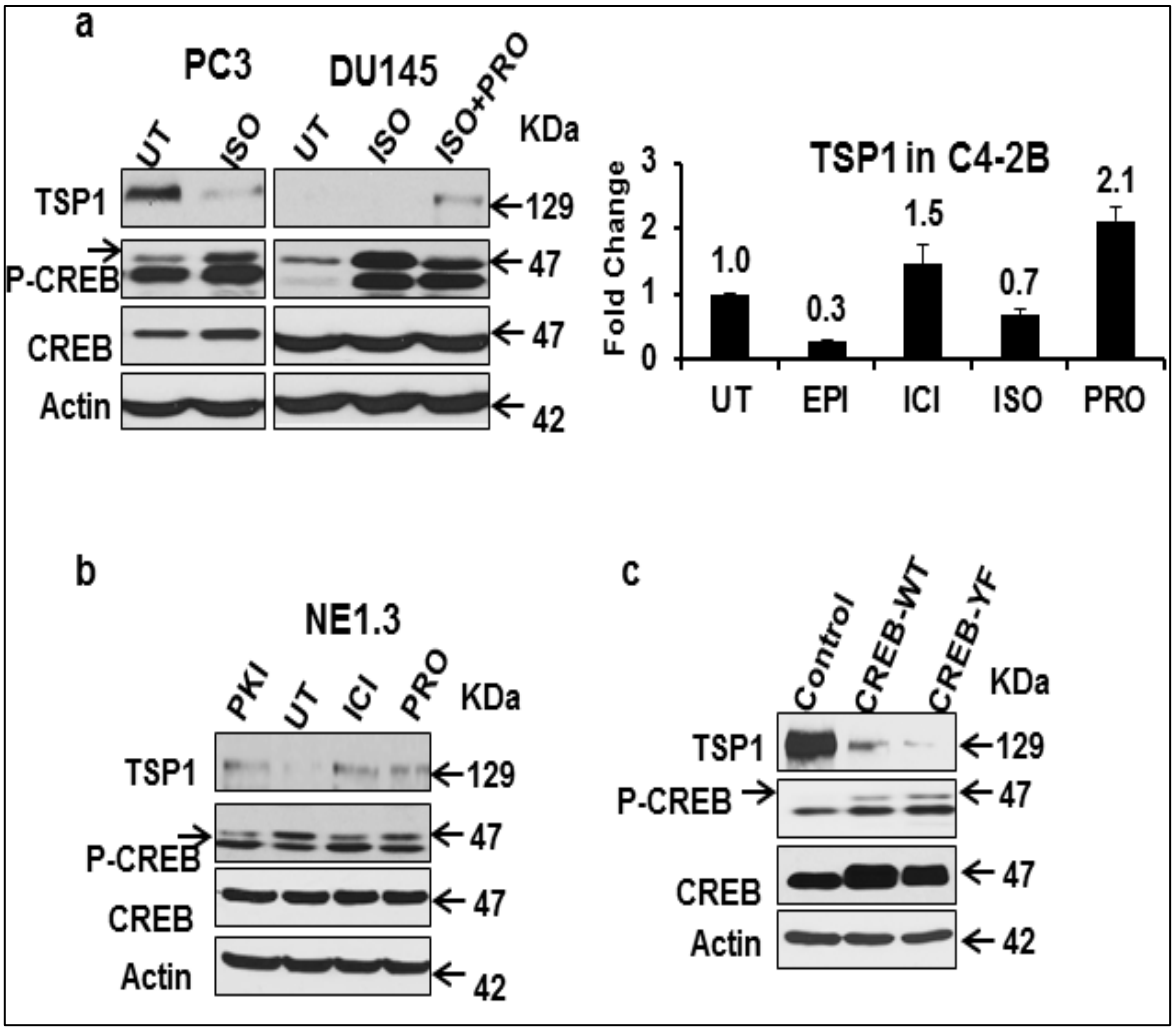

Figure 4.11. β-adrenergic signaling regulates TSP1. (A) PC3 and DU145 cells were treated with 10 μM ISO, or 10 μM ISO + 10 μM PRO for 4 hours, followed by western blotting for TSP1 protein expression. LNCaP C4-2B cells were treated with 10 μM ISO, PRO, epinephrine (EPI, β-adrenergic receptor agonist), or ICI 118 551 (ICI, β-adrenergic receptor antagonist) for 16 hours, followed by quantitative RT-PCR assays. Y-axis shows the relative fold changes in expression, normalized to GAPDH. **(B)** LNCaP-derived neuroendocrine prostate cancer cells NE1.3 were treated with 10 μM PKI (PKA inhibitor), ICI or PRO for 4 hours to inhibit CREB

activation, followed by western blotting. **(C)** Western blotting shows that TSP1 expression was down-regulated when wild-type CREB or constitutively active mutant of CREB Y134F was overexpressed in PC3 cells.

Moreover, TSP1 expression was negatively correlated with CREB in ~1,000 human cancer cell lines in the *CCLE* database[145](*Spearman's rank correlation* coefficient Rho= -0.30, P <2.92e-20) (**Fig. 4.12a**). In addition to these *in vitro* findings, activation of β-adrenergic signaling suppressed TSP1 expression in LNCaP and PC3 mouse xenograft tumors (**Fig. 4.12 b-c**). To test whether TSP1 suppression is critical for ISO induced *in vitro* angiogenesis, conditioned media from ISO-treated PC3 cells with or without added TSP1 peptides were used in tube formation assays of SVEC4-10 cells. We observed that the addition of TSP1 peptides inhibited tube formation of SVEC4-10 cells induced by ISO treatment of PC3 cells (**Fig. 4.13**). These results indicate that activation of β-adrenergic signaling pathway suppresses TSP1 expression to induce angiogenesis.

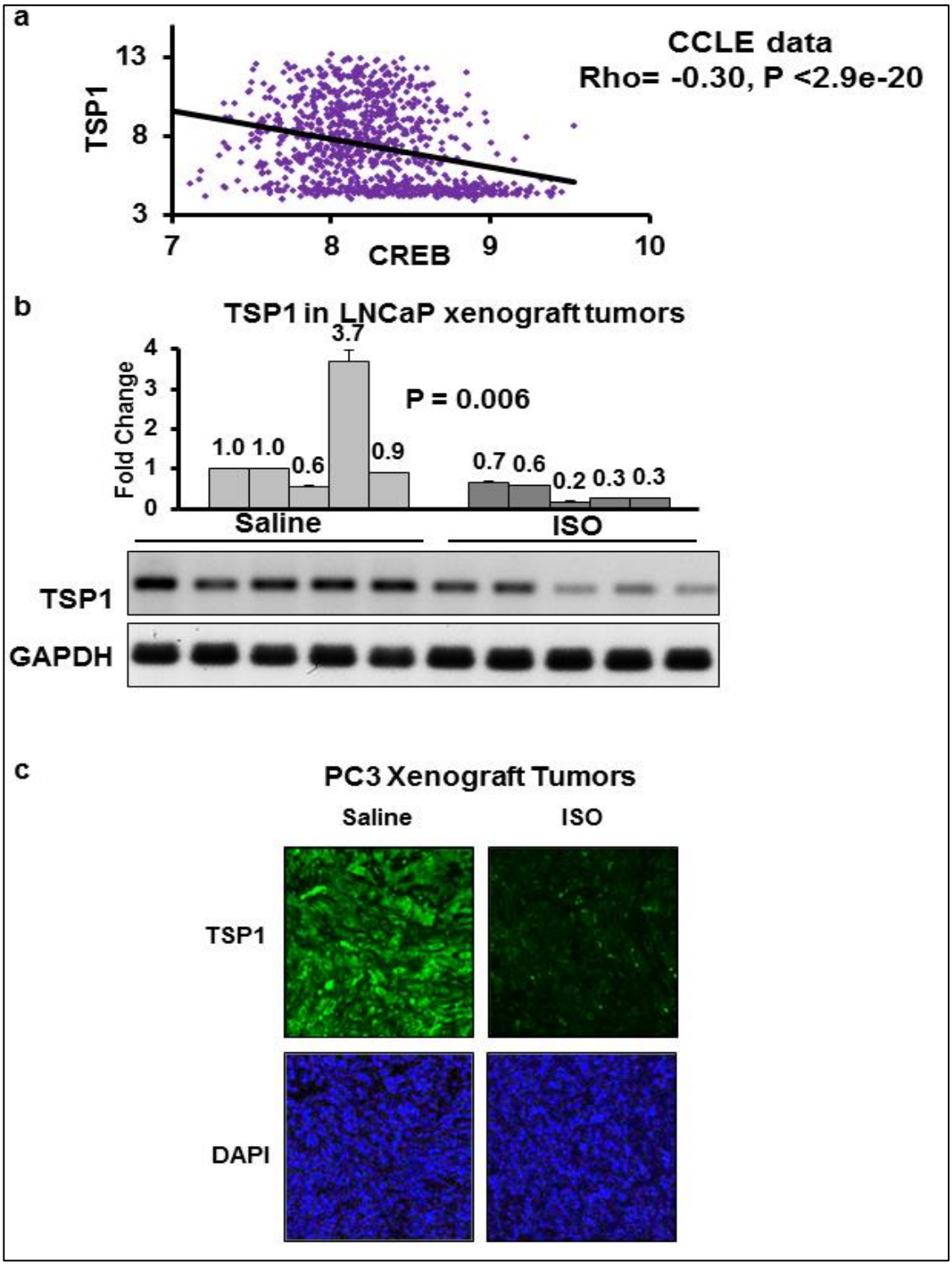

Figure 4.12. β-adrenergic signaling regulates TSP1. (a) Analysis of ~1000 human cancer cell lines in the CCLE database showed that the expressions of TSP1 and

CREB are negatively correlated. **(b)** Quantitative RT-PCR result (**top**) shows that TSP1 expression is down-regulated in LNCaP xenograft tumors from mice treated with ISO. PCR products were also analyzed by DNA gel electrophoresis (**bottom**). Activation of beta-adrenergic signaling suppresses TSP1 expression. A representative image for immunofluorescent (IF) staining of PC3 xenograft tumor tissues shows that ISO treatment suppressed the levels of TSP1 (green). Tissue sections of multiple tumors from the untreated and ISO groups were stained for TSP1. DAPI was used to visualize cell nuclei.

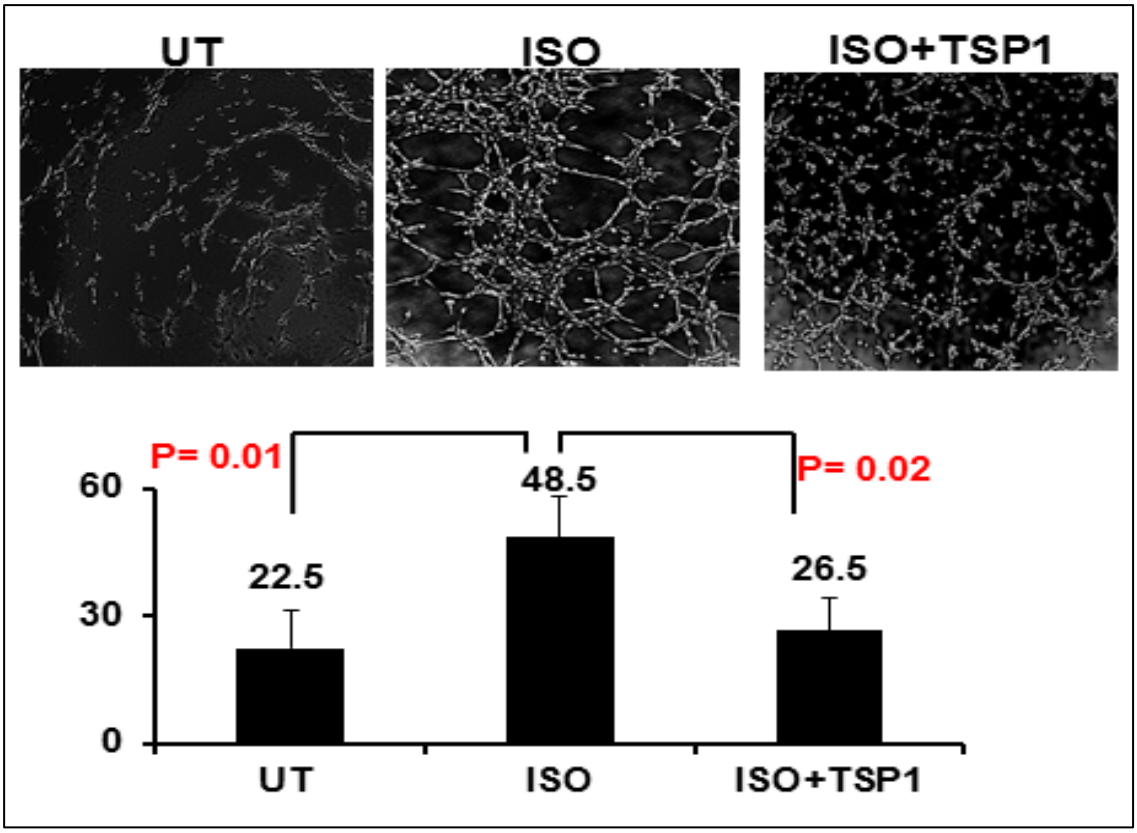

Figure 4.13. Suppression of TSP1 is necessary for β-adrenergic signaling to induce angiogenesis. 10 μM TSP1 peptides were added to the conditioned media from ISO-treated PC3 cells, which was then used to culture SVEC cells in tube formation assay. Representative images show that ISO treatment of PC3 cells could not increase SVEC tube formation in the presence of TSP1 peptides. Tube formation was quantified as number of nodes/branches per field, 3 fields per well, duplicates per sample. All the P-values were calculated using Student's t-test. The assays have been conducted twice with comparable results.

Suppression of TSP1 by β-adrenergic signaling is mediated by HDAC2

After establishing that the suppression of TSP1 expression is one critical mechanism through which the β-adrenergic signaling pathway induces angiogenesis, we tested if HDAC2 acts as a mediator for β-adrenergic signaling to suppress TSP1. Consistent with the literature[170-172], HDAC inhibitor TSA induced TSP1 expression in PC3 cells (**Fig. 4.14a**). Notably, while ISO treatment alone suppressed TSP1 expression, ISO was unable to repress TSP1 expression in the presence of TSA (**Fig. 4.14a**). This indicates that HDACs act as critical mediators of β-adrenergic signaling pathway in regulating TSP1 expression.

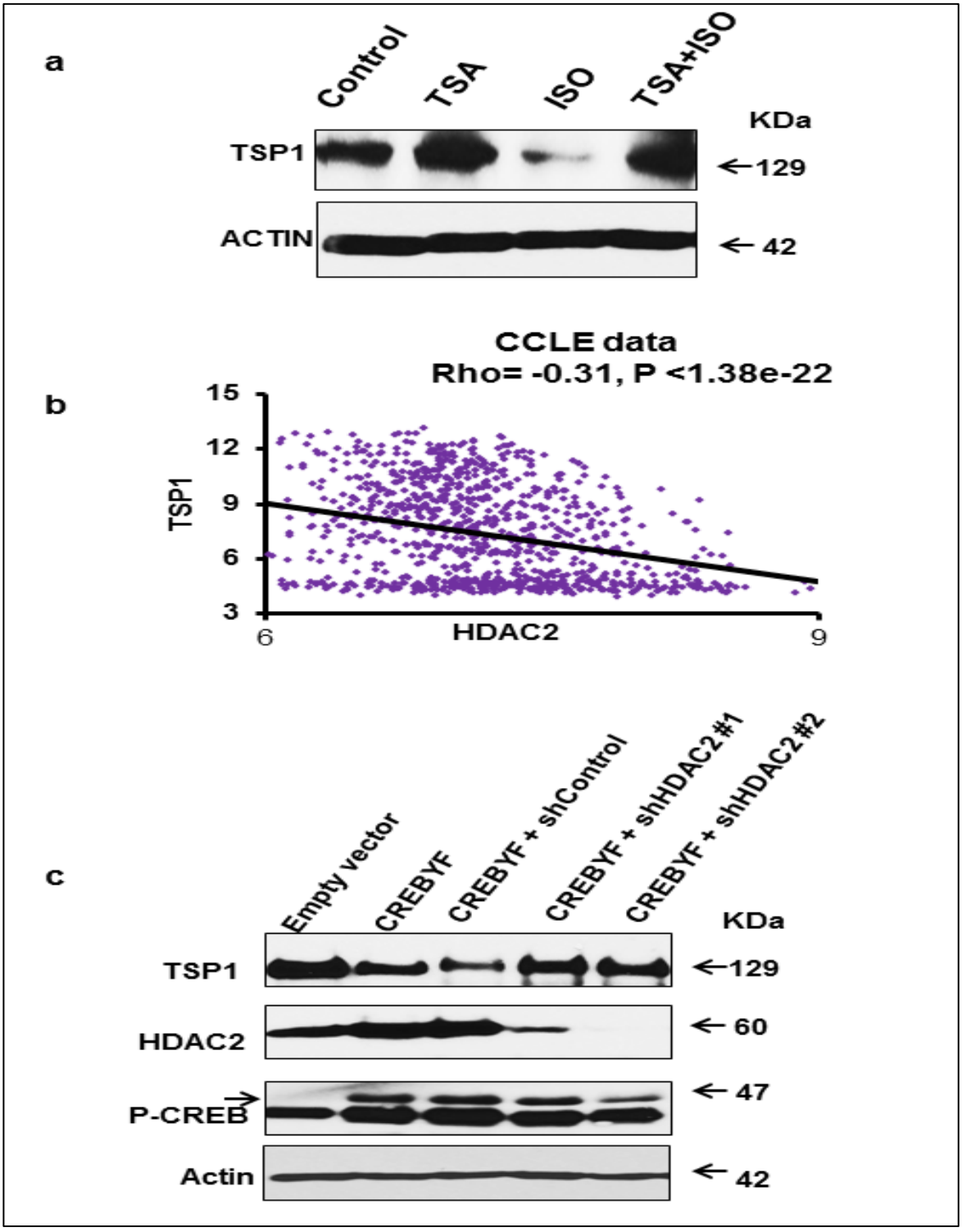

Figure 4.14. Suppression of TSP1 by β-adrenergic signaling is mediated by HDAC2. (A) PC3 cells were treated with 10 μM ISO with or without 0.3 μM

Trichostatin A (TSA, HDAC inhibitor) for 24 hours. Western blotting results show that, while TSA and ISO alone induced and reduced TSP1, respectively, ISO was unable to suppress TSP1 in the presence of TSA. **(B)** Analysis of ~1000 human cancer cell lines in the CCLE database showed that the expressions of TSP1 and HDAC2 are negatively correlated. **(C)** Effects of HDAC2 downregulation on TSP1 expression in CREB overexpressed PC3 cells. While the overexpression of constitutively active CREB Y134F induced HDAC2 and suppressed TSP1 expressions, downregulation of HDAC2 rescued the TSP1 repression by CREB.

We next investigated a specific role of HDAC2 in repressing TSP1 expression. Analysis of the CCLE database[145] showed a strong negative correlation between TSP1 and several HDACs, among which HDAC2 is the strongest one (*Spearman's rank correlation* coefficient Rho= -0.31, P <1.38e-22) (**Fig. 4.14b** and Supplementary **Fig. 4.7b**), which suggests that HDAC2 is a major HDAC repressing TSP1 expression. To identify the specific role of HDAC2, we down-regulated HDAC2 in the PC3 cells overexpressing constitutively active CREB and observed that, while overexpression of constitutively active CREB suppressed TSP1 expression, down-regulation of HDAC2 in these CREB-overexpressing PC3 cells restored TSP1 expression (**Fig. 4.14c**). Upon confirming that HDAC2 acts as a mediator for β-adrenergic signaling to repress TSP1 expression, we next investigated the mechanism through which HDAC2 regulates TSP1 expression. We observed strong histone H3K27 acetylation marks near TSP1 promoter in the UCSC Genome Browser (**Fig. 4.15a**), suggesting that HDACs may be repressing the TSP1 expression by removing H3K27Ac activation marks on TSP1 promoter. ChIP-PCR assays were performed using anti-HDAC2 antibody to pull down HDAC2 bound chromatin and PCR using primers for the TSP1 promoter around these H3K27Ac marks. As expected, HDAC2 bound to the TSP1 promoter. Moreover, the interaction of HDAC2 with proteins binding directly to TSP1 promoter increased upon the activation of β-adrenergic signaling pathway by ISO treatment in PC3 and DU145 cells, which was abrogated by additional treatment of β-adrenergic antagonist PRO (**Fig. 4.15 a-b**). These results indicate that HDAC2 epigenetically suppresses TSP1

expression and it acts as a critical downstream mediator of β-adrenergic signaling pathway in suppressing TSP1 and inducing angiogenesis.

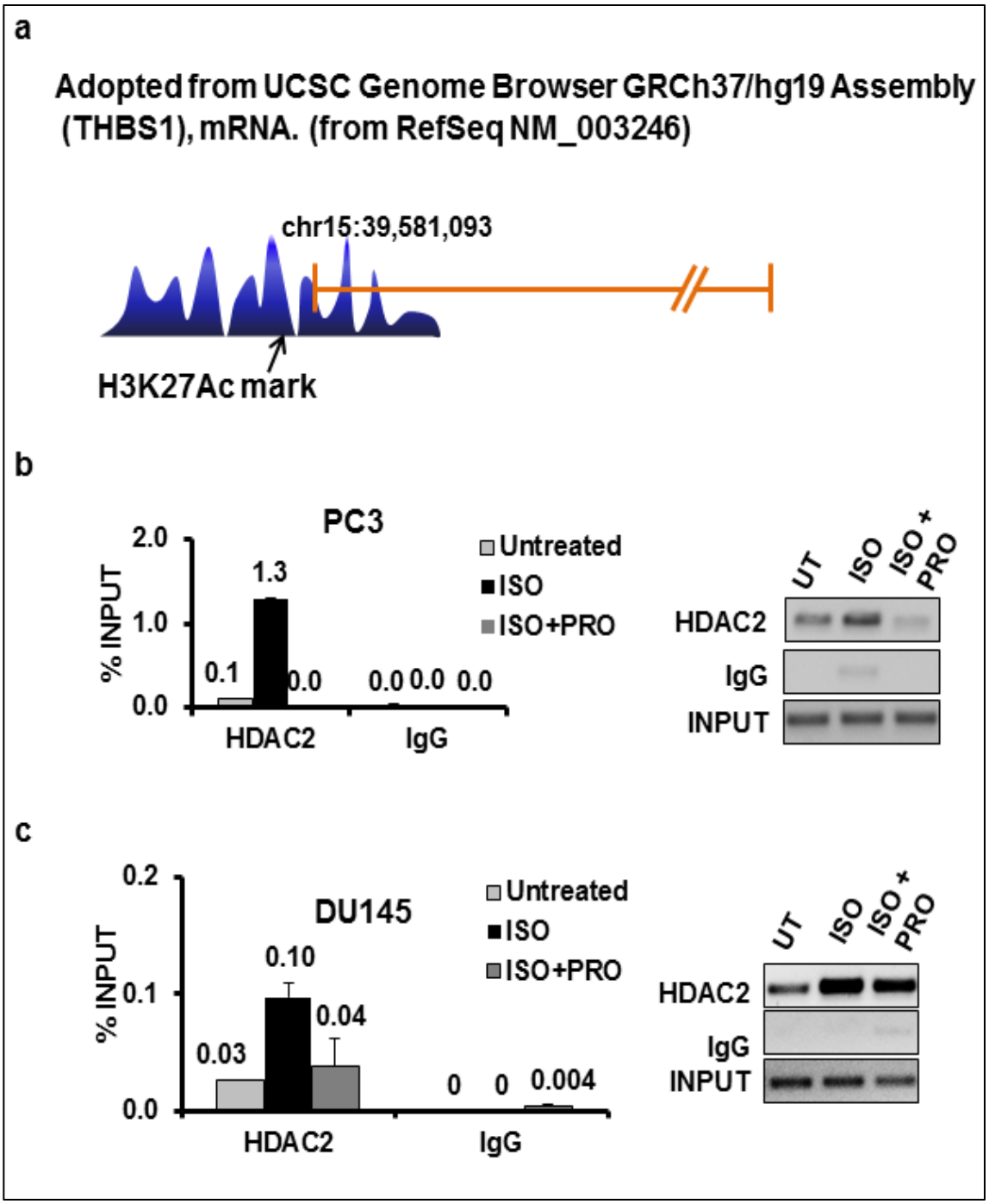

Figure 4.15. HDAC2 binds to TSP1 promoter and suppresses its expression. **(a)** TSP1 gene promoter was visualized in the UCSC genome browser GRCh37/hg19 Assembly. A strong H3K27 histone acetylation mark was observed at the promoter region. A schematic of the original UCSC genome browser

GRCh37/hg19 assembly is shown. **(b-c)** PC3 and DU145 cells were treated with 10 μM ISO, or ISO+PRO for 4 hours. A ChIP assay was conducted using anti-HDAC2 antibody or IgG control, followed by PCR to amplify the TSP1 promoter region around the H3K27Ac mark. The ChIP-PCR result was presented as % of the input on Y-axis, which was further confirmed by DNA gel electrophoresis. All the results were confirmed by reproducing at least once.

Summary

In summary, our study demonstrates that HDAC2 is a new target of CREB and HDAC2 induction is critical for tumor angiogenesis promoted by β-adrenergic signaling activation, which is mediated by HDAC2 suppression of TSP1. Taken together, these results support a new model, as illustrated in **Fig. 8**, that chronic stress and β-adrenergic signaling induce angiogenesis and prostate tumor growth, at least in part, through an unexpected role of CREB in directly inducing HDAC2 expression that in turn epigenetically represses a potent anti-angiogenic protein, TSP1.

Chapter Five:

Discussion

ADT, CREB, GRK3 and Neuroendocrine Prostate Cancer.

Neuroendocrine prostate cancer causes approximately 25% of all the prostate cancer deaths[56,99-101]. It has been recognized that incidence of this aggressive variant of castration-resistant prostate cancer (CRPC) with neuroendocrine phenotype (NEPC) has been previously underestimated[102] and a better understanding of the molecular events underlying NEPC development is urgently needed to develop a therapeutic solution for CRPC/NEPC.

CREB activation has been shown to play a critical role in neuroendocrine differentiation (NED) of prostate cancer cells[46,47,82-86]. However, the downstream pathways and targets of CREB in NEPC cells are still incompletely understood. Here, we have demonstrated that GRK3 is expressed higher in cells and genetically engineered mouse (GEM) models of NEPC. Furthermore, GRK3 cDNA overexpression induces NE markers in prostate cancer cells in a kinase activity dependent manner, while its silencing reduces NE marker expression and reverses NE morphology in NEPC cells. These results indicate that GRK3 is a critical regulator for NEPC cells and that GRK3 contributes to prostate cancer progression at least in part by promoting the development of NEPC, an aggressive subtype of prostate cancers.

Mechanistically, we found that GRK3 expression is induced by chemical activators of CREB or CREB cDNA overexpression. Further, a ChIP-PCR confirmed that CREB binds to GRK3 promoter and this binding of CREB changes in response to its activation and inhibition. On the other hand, silencing GRK3 abrogates CREB induction of NE markers in prostate cancer cells. Furthermore, GRK3 expression

has a positive correlation with CREB expression and activity in broad human cancer cell lines (CCLE) and human prostate cancer tissues. Together, these results establish that GRK3 is a direct target of CREB activation in prostate cancer cells.

Previously, GRK3 has been shown to be up-regulated by chronic treatments with stress hormones, adrenaline and corticotropin release factor (CRF), in human neuroblastoma BE (2)-C cells and Y79 retinoblastoma cells. These two stress hormones are known to signal G-protein coupled receptors (GPCRs), beta adrenergic receptor (ADRB) and CRF1 receptor, and GPCR activation in turn leads to elevated cAMP levels. However, the mechanisms downstream of cAMP, and leading to GRK3 expression have not been studied before. We have addressed this question in our study, showing that downstream of stress and cAMP, CREB binds to GRK3 promoter and activates its expression.

GRKs phosphorylate and desensitize GPCRs upon agonist stimulation. Therefore, up-regulation of GRK3 by adrenaline was considered as a negative feedback regulation to control the activation of beta adrenergic receptors and CREB[182]. However, for the first time, we have demonstrated that GRK3 is a critical mediator for ADRB2 activated CREB to induce neuroendocrine differentiation of prostate cancer cells. Furthermore, we also show a positive correlation between the expression of GRK3 and CREB in ~1000 cell lines of multiple cancer types. These results introduce a new paradigm that CREB/GRK3 axis mediates the effect of ADRB signaling and that it is active in many cancer types.

Previously, our lab used unbiased shRNA and cDNA screening of hundreds of human kinases to show that GRK3 is a new critical activator of prostate cancer

progression[91]. It was found that GRK3 is necessary for the survival and proliferation of metastatic cancer cells *in vitro* and *in vivo* and, that it is sufficient to promote primary tumor growth in the prostate and metastases in soft tissues. Through immunohistochemistry staining for a large tissue array from human prostate cancer patients, it was shown that GRK3 is overexpressed in human prostate tumors, especially in soft tissue metastases[91]. Therefore, the data presented in our previous[91] and current studies suggest that targeting GRK3 may be a viable approach to inhibit prostate cancer progression and NEPC development.

Interestingly, the role of GRK3 in prostate cancer development is dependent on its kinase activity. While GRK3 suppressed the expression of tumor suppressors thrombospondin 1 (TSP1) and plasminogen activator inhibitor 2 (PAI2)[91], the kinase-inactive mutant of GRK3 could not suppress TPS1 and PAI2. In this study, we showed that the kinase-inactive mutant of GRK3 could not induce the NE marker expression in prostate cancer cells, suggesting that the GRK3 kinase activity is necessary to induce neuroendocrine differentiation. These results support the rationale to identify GRK3 kinase inhibitors as candidates for new cancer drugs.

Kinases are known to be druggable[183,184] and although there was initial skepticism about their specificity, highly selective small molecule kinase inhibitors have been successfully developed[185]. Several kinase inhibitors have been approved as cancer therapeutics[186-190] and several hundred more are being studied as potential therapeutics [185,191]. The greatest clinical success of these kinase inhibitors has been seen in cases where the inhibition of kinase activity has shown strong phenotypic changes e.g. cell survival[192] or, reversal of NE phenotypes and reduced

proliferation, as we have shown[97]. An example of successful use of such kinase inhibitors was imatinib (Gleevec; Novartis)[193] in treatment of chronic myelogenous leukemia (CML). This phenomenon, where cancer cells are dependent on an overexpressed/hyperactive gene is called oncogene addiction[194]. Our results show such dependency of neuroendocrine cells on GRK3 expression as well as its kinase activity. Therefore, we believe that GRK3 specific kinase inhibitors could be developed in future as a potential therapeutic against NEPC.

Downstream mechanisms of GRK3, resulting in the expression of NE markers, have not been studied yet. Since GRK3 is a GPCR kinase, it may regulate a critical GPCR signaling pathway in NEPC development. In addition to GPCRs, GRKs are known to phosphorylate directly, or facilitate the phosphorylation of, non-GPCR targets[195-197]. Role of GRK3 in NEPC progression could be understood future by further studying these possible mechanisms.

Our results demonstrate that GRK3 is a new activator for neuroendocrine phenotypes and ADT resistance in prostate cancer cells. It is a direct target and a critical mediator of activated CREB in promoting NE differentiation. These results expand our knowledge of NEPC development, prostate cancer progression, and GRK3 as a prospective novel drug target for aggressive prostate cancers.

β-adrenergic signaling, CREB, HDAC2, TSP1 and Angiogenesis.

β-adrenergic signaling activated by chronic behavioral stress has been shown to promote cancer progression and angiogenesis[18,23,198]. However, the downstream molecular effectors involved and their regulations of this pathway are still not completely known. In particular, the involvement of epigenetic regulation by HDACs in β-adrenergic signaling promoted cancer progression is unclear. While HDAC2 is shown to be necessary to mediate the effects of chronic stress in inducing cardiac hypertrophy[90], how stress and the β-adrenergic signaling regulates HDAC2 and whether HDAC2 mediates stress promoted cancer progression are unknown.

In this study, we have investigated the molecular bases of angiogenesis and cancer progression promoted by chronic stress and β-adrenergic activation *in vitro* as well as in xenograft mouse models. In particular, we have studied the role, regulation and mechanism of action of HDAC2 in these processes. We have demonstrated that β-adrenergic signaling up-regulates HDAC2 and down-regulates TSP1 expressions *in vitro* and *in vivo* in mouse xenografts. Downstream of β-adrenergic signaling, CREB was uncovered to directly regulate HDAC2 expression. We have also demonstrated that epigenetic suppression of TSP1 by HDAC2 is critical for angiogenesis enhanced by β-adrenergic signaling.

Our finding that CREB binds to HDAC2 promoter and induces its expression is intriguing. The current paradigm is that activated CREB recruits CREB binding protein (CBP) to activate transcription of its target genes[199-203]. Since CBP is a histone acetyltransferase (HAT), it is counterintuitive that CREB elevates HDAC2 expression, because increased HDAC activity presumably counteracts CBP and

decreases CREB-dependent transcription. One possible explanation is that the induction of HDAC2 by CREB is a negative feedback regulation to control CREB-activated transcription. However, our results support a new model, showing that HDAC2 is a critical mediator for CREB in TSP1 repression and enhancing angiogenesis. This is consistent with a study by Fass *et al.,* showing that HDACs can either repress or enhance CREB activity by differentially regulating CREB target genes[204]. To further develop this new paradigm, it will be worthwhile in the future to examine the changes in global gene expression as well as in the CBP and HDAC2 epigenetic landscapes upon the activation and inhibition of β-adrenergic signaling and PKA/CREB activation.

β-adrenergic signaling and activation of PKA/CREB pathways have been shown to promote angiogenesis through induction of VEGF in ovarian cancer cells[23]. Angiogenesis often involves changes in the levels of both pro-angiogenic and anti-angiogenic proteins[205-210]. While ADRB2/PKA/CREB pathway was shown to induce the pro-angiogenic VEGF, it was unclear what anti-angiogenic proteins are repressed by β-adrenergic signaling. Our study contributes to filling this gap by demonstrating that β-adrenergic signaling represses a potent anti-angiogenic protein – TSP1. Interestingly, a few studies have demonstrated that TSP1 signaling, through its receptors CD36 or CD47, inhibits the PKA/CREB pathway in platelet activation as well as in T cells and breast cancer cells [211,212]. With our finding that CREB represses TSP1 expression, an antagonism can be postulated between PKA/CREB and TSP1 signaling in certain biological processes.

HDAC inhibitors are being tested as anti-angiogenesis therapeutics[213-216] and are reported to induce TSP1 expression. TSP1 expression is regulated largely at the transcriptional level and it has been established that HDAC inhibitors induce the expression of TSP1[170-172,214,217,218]. In the literature, it remained unclear how HDAC inhibitors (HDACi) induce TSP1 expression and no specific HDACs were implicated in TSP1 regulation[170-172,214,217,218]. Kang et al., have shown that CCAAT box in the promoter region of TSP1 is required for its induction upon treatment with TSA[217]. However, to our knowledge, this is the first study to report that HDAC2 binds to TSP1 promoter and represses its expression in prostate cancer cells. It remains to be determined if other HDACs also contribute to the CREB induction of angiogenesis and TSP1 repression. The global gene expression correlation between CREB and HDACs suggest that HDAC2 is one of the major HDACs involved, which is consistent with a critical role of HDAC2 in stress induced congestive heart failure[90]. In addition to TSP1 up-regulation, HDACi are shown to suppress the expression of VEGF and bFGF[219]. However, the mechanisms of this regulation are not clear. Our finding that HDAC2 represses TSP1 expression by binding to its promoter expands our knowledge of the mechanisms of HDACi as angiogenesis inhibitors.

Hassan S *et al* demonstrated that behavioral stress promotes prostate cancer progression through ADRB2/PKA/BAD pathway[18]. However, unlike Thaker PH *et al*[23], no increased angiogenesis was observed in their stress-promoted prostate cancer model. As Hassan S *et al* pointed out, C4-2, the mouse model they used for the study, is highly vascularized and therefore, it was not possible to study the

increased angiogenesis[18]. Using different prostate cancer xenograft models (PC3 and LNCaP), our data indicated that the CREB/HDAC2/TSP1 axis mediates the effect of β-adrenergic signaling on angiogenesis, which elucidates another critical mechanism underlying the activation of adrenergic signaling in promoting prostate cancer progression.

These results advance established knowledge in the field of chronic behavioral stress, epigenetic regulation and prostate cancer progression. Future research will investigate the activation of the ADRB2/CREB/HDAC2/TSP1 pathway in human prostate cancer samples and determine whether TSP1 can be developed as a potential biomarker to monitor the efficacy of β-blocker or HDACi in cancer therapy.

Novel Mechanisms of β-Adrenergic Signaling in Prostate Cancer Progression.

β-adrenergic signaling has been found to be responsible for the progression of breast[19], colon and pancreatic[20], lung[21], skin[22], ovarian[23] cancers. Its inhibition by β-blocker use is correlated with better cancer prognosis[24-34] and reduced cancer related mortality[24-34]. However, use of β-blockers to treat cancers has been limited due to lack of understanding of the underlying mechanisms. Therefore, mechanisms of β-adrenergic signaling in prostate cancer progression need to be studied further. Here, we have introduced two new pathways acting downstream of ADRB2/CREB axis. CREB/HDAC2/TSP1 pathway is responsible for induction of tumor angiogenesis, while CREB/GRK3 axis leads to tumor angiogenesis and neuroendocrine prostate cancer progression. We have introduced a new paradigm that β-adrenergic signaling and epigenetic gene expression regulation may be working synergistically resulting in cancer progression. The results from our study not only significantly advance current understanding of the pathways involved in β-adrenergic signaling, but also provide with novel molecular candidates with therapeutic as well as biomarker potential.

Future Direction

GRK3 has been shown to be a critical regulator for prostate cancer progression in a previous[91] and our present[97] study. We have also shown that GRK3 expression is regulated by ADT-activated CREB, leading to neuroendocrine differentiation of prostate cancer cells. However, downstream mechanisms of GRK3 are yet to be studied. Since the primary substrate of GRK3 is GPCRs, it is possible that it phosphorylates a GPCR involved in NEPC signaling. It is also possible that GRK3 phosphorylates a non-GPCR target or act as a scaffold to facilitate the interactions between a protein complex. In future, these mechanisms of GRK3 will be studied.

Another direction for future work on CREB/GRK3/NEPC axis is the development of GRK3 kinase inhibitors as therapeutics. We have recently conducted a kinase inhibitor screen and have shortlisted highly potent GRK3 kinase inhibitors. We will study the effect of these inhibitors on prostate cancer progression *in vitro* and *in vivo*.

We are preparing to use a large array of prostate cancer patient tissue samples to validate the CREB/HDAC2/TSP1 axis in human patients. We will perform immunohistochemistry/immunofluorescence staining to study the expressions and correlation between p-CREB, HDAC2 and TSP1 proteins. Based on the results from this study, TSP1 can be developed as a potential biomarker to monitor the efficacy of β-blocker or HDACi in cancer therapy in future.

We will also study the changes in global gene expression as well as in the CBP and HDAC2 epigenetic landscapes upon the activation and inhibition of β-

adrenergic signaling and PKA/CREB activation. This study will uncover the mechanisms of synergy between epigenetic regulations by HDAC2 and CBP, new molecular regulators of ADRB2 signaling, as well as potential novel targets for development of new therapeutics or biomarkers.

It has been shown recently that Epac-Rap1 pathway, activated by ADRB/camp, induce TSP1 expression in endothelial cells and fibroblasts[220], independent of the PKA/CREB pathway. This suggests that β-adrenergic signaling induces TSP1 expression in endothelial cells and fibroblasts, while we have shown that the PKA/CREB pathway suppresses TSP1 expression in epithelial cells of prostate cancer. In future, we will study the synergy between these two regulations, which will reveal vital information about the role of tumor microenvironment in cancer progression.

Bibliography

1. Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. & Bray, F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **136**, E359-386 (2015).
2. Luo, J., Beer, T. M. & Graff, J. N. Treatment of Nonmetastatic Castration-Resistant Prostate Cancer. *Oncology (Williston Park, N.Y.)* **30**, 336-344 (2016).
3. Pound, C. R., Partin, A. W., Eisenberger, M. A., Chan, D. W., Pearson, J. D. & Walsh, P. C. Natural history of progression after PSA elevation following radical prostatectomy. *Jama* **281**, 1591-1597 (1999).
4. Huggins, C. & Hodges, C. V. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA: a cancer journal for clinicians* **22**, 232-240 (1972).
5. Huggins, C. & Hodges, C. V. Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol* **167**, 948-951; discussion 952 (2002).
6. Huggins, C. & Hodges, C. V. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol* **168**, 9-12 (2002).
7. Bubendorf, L., Schopfer, A., Wagner, U., Sauter, G., Moch, H., Willi, N., Gasser, T. C. & Mihatsch, M. J. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Hum Pathol* **31**, 578-583 (2000).
8. Beer, T. M. & Tombal, B. Enzalutamide in metastatic prostate cancer before chemotherapy. *The New England journal of medicine* **371**, 1755-1756 (2014).
9. Froehner, M. & Wirth, M. P. Enzalutamide in metastatic prostate cancer before chemotherapy. *The New England journal of medicine* **371**, 1755 (2014).
10. Beer, T. M., Armstrong, A. J., Rathkopf, D. E., Loriot, Y., Sternberg, C. N., Higano, C. S., Iversen, P., Bhattacharya, S., Carles, J., Chowdhury, S., Davis, I. D., de Bono, J. S., Evans, C. P., Fizazi, K., Joshua, A. M., Kim, C. S., Kimura, G., Mainwaring, P., Mansbach, H., Miller, K., Noonberg, S. B., Perabo, F., Phung, D., Saad, F., Scher, H. I., Taplin, M. E., Venner, P. M. & Tombal, B. Enzalutamide in metastatic prostate cancer before chemotherapy. *The New England journal of medicine* **371**, 424-433 (2014).
11. Beltran, H., Rickman, D. S., Park, K., Chae, S. S., Sboner, A., MacDonald, T. Y., Wang, Y., Sheikh, K. L., Terry, S., Tagawa, S. T., Dhir, R., Nelson, J. B., de la Taille, A., Allory, Y., Gerstein, M. B., Perner, S., Pienta, K. J., Chinnaiyan, A. M., Wang, Y., Collins, C. C., Gleave, M. E., Demichelis, F., Nanus, D. M. & Rubin, M. A. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. *Cancer Discov* **1**, 487-495 (2011).
12. Beltran, H., Tomlins, S., Aparicio, A., Arora, V., Rickman, D., Ayala, G., Huang, J., True, L., Gleave, M. E., Soule, H., Logothetis, C. & Rubin, M. A. Aggressive variants of castration-resistant prostate cancer. *Clin Cancer Res* **20**, 2846-2850 (2014).
13. Conteduca, V., Aieta, M., Amadori, D. & De Giorgi, U. Neuroendocrine differentiation in prostate cancer: current and emerging therapy strategies. *Critical reviews in oncology/hematology* **92**, 11-24 (2014).
14. Jongsma, J., Oomen, M. H., Noordzij, M. A., Van Weerden, W. M., Martens, G. J., van der Kwast, T. H., Schroder, F. H. & van Steenbrugge, G. J. Kinetics of neuroendocrine differentiation in an androgen-dependent human prostate xenograft model. *Am J Pathol* **154**, 543-551 (1999).
15. Terry, S. & Beltran, H. The many faces of neuroendocrine differentiation in prostate cancer progression. *Frontiers in oncology* **4**, 60 (2014).

16. Vlachostergios, P. J. & Papandreou, C. N. Targeting neuroendocrine prostate cancer: molecular and clinical perspectives. *Frontiers in oncology* **5**, 6 (2015).
17. Braadland, P. R., Ramberg, H., Grytli, H. H. & Tasken, K. A. beta-Adrenergic Receptor Signaling in Prostate Cancer. *Frontiers in oncology* **4**, 375 (2014).
18. Hassan, S., Karpova, Y., Baiz, D., Yancey, D., Pullikuth, A., Flores, A., Register, T., Cline, J. M., D'Agostino, R., Jr., Danial, N., Datta, S. R. & Kulik, G. Behavioral stress accelerates prostate cancer development in mice. *J Clin Invest* **123**, 874-886 (2013).
19. Obeid, E. I. & Conzen, S. D. The role of adrenergic signaling in breast cancer biology. *Cancer biomarkers : section A of Disease markers* **13**, 161-169 (2013).
20. Hefner, J., Csef, H. & Kunzmann, V. [Stress and pancreatic carcinoma--beta-adrenergic signaling and tumor biology]. *Deutsche medizinische Wochenschrift (1946)* **139**, 334-338 (2014).
21. Tang, J., Li, Z., Lu, L. & Cho, C. H. beta-Adrenergic system, a backstage manipulator regulating tumour progression and drug target in cancer therapy. *Seminars in cancer biology* **23**, 533-542 (2013).
22. Yang, E. V. & Eubank, T. D. The impact of adrenergic signaling in skin cancer progression: possible repurposing of beta-blockers for treatment of skin cancer. *Cancer biomarkers : section A of Disease markers* **13**, 155-160 (2013).
23. Thaker, P. H., Han, L. Y., Kamat, A. A., Arevalo, J. M., Takahashi, R., Lu, C., Jennings, N. B., Armaiz-Pena, G., Bankson, J. A., Ravoori, M., Merritt, W. M., Lin, Y. G., Mangala, L. S., Kim, T. J., Coleman, R. L., Landen, C. N., Li, Y., Felix, E., Sanguino, A. M., Newman, R. A., Lloyd, M., Gershenson, D. M., Kundra, V., Lopez-Berestein, G., Lutgendorf, S. K., Cole, S. W. & Sood, A. K. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* **12**, 939-944 (2006).
24. Wu, J. W., Boudreau, D. M., Park, Y., Simonds, N. I. & Freedman, A. N. Commonly used diabetes and cardiovascular medications and cancer recurrence and cancer-specific mortality: a review of the literature. *Expert opinion on drug safety* **13**, 1071-1099 (2014).
25. Choi, C. H., Song, T., Kim, T. H., Choi, J. K., Park, J. Y., Yoon, A., Lee, Y. Y., Kim, T. J., Bae, D. S., Lee, J. W. & Kim, B. G. Meta-analysis of the effects of beta blocker on survival time in cancer patients. *Journal of cancer research and clinical oncology* **140**, 1179-1188 (2014).
26. Grytli, H. H., Fagerland, M. W., Fossa, S. D. & Tasken, K. A. Association between use of beta-blockers and prostate cancer-specific survival: a cohort study of 3561 prostate cancer patients with high-risk or metastatic disease. *Eur Urol* **65**, 635-641 (2014).
27. Diaz, E. S., Karlan, B. Y. & Li, A. J. Impact of beta blockers on epithelial ovarian cancer survival. *Gynecologic oncology* **127**, 375-378 (2012).
28. Lemeshow, S., Sorensen, H. T., Phillips, G., Yang, E. V., Antonsen, S., Riis, A. H., Lesinski, G. B., Jackson, R. & Glaser, R. beta-Blockers and survival among Danish patients with malignant melanoma: a population-based cohort study. *Cancer Epidemiol Biomarkers Prev* **20**, 2273-2279 (2011).
29. De Giorgi, V., Grazzini, M., Gandini, S., Benemei, S., Lotti, T., Marchionni, N. & Geppetti, P. Treatment with beta-blockers and reduced disease progression in patients with thick melanoma. *Archives of internal medicine* **171**, 779-781 (2011).
30. Powe, D. G., Voss, M. J., Zanker, K. S., Habashy, H. O., Green, A. R., Ellis, I. O. & Entschladen, F. Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. *Oncotarget* **1**, 628-638 (2010).
31. Melhem-Bertrandt, A., Chavez-Macgregor, M., Lei, X., Brown, E. N., Lee, R. T., Meric-Bernstam, F., Sood, A. K., Conzen, S. D., Hortobagyi, G. N. & Gonzalez-Angulo, A. M. Beta-

blocker use is associated with improved relapse-free survival in patients with triple-negative breast cancer. *J Clin Oncol* **29**, 2645-2652 (2011).

32. Barron, T. I., Connolly, R. M., Sharp, L., Bennett, K. & Visvanathan, K. Beta blockers and breast cancer mortality: a population- based study. *J Clin Oncol* **29**, 2635-2644 (2011).
33. Grytli, H. H., Fagerland, M. W., Fossa, S. D. & Tasken, K. A. Association Between Use of beta-Blockers and Prostate Cancer-Specific Survival: A Cohort Study of 3561 Prostate Cancer Patients with High-Risk or Metastatic Disease. *Eur Urol* (2013).
34. Grytli, H. H., Fagerland, M. W., Fossa, S. D., Tasken, K. A. & Haheim, L. L. Use of beta-blockers is associated with prostate cancer-specific survival in prostate cancer patients on androgen deprivation therapy. *Prostate* **73**, 250-260 (2013).
35. Cole, S. W. & Sood, A. K. Molecular pathways: beta-adrenergic signaling in cancer. *Clin Cancer Res* **18**, 1201-1206 (2012).
36. Goepel, M., Wittmann, A., Rubben, H. & Michel, M. C. Comparison of adrenoceptor subtype expression in porcine and human bladder and prostate. *Urological research* **25**, 199-206 (1997).
37. Penn, R. B., Frielle, T., McCullough, J. R., Aberg, G. & Benovic, J. L. Comparison of R-, S-, and RS-albuterol interaction with human beta 1- and beta 2-adrenergic receptors. *Clinical reviews in allergy & immunology* **14**, 37-45 (1996).
38. Ramos-Jimenez, J., Soria-Jasso, L. E., Lopez-Colombo, A., Reyes-Esparza, J. A., Camacho, J. & Arias-Montano, J. A. Histamine augments beta2-adrenoceptor-induced cyclic AMP accumulation in human prostate cancer cells DU-145 independently of known histamine receptors. *Biochemical pharmacology* **73**, 814-823 (2007).
39. Nagmani, R., Pasco, D. S., Salas, R. D. & Feller, D. R. Evaluation of beta-adrenergic receptor subtypes in the human prostate cancer cell line-LNCaP. *Biochemical pharmacology* **65**, 1489-1494 (2003).
40. Ramberg, H., Eide, T., Krobert, K. A., Levy, F. O., Dizeyi, N., Bjartell, A. S., Abrahamsson, P. A. & Tasken, K. A. Hormonal regulation of beta2-adrenergic receptor level in prostate cancer. *Prostate* **68**, 1133-1142 (2008).
41. Yu, J., Cao, Q., Mehra, R., Laxman, B., Yu, J., Tomlins, S. A., Creighton, C. J., Dhanasekaran, S. M., Shen, R., Chen, G., Morris, D. S., Marquez, V. E., Shah, R. B., Ghosh, D., Varambally, S. & Chinnaiyan, A. M. Integrative genomics analysis reveals silencing of beta-adrenergic signaling by polycomb in prostate cancer. *Cancer Cell* **12**, 419-431 (2007).
42. Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J., Heuer, M. L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A. P., Sander, C. & Schultz, N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* **2**, 401-404 (2012).
43. Palm, D., Lang, K., Niggemann, B., Drell, T. L. th, Masur, K., Zaenker, K. S. & Entschladen, F. The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. *Int J Cancer* **118**, 2744-2749 (2006).
44. Zepp, E. A. & Thomas, J. A. Effect of androgens on isoproterenol-induced increases in mouse accessory sex organ cyclic AMP in vitro. *Biochemical pharmacology* **27**, 465-468 (1978).
45. Bang, Y. J., Pirnia, F., Fang, W. G., Kang, W. K., Sartor, O., Whitesell, L., Ha, M. J., Tsokos, M., Sheahan, M. D., Nguyen, P., Niklinski, W. T., Myers, C. E. & Trepel, J. B. Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. *Proc Natl Acad Sci U S A* **91**, 5330-5334 (1994).
46. Cox, M. E., Deeble, P. D., Bissonette, E. A. & Parsons, S. J. Activated 3',5'-cyclic AMP-dependent protein kinase is sufficient to induce neuroendocrine-like differentiation of the LNCaP prostate tumor cell line. *J Biol Chem* **275**, 13812-13818 (2000).

47. Cox, M. E., Deeble, P. D., Lakhani, S. & Parsons, S. J. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. *Cancer Res* **59**, 3821-3830 (1999).
48. Yuan, T. C., Veeramani, S. & Lin, M. F. Neuroendocrine-like prostate cancer cells: neuroendocrine transdifferentiation of prostate adenocarcinoma cells. *Endocr Relat Cancer* **14**, 531-547 (2007).
49. Turbat-Herrera, E. A., Herrera, G. A., Gore, I., Lott, R. L., Grizzle, W. E. & Bonnin, J. M. Neuroendocrine differentiation in prostatic carcinomas. A retrospective autopsy study. *Archives of pathology & laboratory medicine* **112**, 1100-1105 (1988).
50. Helpap, B. Morphology and therapeutic strategies for neuroendocrine tumors of the genitourinary tract. *Cancer* **95**, 1415-1420 (2002).
51. Tanaka, M., Suzuki, Y., Takaoka, K., Suzuki, N., Murakami, S., Matsuzaki, O. & Shimazaki, J. Progression of prostate cancer to neuroendocrine cell tumor. *International journal of urology : official journal of the Japanese Urological Association* **8**, 431-436; discussion 437 (2001).
52. Shah, R. B., Mehra, R., Chinnaiyan, A. M., Shen, R., Ghosh, D., Zhou, M., Macvicar, G. R., Varambally, S., Harwood, J., Bismar, T. A., Kim, R., Rubin, M. A. & Pienta, K. J. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* **64**, 9209-9216 (2004).
53. Hu, C. D., Choo, R. & Huang, J. Neuroendocrine differentiation in prostate cancer: a mechanism of radioresistance and treatment failure. *Frontiers in oncology* **5**, 90 (2015).
54. Komiya, A., Yasuda, K., Watanabe, A., Fujiuchi, Y., Tsuzuki, T. & Fuse, H. The prognostic significance of loss of the androgen receptor and neuroendocrine differentiation in prostate biopsy specimens among castration-resistant prostate cancer patients. *Molecular and clinical oncology* **1**, 257-262 (2013).
55. Vignani, F., Russo, L., Tucci, M., Motta, M., Vellani, G., Tampellini, M., Papotti, M., Dogliotti, L. & Berruti, A. Why castration-resistant prostate cancer patients with neuroendocrine differentiation should be addressed to a cisplatin-based regimen. *Ann Oncol* **20**, 2019-2020 (2009).
56. Beltran, H., Tagawa, S. T., Park, K., MacDonald, T., Milowsky, M. I., Mosquera, J. M., Rubin, M. A. & Nanus, D. M. Challenges in recognizing treatment-related neuroendocrine prostate cancer. *J Clin Oncol* **30**, e386-389 (2012).
57. Goodman, O. B., Jr., Flaig, T. W., Molina, A., Mulders, P. F., Fizazi, K., Suttmann, H., Li, J., Kheoh, T., de Bono, J. S. & Scher, H. I. Exploratory analysis of the visceral disease subgroup in a phase III study of abiraterone acetate in metastatic castration-resistant prostate cancer. *Prostate cancer and prostatic diseases* **17**, 34-39 (2014).
58. Scher, H. I., Fizazi, K., Saad, F., Taplin, M. E., Sternberg, C. N., Miller, K., de Wit, R., Mulders, P., Chi, K. N., Shore, N. D., Armstrong, A. J., Flaig, T. W., Flechon, A., Mainwaring, P., Fleming, M., Hainsworth, J. D., Hirmand, M., Selby, B., Seely, L. & de Bono, J. S. Increased survival with enzalutamide in prostate cancer after chemotherapy. *The New England journal of medicine* **367**, 1187-1197 (2012).
59. Tan, H. L., Sood, A., Rahimi, H. A., Wang, W., Gupta, N., Hicks, J., Mosier, S., Gocke, C. D., Epstein, J. I., Netto, G. J., Liu, W., Isaacs, W. B., De Marzo, A. M. & Lotan, T. L. Rb loss is characteristic of prostatic small cell neuroendocrine carcinoma. *Clin Cancer Res* **20**, 890-903 (2014).
60. Folkman, J. Tumor angiogenesis. *Advances in cancer research* **19**, 331-358 (1974).
61. Folkman, J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**, 1182-1186 (1971).

62. Augustin, H. G. Commentary on Folkman: "How Is Blood Vessel Growth Regulated in Normal and Neoplastic Tissue?". *Cancer Res* **76**, 2854-2856 (2016).
63. Folkman, J. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Res* **46**, 467-473 (1986).
64. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
65. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
66. Park, S. Y., Kang, J. H., Jeong, K. J., Lee, J., Han, J. W., Choi, W. S., Kim, Y. K., Kang, J., Park, C. G. & Lee, H. Y. Norepinephrine induces VEGF expression and angiogenesis by a hypoxia-inducible factor-1alpha protein-dependent mechanism. *Int J Cancer* **128**, 2306-2316 (2011).
67. Bavadekar, Supriya, Budajaja, Faten, Patel, Kartik & Vansal, Sandeep. Epinephrine stimulates secretion of VEGF by human prostate cancer cells, LNCaP, through a beta2-adrenergic receptor-mediated pathway. *The FASEB Journal* **27**, 1105.1111 (2013).
68. Plecas, B., Glavaski, A. & Solarovic, T. Propranolol treatment affects ventral prostate blood vessels and serum testosterone concentrations in adult rats. *Andrologia* **29**, 109-114 (1997).
69. Borre, M., Nerstrom, B. & Overgaard, J. Association between immunohistochemical expression of vascular endothelial growth factor (VEGF), VEGF-expressing neuroendocrine-differentiated tumor cells, and outcome in prostate cancer patients subjected to watchful waiting. *Clin Cancer Res* **6**, 1882-1890 (2000).
70. Grobholz, R., Bohrer, M. H., Siegsmund, M., Junemann, K. P., Bleyl, U. & Woenckhaus, M. Correlation between neovascularisation and neuroendocrine differentiation in prostatic carcinoma. *Pathology, research and practice* **196**, 277-284 (2000).
71. Heinrich, E., Trojan, L., Friedrich, D., Voss, M., Weiss, C., Michel, M. S. & Grobholz, R. Neuroendocrine tumor cells in prostate cancer: evaluation of the neurosecretory products serotonin, bombesin, and gastrin - impact on angiogenesis and clinical follow-up. *Prostate* **71**, 1752-1758 (2011).
72. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. & Graybiel, A. M. A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**, 2275-2279 (1998).
73. Gloerich, M. & Bos, J. L. Epac: defining a new mechanism for cAMP action. *Annual review of pharmacology and toxicology* **50**, 355-375 (2010).
74. Banerjee, U. & Cheng, X. Exchange protein directly activated by cAMP encoded by the mammalian rapgef3 gene: Structure, function and therapeutics. *Gene* **570**, 157-167 (2015).
75. Almahariq, M., Mei, F. C. & Cheng, X. Cyclic AMP sensor EPAC proteins and energy homeostasis. *Trends in endocrinology and metabolism: TEM* **25**, 60-71 (2014).
76. Cheng, X., Ji, Z., Tsalkova, T. & Mei, F. Epac and PKA: a tale of two intracellular cAMP receptors. *Acta biochimica et biophysica Sinica* **40**, 651-662 (2008).
77. Sastry, K. S., Karpova, Y., Prokopovich, S., Smith, A. J., Essau, B., Gersappe, A., Carson, J. P., Weber, M. J., Register, T. C., Chen, Y. Q., Penn, R. B. & Kulik, G. Epinephrine protects cancer cells from apoptosis via activation of cAMP-dependent protein kinase and BAD phosphorylation. *J Biol Chem* **282**, 14094-14100 (2007).
78. Wells, C. M., Whale, A. D., Parsons, M., Masters, J. R. & Jones, G. E. PAK4: a pluripotent kinase that regulates prostate cancer cell adhesion. *Journal of cell science* **123**, 1663-1673 (2010).
79. Farini, D., Puglianiello, A., Mammi, C., Siracusa, G. & Moretti, C. Dual effect of pituitary adenylate cyclase activating polypeptide on prostate tumor LNCaP cells: short- and long-term exposure affect proliferation and neuroendocrine differentiation. *Endocrinology* **144**, 1631-1643 (2003).

80. Goodin, J. L. & Rutherford, C. L. Identification of differentially expressed genes during cyclic adenosine monophosphate-induced neuroendocrine differentiation in the human prostatic adenocarcinoma cell line LNCaP. *Molecular carcinogenesis* **33**, 88-98 (2002).
81. Zelivianski, S., Verni, M., Moore, C., Kondrikov, D., Taylor, R. & Lin, M. F. Multipathways for transdifferentiation of human prostate cancer cells into neuroendocrine-like phenotype. *Biochim Biophys Acta* **1539**, 28-43 (2001).
82. Deeble, P. D., Cox, M. E., Frierson, H. F., Jr., Sikes, R. A., Palmer, J. B., Davidson, R. J., Casarez, E. V., Amorino, G. P. & Parsons, S. J. Androgen-independent growth and tumorigenesis of prostate cancer cells are enhanced by the presence of PKA-differentiated neuroendocrine cells. *Cancer Res* **67**, 3663-3672 (2007).
83. Deeble, P. D., Murphy, D. J., Parsons, S. J. & Cox, M. E. Interleukin-6- and cyclic AMP-mediated signaling potentiates neuroendocrine differentiation of LNCaP prostate tumor cells. *Mol Cell Biol* **21**, 8471-8482 (2001).
84. Deng, X., Elzey, B. D., Poulson, J. M., Morrison, W. B., Ko, S. C., Hahn, N. M., Ratliff, T. L. & Hu, C. D. Ionizing radiation induces neuroendocrine differentiation of prostate cancer cells in vitro, in vivo and in prostate cancer patients. *Am J Cancer Res* **1**, 834-844 (2011).
85. Deng, X., Liu, H., Huang, J., Cheng, L., Keller, E. T., Parsons, S. J. & Hu, C. D. Ionizing radiation induces prostate cancer neuroendocrine differentiation through interplay of CREB and ATF2: implications for disease progression. *Cancer Res* **68**, 9663-9670 (2008).
86. Suarez, C. D., Deng, X. & Hu, C. D. Targeting CREB inhibits radiation-induced neuroendocrine differentiation and increases radiation-induced cell death in prostate cancer cells. *Am J Cancer Res* **4**, 850-861 (2014).
87. Antos, C. L., McKinsey, T. A., Dreitz, M., Hollingsworth, L. M., Zhang, C. L., Schreiber, K., Rindt, H., Gorczynski, R. J. & Olson, E. N. Dose-dependent blockade to cardiomyocyte hypertrophy by histone deacetylase inhibitors. *J Biol Chem* **278**, 28930-28937 (2003).
88. Kee, H. J., Sohn, I. S., Nam, K. I., Park, J. E., Qian, Y. R., Yin, Z., Ahn, Y., Jeong, M. H., Bang, Y. J., Kim, N., Kim, J. K., Kim, K. K., Epstein, J. A. & Kook, H. Inhibition of histone deacetylation blocks cardiac hypertrophy induced by angiotensin II infusion and aortic banding. *Circulation* **113**, 51-59 (2006).
89. Kong, Y., Tannous, P., Lu, G., Berenji, K., Rothermel, B. A., Olson, E. N. & Hill, J. A. Suppression of class I and II histone deacetylases blunts pressure-overload cardiac hypertrophy. *Circulation* **113**, 2579-2588 (2006).
90. Trivedi, C. M., Luo, Y., Yin, Z., Zhang, M., Zhu, W., Wang, T., Floss, T., Goettlicher, M., Noppinger, P. R., Wurst, W., Ferrari, V. A., Abrams, C. S., Gruber, P. J. & Epstein, J. A. Hdac2 regulates the cardiac hypertrophic response by modulating Gsk3 beta activity. *Nat Med* **13**, 324-331 (2007).
91. Li, W., Ai, N., Wang, S., Bhattacharya, N., Vrbanac, V., Collins, M., Signoretti, S., Hu, Y., Boyce, F. M., Gravdal, K., Halvorsen, O. J., Nalwoga, H., Akslen, L. A., Harlow, E. & Watnick, R. S. GRK3 is essential for metastatic cells and promotes prostate tumor progression. *Proc Natl Acad Sci U S A* (2014).
92. Pettaway, C. A., Pathak, S., Greene, G., Ramirez, E., Wilson, M. R., Killion, J. J. & Fidler, I. J. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin Cancer Res* **2**, 1627-1636 (1996).
93. Yuan, T. C., Veeramani, S., Lin, F. F., Kondrikou, D., Zelivianski, S., Igawa, T., Karan, D., Batra, S. K. & Lin, M. F. Androgen deprivation induces human prostate epithelial neuroendocrine differentiation of androgen-sensitive LNCaP cells. *Endocr Relat Cancer* **13**, 151-167 (2006).
94. Moffat, J., Grueneberg, D. A., Yang, X., Kim, S. Y., Kloepfer, A. M., Hinkle, G., Piqani, B., Eisenhaure, T. M., Luo, B., Grenier, J. K., Carpenter, A. E., Foo, S. Y., Stewart, S. A., Stockwell,

B. R., Hacohen, N., Hahn, W. C., Lander, E. S., Sabatini, D. M. & Root, D. E. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* **124**, 1283-1298 (2006).

95. Du, K., Asahara, H., Jhala, U. S., Wagner, B. L. & Montminy, M. Characterization of a CREB gain-of-function mutant with constitutive transcriptional activity in vivo. *Mol Cell Biol* **20**, 4320-4327 (2000).
96. Li, L., Liu, C., Amato, R. J., Chang, J. T., Du, G. & Li, W. CDKL2 promotes epithelial-mesenchymal transition and breast cancer progression. *Oncotarget* **5**, 10840-10853 (2014).
97. Sang, M., Hulsurkar, M., Zhang, X., Song, H., Zheng, D., Zhang, Y., Li, M., Xu, J., Zhang, S., Ittmann, M. & Li, W. GRK3 is a direct target of CREB activation and regulates neuroendocrine differentiation of prostate cancer cells. *Oncotarget* (2016).
98. Gao, J., Aksoy, B. A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S. O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C. & Schultz, N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* **6**, pl1 (2013).
99. Aparicio, A., Logothetis, C. J. & Maity, S. N. Understanding the lethal variant of prostate cancer: power of examining extremes. *Cancer Discov* **1**, 466-468 (2011).
100. Hirano, D., Okada, Y., Minei, S., Takimoto, Y. & Nemoto, N. Neuroendocrine differentiation in hormone refractory prostate cancer following androgen deprivation therapy. *Eur Urol* **45**, 586-592; discussion 592 (2004).
101. Papandreou, C. N., Daliani, D. D., Thall, P. F., Tu, S. M., Wang, X., Reyes, A., Troncoso, P. & Logothetis, C. J. Results of a phase II study with doxorubicin, etoposide, and cisplatin in patients with fully characterized small-cell carcinoma of the prostate. *J Clin Oncol* **20**, 3072-3080 (2002).
102. Wang, H. T., Yao, Y. H., Li, B. G., Tang, Y., Chang, J. W. & Zhang, J. Neuroendocrine Prostate Cancer (NEPC) progressing from conventional prostatic adenocarcinoma: factors associated with time to development of NEPC and survival from NEPC diagnosis-a systematic review and pooled analysis. *J Clin Oncol* **32**, 3383-3390 (2014).
103. Wyatt, A. W., Mo, F., Wang, K., McConeghy, B., Brahmbhatt, S., Jong, L., Mitchell, D. M., Johnston, R. L., Haegert, A., Li, E., Liew, J., Yeung, J., Shrestha, R., Lapuk, A. V., McPherson, A., Shukin, R., Bell, R. H., Anderson, S., Bishop, J., Hurtado-Coll, A., Xiao, H., Chinnaiyan, A. M., Mehra, R., Lin, D., Wang, Y., Fazli, L., Gleave, M. E., Volik, S. V. & Collins, C. C. Heterogeneity in the inter-tumor transcriptome of high risk prostate cancer. *Genome biology* **15**, 426 (2014).
104. Ismail, A. Hr, Landry, F., Aprikian, A. G. & Chevalier, S. Androgen ablation promotes neuroendocrine cell differentiation in dog and human prostate. *Prostate* **51**, 117-125 (2002).
105. Ito, T., Yamamoto, S., Ohno, Y., Namiki, K., Aizawa, T., Akiyama, A. & Tachibana, M. Up-regulation of neuroendocrine differentiation in prostate cancer after androgen deprivation therapy, degree and androgen independence. *Oncol Rep* **8**, 1221-1224 (2001).
106. Shen, R., Dorai, T., Szaboles, M., Katz, A. E., Olsson, C. A. & Buttyan, R. Transdifferentiation of cultured human prostate cancer cells to a neuroendocrine cell phenotype in a hormone-depleted medium. *Urologic oncology* **3**, 67-75 (1997).
107. Li, Z., Chen, C. J., Wang, J. K., Hsia, E., Li, W., Squires, J., Sun, Y. & Huang, J. Neuroendocrine differentiation of prostate cancer. *Asian journal of andrology* **15**, 328-332 (2013).
108. Theodoropoulos, V. E., Tsigka, A., Mihalopoulou, A., Tsoukala, V., Lazaris, A. C., Patsouris, E. & Ghikonti, I. Evaluation of neuroendocrine staining and androgen receptor expression in incidental prostatic adenocarcinoma: prognostic implications. *Urology* **66**, 897-902 (2005).

109. Choi, S. Y., Lin, D., Gout, P. W., Collins, C. C., Xu, Y. & Wang, Y. Lessons from patient-derived xenografts for better in vitro modeling of human cancer. *Advanced drug delivery reviews* **79-80**, 222-237 (2014).
110. Lin, D., Wyatt, A. W., Xue, H., Wang, Y., Dong, X., Haegert, A., Wu, R., Brahmbhatt, S., Mo, F., Jong, L., Bell, R. H., Anderson, S., Hurtado-Coll, A., Fazli, L., Sharma, M., Beltran, H., Rubin, M., Cox, M., Gout, P. W., Morris, J., Goldenberg, L., Volik, S. V., Gleave, M. E., Collins, C. C. & Wang, Y. High fidelity patient-derived xenografts for accelerating prostate cancer discovery and drug development. *Cancer Res* **74**, 1272-1283 (2014).
111. Lonze, B. E. & Ginty, D. D. Function and regulation of CREB family transcription factors in the nervous system. *Neuron* **35**, 605-623 (2002).
112. Esparza, S. D., Chang, J., Shankar, D. B., Zhang, B., Nelson, S. F. & Sakamoto, K. M. CREB regulates Meis1 expression in normal and malignant hematopoietic cells. *Leukemia* **22**, 665-667 (2008).
113. Hughes-Fulford, M., Sugano, E., Schopper, T., Li, C. F., Boonyaratanakornkit, J. B. & Cogoli, A. Early immune response and regulation of IL-2 receptor subunits. *Cell Signal* **17**, 1111-1124 (2005).
114. Silva, A. J., Kogan, J. H., Frankland, P. W. & Kida, S. CREB and memory. *Annual review of neuroscience* **21**, 127-148 (1998).
115. Bito, H., Deisseroth, K. & Tsien, R. W. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. *Cell* **87**, 1203-1214 (1996).
116. Pitcher, J. A., Freedman, N. J. & Lefkowitz, R. J. G protein-coupled receptor kinases. *Annu Rev Biochem* **67**, 653-692 (1998).
117. Ribas, C., Penela, P., Murga, C., Salcedo, A., Garcia-Hoz, C., Jurado-Pueyo, M., Aymerich, I. & Mayor, F., Jr. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. *Biochim Biophys Acta* **1768**, 913-922 (2007).
118. Miller, W. E., Houtz, D. A., Nelson, C. D., Kolattukudy, P. E. & Lefkowitz, R. J. G-protein-coupled receptor (GPCR) kinase phosphorylation and beta-arrestin recruitment regulate the constitutive signaling activity of the human cytomegalovirus US28 GPCR. *J Biol Chem* **278**, 21663-21671 (2003).
119. Penela, P., Ribas, C. & Mayor, F., Jr. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal* **15**, 973-981 (2003).
120. Reiter, E. & Lefkowitz, R. J. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in endocrinology and metabolism: TEM* **17**, 159-165 (2006).
121. Hausdorff, W. P., Caron, M. G. & Lefkowitz, R. J. Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **4**, 2881-2889 (1990).
122. Freedman, N. J., Liggett, S. B., Drachman, D. E., Pei, G., Caron, M. G. & Lefkowitz, R. J. Phosphorylation and desensitization of the human beta 1-adrenergic receptor. Involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase. *J Biol Chem* **270**, 17953-17961 (1995).
123. O'Hayre, M., Degese, M. S. & Gutkind, J. S. Novel insights into G protein and G protein-coupled receptor signaling in cancer. *Current opinion in cell biology* **27**, 126-135 (2014).
124. Heisler, S. & Reisine, T. Forskolin stimulates adenylate cyclase activity, cyclic AMP accumulation, and adrenocorticotropin secretion from mouse anterior pituitary tumor cells. *J Neurochem* **42**, 1659-1666 (1984).
125. Feldman, B. J. & Feldman, D. The development of androgen-independent prostate cancer. *Nat Rev Cancer* **1**, 34-45 (2001).

126. Nouri, M., Ratther, E., Stylianou, N., Nelson, C. C., Hollier, B. G. & Williams, E. D. Androgen-targeted therapy-induced epithelial mesenchymal plasticity and neuroendocrine transdifferentiation in prostate cancer: an opportunity for intervention. *Frontiers in oncology* **4**, 370 (2014).
127. Zhang, X. Q., Kondrikov, D., Yuan, T. C., Lin, F. F., Hansen, J. & Lin, M. F. Receptor protein tyrosine phosphatase alpha signaling is involved in androgen depletion-induced neuroendocrine differentiation of androgen-sensitive LNCaP human prostate cancer cells. *Oncogene* **22**, 6704-6716 (2003).
128. Mertz, K. D., Setlur, S. R., Dhanasekaran, S. M., Demichelis, F., Perner, S., Tomlins, S., Tchinda, J., Laxman, B., Vessella, R. L., Beroukhim, R., Lee, C., Chinnaiyan, A. M. & Rubin, M. A. Molecular characterization of TMPRSS2-ERG gene fusion in the NCI-H660 prostate cancer cell line: a new perspective for an old model. *Neoplasia (New York, N.Y.)* **9**, 200-206 (2007).
129. D'Antonio, J. M., Ma, C., Monzon, F. A. & Pflug, B. R. Longitudinal analysis of androgen deprivation of prostate cancer cells identifies pathways to androgen independence. *Prostate* **68**, 698-714 (2008).
130. Akamatsu, S., Wyatt, A. W., Lin, D., Lysakowski, S., Zhang, F., Kim, S., Tse, C., Wang, K., Mo, F., Haegert, A., Brahmbhatt, S., Bell, R., Adomat, H., Kawai, Y., Xue, H., Dong, X., Fazli, L., Tsai, H., Lotan, T. L., Kossai, M., Mosquera, J. M., Rubin, M. A., Beltran, H., Zoubeidi, A., Wang, Y., Gleave, M. E. & Collins, C. C. The Placental Gene PEG10 Promotes Progression of Neuroendocrine Prostate Cancer. *Cell reports* **12**, 922-936 (2015).
131. Gingrich, J. R., Barrios, R. J., Foster, B. A. & Greenberg, N. M. Pathologic progression of autochthonous prostate cancer in the TRAMP model. *Prostate cancer and prostatic diseases* **2**, 70-75 (1999).
132. Hurwitz, A. A., Foster, B. A., Allison, J. P., Greenberg, N. M. & Kwon, E. D. The TRAMP mouse as a model for prostate cancer. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 20**, Unit 20.25 (2001).
133. Aytes, A., Mitrofanova, A., Lefebvre, C., Alvarez, M. J., Castillo-Martin, M., Zheng, T., Eastham, J. A., Gopalan, A., Pienta, K. J., Shen, M. M., Califano, A. & Abate-Shen, C. Cross-species regulatory network analysis identifies a synergistic interaction between FOXM1 and CENPF that drives prostate cancer malignancy. *Cancer Cell* **25**, 638-651 (2014).
134. Lucas, J. M., Heinlein, C., Kim, T., Hernandez, S. A., Malik, M. S., True, L. D., Morrissey, C., Corey, E., Montgomery, B., Mostaghel, E., Clegg, N., Coleman, I., Brown, C. M., Schneider, E. L., Craik, C., Simon, J. A., Bedalov, A. & Nelson, P. S. The androgen-regulated protease TMPRSS2 activates a proteolytic cascade involving components of the tumor microenvironment and promotes prostate cancer metastasis. *Cancer Discov* **4**, 1310-1325 (2014).
135. Xiao, X., Li, B. X., Mitton, B., Ikeda, A. & Sakamoto, K. M. Targeting CREB for cancer therapy: friend or foe. *Curr Cancer Drug Targets* **10**, 384-391 (2010).
136. Wu, G. Y., Deisseroth, K. & Tsien, R. W. Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. *Proc Natl Acad Sci U S A* **98**, 2808-2813 (2001).
137. Parker, D., Ferreri, K., Nakajima, T., LaMorte, V. J., Evans, R., Koerber, S. C., Hoeger, C. & Montminy, M. R. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol Cell Biol* **16**, 694-703 (1996).
138. Gonzalez, G. A. & Montminy, M. R. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675-680 (1989).

139. Roseboom, P. H. & Klein, D. C. Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: primary role of a beta-adrenergic receptor/cyclic AMP mechanism. *Molecular pharmacology* **47**, 439-449 (1995).
140. Yuan, Q., Harley, C. W., Bruce, J. C., Darby-King, A. & McLean, J. H. Isoproterenol increases CREB phosphorylation and olfactory nerve-evoked potentials in normal and 5-HT-depleted olfactory bulbs in rat pups only at doses that produce odor preference learning. *Learning & memory (Cold Spring Harbor, N.Y.)* **7**, 413-421 (2000).
141. Ozgen, N., Obreztchikova, M., Guo, J., Elouardighi, H., Dorn, G. W., 2nd, Wilson, B. A. & Steinberg, S. F. Protein kinase D links Gq-coupled receptors to cAMP response element-binding protein (CREB)-Ser133 phosphorylation in the heart. *J Biol Chem* **283**, 17009-17019 (2008).
142. Zhang, D., Ma, Q. Y., Hu, H. T. & Zhang, M. beta2-adrenergic antagonists suppress pancreatic cancer cell invasion by inhibiting CREB, NFkappaB and AP-1. *Cancer biology & therapy* **10**, 19-29 (2010).
143. Yu, Y. P., Landsittel, D., Jing, L., Nelson, J., Ren, B., Liu, L., McDonald, C., Thomas, R., Dhir, R., Finkelstein, S., Michalopoulos, G., Becich, M. & Luo, J. H. Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. *J Clin Oncol* **22**, 2790-2799 (2004).
144. Taylor, B. S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B. S., Arora, V. K., Kaushik, P., Cerami, E., Reva, B., Antipin, Y., Mitsiades, N., Landers, T., Dolgalev, I., Major, J. E., Wilson, M., Socci, N. D., Lash, A. E., Heguy, A., Eastham, J. A., Scher, H. I., Reuter, V. E., Scardino, P. T., Sander, C., Sawyers, C. L. & Gerald, W. L. Integrative genomic profiling of human prostate cancer. *Cancer Cell* **18**, 11-22 (2010).
145. Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A. A., Kim, S., Wilson, C. J., Lehar, J., Kryukov, G. V., Sonkin, D., Reddy, A., Liu, M., Murray, L., Berger, M. F., Monahan, J. E., Morais, P., Meltzer, J., Korejwa, A., Jane-Valbuena, J., Mapa, F. A., Thibault, J., Bric-Furlong, E., Raman, P., Shipway, A., Engels, I. H., Cheng, J., Yu, G. K., Yu, J., Aspesi, P., Jr., de Silva, M., Jagtap, K., Jones, M. D., Wang, L., Hatton, C., Palescandolo, E., Gupta, S., Mahan, S., Sougnez, C., Onofrio, R. C., Liefeld, T., MacConaill, L., Winckler, W., Reich, M., Li, N., Mesirov, J. P., Gabriel, S. B., Getz, G., Ardlie, K., Chan, V., Myer, V. E., Weber, B. L., Porter, J., Warmuth, M., Finan, P., Harris, J. L., Meyerson, M., Golub, T. R., Morrissey, M. P., Sellers, W. R., Schlegel, R. & Garraway, L. A. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603-607 (2012).
146. Diviani, D., Lattion, A. L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L. & Cotecchia, S. Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the alpha1B-adrenergic receptor. *J Biol Chem* **271**, 5049-5058 (1996).
147. Nordin, A., Wang, W., Welen, K. & Damber, J. E. Midkine is associated with neuroendocrine differentiation in castration-resistant prostate cancer. *Prostate* **73**, 657-667 (2013).
148. Zhang, X., Coleman, I. M., Brown, L. G., True, L. D., Kollath, L., Lucas, J. M., Lam, H. M., Dumpit, R., Corey, E., Chery, L., Lakely, B., Higano, C. S., Montgomery, B., Roudier, M., Lange, P. H., Nelson, P. S., Vessella, R. L. & Morrissey, C. SRRM4 Expression and the Loss of REST Activity May Promote the Emergence of the Neuroendocrine Phenotype in Castration-Resistant Prostate Cancer. *Clin Cancer Res* **21**, 4698-4708 (2015).
149. Krantz, D. S. & McCeney, M. K. Effects of psychological and social factors on organic disease: a critical assessment of research on coronary heart disease. *Annual review of psychology* **53**, 341-369 (2002).
150. Lichtman, J. H., Bigger, J. T., Jr., Blumenthal, J. A., Frasure-Smith, N., Kaufmann, P. G., Lesperance, F., Mark, D. B., Sheps, D. S., Taylor, C. B. & Froelicher, E. S. Depression and

coronary heart disease: recommendations for screening, referral, and treatment: a science advisory from the American Heart Association Prevention Committee of the Council on Cardiovascular Nursing, Council on Clinical Cardiology, Council on Epidemiology and Prevention, and Interdisciplinary Council on Quality of Care and Outcomes Research: endorsed by the American Psychiatric Association. *Circulation* **118**, 1768-1775 (2008).

151. Antoni, M. H., Lutgendorf, S. K., Cole, S. W., Dhabhar, F. S., Sephton, S. E., McDonald, P. G., Stefanek, M. & Sood, A. K. The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nat Rev Cancer* **6**, 240-248 (2006).
152. Holmes, S., Griffith, E. J., Musto, G. & Minuk, G. Y. Antihypertensive medications and survival in patients with cancer: A population-based retrospective cohort study. *Cancer Epidemiol* (2013).
153. Wang, H. M., Liao, Z. X., Komaki, R., Welsh, J. W., O'Reilly, M. S., Chang, J. Y., Zhuang, Y., Levy, L. B., Lu, C. & Gomez, D. R. Improved survival outcomes with the incidental use of beta-blockers among patients with non-small-cell lung cancer treated with definitive radiation therapy. *Ann Oncol* **24**, 1312-1319 (2013).
154. Akey, C. W. & Luger, K. Histone chaperones and nucleosome assembly. *Current opinion in structural biology* **13**, 6-14 (2003).
155. Richmond, T. J. & Davey, C. A. The structure of DNA in the nucleosome core. *Nature* **423**, 145-150 (2003).
156. Reik, W., Dean, W. & Walter, J. Epigenetic reprogramming in mammalian development. *Science* **293**, 1089-1093 (2001).
157. Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics* **33 Suppl**, 245-254 (2003).
158. Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. *Cell research* **21**, 381-395 (2011).
159. Minucci, S. & Pelicci, P. G. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* **6**, 38-51 (2006).
160. Yang, X. J. & Seto, E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene* **26**, 5310-5318 (2007).
161. de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S. & van Kuilenburg, A. B. Histone deacetylases (HDACs): characterization of the classical HDAC family. *The Biochemical journal* **370**, 737-749 (2003).
162. Cress, W. D. & Seto, E. Histone deacetylases, transcriptional control, and cancer. *Journal of cellular physiology* **184**, 1-16 (2000).
163. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A. & Bouck, N. P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A* **87**, 6624-6628 (1990).
164. Hsu, S. C., Volpert, O. V., Steck, P. A., Mikkelsen, T., Polverini, P. J., Rao, S., Chou, P. & Bouck, N. P. Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. *Cancer Res* **56**, 5684-5691 (1996).
165. Lawler, J. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *Journal of cellular and molecular medicine* **6**, 1-12 (2002).
166. Margossian, S. S., Lawler, J. W. & Slayter, H. S. Physical characterization of platelet thrombospondin. *J Biol Chem* **256**, 7495-7500 (1981).
167. McPherson, J., Sage, H. & Bornstein, P. Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture. Apparent identity with platelet thrombospondin. *J Biol Chem* **256**, 11330-11336 (1981).

168. Lawler, J. W., Slayter, H. S. & Coligan, J. E. Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J Biol Chem* **253**, 8609-8616 (1978).
169. Lawler, P. R. & Lawler, J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. *Cold Spring Harbor perspectives in medicine* **2**, a006627 (2012).
170. Byler, T. K., Leocadio, D., Shapiro, O., Bratslavsky, G., Stodgell, C. J., Wood, R. W., Messing, E. M. & Reeder, J. E. Valproic acid decreases urothelial cancer cell proliferation and induces thrombospondin-1 expression. *BMC urology* **12**, 21 (2012).
171. Duvic, M., Talpur, R., Ni, X., Zhang, C., Hazarika, P., Kelly, C., Chiao, J. H., Reilly, J. F., Ricker, J. L., Richon, V. M. & Frankel, S. R. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood* **109**, 31-39 (2007).
172. Yang, Q., Tian, Y., Liu, S., Zeine, R., Chlenski, A., Salwen, H. R., Henkin, J. & Cohn, S. L. Thrombospondin-1 peptide ABT-510 combined with valproic acid is an effective antiangiogenesis strategy in neuroblastoma. *Cancer Res* **67**, 1716-1724 (2007).
173. de Graaf, C. & Rognan, D. Selective structure-based virtual screening for full and partial agonists of the beta2 adrenergic receptor. *Journal of medicinal chemistry* **51**, 4978-4985 (2008).
174. Dishy, V., Sofowora, G. G., Xie, H. G., Kim, R. B., Byrne, D. W., Stein, C. M. & Wood, A. J. The effect of common polymorphisms of the beta2-adrenergic receptor on agonist-mediated vascular desensitization. *The New England journal of medicine* **345**, 1030-1035 (2001).
175. Graupera, M., Guillermet-Guibert, J., Foukas, L. C., Phng, L. K., Cain, R. J., Salpekar, A., Pearce, W., Meek, S., Millan, J., Cutillas, P. R., Smith, A. J., Ridley, A. J., Ruhrberg, C., Gerhardt, H. & Vanhaesebroeck, B. Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. *Nature* **453**, 662-666 (2008).
176. Walchli, T., Mateos, J. M., Weinman, O., Babic, D., Regli, L., Hoerstrup, S. P., Gerhardt, H., Schwab, M. E. & Vogel, J. Quantitative assessment of angiogenesis, perfused blood vessels and endothelial tip cells in the postnatal mouse brain. *Nature protocols* **10**, 53-74 (2015).
177. Staton, C. A., Stribbling, S. M., Tazzyman, S., Hughes, R., Brown, N. J. & Lewis, C. E. Current methods for assaying angiogenesis in vitro and in vivo. *Int J Exp Pathol* **85**, 233-248 (2004).
178. Auerbach, R., Lewis, R., Shinners, B., Kubai, L. & Akhtar, N. Angiogenesis assays: a critical overview. *Clinical chemistry* **49**, 32-40 (2003).
179. Arnaoutova, I. & Kleinman, H. K. In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. *Nature protocols* **5**, 628-635 (2010).
180. The Molecular Taxonomy of Primary Prostate Cancer. *Cell* **163**, 1011-1025 (2015).
181. Robinson, D., Van Allen, E. M., Wu, Y. M., Schultz, N., Lonigro, R. J., Mosquera, J. M., Montgomery, B., Taplin, M. E., Pritchard, C. C., Attard, G., Beltran, H., Abida, W., Bradley, R. K., Vinson, J., Cao, X., Vats, P., Kunju, L. P., Hussain, M., Feng, F. Y., Tomlins, S. A., Cooney, K. A., Smith, D. C., Brennan, C., Siddiqui, J., Mehra, R., Chen, Y., Rathkopf, D. E., Morris, M. J., Solomon, S. B., Durack, J. C., Reuter, V. E., Gopalan, A., Gao, J., Loda, M., Lis, R. T., Bowden, M., Balk, S. P., Gaviola, G., Sougnez, C., Gupta, M., Yu, E. Y., Mostaghel, E. A., Cheng, H. H., Mulcahy, H., True, L. D., Plymate, S. R., Dvinge, H., Ferraldeschi, R., Flohr, P., Miranda, S., Zafeiriou, Z., Tunariu, N., Mateo, J., Perez-Lopez, R., Demichelis, F., Robinson, B. D., Schiffman, M., Nanus, D. M., Tagawa, S. T., Sigaras, A., Eng, K. W., Elemento, O., Sboner, A., Heath, E. I., Scher, H. I., Pienta, K. J., Kantoff, P., de Bono, J. S., Rubin, M. A., Nelson, P. S., Garraway, L. A., Sawyers, C. L. & Chinnaiyan, A. M. Integrative clinical genomics of advanced prostate cancer. *Cell* **161**, 1215-1228 (2015).
182. Dautzenberg, F. M., Wille, S., Braun, S. & Hauger, R. L. GRK3 regulation during CRF- and urocortin-induced CRF1 receptor desensitization. *Biochem Biophys Res Commun* **298**, 303-308 (2002).

183. Klebl, B. M. & Muller, G. Second-generation kinase inhibitors. *Expert opinion on therapeutic targets* **9**, 975-993 (2005).
184. Perot, S., Sperandio, O., Miteva, M. A., Camproux, A. C. & Villoutreix, B. O. Druggable pockets and binding site centric chemical space: a paradigm shift in drug discovery. *Drug discovery today* **15**, 656-667 (2010).
185. Janne, P. A., Gray, N. & Settleman, J. Factors underlying sensitivity of cancers to small-molecule kinase inhibitors. *Nature reviews. Drug discovery* **8**, 709-723 (2009).
186. Arora, A. & Scholar, E. M. Role of tyrosine kinase inhibitors in cancer therapy. *The Journal of pharmacology and experimental therapeutics* **315**, 971-979 (2005).
187. Chessum, N., Jones, K., Pasqua, E. & Tucker, M. Recent advances in cancer therapeutics. *Progress in medicinal chemistry* **54**, 1-63 (2015).
188. Noble, M. E., Endicott, J. A. & Johnson, L. N. Protein kinase inhibitors: insights into drug design from structure. *Science* **303**, 1800-1805 (2004).
189. Sawyer, T. K. Cancer metastasis therapeutic targets and drug discovery: emerging small-molecule protein kinase inhibitors. *Expert Opin Investig Drugs* **13**, 1-19 (2004).
190. Sawyers, C. Targeted cancer therapy. *Nature* **432**, 294-297 (2004).
191. Zhang, J., Yang, P. L. & Gray, N. S. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**, 28-39 (2009).
192. Sharma, S. V. & Settleman, J. Oncogene addiction: setting the stage for molecularly targeted cancer therapy. *Genes & development* **21**, 3214-3231 (2007).
193. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S. & Sawyers, C. L. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *The New England journal of medicine* **344**, 1031-1037 (2001).
194. Weinstein, I. B. & Joe, A. Oncogene addiction. *Cancer Res* **68**, 3077-3080; discussion 3080 (2008).
195. Cant, S. H. & Pitcher, J. A. G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. *Molecular biology of the cell* **16**, 3088-3099 (2005).
196. Penela, P., Murga, C., Ribas, C., Lafarga, V. & Mayor, F., Jr. The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. *Br J Pharmacol* **160**, 821-832 (2010).
197. Pitcher, J. A., Hall, R. A., Daaka, Y., Zhang, J., Ferguson, S. S., Hester, S., Miller, S., Caron, M. G., Lefkowitz, R. J. & Barak, L. S. The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J Biol Chem* **273**, 12316-12324 (1998).
198. Nagaraja, A. S., Armaiz-Pena, G. N., Lutgendorf, S. K. & Sood, A. K. Why stress is BAD for cancer patients. *J Clin Invest* **123**, 558-560 (2013).
199. Bouchal, J., Santer, F. R., Hoschele, P. P., Tomastikova, E., Neuwirt, H. & Culig, Z. Transcriptional coactivators p300 and CBP stimulate estrogen receptor-beta signaling and regulate cellular events in prostate cancer. *Prostate* **71**, 431-437.
200. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R. & Goodman, R. H. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859 (1993).
201. Goodman, R. H. & Smolik, S. CBP/p300 in cell growth, transformation, and development. *Genes & development* **14**, 1553-1577 (2000).
202. Hardingham, G. E., Chawla, S., Cruzalegui, F. H. & Bading, H. Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. *Neuron* **22**, 789-798 (1999).

203. Kwok, R. P., Laurance, M. E., Lundblad, J. R., Goldman, P. S., Shih, H., Connor, L. M., Marriott, S. J. & Goodman, R. H. Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP. *Nature* **380**, 642-646 (1996).
204. Fass, D. M., Butler, J. E. & Goodman, R. H. Deacetylase activity is required for cAMP activation of a subset of CREB target genes. *J Biol Chem* **278**, 43014-43019 (2003).
205. Li, B. & Xiu, R. Angiogenesis: from molecular mechanisms to translational implications. *Clinical hemorheology and microcirculation* **54**, 345-355 (2013).
206. Bisacchi, D., Benelli, R., Vanzetto, C., Ferrari, N., Tosetti, F. & Albini, A. Anti-angiogenesis and angioprevention: mechanisms, problems and perspectives. *Cancer detection and prevention* **27**, 229-238 (2003).
207. Wojtukiewicz, M. Z., Sierko, E., Klement, P. & Rak, J. The hemostatic system and angiogenesis in malignancy. *Neoplasia (New York, N.Y.)* **3**, 371-384 (2001).
208. Ferrara, N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* **2**, 795-803 (2002).
209. Furuya, M., Nishiyama, M., Kasuya, Y., Kimura, S. & Ishikura, H. Pathophysiology of tumor neovascularization. *Vascular health and risk management* **1**, 277-290 (2005).
210. Bottos, A. & Bardelli, A. Oncogenes and angiogenesis: a way to personalize anti-angiogenic therapy? *Cellular and molecular life sciences : CMLS* **70**, 4131-4140 (2013).
211. Roberts, W., Magwenzi, S., Aburima, A. & Naseem, K. M. Thrombospondin-1 induces platelet activation through CD36-dependent inhibition of the cAMP/protein kinase A signaling cascade. *Blood* **116**, 4297-4306 (2010).
212. Manna, P. P. & Frazier, W. A. CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. *Cancer Res* **64**, 1026-1036 (2004).
213. Chou, C. W. & Chen, C. C. HDAC inhibition upregulates the expression of angiostatic ADAMTS1. *FEBS Lett* **582**, 4059-4065 (2008).
214. Ellis, L., Hammers, H. & Pili, R. Targeting tumor angiogenesis with histone deacetylase inhibitors. *Cancer Lett* **280**, 145-153 (2009).
215. Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W. & Kim, K. W. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med* **7**, 437-443 (2001).
216. West, A. C. & Johnstone, R. W. New and emerging HDAC inhibitors for cancer treatment. *J Clin Invest* **124**, 30-39 (2014).
217. Kang, J. H., Kim, M. J., Chang, S. Y., Sim, S. S., Kim, M. S. & Jo, Y. H. CCAAT box is required for the induction of human thrombospondin-1 gene by trichostatin A. *Journal of cellular biochemistry* **104**, 1192-1203 (2008).
218. Mirochnik, Y., Kwiatek, A. & Volpert, O. V. Thrombospondin and apoptosis: molecular mechanisms and use for design of complementation treatments. *Current drug targets* **9**, 851-862 (2008).
219. Sasakawa, Y., Naoe, Y., Noto, T., Inoue, T., Sasakawa, T., Matsuo, M., Manda, T. & Mutoh, S. Antitumor efficacy of FK228, a novel histone deacetylase inhibitor, depends on the effect on expression of angiogenesis factors. *Biochemical pharmacology* **66**, 897-906 (2003).
220. Doebele, R. C., Schulze-Hoepfner, F. T., Hong, J., Chlenski, A., Zeitlin, B. D., Goel, K., Gomes, S., Liu, Y., Abe, M. K., Nor, J. E., Lingen, M. W. & Rosner, M. R. A novel interplay between Epac/Rap1 and mitogen-activated protein kinase kinase 5/extracellular signal-regulated kinase 5 (MEK5/ERK5) regulates thrombospondin to control angiogenesis. *Blood* **114**, 4592-4600 (2009).

Vita

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