TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1057

MEDICA - ODONTOLOGICA

IDENTIFICATION OF EPIGENETIC TARGETS IN PROSTATE CANCER FOR THERAPEUTIC DEVELOPMENT

by

Mari Björkman

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2013

From the Institute of Biomedicine, Department of Pharmacology. Drug Development
and Therapeutics, University of Turku, VTT Medical Biotechnology, Turku Centre for Biotechnology,
FinPharma Doctoral Program Drug Discovery, Turku Finland

Supervised by

Docent Matthias Nees
VTT Medical Biotechnology and Turku Centre for Biotechnology
University of Turku
Turku, Finland

and

Professor Olli Kallioniemi
Institute for Molecular Medicine Finland (FIMM)
University of Helsinki
Helsinki, Finland

Reviewed by

Professor Pirkko Vihko MD, PhD
Institute of Clinical Medicine, Division of Clinical Chemistry
University of Helsinki
Helsinki, Finland

and

Docent Tiina Jääskeläinen, PhD
Institute of Biomedicine
University of Eastern Finland
Kuopio, Finland

Dissertation opponent

Docent Bernard Händler, PhD
Global Drug Discovery
Bayer HealthCare
Berlin, Germany

ISBN 978-951-29-5304-2 (PRINT)
ISBN 978-951-29-5305-9 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2013

To my family

"Cancer exploits the fundamental logic of evolution unlike any other illness"

-Siddharta Mukherjee:
The Emperor of All Maladies

Mari Björkman

Identification of epigenetic targets in prostate cancer for therapeutic development

University of Turku, Institute of Biomedicine, Department of Pharmacology. Drug Development and Therapeutics, VTT Medical Biotechnology, FinPharma Doctoral Program Drug Discovery, Turku Finland

Annales Universitatis Turkuensis, Medica-Odontologica

Painosalama Oy, Turku, Finland, 2013

ABSTRACT

Recurrent castration resistant prostate cancer remains a challenge for cancer therapies and novel treatment options in addition to current anti-androgen and mitosis inhibitors are needed. Aberrations in epigenetic enzymes and chromatin binding proteins have been linked to prostate cancer and they may form a novel class of drug targets in the future. In this thesis we systematically evaluated the epigenenome as a prostate cancer drug target. We functionally silenced 615 known and putative epigenetically active protein coding genes in prostate cancer cell lines using high throughput RNAi screening and evaluated the effects on cell proliferation, androgen receptor (AR) expression and histone patterns. Histone deacetylases (HDACs) were found to regulate AR expression. Furthermore, HDAC inhibitors reduced AR signaling and inhibited synergistically with androgen deprivation prostate cancer cell proliferation. In particular, TMPRSS2-EGR fusion gene positive prostate cancer cell lines were sensitive to combined HDAC and AR inhibition, which may partly be related to the dependency of a fusion gene induced epigenetic pathway. Histone demethylases (HDMs) were identified to regulate prostate cancer cell line proliferation. We discovered a novel histone JmjC-domain histone demethylase PHF8 to be highly expressed in high grade prostate cancers and mediate cell proliferation, migration and invasion in *in vitro* models. Additionally, we explored novel HDM inhibitor chemical structures using virtual screening methods. The structures best fitting to the active pocket of KDM4A were tested for enzyme inhibition and prostate cancer cell proliferation activity *in vitro*. In conclusion, our results show that prostate cancer may efficiently be targeted with combined AR and HDAC inhibition which is also currently being tested in clinical trials. HDMs were identified as another feasible novel drug target class. Future studies in representative animal models and development of specific inhibitors may reveal HDMs full potential in prostate cancer therapy.

Keywords: prostate cancer, drug target, epigenetic, histone demethylase, histone deacetylase, androgen receptor, RNA interference, high throughput screening

Mari Björkman

Epigeneettisten lääkeainekehityskohteiden tunnistaminen eturauhassyövässä.

Turun yliopisto, Biolääketieteen laitos, Farmakologia, lääkekehitys ja lääkehoito, VTT Lääkekehityksen biotekniikka, Suomen lääkekehityksen tohtoriohjelma Lääkekehitys, Turku

Annales Universitatis Turkuensis, Medica-Odontologica

Painosalama Oy, Turku, 2013

TIIVISTELMÄ

Eturauhassyövän hoidon haasteena on edelleen syövän uusiutuminen ja vastustuskyky nykyisille anti-androgeeni- ja mitoosi-inhibiittoreille. Uusia hoitovaihtoehtoja etsitäänkin kiivaasti. Epigeneettisten entsyymien ja kromatiiniin sitoutuvien proteiinien on havaittu eturauhassyövissä ilmentyvän normaalikudoksesta poikkeavalla tavalla. Tässä väitöstutkimuksessa kartoitimme systemaattisesti epigeneettisten proteiinien soveltuvuutta eturauhassyövän lääkeainekohteiksi. Tutkimuksessa hiljennettiin eturauhassyöpäsolulinjoissa 615 tunnettua tai oletettua epigeneettisesti aktiivista proteiinia koodaavaa geeniä tehoseulonta-RNAintereferenssi(RNAi)-menetelmällä ja määritettiin hiljentämisen vaikutuksia solujen kasvuun sekä androgeenireseptorin (AR) ja histonimarkkereiden ilmenemiseen. Tutkimuksessamme osoitettiin, että histonideasetylaasit (HDAC:t) säätelevät AR:n ilmentymistä eturauhassyöpäsolulinjoissa. Lisäksi HDAC inhibiittorit vähensivät AR-välitteistä signalointia ja estivät eturauhassyöpäsolujen kasvua erityisesti matalissa androgeenipitoisuuksissa. Erityisesti TMPRSS2-ERG fuusiogeeniä ilmentävät eturauhassyöpäsolulinjat olivat herkistyneitä yhdistelmähoidolle, mikä saattaa osaksi liittyä fuusiogeenin aiheuttamaan riippuvuuteen epigeneettisistä mekanismeista. Histonidemetylaasien (HDM) taas huomattiin säätelevän eturauhassyöpäsolujen kasvua. Havaitsimme myös eturauhassyövässä entuudestaan tuntemattoman HDM:n PHF8:n ilmentyvän histologisesti pahanlaatuisissa syöpäkasvaimissa sekä välittävän solujen kasvua, liikkumista ja invaasiota soluviljelymalleissa. Lisäksi tässä tutkimuksessa virtuaaliseulottiin uusia HDM inhibiittoreita ja KDM4A:n aktiiviseen keskukseen parhaiten sitoutuvien yhdisteiden testattiin *in vitro*. Tutkimuksen tulokset osoittavat, että yhdistetty anti-androgeeni ja HDAC inhibiittorihoito saattaisi tulevaisuudessa tehota pelkkää anti-androgeeniterapiaa paremmin eturauhassyöpään. Tätä yhdistemähoitoa testataankin parhaillaan kliinisissä kokeissa. HDM:t tunnistettiin toiseksi lupaavaksi uudeksi eturauhassyövän lääkeainekohdeluokaksi. Jatkotutkimukset ennustavissa eläimalleissa sekä spesifisten inhibiittorien kehittäminen paljastavat toivottavasti tulevaisuudessa HDM:en koko potentiaalin eturauhassyövän lääkeainekohteina.

Avainsanat: eturauhassyöpä, lääkeainekohde, epigenetiikka, histonidemetylaasi, histonideasetylaasi, androgeenireseptori, RNA interferenssi, tehoseulonta

TABLE OF CONTENTS

ABBREVIATIONS

2,4-PDCA	2,4-pyridine di-carboxylic acid
AKT	v-akt murine thymoma viral oncogene homolog
APC	adenomatous polyposis coli
AR	androgen receptor
ATRA	all-trans retinoic acid
cPARP	cleaved poly(ADP-ribose) polymerase
CpG	cytosine-guanine repeats
CRPC	castrate resistant prostate cancer
CSMA	cell spot microarray
CTCL	cutaneous T-cell lymphoma
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1
DICER	double stranded RNA endonuclease
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EGCG	(-)-epigallocatechin-3-gallate
ERG	v-ets erythroplastosis virus E26 oncogene homolog (avian)
ETV	ets variant
EZH2	enhancer of zeste homolog 2
FAD	flavin adenine dinucleotide
FDA	U.S. Food and Drug Administration
GNAT	Gcn-5-related N-acetyltransferase
GSTP1	Glutathione S-transferase pi 1
HDAC	histone deacetylase
HMD	histone demethylase
HMT	histone methyltransferase
HPSE	heparanase
HTS	high throughput screening
JMJD	jumonji domain containing
KAT	histone lysine acetyltrasnferase
KMD	histone lysine demethylase

KMT	histone lysine methyltransferase
LHRH	luteinizing hormone-releasing hormone
MAO	monoamine oxidase
MBD	methyl-CpG-binding proteins
MeCP2	methyl CpG binding protein 2
MMP-2	matrix metalloproteinase-2
mRNA	messenger ribonucleic acid
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NAD	Nicotinamide adenine dinucleotide
NCOR	nuclear receptor corepressor
NOG	N-oxalyl-glycine
NuRD	nucleosome remodeling and deacetylase complex
PHD	plant homeodomain
PHF8	plant homeodomain finger protein 8
PHLPP	phosphatase PH domain and leucine rich repeat protein
PI3K	phosphatidylinositol 3-kinase
PIN	prostatic intraepithelial neoplasia
PRMT	protein arginine methyltransferase
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
RANKL	Receptor activator of nuclear factor kappa-B ligand
RASSF1A	Ras association (RalGDS/AF-6) domain family member 1
RISC	RNA induced silencing complex
RKIP	Raf-1 kinase inhibitor protein
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
SAR	structure activity relationship
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SIRT	sirtuin
SMRT	silencing mediator for retinoid and thyroid hormone receptors
TMPRSS2	transmembrane protease, serine 2
TRAMP	transgenic adenocarcinoma of mouse prostate

TSA	trichostatin A
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
WNT	wingless-type MMTV integration site family
WNT5A	wingless-type MMTV integration site family, member 5A
ZBTB	zinc finger and BTB domain containing

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the original publications, which are reffered to in the text by the Roman numerals I-IV. The original publications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I **Björkman M**, Ostling P, Härmä V, Virtanen J, Mpindi JP, Rantala J, Mirtti T, Vesterinen T, Lundin M, Sankila A, Rannikko A, Kaivanto E, Kohonen P, Kallioniemi O, Nees M. Systematic knockdown of epigenetic enzymes identifies a novel histone demethylase PHF8 overexpressed in prostate cancer with an impact on cell proliferation, migration and invasion. Oncogene. 2012 31(29): 3444-3456.

II **Björkman M**, Rantala J, Nees M, Kallioniemi O. Epigenetics of prostate cancer and the prospect of identification of novel drug targets by RNAi screening of epigenetic enzymes. Epigenomics. 2010 2(5):683-9.

III **Björkman M**, Iljin K, Halonen P, Sara H, Kaivanto E, Nees M, Kallioniemi O. Defining the molecular action of HDAC inhibitors and synergism with androgen deprivation in ERG-positive prostate cancer. Int J Cancer. 2008 123(12):2774-81.

IV **Björkman M**, Tiikkainen P, Mercurio C, Kohonen P, Pérez-Santín E, Altucci L, Minucci S, Poso A, de Lera AR, Nees M, Kallioniemi O. High throughput virtual and cell-based screenings identify novel small molecule structures inhibiting histone demethylase Jumonji domain-containing protein 2A. Submitted.

1 INTRODUCTION

Prostate cancer is the third most frequently diagnosed cancer and the second leading cause of cancer related deaths among Western men (Simard et al. 2012). The prostate cancer mortality rates have remained high despite the advances in detection and intervention methods. The main challenge in prostate cancer therapy is the development of castration resistant disease which is unresponsive to anti-androgen therapy (Hugosson et al. 2010). Over the recent five to ten years, it has become evident that prostate cancer is not only caused by progressive gene level aberrations (mutations, amplifications, deletions) but also by epigenetic modifications of chromatin and histone tails (Jeronimo et al. 2011). Aberrations in epigenetic modification patterns are clonally inherited and have been discovered already in premalignant lesions of prostate. Both silencing hypermethylation of key tumor-suppressor gene promoters and global genome hypomethylation, possibly leading to oncogene activation, have been detected in prostate cancer (Perry et al. 2006). Also global loss of Histone H3 and H4 acetylation and changes in histone methylation patterns have been observed in prostate cancer (Ellinger et al. 2010, Bianco-Miotto et al. 2010). All these chromatin modifications are introduced and modified by a diverse set of epigenetic enzymes whose expression has also been discovered to be altered in cancer tissues (Abbas and Gupta 2008, Albert and Helin 2010). Together these chromatin modification patterns and the enzymes estabilishing them form prostate cancer epigenome which is emerging as a novel class of drug targets in lethal prostate cancer.

In this thesis, we aimed to explore prostate cancer epigenome as drug target. We studied the significance of all known and predicted epigenetically active enzymes (615 genes) for prostate cancer proliferation, apoptosis, androgen receptor (AR) expression and histone acetylation and methylation patterns, using high throughput RNAi screening. These studies revealed that silencing of histone deacetylases reduced AR expression. We had also previously shown that TMPRSS2-ERG fusion gene positive subset of prostate cancers overexpress histone deacetylase 1 (HDAC1). This led us to test the hypothesis that the fusion gene positive prostate cancer subset would be particularly sensitive to histone deacetylase inhibitors in combination with anti-androgens.

Silencing of histone demethylases inhibited prostate cancer cell line proliferation in our epigenome-wide siRNA screen. Further studies on histone demethylases role in prostate cancer identified the novel histone demethylase PHF8 to be overexpressed in prostate cancer. PHF8 mediates cell proliferation, motility and invasion and its overexpression was here linked to high grade prostate cancer for the first time. Additionally, we searched for novel histone demethylase inhibitors. Our computational screenings based on chemoinformatics and cell based assays identified a group of novel chemical structures inhibiting histone demethylases. Together, these studies offer a systematic view on the epigenetic enzymes contributing to prostate cancer and evaluate the targeting the main epigenetic enzyme classes as novel concepts in prostate cancer therapy.

2 REVIEW OF THE LITERATURE

2.1 Introduction to drug discovery

Drug discovery can be divided into several independent steps, beginning with identification of a suitable target. This drug target is typically a biological macromolecule, holding a promise to be functionally significant for a disease state e.g. cancer progression (Figure 1) (Carragher et al. 2012). Drug targets are in most cases proteins, but recent development of interfering RNA delivery techniques may emphasize also disease genes (mRNA) as direct drug targets in the future (Sams-Dodd 2005). The ideal drug target should also be a target for a small molecule inhibitor drug i.e. be druggable. Target druggability, especially in small molecule and monoclonal antibody development, is typically understood as a target macromolecule that has a binding pocket or an epitope with good affinity for compounds and antibodies (Wikber et al.).

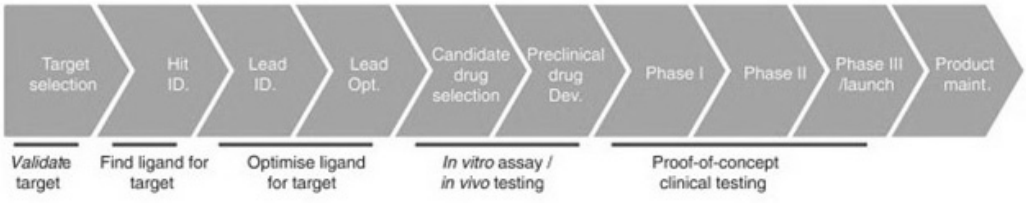

Figure 1. Phases of drug discovery. Adapted and modified from Carregher et al. 2012 Drug Discovery Today.

Target identification and validation in pre-clinical cellular and animal models is usually followed by the synthesis of an array of chemical structures inhibiting the target (ligands). These are then assayed for their activity and potency primarily in cell-free *in vitro* assays (Sams-Dodd 2005). After identification of the most potent ligands, the compound properties are further optimized through iterative medicinal chemistry and modified chemical entities are tested *in vitro* and *in vivo*. These activities lead to the identification of a candidate drug which can further proceed into preclinical drug development (formulation optimization, toxicity testing etc.) (Carragher et al. 2012). Finally, the ideal compound may enter into clinical trials where the final proof-of-concept is tested: efficacy, superiority in comparison to existing therapies, safety and side-effects in human probands with the preferred disease indication.

2.1.1 Early phases of drug discovery- from target to hit compound identification

Drug discovery starts with the identification of a functionally relevant, disease-driving molecule that is causally involved in a certain disease phenotype. Before targeted therapies, drug targets were typically identified based on phenotypic and biochemical

assays and the knowledge of the molecular mechanisms of the disease was often a limited. Post-genome era drug discovery often exploits functional genomics, such as RNA interference (RNAi), in cell-based high throughput screens. Additionally, genomic, transcriptomic, proteomic and metabolomic information is generated to explore key pathways driving the disease phenotype. Target-based drug discovery approaches have also been evaluated as the most successful in identification of novel cancer therapeutics (Swinney and Anthony 2011).

2.1.1.1 RNAi screening in target discovery

RNAi represents an evolutionary conserved, sequence-specific and post-transcriptional gene silencing mechanism. RNAi is a cellular defense mechanism against viral parasites and transposomal DNA insertions and it ensures the correct expression of the repetitive DNA elements and microRNA (miRNA) guided epigenetic gene regulation (Meister and Tuschl 2004). RNAi is triggered by double stranded RNA (dsRNA) which is processed into short RNA duplexes. These duplexes are further processed into small interfering RNAs (siRNAs) by a ribonuclease called DICER. The resulting siRNA strands enter to the RNA-induced silencing protein complex (RISC) which binds and cleaves a complementary messenger RNA (mRNA). Silencing of gene expression can also occur during protein translation (Rana 2007). Here, RISC carries a guide strand that does not have perfect complementarities with the mRNA. These messages are sequestered in cytoplasmic foci containing translationally repressed mRNA-protein complexes called P-bodies which are subsequently degraded.

Loss-of-function testing (screening) with RNAi libraries offers a straightforward tool for evaluating gene function in e.g. cancer cell lines. RNAi screens can take two basic forms: arrayed screening approaches, and pooled library selections. In the latter, viral/plasmid based short hairpin RNAs (shRNAs) are barcoded with a unique DNA barcode and the cells are infected/transfected with pools of shRNAs (Ngo et al. 2006). Cells are typically exposed to some selective pressure (e.g. a drug), and the resistant population barcodes are analyzed by sequencing. The advantage of shRNA libraries is their high knock-down efficiency, but their use is limited by the off-target effects of pooling, infection/transfection variability, inequality in viral/plasmid preparations and difficulties in identification of which shRNA affected the most in the observed endpoint (Kassner 2008).

Arrayed siRNA screening is the most common form of RNAi screening. Arrayed screening can either be performed on microtiter plates or sh/siRNAs can be printed together with the transfection reagent on cell microarrays. Arrayed siRNA screening typically uses commercial siRNA libraries, available through various vendors (Chatterjee-Kishore and Miller 2005). Commercial siRNA libraries are the most convenient and straightforward solutions to use, but the effect of gene silencing is typically limited to a time interval between 24 to 144 hours, which requires that the gene/protein kinetics measured for readout have to be relatively short. Cell lines used in plate-based screens need to be

readily transfectable with lipid-based siRNA delivery reagents. In contrast, viral based delivery of shRNAs usually guarantees better transfection efficacy in larger repertoire of cell lines, but the library preparations and requirements for safety level II virus facilities can pose a hinder to wider use of shRNAs.

In a typical plate-based siRNA screen cultured cells are dispensed onto wells of 96 or 384-well microtiter plates using laboratory automation and robotics. Due to the off-target effects related to siRNA promiscuity, partial target gene knockdown and the effect of delivery agents it is recommended to target a gene with two to four individual siRNAs in the screens (Jackson et al. 2003). SiRNAs are mixed with the transfection reagent and one siRNA is applied per well. Cells are exposed to the reagents for 48 to 72 hours to allow optimal conditions for RNAi-mediated gene silencing. A wide array of phenotypic assays can be performed following the siRNA transfections, including various endpoint measurements such as programmed cell death/aptoptosis, functional reporter assays and high throughput imaging based on immunofluorescence staining.

The recently developed cell spot microarray (CSMA) technology allow large scale RNAi screens to be performed with increased experimental throughput and reduced screening costs, in comparison to microtiter plate RNAi screens (Rantala et al. 2011). In optimized settings, siRNAs are complexed with lipid-based transfection agents and extracellular matrix components and printed on microplate size arrays using contact printing. Adherent cell lines are laid out on top of the arrays, allowing cells to attach only to siRNA-containing spots resulting in reverse-transfection. Gene silencing effects on CSMA can then be assayed with immunofluorescence staining and high throughput microscopic imaging for any desired marker protein.

RNAi screening is an unbiased exploratory starting point for drug target discovery but the significance of the target validation in relevant *in vitro* and *in vivo* models should be emphasized. One should bear in mind that the gene knockdown phenotype achieved with RNAi may not directly mimic the specific effect of a drug inhibiting a certain functional protein domain (e.g. kinase or histone modification catalyzing domain), leaving other interacting domains active. The presence of closely related gene/protein homologs can also lead to false negative discoveries in RNAi screening, which has been observed with the known drug targets involved in particular phenotype (Kassner 2008).

With these limitations in mind, RNAi screening has been successfully used in drug target discovery for e.g. renal clear cell carcinoma, acute myeloid leukemia and breast cancer (Ding et al. 2011, Zuber et al. 2011, Giamas et al. 2011). RNAi has also been proved powerful in identification of drug resistance pathways to commonly used chemotherapeutic agents like doxorubicin, cisplatin, 5-fluorouracil and paclitaxel (Swanton et al. 2007, Whitehurst et al. 2007, Iorns et al. 2008, Bartz et al. 2006) and in studying mechanism of action of established cancer therapies, like trastuzumab and PARP inhibitors (Berns et al. 2007, Turner et al. 2008).

2.1.1.2 Ligand screening for hit compounds

After a promising drug target has been identified and its role in disease phenotype has been characterized, a small molecule ligand with good pharmacological properties and possibilities for further medicinal chemistry optimizations is developed. Several different screening methods can be used for hit ligand identification. Three of the most commonly used methods are High Throughput Screening (HTS), Fragment Based Screening and Virtual Screening. Ligand generation in industrial settings evaluates also compounds legal protection by patents since without this it is generally impossible for a company to enter drug development process which is estimated to cost 1.4 billion dollars and take 10 to 15 years in total (Paul et al. 2010).

HTS screening can be based on cell lines expressing the target protein or it can detect direct inhibition of a reaction catalyzed by the target protein. This usually requires extensive beforehand investments into the medicinal chemistry, laboratory robotics and the assay development. Fragment based screening uses the ligand bound crystal structure of the target protein and aims to identify small compound fragments (pharmacophores) which may bind to substructures of the binding pocket (Hajduk and Greer 2007). When a sufficient number of fragments covering the entire binding pocket have been identified, they can be combined for a candidate compound after their binding modes have been determined by X-ray crystallography. The advantage of fragment based screening in comparison to HTS is that smaller numbers of compounds need to be screened and synthesized.

Virtual screening is a computational method and compounds identified with this method are said to be developed *in silico*. Here, the structural data of libraries of compounds are collected and fitted computationally into the target 3-dimesional (3D) crystal structure, using a series of selected docking algorithms. The effectiveness of a given compound against the target has to be further tested *in vitro*, using biochemical and cell-based assays (Villoutreix et al. 2009). These tests also give sufficient guidance for the structure-activity relationship (SAR) studies which can be used to chemically modify the compound, resulting in more potent or more specific derivatives.

2.2 Prostate cancer

2.2.1 Current clinical practice

Prostate cancer is the second most common cause of male cancer related deaths in the Western world (Simard et al. 2012). This is a quite dramatic frequency, considering that prostate cancer is typically a slowly progressing cancer. It may take up to 15 years from the initial detection (e.g. by biomarkers such as prostate-specific antigen PSA) to the development of clinically symptomatic prostate cancer. One out of seven patients develops castration resistant prostate cancer (CRPC) which is unresponsive to androgen ablation therapy (Hugosson et al. 2010). Standard treatment options depending on the level of PSA or histological Gleason grading of the primary tumor are watchful waiting,

radical prostatectomy, radiotherapy and/or medical castration (with luteinizing hormone-releasing hormone (LHRH) agonists) often combined with androgen receptor (AR) antagonists (like flutamide and bicalutamide). The high cancer mortality rate is mainly related to the poor management options for CRPC. Until 2010, the therapy options for often symptomatic and painful metastatic CRPC were limited to the bisphosphonate zoledronic acid, reducing skeletal events (fractures, pain caused by the bone metastases) (Saad 2002) and tubulin-binding anti-mitotic taxane docetaxel (Tannock et al. 2004). These compounds are mostly palliative and only alleviate symptoms, median overall survival being around 17 months under these therapies.

2.2.2 Emerging prostate cancer therapies

Since 2010, multiple clinical trials addressing metastatic CRPC have been published with favorable results. Sipuleucel-T, which is an immunotherapeutic autologous CD54-positive dendritic-cell vaccine, was shown to significantly improve overall survival in metastatic CRPC patients (Kantoff et al. 2010). Cabazitaxel, a novel semi-synthetic taxane, increased the median survival by >3 moths and showed significant efficacy after docetaxel failure (de Bono et al. 2010). Denosumab, a human monoclonal antibody against RANKL, was shown to significantly delay the time to emergence of skeletal-related adverse events in metastatic CRPC, compared to zolendronic acid (Fizazi et al. 2011). Palliation of bone pain and improved overall survival in metastatic CRPC has also been achieved with the radium-223 radioisotope (Nilsson et al. 2007, Nilsson et al. 2012).

However, the clinical trial results obtained with compounds targeting AR signaling have gained the most attention. MDV-3100 (enzalutamide), a novel AR antagonist reducing ligand binding and AR nuclear accumulation, resulted in at least 50% PSA decline in both docetaxel naïve and –treated patients (Scher et al. 2010). Phase III trials with MDV-3100 were successfully completed in 2010 and it has been announced that MDV-3100 improves overall survival in chemotherapy treated CRPC patients by almost 5 months (http://investors.medivation.com/ releasedetail.cfm?ReleaseID=620500). MDV-3100 is expected to receive U.S. Food and Drug Administration's (FDA) approval soon. In addition, abiraterone acetate which blocks a key step of androgen synthesis catalyzed by 17,20 lyase (CYP17A1), received FDA approval in 2011 for the treatment of metastatic CRPC patients who have failed chemotherapy. Abiraterone acetate resulted in an almost 4 month overall survival benefit in clinical trials (de Bono et al. 2011). Many other compounds targeting AR signaling are currently in the clinical development, including other 17,20 lyase inhibitors (TAK-700, TOK-001) and AR antagonists (ODM-201, ARN-509). Despite these advancements, part of the CRPC patient population is inherently resistant also to the new therapeutics or the resistance develops during the course of treatment. The reasons for these therapy resistance mechanisms may be related to diversity and genetic heterogeneity of CRPC genomes and plasticity of the cancers (Grasso et al. 2012, Zhang et al. 2011). Thorough understanding of prostate cancer molecular biology may thus open new treatment options.

2.2.3 Molecular biology of prostate cancer

Despite the recent advancements in the treatment of metastatic CRPC, genomic instability and intratumoral heterogeneity of cancer cells can lead to progression of the disease and treatment resistance approximately within 2 to 3 years. Thus, detailed knowledge of the molecular origins of the prostate cancer is thought to provide novel means for targeted therapies. The current understanding of the main molecular mechanisms important for prostate cancer is reviewed below.

2.2.3.1 Androgen receptor

Prostate cancer has been known to be sensitive to androgen signaling already since the 1940's (Huggins and Hodges 2002). Despite the development of numerous AR antagonists and LHRH agonists and the excellent initial response to them, CRPC remarkably remains dependent on AR signaling. Castration resistance has been shown to develop via multiple mechanisms leading to constitutive activation of the AR and downstream pathways. Thus, AR signaling continues in CRPC or is even enhanced in the presence of androgen deprivation therapy. This is commonly achieved through amplification of the genomic AR and overexpression of the AR protein, which enables very low levels of androgens to activate AR (Waltering et al. 2009). AR amplifications have been found in up to 80% of CRPC samples (Visakorpi et al. 1995).

Additionally, various AR mutations have been found in 10-30% of anti-androgen treated CRPCs (Waltering et al. 2012). Mutations of the AR ligand binding domain can lead to its constitutive activation by alternative ligands like glucocorticoids and estrogens or increase its affinity for androgens (Steketee et al. 2002). AR mutations can also develop in response to AR antagonist therapy like flutamide and bicalutamide, transforming these compounds from antagonists into agonists (Steinkamp et al. 2009).

Constitutively active AR can also be gained through alternative splicing of AR exon leading to truncation of COOH terminal domain of the receptor protein (Dehm et al. 2008, Sun et al. 2010). AR splice variants have been shown to be enriched in CRPC metastases and are associated with poor patient survival (Sun et al. 2010, Hornberg et al. 2011). AR activation can also take place through aberrant activation of cytochrome p450 enzyme mediated intratumoral steroid synthesis, typically in response to CYP17A1 inhibitor therapy (Cai et al. 2011, Mostaghel et al. 2011, Attard et al. 2012). Due to the various mechanisms that enable AR signaling under anti-androgen therapy, development of novel AR inhibitors and alternative AR signaling targeting strategies is ongoing.

2.2.3.2 PTEN loss and PI3K pathway activation

Phosphatase and tensin homolog (PTEN) loss and phosphoinositide 3-kinase (PI3K) pathway activation are among the most frequent genetic alterations in prostate cancer (Taylor et al. 2010). PTEN inactivation may also offer yet another escape route for androgen deprivation therapy in CRPC (Ham et al. 2009). Interestingly, supporting

this observation it was recently discovered that androgen deprivation therapy may lead to constitutive activation of the PI3K pathway signaling in a PTEN loss background. AR inhibition was found to reduce the levels of phosphatase PH domain and leucine rich repeat protein phosphatase (PHLPP) which dephosphorylates and inactivates v-akt murine thymoma viral oncogene homolog (AKT) leading to increased AKT activation (Mulholland et al. 2011). This mechanism may emphasize the androgen independency of the CRPC and survival through the PI3K pathway. There seems to be a complex reciprocal feedback mechanism between the AR and PI3K signaling pathways, and pre-clinical evidence shows that simultaneous pharmacological blocking of both of these pathways may offer a novel and effective way to tackle CRPC (Carver et al. 2011).

2.2.3.3 TMPRSS2-ETS gene fusions

Approximately 40-70% of all prostate cancer samples contain a fusion gene, joining the promoter region and few first exons of the AR-regulated transmembrane serine protease 2 (TMPRSS2) with ETS transcription factors (Tomlins et al. 2005). This leads to the overexpression of ETS oncogenes not present in normal prostate. TMPRSS2 is most frequently joined with the v-ets erythroblastosis virus E26 oncogene homolog, avian (ERG), followed by ets variant 1 (ETV1), ETV4 and ETV5 (Mehra et al. 2007, Helgeson et al. 2008). ETS gene fusions are found already in the premalignant lesions of the prostate and may induce prostatic intraepithelial neoplasia (PIN) in a transgenic mouse model (Tomlins et al. 2008). ETS fusion genes are not considered to be tumorigenic by themselves, but are frequently associated with PTEN loss in prostate cancers (Carver et al. 2009, Han et al. 2009, King et al. 2009). PTEN loss has also been shown to be required for the development of prostatic adenocarcinoma in transgenic ETS fusion gene mouse model.

Aberrant ERG oncogene expression has been linked to the promotion of multiple cancer-associated signaling pathways. These include increased expression of plasminogen (Tomlins et al. 2008), MYC (Sun et al. 2010, Sun et al. 2008), EZH2 (Kunderfranco et al. 2010), and activation of PI3K (Carver et al. 2011, Carver et al. 2009, Han et al. 2009, King et al. 2009) and Wnt signaling (Gupta et al. 2010) as well as HDAC1 overexpression and epigenetic reprogramming (Iljin et al. 2006, Friedlander et al. 2012). All of the identified pathways may offer novel opportunities for targeted therapy of prostate cancer in the future.

2.3 The epigenome and cancer

The genomic DNA in interphase cells is wrapped around histone octamers. Translation of this genetic information from DNA to mRNA to proteins performing cellular functions is not only dictated by the gene promoter sequence. Additionally, chemical modifications of both the DNA strand and/or the histones can affect chromatin density and accessibility to transcriptional protein complexes and thus epigenetically

regulate transcription. The epigenome consists of the tissue specific combination of DNA methylation, histone modifications, nucleosome remodeling proteins and non-coding RNAs which together contribute to heritable regulation of gene expression (Rodriguez-Paredes and Esteller 2011). Various chromatin modifications and enzymes catalyzing modifications particularly of histones H3 and H4 are summarized in Figure 2. The epigenome ensures that temporal and spatial activation or silencing of specific genes in a cell-type-specific pattern remain stable over many cell generations, long after inductive developmental signals have disappeared (Berdasco and Esteller 2010). Since these epigenetic mechanisms in the normal cells limit the pluripotency and the regenerative properties of cells, their aberrations are associated with uncontrolled cell division and spreading of cancer (Baylin and Jones 2011). The various epigenetic mechanisms and their contribution to prostate cancer have been studied extensively over the recent years.

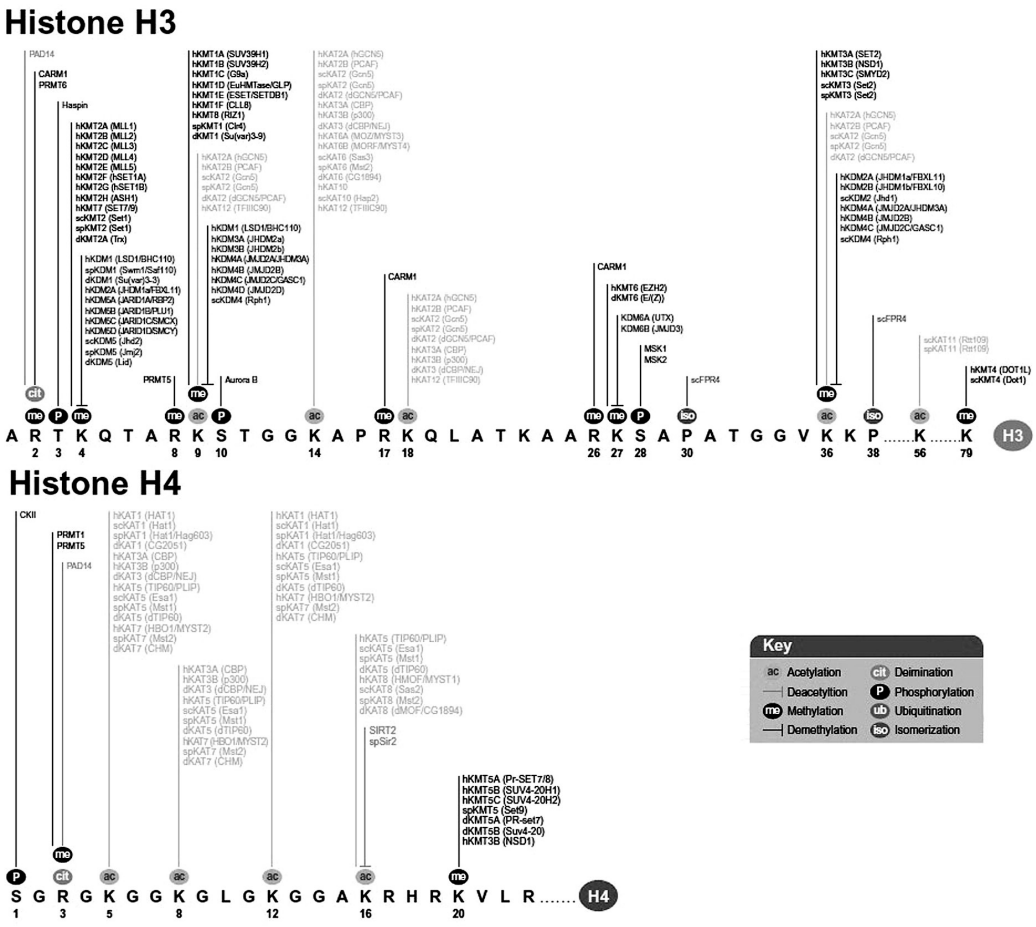

Figure 2. Histone modifications of histone H3 and H4 and the enzymes catalyzing these modifications. Figure adapted and modified from http://www.abcam.com/index.html?pageconfig=resource&rid=11924

2.3.1 DNA methylation and prostate cancer

DNA cytosine (C) methylation in CpG dinucleotides is a common biochemical modification of eukaryotic DNA and linked to transcriptionally inactive chromatin. Around 1% of the human genome consists of short, CpG-dense sequences called the CpG islands (Takai and Jones 2002). 60-90% of these CpG dinucleotides are methylated in differentiated cells. The majority of CpG methylation occurs at the intragenic and intergenic non-coding regions and ensures the transcriptional silencing of the repetitive and transposable elements and alternative promoters (Meissner et al. 2008). Also non-CpG sites in the gene bodies have been found hypermethylated in embryonic stem cells, which may mark and maintain their pluripotency (Lister et al. 2009).

In contrast, the CpG islands within the functional gene promoters are normally unmethylated and thus active, with the exception of imprinted genes and along the inactivated X-chromosome. Promoter hypermethylation effectively inhibits the initiation of gene transcription by two mechanisms. Transcription factor binding is directly excluded by the inability of transcription initiation complex to bind to methylated DNA (Baylin 2005). In addition, methylated promoters attract methyl-CpG-binding proteins (MBDs) that interact with histone deacetylases (HDACs) and the nucleosomal remodeling complex (NuRD) to condensate the chromatin structure and render the promoter inaccessible to transcription initiation factors (Jones et al. 1998).

Aberrant DNA hypomethylation and hypermethylation is a common anomaly in prostate cancer. Compared to normal cells, certain tumor suppressor gene promoters are densely hypermethylated (Park et al. 2007) whereas oncogenes are frequently devoid of methylation (Wang et al. 2007). Prototype tumor suppressor genes that are frequently reported to be silenced by hypermethylation and linked to clinical outcome in prostate cancer include Glutathione S-transferase pi 1 (GSTP1) (Lee et al. 1994), Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A), and adenomatous polyposis coli (APC) (Maruyama et al. 2002, Liu et al. 2011). Also AR expression has been detected to be suppressed by promoter hypermethylation in cell lines and primary prostate and CRPC tumors (Jarrard et al. 1998, Reibenwein et al. 2007). However, the difference between AR hypermethylation levels in normal prostate and prostate tumors was considered to be only moderate and statistically insignificant. Additionally, low PTEN levels without an associated deletion were shown in multiple prostate cancer xenograft and cell line models (Whang et al. 1998). This may be related to PTEN methylation since DNA methyltransferase inhibitor 5-azadeoxycytidine could restore mRNA expression. However, PTEN silencing in prostate cancer by promoter hypermethylation has not been confirmed in large patient cohorts. Hypomethylation activated oncogenes include wingless-type MMTV integration site family, member 5A (WNT5A) (Wang et al. 2007), urokinase-type plasminogen activator (uPA), matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF) and extracellular matrix degrading enzyme heparanase (HPSE) which may contribute in prostate cancer invasion (Shukeir et al. 2006, Ogishima et al. 2005).

Cancer genomes are also globally hypomethylated, an observation that has been associated with genomic instability (Eden et al. 2003). This instability may arise from decondensation of the chromatin into permissive conformations that allow or promote recombination events (Tuck-Muller et al. 2000) or result in the activation of proviral and retrotransposone repeats (Steinhoff and Schulz 2003). In prostate cancer, DNA hypomethylation is detected at later stages of cancer development and may contribute to tumor heterogeneity (Yegnasubramanian et al. 2008)

2.3.1.1 DNA methyltransferases

DNA methylations are catalyzed by DNA methyltransferaces (DNMTs). DNA methylation patterns are tissue-specific and clonally preserved to daughter cells during mitosis through postreplicative methylation of the newly synthesized DNA strand (Stein et al. 1982). DNMTs can be divided to maintenance methylases (DNTM1) and *de novo* methylases (DNMT3A and DNMT3B). DNMT1s copy the methylation patterns to the newly synthesized strand during S-phase of cell division using hemimethylated DNA as a template (Leonhardt et al. 1992). DNMT3A and DNMT3B have both maintenance and *de novo* methylation activities and are thought to accomplish massive methylation pattern regeneration during embryogenesis (Chen et al. 2003). DNMTs can also recruit histone deacetylases (HDACs) and other chromatin binding proteins to promoter sites and thus repress transcription independently of their methyltransferase activity (Fuks et al. 2001). This may assist in maintaining the gene silencing throughout a series of cell divisions, since HDACs bound to the replication fork may hypoacetylate histones at newly assembled nucleosomes.

Deregulated expression of mainly DNMT1 has been reported in prostate cancer and cancer cell lines (Patra et al. 2002). Studies with transgenic adenocarcinomas of the mouse prostate (TRAMP) models suggest that DNMTs are overexpressed in prostatic intraepithelial neoplasia and well-differentiated tumors, but not in poorly differentiated tumors (Morey Kinney et al. 2008). However, a possible correlation of DNMT expression with prostate cancer outcome has not been studied.

2.3.1.2 Methyl-binding domain proteins

Methyl-binding domain proteins (MBDs) are transcriptional repressors that specifically recognize DNA 5-methylcytosines introduced by DNMTs. MBDs are commonly found on hypermethylated promoters in a gene and cell-type specific manner (Lopez-Serra et al. 2006). This protein group consists of MBDs 1-4 and MeCP2 (Hendrich, Bird 1998). Also the zinc finger proteins KAISO, ZBTB4 and ZBTB38 repress transcription by binding methylated DNA (Filion et al. 2006). The effect of methylated DNA binding proteins is mainly mediated through the recruitment of repressor complexes containing HDACs (Jones et al. 1998) and histone methyltransferases (HMTs) (Fuks et al. 2001, Fuks et al. 2003). Thus MBDs can be viewed as a molecular bridge between DNA methylation and histone modifications in transcriptional regulation.

MBDs and MeCP2 protein expression have not been detected in prostate cancer samples although the study cohort was relatively small (Patra et al. 2003). However, MBD2 silencing in cell line models has been linked to less invasive *in vitro* behavior and reduced xenograft size (Shukeir et al. 2006). High MBD4 mRNA expression was also a part of gene signature discriminating benign and malignant prostate samples (Bianco-Miotto et al. 2010). Interestingly, MeCP2 appears to be required for prostate cancer cell line growth and its overexpression promotes androgen independent proliferation possibly through c-myc activation (Bernard et al. 2006). However, studies of the role of MeCP2 and other MBDs in larger set of clinical prostate tumors and *in vivo* models are required to reveal their contribution to the development of CRPC.

2.3.2 Histone acetylation and methylation in prostate cancer

Chromatin is packed as nucleosome repeat units, composed of histone octamers that consist of the four core histones (H2A, H2B, H3 and H4), and an average of 147 nucleotides of DNA that are coiled around the core. Nucleosomes are progressively packed in higher order structures, ultimately forming highly condensed metaphase chromosomes. Histone N-terminal tails, which protrude from the nucleosome, may undergo a large number of reversible post-translational modifications (Figure 2). These include acetylation, methylation, phosphorylation, ubiquitinylation, poly-ADP ribosylation, sumoylation, carbonylation and glycosylation. These modifications form an integral part of transcriptional regulation and aberrant histone modification patterns have frequently been connected to cancer development.

Histone tail lysine acetylation is typically associated with an open, active conformation of the chromatin. Especially acetylation of lysines of histone H3 and H4 (H3K and H4K, respectively) is linked to gene activation (Kouzarides 2007). Histone lysines are typically methylated at H3K4, H3K36 and H3K79 which are associated with active regions of chromatin. In contrast, methylation of H3K9, H3K27 and H4K20 are generally found in silenced regions. Lysine methylation does not affect the overall charge of the histone molecule, but these marks are recognized and bound by effector proteins that specifically recognize methylated lysine residues and regulate chromatin function. Histone tails can also be methylated from their arginine residues. Arginines can be either symmetrically or a-symmetrically mono-methylated or dimethylated. These arginine methylations can either activate or repress gene transcription depending on the methylated amino acid and the number of methyl groups attached to a certain residue (monomethylated, dimethylated or trimethylated). Histone tail arginines are typically methylated on residues 2, 8, 17 and 26 of histone H3 (H3R2, H3R8, H3R17 and H3R26) and residue 3 of histone H4 (H4R3) in mammals (Klose and Zhang 2007).

Histone modifications are frequently altered in prostate cancer and they may be used for disease outcome predictions (Seligson et al. 2005). Histone H3K4 monomethylation, H3K9 di- and trimethylation and global H3 and H4 acetylation levels are significantly

lower in prostate cancer than in nonmalignant tissues (Ellinger et al. 2010). However, CRPCs have elevated levels of histone H3K4 mono-, di-and trimethylation. In another study, high global levels of acetylated H3K18 and dimethylated H3K4 predicted prostate tumor recurrence (Bianco-Miotto et al. 2010). Additionally, elevated levels of trimethylated H3K4 and H3K27 along with reduced levels of dimethylated H3K9 have been associated with poor prognosis in prostate cancer patients (Kondo et al. 2008, Seligson et al. 2009, Yu et al. 2007). Simultaneously, multiple enzymes catalyzing the addition and removal of these modifications are found aberrantly expressed in prostate cancer.

2.3.2.1 Histone acetyltransferases

Histone acetylation is currently the best studied epigenetic modification. This modification is introduced to the lysine (K) residues of the histone tails by histone acetyltransferases (KATs). Histone acetylation leads to local chromatin expansion through diminished positive charge of N-terminal tails and DNA binding. This has been demonstrated to correlate with transcriptional activity, which may be due to an increased accessibility of the DNA to regulatory proteins (Roth et al. 2001). KATs are divided into several superfamilies based on their conserved acetylation-related structural motifs. These include GCN5-related N-acetyltransferase (GNAT) family, the MYST family (named for its members MOZ, Ybf2/Sas3, Sas2 and Tip60) and p300/CBP. Also a few nuclear receptor cofactors (p160 family) and basal transcription factors (TFIIIC90, KAT12) show protein and histone acetylation activity. KATs can also acetylate other proteins such as transcription factors, transcriptional co-activators, structural proteins, polyamines and one family of nuclear importin (Roth et al. 2001). KATs function as protein complex with other KATs, chromatin remodeling factors like ATP-dependent Swi/Snf-like complexes and transcriptional activators which together acetylate histone lysine residues at specific gene locus and activate transcription.

KATs can directly activate AR and AR mediated signaling by acetylation and forming co-activator complexes with the receptor (Faus and Haendler 2006). KATs p160, CBP (KAT3A), p300 (KAT3B) and PCAF (KAT2B) are directly recruited by agonist-bound AR to PSA promoter and are required for its transcriptional activation (Shang et al. 2002). Especially P300 may have a mechanistic role in CRPC development since its expression is elevated in response to androgen deprivation in prostate cancer cell line models (Heemers et al. 2007). Accordingly, increased expression of p300 in clinical prostate tumors has been linked to higher grade cancers and an increased risk for PSA relapse (Isharwal et al. 2008). P300 may qualify also as a CRPC drug target in the future since preclinical results show that p300 ablation may reduce prostate cancer cell proliferation and invasion (Santer et al. 2011).

Members of the p160 family of steroid receptor co-activators (SRCs) have also been identified as AR co-activators. High SRC-1 (NCOA1) protein levels have been found in recurrent prostate cancers (Gregory et al. 2001). High SRC-1 levels also correlated

with aggressive behavior of clinically localized androgen-dependent prostate cancers possibly contributing to castration resistant phenotype observed in cell lines (Agoulnik et al. 2005). Also SRC-3 (NCOA3) has been shown to be overexpressed in prostate cancer and regulate proliferation of prostate cancer cell lines (Zhou et al. 2005). In addition, the MYST family member nuclear receptor co-activator Tip60 (KAT5) was shown to accumulate in cell nuclei in castration resistant prostate tumors, most likely in response to androgen deprivation, as shown in cell lines and animal models (Halkidou et al. 2003). However, aberrant expression or genetic abnormalities of KATs have not yet been linked to the observed reduced histone acetylation levels in prostate cancer.

2.3.2.2 Histone deacetylases

Histone deacetylases (HDACs) remove acetyl groups, thus enabling ionic interactions between positively charged N-terminal histone tails and the negatively charged DNA phosphate backbone to occur. This leads to condensed chromatin and repressed transcription (Glozak and Seto 2007). Histone deacetylases are divided into four classes (I-IV) (Haberland et al. 2009). HDACs function in multi-subunit transcriptional co-repressor complexes that transcription factors recruit sequence specifically to gene promoters. Expression and *in vivo* knock-out phenotype of HDAC superfamily are summarized in Table I. Class I HDACs (HDACs 1-3 and -8) are predominantly located in the nucleus and are ubiquitously expressed. HDAC-1 and -2 are nearly identical and are generally found in repressive complexes like Sin3, NuRD, CoREST and PRC2 (Yang and Seto 2003). HDAC-3 is found in distinct complexes like N-CoR-SMRT, whereas HDAC-8 has not been found to be part of any repression protein complex (Yang and Seto 2008). Class IIa HDACs (HDAC-4, -5, -7, -9 and -10) are highly specifically expressed in certain tissues and show both nuclear and cytoplasmic expression. Very little is known about HDAC-10, but others may mediate transcriptional repression by recruiting transcription factors such as myocyte enhancer factor 2 (MEF2), the chaperone protein 14-3-3 and class I HDACs (Haberland et al. 2009). The class IIb member HDAC-6 is the main cytoplasmic deacetylase in mammalian cells and known to directly acetylate cytoskeletal proteins such as α-tubulin and cortactin (Zhang et al. 2008). Class III Sirtuins are not structurally related to other HDACs and require nicotinamide adenine dinucleotide (NAD+) as a cofactor. There are altogether seven different sirtuins which subcellular localization varies from mitochondrial to nucleus. Sirtuins have been strongly linked to metabolic regulation and also target non-histone proteins (Schwer and Verdin 2008). HDAC11 is the only one Class IV HDAC, and is mainly expressed in brain, heart, muscle, kidney and testis. The function of HCAD11 is still unknown.

Table I. Histone deacetylase superfamily protein expression, function and *in vivo* phenotype.

Protein	Class	Expression	Knock-out phenotype
HDAC1	I	Ubiquitous	Proliferation defects
HDAC2	I	Ubiquitous	Cardiac malformation
HDAC3	I	Ubiquitous	Gastrulation defects, disruption of lipid and cholesterol homeostatis
HDAC4	IIa	Brain, skeletal growth plates	Chondrocyte differentiation defects
HDAC5	IIa	Muscle, heart, brain	Excessive stress induced cardiac hypertrophy
HDAC6	IIb	Ubiquitous	Increased tubulin acetylation, no apparent phenotype
HDAC7	IIa	Endothelial cells and thymocytes	Endothelial dysfunction and vascular disruption
HDAC8	I	Ubiquitous	Craniofacial defects
HDAC9	IIa	Muscle, heart, brain	Excessive stress induced cardiac hypertrophy
HDAC10	IIb	Ubiquitous	-
HDAC11	IV	Brain, heart, muscle, kidney, testis	-

AR antagonists (like bicalutamide), that do not completely inhibit AR nuclear translocation, recruit repressive protein complexes like NCoR and SMRT together with HDACs to AR regulated gene promoters (Rosenfeld et al. 2006). However, HDACs may also be required for the transcriptional activation of AR regulated genes. HDAC1-3 are frequently overexpressed in prostate cancer and linked to PSA relapse (Weichert et al. 2008, Halkidou et al. 2004b). Additionally, HDAC inhibitors have antiproliferative effects particularly in AR expressing prostate cancer cell lines (Butler et al. 2000, Rokhlin et al. 2006). HDAC inhibition (especially silencing of HDAC-1 and -3) has been found to directly downregulate AR protein levels and inhibit AR target gene expression (Welsbie et al. 2009). Nuclear expression of HDAC-4 has also been detected in CRPC (Halkidou et al. 2004a) and was found to inhibit AR activity through receptor sumoylation (Yang et al. 2011). These findings imply that elevated levels of HDAC4 may contribute to development of castration resistant disease.

Also elevated SIRT-1 protein levels have been found in prostate tumors and TRAMP prostate cancer mouse models (Huffman et al. 2007). SIRT-1 has been shown to mediate antagonist bound AR target gene (Dai et al. 2007) and E-cadherin transcriptional suppression (Byles et al. 2012) in prostate cancer cell lines. This suggests an intriguing hypothesis that SIRT-1 may contribute to CRPC invasion and possibly epithelial to mesenchymal transformation (EMT).

2.3.2.3 Histone methyltransferases

Histone arginine methylation is catalyzed by the protein arginine methyltransferase (PRMT) class of histone methyltransferases (HMTs) and partly both activates and represses chromatin. However, histone lysines are primarily methylated by a family

of proteins (KMTs) that contain a SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain, like DOT1L (KMT4). In humans, 49 HMTs have been identified which often reside in the same protein complexes together with histone demethylases. This allows switching between transcriptional states by erasing the pre-existing marks, followed by their replacement with new chromatin modifications (Albert and Helin 2010). HMTs are crucial for embryonic development, and aberrant expression or mutation of about half of them has been linked to cancer and other diseases.

Enhancer of zeste homolog 2 (EZH2, KMT6) is overexpressed and amplified in metastatic CRPC and its silencing attenuated cell proliferation in prostate cancer cell lines (Varambally et al. 2002, Saramaki et al. 2006). EZH2 is a part of the PRC2 protein complex, which catalyzes repressive methylation of H3K27. EZH2 overexpression may lead to silencing of AR regulated genes, and metastasis suppressor Raf-1 kinase inhibitor protein (RKIP) mediating metastatic and dedifferentiated CRPC phenotype (Zhao et al. 2012, Ren et al. 2012). Also co-activator-associated arginine methyltransferase 1 (CARM1, PRMT4) is a transcriptional co-activator of AR. CARM1 has been found to be overexpressed in CRPC, where it is thought to activate AR dependent genes and prostate cancer cell line proliferation under low androgen conditions such as anti-androgen therapy (Hong et al. 2004, Majumder et al. 2006). Recently, multiple myeloid/lymphoid or mixed-lineage leukemia (MLLs, KMT2s) H3K4 methyltransferase genes were identified to be mutated recurrently in metastatic CRPC (Grasso et al. 2012). Also these proteins interact with the AR, possibly affecting AR signaling in CRPC. Additionally, SET9 (KMT7) has been shown to directly methylate AR and mediate AR target gene activation by enhancing H3K4 mono-methylation. SET9 nuclear expression is elevated in prostate cancer tissues compared to normal prostate (Gaughan et al. 2011, Ko et al. 2011).

2.3.2.4 Histone demethylases

Histone arginine methylation can be removed by a family of four peptidylarginine deiminases (PADs), using a hydrolase reaction that converts methylarginine to citrulline (Klose and Zhang 2007). Histone lysines are demethylated by two types of lysine demethylases (KDMs), using either flavin adenine dinucleotide (FAD) as a cofactor and producing unmethylated lysines, or iron ions and α-ketoglutarate as cofactors to catalyze the hydroxylation of lysine methylamine groups (Cloos et al. 2008). The first discovered histone KDM was the FAD-dependent amine oxidase lysine-specific histone demethylase 1 (LSD1, KDM1a, AOF2) (Shi et al. 2004). This and its recently found homolog LSD2 (KDM1b) (Fang et al. 2010) demethylate di- and monomethylated H3K4. However, removal of trimethylated histone lysines are preferably catalyzed by Jumonji-domain (JmjC) enzymes. There are 27 different JmjC-domain enzymes in human genome which specifically demethylate histone H3 lysine and arginine residues. These enzymes and the modification which removal they catalyse are summarized in Table II.

Interestingly, somatic mutations of JARID1A (KDM5A), JARID1C (KDM5C) and UTX (KDM6A) have been found in cancer tissues (Futreal et al. 2004), implicating that silencing of these KDMs may provide growth advantage to tumor cells. In prostate cancer, eight histone demethylases have been reported as overexpressed and they are summarized in Table II and by Stratmann and Haendler 2012. UTX (KDM6A) and JARID2 copy number alterations and point mutations were also recently identified in CRPC, but their functional significance remains to be established (Grasso et al. 2012).

Table II. Histone demethylases with known substrate specificity along with reported overexpression in prostate cancer and interaction with AR.

Official name	Alternative name	Substrate specificity	Overexpressed in prostate cancer	Interacts with AR
KDM1a	LSD1	H3K4me1/me2	X	X
KDM1b	LSD2	H3K4me1/me2		
KDM2A	FBXL11	H3K36me1/me2		
KDM2B	FBXL10	H3K36me1/me2, H3K4me3		
KDM3A	JMJD1A	H3K9me1/me2	X	X
KDM3B	JMJD1B	H3K9me1/me2		
KDM3C	JMJD1C	H3K9me1/me2	X	X
KDM4A	JMJD2A	H3K9me2/me3, H3K36me2/me3	X	X
KDM4B	JMJD2C	H3K9me2/me3, H3K36me2/me3	X	
KDM4C	JMJD2B	H3K9me2/me3, H3K36me2/me3	X	X
KDM4D	JMJD2D	H3K9me2/me3, H3K36me2/me3		X
KDM5A	JARID1A	H4K4me2/me3		
KDM5B	JARID1B	H4K4me2/me3	X	X
KDM5C	JARID1C	H4K4me2/me3		
KDM5D	JARID1D	H4K4me2/me3		
JARID2	JMJ			
KDM6A	UTX	H3K27me2/me3		
KDM6B	JMJD3	H3K27me2/me3	X	
UTY	UTY1			
PHF2	JHDM1E	H3K9me2		
PHF8	ZNF422	H3K9me1/me2, H3K27me2, H4K20me1		
HR	ALUNC			
JMJD4	FLJ12517			
KDM8	JMJD5	H3K36me2		
JMJD6	PTDSR	H3R2me2, H4R3me2		
HSPBAP1	PASS1			
HIF1AN	FIH1			
JMJD7				
PLA2G4B	CPLA2-β			

Several histone demethylases have also been identified as AR co-factors (Table II, Stratmann and Haendler 2012). LSD1 can remove mono- and dimethylated H3K4 marks and may act as a H3K9 demethylase in complex with AR, thus leading to AR target gene activation (Shi et al. 2004, Metzger et al. 2005). Elevated LSD1 levels have been linked to an increased risk for prostate cancer relapse (Kahl et al. 2006). Several KDMs have also been shown to interact with AR and mediate reporter gene activation. These include JARID1B, a H3K4 demethylase (Xiang et al. 2007), the H3K9 mono- and didemethylase JMJD1A (Yamane et al. 2006, Wolf et al. 2007), a splice variant of the H3K9 demethylase JMJD1C (Wolf et al. 2007), and H3K9 demethylases JMJD2A and JMJD2D (Shin and Janknecht 2007). Additionally, JMJD2C functions as an AR co-activator in complex with LSD1 and upregulates AR target genes by specifically removing H3K9 methylation marks (Wissmann et al. 2007).

2.3.3 Histone modification binding proteins in prostate cancer

The above summarized enzymes accomplish the dynamic histone modifications. These biochemical marks are then recognized and bound by various classes of histone binding proteins, sometimes referred to as histone code "effector" or "reader" proteins (Rodriguez-Paredes and Esteller 2011), which ultimately dictate the activity of the chromatin. Histone binding proteins have been divided into several classes based on their conserved protein domains. It should be noted that the same protein can contain different chromatin binding domains along with histone modification domains, like KDM or HDAC domain. The main histone binding protein domains and their functional association with prostate cancer are briefly summarized below and in Figure 3.

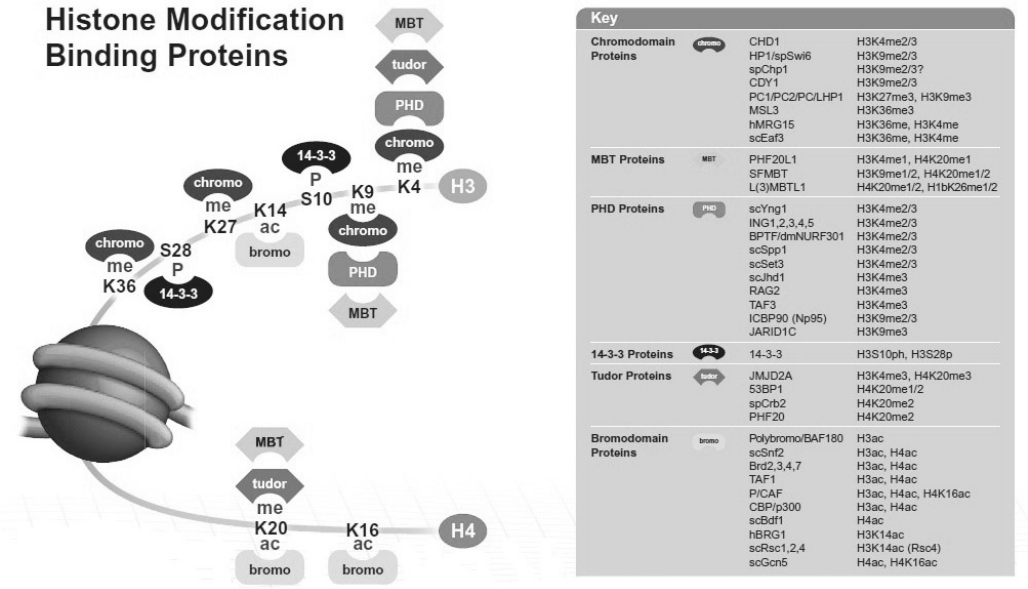

Figure 3. Histone modification binding proteins. Figure adapted and modified from http://www.abcam.com/index.html?pageconfig=resource&rid=11924

2.3.3.1 Chromodomain proteins

Chromodomain containing proteins recognize and bind mainly H3K9 di- and trimethylated histones, and mediate the formation of transcriptionally silenced and inactive heterochromatin (Grewal and Jia 2007). However, some of the chromodomain proteins can also bind methylated H3K4 and contribute to transcriptional activation. The chromodomain helicase DNA-binding protein 1 (CHD1) has been identified as deleted or mutated or showing reduced expression levels in a subset of primary prostate tumors and cell lines (Huang et al. 2011). Additionally, CHD1 silencing in non-tumorigenic prostate cell lines was linked to increased invasion.

2.3.3.2 MBT proteins

Malignant brain tumor domain (MBT) binds mono- and dimethylated lysines. This protein domain is primarily found in Polycomb group (PcG) proteins as well as in the lethal 3 malignant brain tumor (l(3)mbt) family of tumor suppressors (Bonasio et al. 2010). MBT proteins are suggested to function in the repression of developmental genes and promotion of cellular identity as well as in chromatin processes affecting the cell cycle, associated with E2F/Rb complexes. No alterations in MBT proteins have been reported in prostate cancer, although deletion of l(3)mbt-like protein 1 (L3MBTL) was reported in myeloid malignancies (Li et al. 2004).

2.3.3.3 PHD proteins

The plant homeodomain (PHD) finger is found in many chromatin-remodeling proteins. Different subsets of PHD finger proteins can either bind to trimethylated H3K4, unmodified histone H3, trimethylated H3K9me3 or various acetylated histone H3 or H4 lysine residues (Musselman and Kutateladze 2009). Over 200 PHD finger proteins are encoded in the human genome, and different mutations and translocations have been linked to a variety of neurological diseases and cancers (Baker et al. 2008).

2.3.3.4 Tudor domain proteins

Methylated histone arginines and H4K20 are recognized by Tudor domain proteins. There are about 30 Tudor domain proteins in the human genome, involved in a spectrum of cellular processes, including RNA metabolism, germ cell development, transposon silencing, DNA damage response, histone modification and chromatin remodeling (Chen et al. 2011). A large group of Tudor domain proteins is primarily expressed in germ cell lines, and was recently identified to interact with arginine-methylated P-element induced wimpy testis (PIWI) proteins. These proteins appear to coordinate the use of PIWI-interacting RNAs (piRNAs) as structural guide factors to silence gene expression during gametogenesis. There are no reports on the role of Tudor domain mutations in cancer, but it should be noted that some histone modifiers like JMJD2A also contain a Tudor domain.

2.3.3.5 Bromodomain proteins

Bromodomain proteins recognize acetylated histone lysine residues and they are found both in chromatin-associated proteins and KATs (Mujtaba et al. 2007). Bromodomain containing protein 4 (BRD4) arise as a putative cancer target since BDR4-NUT fusion gene associated to rare midline carcinoma (French et al. 2003) was found to be specifically displaced from chromatin by a novel combound VQ1 (Filippakopoulos et al. 2010). No mutations have been reported on bromodomain region of the proteins in prostate cancers. However, bromodomain containing KATs CBP, p300 and PCAF have been identified as AR co-regulators and their expression is elevated in prostate tumors as previously discussed (Shang et al. 2002, Heemers et al. 2007, Isharwal et al. 2008).

2.3.3.6 PWWP proteins

PWWP domain (named after a conserved Pro-Trp-Trp-Pro motif) has the capability to act both as a protein interaction and a histone methylation binding domain. PWWP domain is found in the human genome in approximately 25 proteins, which are involved in DNA methylation, DNA repair and regulation of transcription. The best-studied PWWP domain containing protein is DNMT3A, which was shown to bind tri-methylated H3K36 through its PWWP domain (Dhayalan et al. 2010). This binding leads to the heterochromatic localization of DNMT3A and possibly guides DNA methylation, since genome-wide DNA methylation patterns overlap with H3K36 methylation (Meissner et al. 2008). The functional role of the PWWP proteins in cancer remains to be studied.

2.3.3.7 14-3-3 proteins

14-3-3 proteins are phosphoserine- or phosphothreonine-binding proteins involved in a multitude of cellular processes, including gene regulation, differentiation, cell cycle progression, and metabolism. 14-3-3- family consists of seven isoforms which are ubiquitously expressed and self-assemble into homo- and heterodimers. They also bind phosphorylated, transcriptionally active histone H3 and interact with KATs like PCAF and MOF (Healy et al. 2011). 14-3-3σ expression is lost or reduced in over 90% of prostatic intraepithelial lesions and prostate adenocarcinomas through promoter methylation (Cheng et al. 2004b, Lodygin et al. 2004).

2.3.4 Micro-RNAs and prostate cancer

Micro-RNAs (miRNA) are short (18 to 25 nucleotides) non-coding RNAs which post-transcriptionally alter the mRNA expression (Ambros 2004). MiRNAs bind to the complementary mRNA UTR sequences and regulate their expression through RISC. Several miRNAs can bind to the same mRNA and likewise the same miRNA can regulate the expression of several mRNAs. MiRNAs are expressed tissue-specifically and they

control various cellular processes including proliferation, differentiation, apoptosis and development.

Several differentially expressed miRNAs have been identified in prostate cancer (Jeronimo et al. 2011). Most notably, overexpression of miR-221, miR-222 and miR-21 was shown to induce androgen independent cell growth in prostate cancer cell lines and *in vivo* models, possibly contributing to CRPC phenotype (Sun et al. 2009, Ribas et al. 2009). Additionally, high expression of miR-34a and -34c were shown to reduce AR protein levels in prostate cancer cell lines and correlate negatively with AR staining in clinical samples (Ostling et al. 2011). MiRNAs can also target chromatin modifying enzymes. Genomic loss of miR-101 has been detected to induce overexpression of histone methyltransferase EZH2 and their expression correlated inversely in clinical prostate tumors (Varambally et al. 2008). MiR-101 expression was also shown to be androgen regulated (Cao et al. 2010) implying that androgen deprivation therapy may contribute to EZH2 overexpression observed in CRPC (Varambally et al. 2002). Interestingly, serum circulating miRNAs (e.g. miR-141 and miR-375) could also be used as a predictive, non-invasive method for prostate cancer diagnostics (Nguyen et al. 2012, Mitchell et al. 2008).

2.4 Epigenetic drugs in cancer therapy

Compounds capable to restore normal epigenetic landscape in cancer cells have been very much in focus over recent years. This is mainly due to the fact that unlike genetic mutations, epigenetic alterations are reversible. To date (2012), four epigenetic drugs have been approved for cancer treatment by the FDA. These include the DNMT inhibitors vizada and decitabine (5-aza- and 5-aza-2'-deoxycytidine, respectively), which are approved for treatment of patients with myelodysplastic syndrome (Garcia et al. 2010), and the HDAC inhibitors vorinostat (suberoylaniline hydroxamid acid) and romidepsin (FK-228), which have been approved for cutaneous T cell lymphoma (CTCL) (O'Connor et al. 2006, Piekarz et al. 2011). A number of other compounds are either under development or have shown efficacy cancer in cell lines. Their epigenetic targets and developmental phase are summarized in Figure 4 and below.

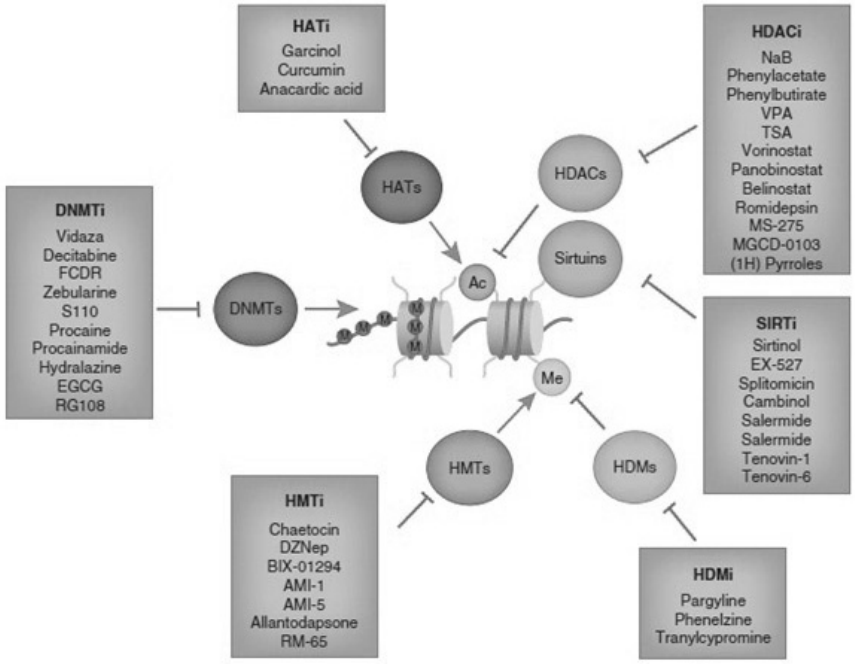

Figure 4. The most important epigenetic drugs for cancer therapy classified based on their particular epigenetic targets. Adapted and modified from Rodriguez-Paredes & Esteller 2011 Nature Medicine.

2.4.1 DNMT inhibitors

In addition to the FDA-approved drugs vizada and decitabine, other nucleoside analogs have been found to inhibit DNTMs. 5-fluoro-2'-deoxycytidine (FdCyd) is in phase II in clinical trials for acute myeloid leukemia and myelodysplastic syndrome in combination with tetrahydrouridine which diminishes FdCyd conversion to its cytotoxic metabolites (ClinicalTrials.gov identifier: NCT00978250) (Beumer et al. 2008). Also some non-nucleotide analogs, like (-)-epigallocatechin-3-gallate (EGCG) and procainamide have been described as micromolar DNMT inhibitors (Fang et al. 2003, Lee et al. 2005).

2.4.2 Histone deacetylase inhibitors

HDAC inhibitors can be divided into four chemically distinct classes: short-chain fatty acids, hydroxamid acids, cyclic peptides and benzamide derivatives. However, the function of all HDAC inhibitors is based on their ability to block the substrate-Zn chelation at the active site of HDACs, thus interfering with their metal-binding domain. Class III HDACs sirtuins require NAD+ at their active sites and are inhibited by nicotinamide analogs, histone peptide competition and SIRT1 protein precipitation (Liu et al. 2009). There are currently no sirtuin inhibitors in clinical trials for cancer.

Short-chain fatty acid HDAC inhibitor phenylbutyrate is a high millimolar HDAC inhibitor which has been studied in clinical trials against both solid and hematological malignancies, and applied as a single agent or in combination with 5-aza-deoxycytidine (Gilbert et al. 2001). Valproate, another short-chain fatty acid HDAC inhibitor, is active against HDACs 1-5, -7 and -9. Valproate has also been studied extensively in combination with various chemotherapy agents, such as all-trans retinoic acid (ATRA), 5-aza-deoxycytidine and radiation therapy, in both solid and hematological malignancies (Soriano et al. 2007). The generally low potency of the short-chain fatty acid HDAC inhibitors may significantly limit their clinical use.

Besides being approved for therapy of myelodysplastic syndromes, the hydroxamid acid HDAC class I and II inhibitor vorinostat is intensely studied for solid tumors in Phase I/II clinical trials both as a single therapy and in combination with 5-aza-deoxycytidine, the proteosomal inhibitor bortezomib, tyrosine kinase inhibitors sorafenib and dasatinib, or ionizing radiation. Interestingly, in prostate cancer vorinostat is also explored in combination with inhibitors against the mammalian target of rapamycin (mTOR) such as temsirolimus (NCT01174199) and androgen deprivation therapy (NCT00589472). Another hydroxamid acid HDAC inhibitor, panobinostat (LBH589), which inhibits HDACs 1-4, -7 and -9, has already been shown to reduce PSA levels in Phase I trials in metastatic CRPC and is under further development (Rathkopf et al. 2010).

Like vorinostat, the FDA approved HDAC cyclic peptide romidepsin inhibiting HDACs 1, 2, 4 and 6 is in clinical trials also for solid tumors. However, it was recently found to be ineffective in Phase II clinical trials against metastatic, chemotherapy-naïve CRPC (Molife et al. 2010).

The benzamide derivative entinostat (MS-275) is a class I selective HDAC inhibitor currently in clinical trials against both solid and hematological malignancies, also in combination with 13-cis retinoic acid, 5-aza-deoxycytidine and the kinase inhibitors imatinib and sorafenib. Interestingly, entinostat was found to activate estrogen receptor α (ERα) and aromatase, and combination treatment with entinostat and aromatase inhibitors has shown promising results in preclinical ERα-negative breast cancer models (Sabnis et al. 2011). This combination treatment is currently being tested in Phase II clinical trials (NCT00828854, NCT01234532).

2.4.3 Histone acetyltransferase inhibitors

HAT inhibitor activity has been found among natural compounds curcumin, garcinol and anacardic acid. Curcumin and garcinol inhibit p300/CBP and PCAF at micromolar concentrations (Balasubramanyam et al. 2004a, Balasubramanyam et al. 2004b). Curcumin has been studied as a cancer preventing agent and therapy option in clinical trials but no results have been reported to date.

2.4.4 Histone methyltransferase inhibitors

KMT activity is inhibited by chaetocin, DZNep and BIX-01294 (Isham et al. 2007, Miranda et al. 2009, Chang et al. 2009). Arginine HMTs PRMT1, -3, -4 and -6 are inhibited by arginine N-methyltransferase inhibitor 1 (AMI-1) (Cheng et al. 2004a). AMI-1 was also identified to inhibit activation of AR and ER promoter constructs. Since CARM1 (PRMT4) has been shown to be overexpressed in CRPC and functions as an AR co-activator (Hong et al. 2004, Majumder et al. 2006), it may be feasible to explore the effects of PRMT inhibition in hormone dependent cancers in the future.

2.4.5 Histone demethylase inhibitors

Several monoamine oxidase inhibitors (MAOi) such as pargyline, phenelzine and tranylcypromine also inhibit LSD1 at millimolar concentrations (Lee et al. 2006). The MAO inhibitor derivative NCL-1 has been shown to have greater selectivity for LSD1 (Ogasawara et al. 2011). Inhibitors based on the N-terminal H3 tail like hydrazine-containing H3 peptides have also been described as more potent LSD1 inhibitors (Culhane et al. 2010).

Most of the inhibitors developed against JmjC-domain KDMs are based on the structural α-ketoglutarate scaffold of the proteins (Lohse et al. 2011a). A number of KDM inhibitors showing micromolar potency have been identified, including N-oxalyl-glycine (NOG) based derivatives (Hamada et al. 2009), 2,4-pyridine di-carboxylic acid (2,4-PDCA) based inhibitors (Thalhammer et al. 2011), hydroxamid acids (Hamada et al. 2010), and a JMJD2C selective substrate-based inhibitor (Lohse et al. 2011b, Nielsen et al. 2012). Specificity towards certain a KDM seems to be possible to obtain and considered an essential trait of these compounds, since diverting cellular functions of various KDMs have been reported. Many KDM inhibitors are now in the hit ligand finding phase and have thus not been tested in clinical trials.

3 AIMS OF THE STUDY

The role of DNA methylation and histone modifications in prostate cancer initiation and progression has been recognized during the recent decade (Perry et al. 2006). Altered expression of multiple epigenetic enzymes responsible for these chromatin modifications has been discovered behind these phenomena, and some of the gene products involved are being explored as potential targets for novel prostate cancer drugs. Here, we wanted to systematically evaluate by RNAi screening as to which epigenetic enzymes have functional significance for several prostate cancer cell phenotypes. We aimed to use multiple phenotypic readouts, such as histone acetylation and methylation, cell proliferation, apoptosis and AR expression and to carry out high throughput RNAi screening of epigenetic enzymes covering all the protein families with known or predicted epigenetic activity. The epigenetic drug target protein classes identified were further validated *in vitro*, using cell-based experiments and specific inhibitors. Furthermore, we aimed to evaluate the mechanism of action of known epigenetic drugs in prostate cancer and to explore novel compound structures inhibiting Jumonji-domain histone demethylases. More specifically, the objectives of this doctoral thesis were:

1. To identify systematically all epigenetic enzymes that have a phenotypic impact on prostate cancer cells by gene knockdown experiments of 615 epigenetic gene targets

2. To study the functional consequences of histone deacetylase inhibitors in TMPRSS2-ERG fusion gene positive prostate cancers.

3. To identify novel chemical structures inhibiting Jumonji-domain histone demethylases by chemoinformatic approaches.

4 MATERIALS AND METHODS

More detailed information on the methods is available in the original publications (I-IV).

4.1 Cell lines

Cell line name	Origin	Tissue of origin	Used in
22rv1	ATCC	prostate adenocarcinoma, AI CWR22 xenograft	IV
CWR1	ATCC	prostate adenocarcinoma, AI CWR22 xenograft	IV
DU-145	ATCC	prostate adenocarcinoma, brain metastasis	I-IV
DuCaP	Adrie van Bokhoven, University Medical Centre Nijmegen, Netherlands	prostate adenocarcinoma, dura mater metastasis	III
EP156T	Warda Rotter, Weizmann Institute, Israel	Primary prostate cell line, hTERT immortalized	III
LAPC-4	ATCC	prostate adenocarcinoma	IV
LNCaP	ATCC	prostate adenocarcinoma, lymph node metastasis	I-IV
MDA-Pca-2b	ATCC	prostate adenocarcinoma, AI bone metastasis	I-IV
PC-3	ATCC	prostate adenocarcinoma, AI bone metastasis	I-IV
PC-3M	ATCC	prostate adenocarcinoma, AI bone metastasis	IV
RWPE-1	ATCC	histologically normal prostate, HVP-18 immortalized	I-IV
VCaP	Adrie van Bokhoven, University Medical Centre Nijmegen, Netherlands	prostate adenocarcinoma, vertebral metastasis	I-IV

4.2 Reagents and chemicals

Reagent	Supplier	Used in
Apo-One	Promega	III
Bicalutamide	Sequoia Research Products	III
CellTiterBlue	Promega	III
CellTiterGlo	Promega	I, IV
Collagen	Inamed Biomaterials	I
Dihydrotestosterone	Sigma-Aldrich	III
Flutamide	Sigma-Aldrich	III
FuGeneHD	Applied Biosciences	I

Reagent	Supplier	Used in
GFR Matrigel	BD Biosciences	I, II
MS-275	Promega	III
Propidium Iodide	Biofellows	I, III
SiLentFect	Bio-Rad Laboratories	I, II
siRNA oligonucleotide libraries	Qiagen	I, II
Trichostatin A	Sigma-Aldrich	III
Triton X-100	Sigma-Aldrich	I, II
Vorinostat (SAHA)	Lucia Altucci, University of Naples, Italy	III

4.3 Antibodies

Antigen	Supplier/ID	Species	Used in
AR	Santa Cruz Biotechnology/H-280	mouse	I-III
cPARP	Cell Signaling Technology/9546S	mouse	I, II
H3K18ac	Abcam	rabbit	I, II
H3K4me2	Abcam	rabbit	I, II
H3K9me2	Abcam	rabbit	I, II
H4K16ac	Abcam	goat	I, II
Histone H3	Abcam	rabbit	III
Ki67	Abcam/ab15580	mouse	I, II
PHF8	Abcam/ab35471	rabbit	I
β-actin	Sigma-Aldrich	mouse	I-III

4.4 Equipment

Equipment and software	Supplier	Used in
384-well standard plates	Corning	I, II
7900HT Real-time PCR System	Applied Biosciences	I, III
Automated liquid dispenser	ThermoFisher	I, III, IV
Automated liquid handling robot	Hamilton	I, II, IV
BD FACSarray Flow cytometer	BD Biosciences	I, III
BeadArray Reader	Illumina	I, III
Bioanalyzer 2100	Agilent Technologicies	I, III
Envision Multilabel plate reader	Perkin Elmer/ Wallac	I, III, IV
GrapPadPrism4 software	GraphPad Software, Inc.	III
Incucyte live cells real-time imager and software	Essen Biosciences	I
Odyssey Infrared Imaging System	LI-COR Biosciences	I, III
Odyssey v2 analysis software	LI-COR Biosciences	I, III
Scanning microscope scan^R	Olympus Biosystems	I, II
Universal probe library assay design center	Roche Diagnostics	I-III
VTT Acca software	VTT	I

4.5 Methodology

Method	Used in
3D cell culture	I
Apoptosis assay	I, III
Bioinformatics	I, III
Cell culture	I-IV
Cell migration path length analysis	I
Cell viability assays	I, III, IV
Compound treatments	I-IV
CSMA	I, II
Flow cytometric analysis	I, III
Gene expression analysis	I, III
High throughput screening	I, II
Histone Demethylation and Formaldehyde Dehydrogenase (FDH) Coupled Assay	IV
Immunoblotting	I, III
Immunofluorescence staining	I, II
Immunohistochemisrtry	I
Live cell microscopy	I
Reverse transcriptase-PCR	I, III
RNA extraction	I, III
RNA interference	I-III
Statistical analysis	I-III
Structure based virtual screening	IV
Tissue microarray	I
Transfection of overexpression construct	I

5 RESULTS

5.1 High-content RNAi screening of 615 epigenetically active proteins in prostate cancer cell lines (I, II)

Despite multiple emerging prostate cancer therapies, the treatment of CRPC remains a challenge (Petrylak 2011). Accumulated evidence for the prostate cancer dependency on epigenetic modification patterns and aberrant expression of epigenetic enzymes suggests that an alternative class of novel drug targets may possibly be found among epigenetic modifiers (Perry et al. 2010).

To evaluate prostate cancer cell line dependencies on known and predicted epigenetically active enzymes, we performed a systematic high througput RNAi screen addressing all 615 epigenetic proteins. Genes were included into the siRNA screening library based Gene Ontology (GO) annotations epigenetics, chromatin remodeling/maintenance, and co-regulatory functions. Genes with at least one epigenetically active protein domain (like bromo, Tudor, JmjC, PHD, HDAC or chromo domain) were included. Additionally, we included genes based on literature meta-searches and protein-protein-interaction data of epigenetic transcriptional regulation complexes (I: Figure 1A, II: Figure 1). Our custom-made siRNA library containing 2 siRNAs against each genes and controls (1328 siRNA altogether) was screened with high throughput CSMA RNAi screening technique in VCaP prostate cancer cell line for evaluating the significance of epigenetic enzymes for cell proliferation (Ki67), survival (cPARP) and AR expression (I: Figure 1B, II: Figure 2). We also studied the regulation of global levels of histone modifications linked to prostate cancer prognosis (H3K4/H3K9 dimethylation and H3K18/H4K16 acetylation) (Seligson et al. 2005, Seligson et al. 2009). Our screen identified 272 siRNAs targeting 231 genes affecting the various endpoints (I: Supplementary Figure S1). Hit siRNAs were grouped by hierarchical clustering and significantly enriched protein domains in each cluster were annotated. We aimed to identify the most significant epigenetic protein domains enriched that affected prostate cancer cell proliferation, survival, AR expression and histone modifications (I: Supplementary Table 2).

5.1.1 Role of histone demethylases and deacetylases in prostate cancer

This approach identified a significant enrichment of PHD-finger domain proteins among siRNAs that reduced cell survival and simultaneously increased global H3K4 dimethylation (I: Figure 1C, Cluster I). Proteins containing an HDAC domain were enriched in a cluster of siRNAs that increased histone acetylation and reduced cell proliferation and AR levels (I: Figure 1C, Cluster II). Additionally, cell proliferation was reduced and H3K9 dimethylation increased by silencing of another set of genes, mainly targeting JmjC-domain containing proteins (I: Figure 1C, Cluster III). In conclusion, these screening results indicated that the most promising epigenetic enzyme classes to

be targeted in prostate cancer may be HDACs and JmjC-domain containing KDMs. Silencing of multiple members of these protein classes significantly reduced AR expression and cell proliferation.

Our large scale primary screen indicated that HDMs are likely to be functionally significant for prostate cancer cell proliferation. This was tested *in vitro* using a microtiter plate-based secondary RNAi screen, targeting all of the 32 human histone demethylases in a panel of cell lines: non-transformed RWPE-1 and prostate cancer cell lines MDA-PCa-2b, LNCaP, VCaP and DU-145. We also explored the mRNA expression of these HDMs in a large number of clinical prostate tumors in the GeneSapiens database, containing gene expression data from 149 normal and 349 prostate adenocarcinoma samples (Kilpinen et al. 2008). Five HDMs were identified to be overexpressed in prostate tumors (JARID1B, PHF8, JMJD1A, JMJD2B, JMJD2A) (I: Table 1). Silencing of PHF8 in these cell lines also reduced prostate cancer cell proliferation (I: Figure 6, Supplemetary Table 4).

PHF8 mRNA was found to be significantly overexpressed in various published prostate cancer gene expression sets, in particular when compared to normal or benign prostate samples (I: Figure 2). PHF8 protein expression was studied using immunohistochemical staining in tissue microarrays (TMA) containing 332 cancer and 90 normal/benign prostate samples. 76% of normal/benign samples showed negative to weak PHF8 expression, whereas 80% of the prostate cancer samples had moderate to strong PHF8 staining (I: Figure 3A and B). Increased PHF8 staining correlated significantly with high Gleason score (I: Figure 3D).

Next, we explored the cellular processes induced by high PHF8 expression in prostate cancer. Gene expression patterns correlating with high PHF8 expression *in vivo* were studied in the GeneSapiens database in 233 prostate tumor samples. MRNA expression of 757 genes correlated significantly with high PHF8 expression. Their functional gene ontology annotations were mainly linked to central nervous system development, followed by regulation of the actin cytoskeleton (I: Supplementary Table 6). Altered gene expression patterns in response to PHF8 silencing were also studied *in vitro* in the LNCaP prostate cancer cell line (I: Figure 4). 62 genes were found to be significantly downregulated in response to PHF8 silencing (I: Supplementary Table 7) and their functional annotation was linked to integrin signaling pathway (I: Supplementary Table 6). Since both actin cytoskeleton regulation and integrin signaling are linked to cellular migration and invasion (Guo and Giancotti 2004), we studied the effects of PHF8 overexpression and silencing on cell migration and invasion in 2D and 3D *in vitro* assays. PHF8 was found to significantly regulate 2D cell migration in time-lapse microscopy path quantitation assay (I: Figure 5A and B). PHF8 silencing also significantly reduced prostate cancer cell spheroid size and invasion in 3D organotypic invasion assay (Harma et al. 2010) (I: Figure 5C and D).

In conclusion, our systematic multiplexed high throughput RNAi screen of 615 epigenetic genes in prostate cancer cell lines identified JmjC-domain HDMs critical for

cell proliferation and HDACs for AR expression. More detailed analysis of silencing 32 HDMs in prostate cancer cell lines revealed a novel HDM PHF8 to mediate cell proliferation in prostate cancer cell lines. PHF8 was identified to be overexpressed in high grade prostate tumors and involved in cell proliferation and motility.

5.2 *In vitro* evaluation of histone deacetylase inhibitors in the treatment of TMPRSS2-ERG fusion gene positive prostate cancers (III)

Approximately 40-70% of all prostate cancer samples contain a gene fusion joining the promoter region and the AR regulated TMPRSS2 with ETS transcription factors like ERG, ETV1, ETV4 and ETV5 leading to their oncogenic overexpression in prostate cancer (Mehra et al. 2007, Helgeson et al. 2008). We have previously found TMPRSS2-ERG overexpression in clinical prostate tumors to be associated with high HDAC-1 expression and pathways related to epigenetic reprogramming (Iljin et al. 2006). In addition, our high throughput RNAi screen detected enrichment of HDAC targeting siRNAs among the hits reducing the AR protein levels in TMPRSS2-ERG expressing VCaP prostate cancer cell line (I: Figure 3C). These evidences suggest that TMPRSS2-ERG positive prostate cancers may be specifically sensitive against HDAC inhibitors, and that HDACs may play a role in the regulation of AR expression. To validate these hypotheses *in vitro*, we compared the proliferation responses of immortalized prostate cell lines with the TMPRSS2-ERG fusion gene expressing and fusion gene negative prostate cancer cell lines. We also compared the mRNA expression profiles induced by different HDAC inhibitors and androgen deprivation in fusion gene positive prostate cancer cell lines. Finally, AR mRNA, protein levels and nuclear localization were studied in response to HDAC inhibitors.

5.2.1 TMPRSS2-ERG positive prostate cancer cell lines are sensitized to HDAC inhibitors

We determined the proliferation IC50 values in response to pan-HDAC inhibitor trichostatin A (TSA) and class I specific entinostat (MS-275) for immortalized prostate epithelial cell lines RWPE-1 and EP156T, TMPRSS2-ERG positive prostate cancer cell lines VCaP and DuCaP and TMPRSS2-ERG negative prostate cancer cell lines LNCaP, PC-3 and DU-145 (III: Table 1). TSA was found to inhibit the ERG-fusion gene positive cell line proliferation at >50-fold and MS-275 >10-fold lower nanomolar concentrations than the fusion gene negative cell lines. HDAC inhibitors had no measurable anti-proliferative effect on immortalized prostate epithelial cell line proliferation.

5.2.2 HDAC inhibitors and androgen deprivation induced gene expression patterns

Next, we studied the mechanisms of HDAC inhibitor induced loss of cell viability in fusion gene expressing prostate cancer cell line VCaP (III: Figure 3). Since fusion poses

ERG expression under AR regulated TMPRSS2 promoter, these gene expression profiles were compared to ones induced by androgen deprivation using charcoal-stripped serum. Also possible combinatorial effects of HDAC inhibitors and androgen deprivation were studied. The association of altered gene expression patterns with cellular pathways was analyzed with the Gene Set Enrichment Analysis algorithm (http://www.broadinstitute.org/gsea/index.jsp). It should be noted that the gene expression profiles induced by TSA and MS-275 differed significantly, thus possibly indicating different modes of action, or toxic and off-target effects. However, in this study we chose to analyze the overlapping gene expression patterns between TSA and MS-275 and called this HDACi response pattern. Both the HDACi and androgen deprivation induced gene expression changes in VCaP cell line were significantly associated with the regulation of cell proliferation (III: Supporting Information Table I). Interestingly, we also found that TSA and androgen deprivation repressed an overlapping pattern of genes including TMPRSS2 and ERG. These genes were even more significantly repressed when androgen deprivation was combined with HDAC inhibitors (III: Figure 3C, Supporting Information Table II). Androgen regulated genes were also suppressed with MS-275 when combined with androgen deprivation, whereas HDAC inhibition with MS-275 alone had no effect. We also noted that HDAC inhibitors and androgen deprivation suppressed the gene expression pattern linked to ERG overexpression in prostate tumors (Iljin et al. 2006) in VCaP cell line (III: Figure 3D). Furthermore, a significant enrichment of the ERG signature gene expression pattern was only present in TMPRSS2-ERG expressing prostate cancer cell lines (III: Figure 3C), thus giving us *in vitro* evidence that this set of genes may be indeed TMPRSS2-ERG regulated.

5.2.3 HDAC inhibitors and androgen deprivation suppress TMPRSS2-ERG fusion gene through synergistic mechanisms targeting the AR

The above described gene expression analyses suggested that both androgen deprivation and HDAC inhibition could not only suppress TMPRSS2-ERG expression, but also a larger set of androgen regulated genes. Thus, we studied whether the HDAC inhibitors could have a more direct effect also on AR expression (III: Figure 4). Pan-HDAC inhibitors like TSA and vorinostat (SAHA) were found to significantly downregulate AR protein levels, whereas the class I specific HDAC inhibitor MS-275 had no significant effect on the AR (III: Figure 4B). All three HDAC inhibitors and androgen deprivation were also observed to suppress ERG mRNA expression in VCaP prostate cancer cell line (II: Figure 4A). The mechanisms of this ERG suppression were studied by analyzing subcellular localization of the AR in response to MS-275 and SAHA. We noted that concentrations of these HDAC inhibitors close to the IC50 values for the blocking of proliferation led to cytoplasmic accumulation of the AR (III: Figure 4C). Interestingly, when combined with the AR antagonist flutamide, synergy in AR cytoplasmic accumulation was noted with HDAC inhibition. Additionally, HDAC inhibition and flutamide synergistigally

enhanced suppression of proliferation in the fusion gene positive prostate cancer cell lines VCaP and DuCaP compared to fusion gene negative/AR positive cell lines like LNCaP (III: Figure 1).

Taken together, these results provide pre-clinical *in vitro* evidence that TMPRSS2-ERG positive cancers may be functionally sensitized to HDAC inhibitors. The suggested molecular mechanisms may work via suppression of AR signaling and downregulation of TMPRSS2/ERG fusion gene expression. Also the synergy observed between androgen deprivation and HDAC inhibitors suggests that combining these two inhibitors might have improved efficacy in prostate cancer therapy.

5.3 High throughput virtual and cell-based screenings for novel small molecule structures inhibiting Jumonji domain-containing histone demethylases (IV)

Various HDMs have been observed to be overexpressed and mutated in prostate cancer (Stratmann and Haendler 2012). HDMs have also been identified as AR cofactors, thus mechanistically linking HDM gene overexpression with progression to CRPC. Additionally, we identified in this thesis that HDMs may mediate prostate cancer cell proliferation, and validated overexpression of the novel HDM PHF8 in high grade prostate tumors. PHF8 also mediates cell invasion *in vitro* (I). In combination, this biological evidence suggest that HDM inhibitors, targeting JmjC-domain KDMs in particular, may represent a novel class of compounds to treat advanced prostate cancer. However, currently most of the identified KDM inhibitors either mimic the oxoglutarate cofactor or the metal chelators. In order to search for novel structures inhibiting JmjC-domain KDMs we performed a chemoinformatic virtual screening searching for JMJD2A ligands coupled with cellular and enzymatic assays.

5.3.1 Virtual ligand-based screening for novel JMJD2A binding chemical structures

We started our chemoinformatic search for inhibitors targeting JmjC-domain by taking Jumonji domain-containing protein 2A (JMJD2A, KDM4A) crystal structure as the starting point. Next, we used chemoinformatic FRED software (Miteva et al. 2005) to virtually dock and screen a library of four million molecules against the putative active site of the protein. A large number of 11,000 compounds satisfied the basic criteria set and were fitting into the active pocket of the enzyme. These molecules were then re-analyzed using the Surflex-Dock docking software (Miteva et al. 2005), in order to reduce the exceedingly large number of hits. The final set of resulting structures was further selected based on the conformational energy values in the bound state, and also on visual inspection. This approach produced a list of 64 compounds which were ordered from commercial vendors (IV: Supplementary Table 1)

5.3.2 Enzyme and cell proliferation inhibition by JMJD2A binding compounds

Next, we tested these 64 chemoinfomatically identified JMJD2A inhibitors *in vitro* for the ability to inhibit recombinant JMJD2A demethylase activity against trimethylated H3K9 peptide substrate. Seven compounds were identified to specifically inhibit >50% of the JMJD2A activity at 1.5 mM concentration (III: Figure 2). These compounds were also tested in the 22rv1, CWR1, LNCaP, VCaP, LAPC-4, DU-145, PC3 and PC3M prostate cancer cell lines, as well as in RWPE1 immortalized prostate cell line for their potency to inhibit cell proliferation. Compound 8 inhibited both the JMJD2A activity and cell proliferation in all tested prostate cancer cell lines (IV: Supporting Information, Figure S1). These results provided a starting point to perform an additional virtual chemoinformatic screen and medicinal chemistry SAR development around the identified lead scaffolds, in order to identify structures that may specifically inhibit the JmjC-domain containing HDMs.

6 DISCUSSION

6.1 Epigenetic proteins in prostate cancer

Our high throughput RNAi screen in VCaP prostate cancer cell line covering most of the epigenetically active enzymes and regulators identified distinct classes of epigenetic modifiers as critical for prostate cancer. PHD finger proteins were shown to reduce cell survival and decrease global H3K4 dimethylation which have been linked to prostate cancer recurrence (Ellinger et al. 2010, Seligson et al. 2005). PHD finger domain is known to recognize and bind non-methylated histone H3 and methylated H3K4, H3K9 and H3K36 histone modifications (Musselman and Kutateladze 2009, Shi et al. 2007). The PHD finger domain is found in over 200 proteins in the human genome, and was found to interact with multiple chromatin remodeling complexes – thus regulating mainly genes and gene networks linked to development. Aberrant expression, gene fusions and mutations of many PHD finger proteins have been associated with immunodeficiency syndromes, cancers and neurological disorders (Baker et al. 2008). Targeting the PHD finger domain with small molecule inhibitors or permeable dominant negative peptides which disrupt the chromatin binding may offer a novel strategy for cancer therapeutics. The specific targets, efficacy and indications of this approach deserve more exploration in relevant *in vitro* and *in vivo* models.

We also identified HDACs as one of the main epigenetic protein classes regulating the levels of AR protein in the VCaP prostate cancer cell line. HDAC inhibitors have been previously shown to reduce AR protein levels and AR-regulated gene transcription (Welsbie et al. 2009), thus suggesting that HDAC inhibitors may be particularly efficient in targeting the advanced CRPC. This is currently tested in clinical trials with HDAC inhibitors vorinostat (NCT01174199, NCT00589472) and panobinostat (LBH589), which was shown to reduce PSA levels in CRPC (Welsbie et al. 2009, Rathkopf et al. 2010).

Furthermore, proliferation of VCaP cells was clearly linked to the function and activity of JmjC-domain histone demethylases. These results were validated in a panel of prostate cancer cell lines, and one immortalized but non-transformed prostate cell line. We also evaluated the expression of all 32 histone demethylases in prostate tumors and discovered the novel histone demethylase PHF8 mRNA and protein to be overexpressed in prostate cancer. PHF8 over-expression is linked to high grade (Gleason 8 and 9) cancers, and regulates prostate cancer cell line proliferation and invasion. PHF8 acts as a transcriptional co-activator which binds to di- and trimethylated H3K4 through its PHD finger domain, and subsequently demethylates dimethylated H3K9 and monomethylated H4K20 (Feng et al. 2010, Qi et al. 2010). Mutations disrupting the function of PHF8 JmjC-domain have been associated with X-linked mental retardation syndrome and cleft lip/cleft palate in humans (Loenarz et al. 2010). Accordingly, PHF8 has been shown to

be important for neuronal differentiation and craniofacial development in animal models (Fortschegger et al. 2010, Kleine-Kohlbrecher et al. 2010, Qi et al. 2010). Our findings suggest that PHF8 should be further evaluated in representative prostate cancer xenograft models to explore its effects on tumor growth and metastasis. These *in vivo* validations may also provide additional information about the feasibility to initiate development and screening for small molecule inhibitors that specifically block the functions of the PHF8 histone demethylase.

6.2 Histone deacetylase inhibitors in TMPRSS2-ERG fusion gene positive prostate cancer therapy

Here, we validated our findings from high throughput RNAi screening where HDACs were found to regulate AR protein levels. This also coincided with our previous discovery that AR driven TMPRSS2-ERG fusion gene expression in prostate tumors may lead to epigenetic reprogramming through high HDAC-1 expression (Iljin et al. 2006). Together, these results suggested that TMPRSS2-ERG positive prostate cancers may be particularly sensitive to HDAC inhibitors, especially when combined with androgen deprivation.

In accordance with the above, HDAC inhibitors and androgen deprivation reduced cell proliferation in a synergistic manner in prostate cancer cell lines. This was found to be mediated through the AR since the pan-HDAC inhibitors TSA and SAHA downregulated AR protein levels and the class I specific HDAC inhibitor MS-275 resulted in translocation of AR into the cell cytoplasm. Cytoplasmic AR localization has been linked to reduced capability to activate target gene transcription. Combined treatment with HDAC inhibitors and anti-androgens flutamide and bicalutamide synergistically sequestered AR in cell cytoplasm and blocked nuclear import. AR expression has been previously described to be downregulated by HDAC inhibitors in prostate cancer cell lines (Welsbie et al. 2009). Also anti-androgens flutamide and bicalutamide have been described to accumulate AR to nuclear membrane fraction in the absence of agonist in cell lines (Whitaker et al. 2004). However, the precise molecular mechanisms of synergy between HDAC inhibitors and anti-androgens in AR nuclear localization remain to be solved. It would also be interesting to study the possible synergy between HDAC inhibitors and second-generation anti-androgens, like MDV-3100 and ARN-509, since these novel compounds have been described to potently prevent AR nuclear localization even in the presence of agonist (Tran et al. 2009, Clegg et al. 2012).

Taken together, these results provide evidence that targeting prostate cancer with a combination of anti-androgens and HDAC inhibitors may improve their efficacy in prostate cancer, especially in TMPRSS2-ERG fusion gene positive subtype. The concept is currently being tested in clinical trials with HDAC inhibitor vorinostat and androgen deprivation therapy (NCT00589472). Combining these two therapies may improve the

prostate cancer response to vorinostat, which showed poor efficacy as a single therapy (Bradley et al. 2009).

6.3 Identification of novel chemical structures inhibiting Jumonji-domain histone demethylases

Our high throughput virtual docking of four million compounds to the JMJD2A active site followed by JMJD2A enzyme inhibition and cell proliferation assays identified seven novel chemical structures. Especially compound 8 reduced cell proliferation in a panel of nine prostate cell lines. The identified novel scaffolds were unrelated to the previously described α-ketoglutarate and N-oxalyl-glycine based derivatives since they do not appear to act as bidentate metal chelators of the oxalylglycine type (Rose et al. 2011).

However, micromolar concentrations of the identified compounds were found to be effective in cellular assays, whereas only millimolar concentrations inhibited JMJD2A in the enzymatic assays. This may be caused by inhibition of the other HDMs beside JMJD2A by the compounds. Alternatively, the identified structures may possess cellular toxicity and unidentified off-target effects; also a combination of both aspects may apply. Increased selectivity towards certain HDMs would be a highly desirable feature for HDM inhibitors, since different HDMs within the same family may have diverse functions. For example, JMJD2C has been identified as an AR co-activator upregulated in metastatic prostate cancer (Wissmann et al. 2007, Cloos et al. 2006), whereas its structurally close family member UTX (KDM6A) may be a tumor suppressor gene frequently mutated in CRPC (Grasso et al. 2012, Tsai et al. 2010). 2,4-pyridine di-carboxylic acid (2,4-PDCA) based inhibitors and catechols have been identified to show significant selectivity for JMJD2C over UTX (Nielsen et al. 2012, Kristensen et al. 2011). Additional virtual chemoinformatic screens around the lead scaffolds identified in our screens may result more potent lead structures that could qualify as specific inhibitors of JmjC-proteins

7 SUMMARY AND CONCLUSIONS

The primary aim of this study was to systematically characterize and evaluate prostate cancer epigenome as a source of novel drug targets using high throughput RNAi screening in cell culture models. In these screens prostate cancer cell proliferation, cell survival, AR expression and histone modifications were identified to be most prominently dependent on HDACs, HDMs and PHD finger proteins suggesting these proteins as most feasible classes for novel drug targeting. Furthermore, our more detailed *in vitro* validations showed that HDAC inhibitors can suppress AR protein expression and signaling. Especially, TMPRSS2-ERG fusion gene expressing prostate cancer cell lines were found to be sensitive to combined HDAC inhibition and androgen deprivation. Thus, combining HDAC inhibitors with anti-androgens, which are a part of a standard prostate cancer care, may enhance their therapeutic efficiency in CRPC. This concept is currenly being tested in ongoing clinical trials which will offer a final proof of concept for the efficacy and safety of HDAC inhibition in the battle against CRPC.

Furthermore, our epigenetic high throughput screens and validations in cell culture models and expression analyses in clinical samples discovered a novel JmjC-domain containing HDM PHF8 to be overexpressed in high grade prostate cancers and regulate cell proliferation and invasion. Substantial amount of literature reports overexpression of several HDMs in prostate cancer and they have also been described as significant AR co-activators. Although multiple HDMs have shown to be crucial for correct embryonic development in knock-out mice and zebrafish animal models, explorations of the HDMs in prostate cancer xenograft or transgenic animal models have not been published. These models would be needed to confirm HDMs role in prostate cancer and their efficacy as drug targets. Animal models would also help to reveal HDMs' contribution to CRPC development.

Target validation of HDMs in prostate cancer would also benefit from studies where their function would be inhibited with not only gene silencing techniques but also with specific and selective HDM inhibitors. Studies with HDM inhibitors would in particular reveal wheter there is an added benefit to target certain HDMs simultaneously to gain efficacy in prostate cancer. Chemical structures inhibiting JmjC-domain HDM were screened chemoinformatically in this thesis work and we identified several novel scaffolds for further development. Additionally, natural product library screenings reported in the literature have identified a few catechols which specifically inhibited HDM subtypes, although their potency was low. An increasing number of HDM crystal structures have recently become available which will hopefully help in the future in structure-based designing of subtype specific and potent HDM inhibitors.

In addition to above-mentioned proof-of-concept studies in prostate cancer, some of the general questions remaining in epigenetic cancer therapy development are the need for

combination therapy and the efficacy of epigenetic inhibitors in solid tumors. Epigenetic enzymes function as multiprotein complexes containing transcription factors, RNA polymerases and several different chromatin modifying proteins. Thus, it remains to be seen if inhibiting just one specific epigenetic enzyme is enough for inhibition of cancer progression. Combination therapy may also be needed to overcome the tremendous genetic and epigenetic plasticity of cancer cells often leading to therapy resistance. It should also be noted that epigenetic inhibitors have been approved for therapy mainly in hematological malignancies. Hematological malignancies contain a large number of rapidly proliferating cells which arise through flaws in their lineage differentiation process. However, solid cancer types accumulate mutations for years thus gaining evolutional growth advantages for smaller mass of proliferating cells. More studies are needed to reveal if sufficient efficacy in solid tumors can be attained with epigenetic therapy and which cancer types would be the most susceptible to respond.

ACKNOWLEDGEMENTS

This study was carried out in the Department of Medical Biotechnology, VTT Technical Research Centre of Finland and in the Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku in 2006-2011.

I am grateful to my supervisors Professor Olli Kallioniemi and Docent Matthias Nees for the opportunity to work and develop as a scientist in a multi-disciplinary, ambitious, international and enthusiastic research team and EU consortium. Your enthusiasm has been contagios and the freedom and independence you have given me as a researcher early on has been valuble for my personal development. I thank Docent Harri Siitari, Docent Kirsi-Marja Oksman-Caldentey and Dr. Richard Fagerström for providing excellent research facilities in the interface of academic and industrial world at VTT-MBT. The Department of Pharmacology, Drug Development and Therapeutics and Professor Mika Scheinin are thanked for interest and support for my thesis studies. FinPharma Doctoral Program Drug Discovery, Dr Eeva Valve and Professor Mika Scheining are acknowledged for providing valuable training courses, scientific meetings, networking opportunities and broadening my view on the various aspects of drug development.

The European Union 6th Framework Programme EPIgenetic TReatment Of Neoplastic disease (EPITRON, LSHC-CT-2005-518417) and especially coordinator Professor Hinrich Gronemeyer and Scientific Administrators Dr. Irene Yujnovsky and Dr. Cecilia Poli are thanked for providing financial support, excellent scientific meetings and research collaboration opportunities which have been essential for this thesis study. Also EPITRON collaborators and co-authors Dr. Ciro Mercurio, Dr. Efren Pérez-Santín, Professor Lucia Altucci, Professor Saverio Minucci, and Professor Angel de Lera are warmly acknowledged for their significant contribution to this study.

My PhD supervisory board members Professor Riitta Lahesmaa and Professor Tapio Visakorpi are thanked for encouragement and evaluation during this project. In addition, Professor Pirkko Vihko and Docent Tiina Jääskeläinen are acknowledged for critical review and valuable comments to this thesis manuscript.

The publications included into this thesis would not have been possible without the efforts of all the co-authors. Thus, I want to warmly thank Päivi Östling, Ville Härmä, Johannnes Virtanen, John-Patrick Mpindi, Juha Rantala, Tuomas Mirtti, Tiina Vesterinen, Mikael Lundin, Anna Sankila, Antti Rannikko, Elisa Kaivanto, Pekka Kohonen, Henri Sara, Kristiina Iljin, Pasi Halonen, Pekka Tiikkainen and Antti Poso for fruitful collaboration and team spirit. Each one of you has thought me tremendously about your special area in science and I feel priviledged that I have had the change to collaborate with such a talented bunch of people.

All the co-workers at VTT-MBT are thanked for their friendship and support, not to forget exting and cheering coffee table discussions and parties.

Anu Moilanen, Reetta Riikonen, Leena Kahala, Tarja Ikonen, Tim Holmström, Riikka Oksala, Jonna Saaristo, Pekka Kallio and Jyrki Lehtimäki from Orion Pharma are acknowledeged for sharing your knowledge on cancer drug development and giving me an opportunity to learn what drug development is for real.

My 2007 LAL mentor Pia Ollikka is thanked for support and encouragement to continue this work at the critical moment.

Most importantly, I want to acknowledge my family. My parents Leena and Kari Peltola and my husband John have been extremely supportive in so many ways during my studies and thesis writing process that none of it would have happened without them. Thank you. Special thanks to my children Oskar and Otto for teaching me the true meaning of love, joy, patience and conscious presence. Thank you also for teaching me the value of good organizational skills and setting ultimate deadlines.

This thesis work has been financially supported by European Union 6th Framework Programme EPIgenetic TReatment Of Neoplastic disease (EPITRON, LSHC-CT-2005-518417) and FinPharma Doctoral Program Drug Discovery. The support is gratefully acknowledged.

Turku, March 2013

Mari Björkman

REFERENCES

Abbas A and Gupta S. (2008). The role of histone deacetylases in prostate cancer. *Epigenetics* 6: 300-309.

Agoulnik IU, Vaid A, Bingman WE,3rd, Erdeme H, Frolov A, Smith CL *et al.* (2005). Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression. *Cancer Res.* 17: 7959-7967.

Albert M and Helin K. (2010). Histone methyltransferases in cancer. *Semin.Cell Dev.Biol.* 2: 209-220.

Ambros V. (2004). The functions of animal microRNAs. *Nature* 7006: 350-355.

Attard G, Reid AH, Auchus RJ, Hughes BA, Cassidy AM, Thompson E *et al.* (2012). Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer. *J.Clin. Endocrinol.Metab.* 2: 507-516.

Baker LA, Allis CD and Wang GG. (2008). PHD fingers in human diseases: disorders arising from misinterpreting epigenetic marks. *Mutat.Res.* 1-2: 3-12.

Balasubramanyam K, Altaf M, Varier RA, Swaminathan V, Ravindran A, Sadhale PP *et al.* (2004). Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J.Biol. Chem.* 32: 33716-33726.

Balasubramanyam K, Varier RA, Altaf M, Swaminathan V, Siddappa NB, Ranga U *et al.* (2004). Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. *J.Biol.Chem.* 49: 51163-51171.

Bartz SR, Zhang Z, Burchard J, Imakura M, Martin M, Palmieri A *et al.* (2006). Small interfering RNA screens reveal enhanced cisplatin cytotoxicity in tumor cells having both BRCA network and TP53 disruptions. *Mol.Cell.Biol.* 24: 9377-9386.

Baylin SB. (2005). DNA methylation and gene silencing in cancer. *Nat.Clin.Pract.Oncol.* S4-11.

Baylin SB and Jones PA. (2011). A decade of exploring the cancer epigenome - biological and translational implications. *Nat.Rev.Cancer.* 10: 726-734.

Berdasco M and Esteller M. (2010). Aberrant epigenetic landscape in cancer: how cellular identity goes awry. *Dev.Cell.* 5: 698-711.

Bernard D, Gil J, Dumont P, Rizzo S, Monte D, Quatannens B *et al.* (2006). The methyl-CpG-binding protein MECP2 is required for prostate cancer cell growth. *Oncogene* 9: 1358-1366.

Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K *et al.* (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer.Cell.* 4: 395-402.

Beumer JH, Parise RA, Newman EM, Doroshow JH, Synold TW, Lenz HJ *et al.* (2008). Concentrations of the DNA methyltransferase inhibitor 5-fluoro-2'-deoxycytidine (FdCyd) and its cytotoxic metabolites in plasma of patients treated with FdCyd and tetrahydrouridine (THU). *Cancer Chemother. Pharmacol.* 2: 363-368.

Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M *et al.* (2010). Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. *Cancer Epidemiol.Biomarkers Prev.* 10: 2611-2622.

Bonasio R, Lecona E and Reinberg D. (2010). MBT domain proteins in development and disease. *Semin. Cell Dev.Biol.* 2: 221-230.

Bradley D, Rathkopf D, Dunn R, Stadler WM, Liu G, Smith DC *et al.* (2009). Vorinostat in advanced prostate cancer patients progressing on prior chemotherapy (National Cancer Institute Trial 6862): trial results and interleukin-6 analysis: a study by the Department of Defense Prostate Cancer Clinical Trial Consortium and University of Chicago Phase 2 Consortium. *Cancer* 23: 5541-5549.

Brough R, Frankum JR, Sims D, Mackay A, Mendes-Pereira AM, Bajrami I *et al.* (2011). Functional Viability Profiles of Breast Cancer. *Cancer.Discov.* 3: 260-273.

Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C *et al.* (2000). Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. *Cancer Res.* 18: 5165-5170.

Byles V, Zhu L, Lovaas JD, Chmilewski LK, Wang J, Faller DV *et al.* (2012). SIRT1 induces EMT by cooperating with EMT transcription factors and enhances prostate cancer cell migration and metastasis. *Oncogene*

Cai C, Chen S, Ng P, Bubley GJ, Nelson PS, Mostaghel EA *et al.* (2011). Intratumoral de novo steroid synthesis activates androgen receptor in castration-resistant prostate cancer and is upregulated by treatment with CYP17A1 inhibitors. *Cancer Res.* 20: 6503-6513.

Cao P, Deng Z, Wan M, Huang W, Cramer SD, Xu J *et al.* (2010). MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta. *Mol.Cancer.* 108.

Carragher NO, Brunton VG and Frame MC. (2012). Combining imaging and pathway profiling: an

alternative approach to cancer drug discovery. *Drug Discov.Today* 5-6: 203-214.

Carver BS, Chapinski C, Wongvipat J, Hieronymus H, Chen Y, Chandarlapaty S *et al.* (2011). Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. *Cancer. Cell.* 5: 575-586.

Carver BS, Tran J, Gopalan A, Chen Z, Shaikh S, Carracedo A *et al.* (2009). Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. *Nat.Genet.* 5: 619-624.

Chang Y, Zhang X, Horton JR, Upadhyay AK, Spannhoff A, Liu J *et al.* (2009). Structural basis for G9a-like protein lysine methyltransferase inhibition by BIX-01294. *Nat.Struct.Mol.Biol.* 3: 312-317.

Chatterjee-Kishore M and Miller CP. (2005). Exploring the sounds of silence: RNAi-mediated gene silencing for target identification and validation. *Drug Discov. Today* 22: 1559-1565.

Chen C, Nott TJ, Jin J and Pawson T. (2011). Deciphering arginine methylation: Tudor tells the tale. *Nat.Rev.Mol. Cell Biol.* 10: 629-642.

Chen T, Ueda Y, Dodge JE, Wang Z and Li E. (2003). Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol.Cell.Biol.* 16: 5594-5605.

Cheng D, Yadav N, King RW, Swanson MS, Weinstein EJ and Bedford MT. (2004). Small molecule regulators of protein arginine methyltransferases. *J.Biol.Chem.* 23: 23892-23899.

Cheng L, Pan CX, Zhang JT, Zhang S, Kinch MS, Li L *et al.* (2004). Loss of 14-3-3sigma in prostate cancer and its precursors. *Clin.Cancer Res.* 9: 3064-3068.

Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, Dilhas A *et al.* (2012). ARN-509: a novel antiandrogen for prostate cancer treatment. *Cancer Res.* 6: 1494-1503.

Cloos PA, Christensen J, Agger K and Helin K. (2008). Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev.* 9: 1115-1140.

Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T *et al.* (2006). The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* 7100: 307-311.

Culhane JC, Wang D, Yen PM and Cole PA. (2010). Comparative analysis of small molecules and histone substrate analogues as LSD1 lysine demethylase inhibitors. *J.Am.Chem.Soc.* 9: 3164-3176.

Dai Y, Ngo D, Forman LW, Qin DC, Jacob J and Faller DV. (2007). Sirtuin 1 is required for antagonist-induced transcriptional repression of androgen-responsive genes by the androgen receptor. *Mol.Endocrinol.* 8: 1807-1821.

de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L *et al.* (2011). Abiraterone and increased survival in metastatic prostate cancer. *N.Engl.J.Med.* 21: 1995-2005.

de Bono JS, Oudard S, Ozguroglu M, Hansen S, Machiels JP, Kocak I *et al.* (2010). Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 9747: 1147-1154.

Dehm SM, Schmidt LJ, Heemers HV, Vessella RL and Tindall DJ. (2008). Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res.* 13: 5469-5477.

Dhayalan A, Rajavelu A, Rathert P, Tamas R, Jurkowska RZ, Ragozin S *et al.* (2010). The Dnmt3a PWWP domain reads histone 3 lysine 36 trimethylation and guides DNA methylation. *J.Biol.Chem.* 34: 26114-26120.

Ding Y, Huang D, Zhang Z, Smith J, Petillo D, Looyenga BD *et al.* (2011). Combined gene expression profiling and RNAi screening in clear cell renal cell carcinoma identify PLK1 and other therapeutic kinase targets. *Cancer Res.* 15: 5225-5234.

Eden A, Gaudet F, Waghmare A and Jaenisch R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 5618: 455.

Ellinger J, Kahl P, von der Gathen J, Rogenhofer S, Heukamp LC, Gutgemann I *et al.* (2010). Global levels of histone modifications predict prostate cancer recurrence. *Prostate* 1: 61-69.

Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H *et al.* (2003). Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.* 22: 7563-7570.

Fang R, Barbera AJ, Xu Y, Rutenberg M, Leonor T, Bi Q *et al.* (2010). Human LSD2/KDM1b/AOF1 regulates gene transcription by modulating intragenic H3K4me2 methylation. *Mol.Cell* 2: 222-233.

Faus H and Haendler B. (2006). Post-translational modifications of steroid receptors. *Biomed. Pharmacother.* 9: 520-528.

Feng W, Yonezawa M, Ye J, Jenuwein T and Grummt I. (2010). PHF8 activates transcription of rRNA genes through H3K4me3 binding and H3K9me1/2 demethylation. *Nat.Struct.Mol.Biol.* 4: 445-450.

Filion GJ, Zhenilo S, Salozhin S, Yamada D, Prokhortchouk E and Defossez PA. (2006). A family of human zinc finger proteins that bind methylated DNA and repress transcription. *Mol.Cell.Biol.* 1: 169-181.

Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O *et al.* (2010). Selective inhibition of BET bromodomains. *Nature* 7327: 1067-1073.

Fizazi K, Carducci M, Smith M, Damiao R, Brown J, Karsh L *et al.* (2011). Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. *Lancet* 9768: 813-822.

Fortschegger K, de Graaf P, Outchkourov NS, van Schaik FM, Timmers HT and Shiekhattar R. (2010). PHF8 targets histone methylation and RNA polymerase II to activate transcription. *Mol.Cell.Biol.* 13: 3286-3298.

French CA, Miyoshi I, Kubonishi I, Grier HE, Perez-Atayde AR and Fletcher JA. (2003). BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. *Cancer Res.* 2: 304-307.

Friedlander TW, Roy R, Tomlins SA, Ngo VT, Kobayashi Y, Azameera A *et al.* (2012). Common structural and epigenetic changes in the genome of castration-resistant prostate cancer. *Cancer Res.* 3: 616-625.

Fuks F, Burgers WA, Godin N, Kasai M and Kouzarides T. (2001). Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J.* 10: 2536-2544.

Fuks F, Hurd PJ, Deplus R and Kouzarides T. (2003). The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res.* 9: 2305-2312.

Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R *et al.* (2004). A census of human cancer genes. *Nat.Rev.Cancer.* 3: 177-183.

Garcia JS, Jain N and Godley LA. (2010). An update on the safety and efficacy of decitabine in the treatment of myelodysplastic syndromes. *Onco Targets Ther.* 1-13.

Gaughan L, Stockley J, Wang N, McCracken SR, Treumann A, Armstrong K *et al.* (2011). Regulation of the androgen receptor by SET9-mediated methylation. *Nucleic Acids Res.* 4: 1266-1279.

Giamas G, Filipovic A, Jacob J, Messier W, Zhang H, Yang D *et al.* (2011). Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer. *Nat.Med.* 6: 715-719.

Gilbert J, Baker SD, Bowling MK, Grochow L, Figg WD, Zabelina Y *et al.* (2001). A phase I dose escalation and bioavailability study of oral sodium phenylbutyrate in patients with refractory solid tumor malignancies. *Clin. Cancer Res.* 8: 2292-2300.

Glozak MA and Seto E. (2007). Histone deacetylases and cancer. *Oncogene* 37: 5420-5432.

Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, Khan AP *et al.* (2012). The mutational landscape of lethal castration-resistant prostate cancer. *Nature*

Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS *et al.* (2001). A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res.* 11: 4315-4319.

Grewal SI and Jia S. (2007). Heterochromatin revisited. *Nat.Rev.Genet.* 1: 35-46.

Guo W and Giancotti FG. (2004). Integrin signalling during tumour progression. *Nat.Rev.Mol.Cell Biol.* 10: 816-826.

Gupta S, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P *et al.* (2010). FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res.* 17: 6735-6745.

Haberland M, Montgomery RL and Olson EN. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat.Rev.Genet.* 1: 32-42.

Hajduk PJ and Greer J. (2007). A decade of fragment-based drug design: strategic advances and lessons learned. *Nat.Rev.Drug Discov.* 3: 211-219.

Halkidou K, Cook S, Leung HY, Neal DE and Robson CN. (2004). Nuclear accumulation of histone deacetylase 4 (HDAC4) coincides with the loss of androgen sensitivity in hormone refractory cancer of the prostate. *Eur.Urol.* 3: 382-9; author reply 389.

Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE and Robson CN. (2004). Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *Prostate* 2: 177-189.

Halkidou K, Gnanapragasam VJ, Mehta PB, Logan IR, Brady ME, Cook S *et al.* (2003). Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene* 16: 2466-2477.

Ham WS, Cho NH, Kim WT, Ju HJ, Lee JS and Choi YD. (2009). Pathological effects of prostate cancer correlate with neuroendocrine differentiation and PTEN expression after bicalutamide monotherapy. *J.Urol.* 4: 1378-1384.

Hamada S, Kim TD, Suzuki T, Itoh Y, Tsumoto H, Nakagawa H *et al.* (2009). Synthesis and activity of N-oxalylglycine and its derivatives as Jumonji C-domain-containing histone lysine demethylase inhibitors. *Bioorg.Med.Chem.Lett.* 10: 2852-2855.

Hamada S, Suzuki T, Mino K, Koseki K, Oehme F, Flamme I *et al.* (2010). Design, synthesis, enzyme-inhibitory activity, and effect on human cancer cells of a novel series of jumonji domain-containing protein 2 histone demethylase inhibitors. *J.Med.Chem.* 15: 5629-5638.

Han B, Mehra R, Lonigro RJ, Wang L, Suleman K, Menon A *et al.* (2009). Fluorescence in situ hybridization study shows association of PTEN deletion with ERG rearrangement during prostate cancer progression. *Mod.Pathol.* 8: 1083-1093.

Harma V, Virtanen J, Makela R, Happonen A, Mpindi JP, Knuuttila M *et al.* (2010). A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. *PLoS One* 5: e10431.

Healy S, Khan DH and Davie JR. (2011). Gene expression regulation through 14-3-3 interactions with histones and HDACs. *Discov.Med.* 59: 349-358.

Heemers HV, Sebo TJ, Debes JD, Regan KM, Raclaw KA, Murphy LM *et al.* (2007). Androgen deprivation increases p300 expression in prostate cancer cells. *Cancer Res.* 7: 3422-3430.

Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR *et al.* (2008). Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. *Cancer Res.* 1: 73-80.

Hendrich B and Bird A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol.Cell.Biol.* 11: 6538-6547.

Hong H, Kao C, Jeng MH, Eble JN, Koch MO, Gardner TA *et al.* (2004). Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status. *Cancer* 1: 83-89.

Hornberg E, Ylitalo EB, Crnalic S, Antti H, Stattin P, Widmark A *et al.* (2011). Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. *PLoS One* 4: e19059.

Huang S, Gulzar ZG, Salari K, Lapointe J, Brooks JD and Pollack JR. (2011). Recurrent deletion of CHD1 in prostate cancer with relevance to cell invasiveness. *Oncogene*

Huffman DM, Grizzle WE, Bamman MM, Kim JS, Eltoum IA, Elgavish A *et al.* (2007). SIRT1 is significantly elevated in mouse and human prostate cancer. *Cancer Res.* 14: 6612-6618.

Huggins C and Hodges CV. (2002). 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.* 1: 9-12.

Hugosson J, Carlsson S, Aus G, Bergdahl S, Khatami A, Lodding P *et al.* (2010). Mortality results from the Goteborg randomised population-based prostate-cancer screening trial. *Lancet Oncol.* 8: 725-732.

Iljin K, Wolf M, Edgren H, Gupta S, Kilpinen S, Skotheim RI *et al.* (2006). TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming. *Cancer Res.* 21: 10242-10246.

Iorns E, Turner NC, Elliott R, Syed N, Garrone O, Gasco M *et al.* (2008). Identification of CDK10 as an important determinant of resistance to endocrine therapy for breast cancer. *Cancer.Cell.* 2: 91-104.

Isham CR, Tibodeau JD, Jin W, Xu R, Timm MM and Bible KC. (2007). Chaetocin: a promising new antimyeloma agent with in vitro and in vivo activity mediated via imposition of oxidative stress. *Blood* 6: 2579-2588.

Isharwal S, Miller MC, Marlow C, Makarov DV, Partin AW and Veltri RW. (2008). P300 (Histone Acetyltransferase) Biomarker Predicts Prostate Cancer Biochemical Recurrence and Correlates with Changes in Epithelia Nuclear Size and Shape. *Prostate* 10: 1097-1104.

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M *et al.* (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat.Biotechnol.* 6: 635-637.

Jarrard DF, Kinoshita H, Shi Y, Sandefur C, Hoff D, Meisner LF *et al.* (1998). Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. *Cancer Res.* 23: 5310-5314.

Jeronimo C, Bastian PJ, Bjartell A, Carbone GM, Catto JW, Clark SJ *et al.* (2011). Epigenetics in prostate cancer: biologic and clinical relevance. *Eur.Urol.* 4: 753-766.

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N *et al.* (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat.Genet.* 2: 187-191.

Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R *et al.* (2006). Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. *Cancer Res.* 23: 11341-11347.

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF *et al.* (2010). Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N.Engl.J.Med.* 5: 411-422.

Kassner PD. (2008). Discovery of novel targets with high throughput RNA interference screening. *Comb.Chem. High Throughput Screen.* 3: 175-184.

Kilpinen S, Autio R, Ojala K, Iljin K, Bucher E, Sara H *et al.* (2008). Systematic bioinformatic analysis of expression levels of 17,330 human genes across 9,783 samples from 175 types of healthy and pathological tissues. *Genome Biol.* 9: R139.

King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH *et al.* (2009). Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. *Nat.Genet.* 5: 524-526.

Kleine-Kohlbrecher D, Christensen J, Vandamme J, Abarrategui I, Bak M, Tommerup N *et al.* (2010). A functional link between the histone demethylase PHF8 and the transcription factor ZNF711 in X-linked mental retardation. *Mol.Cell* 2: 165-178.

Klose RJ and Zhang Y. (2007). Regulation of histone methylation by demethylimination and demethylation. *Nat.Rev.Mol.Cell Biol.* 4: 307-318.

Ko S, Ahn J, Song CS, Kim S, Knapczyk-Stwora K and Chatterjee B. (2011). Lysine methylation and

functional modulation of androgen receptor by Set9 methyltransferase. *Mol.Endocrinol.* 3: 433-444.

Kondo Y, Shen L, Cheng AS, Ahmed S, Boumber Y, Charo C *et al.* (2008). Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. *Nat.Genet.* 6: 741-750.

Kouzarides T. (2007). Chromatin modifications and their function. *Cell* 4: 693-705.

Kristensen JB, Nielsen AL, Jorgensen L, Kristensen LH, Helgstrand C, Juknaite L *et al.* (2011). Enzyme kinetic studies of histone demethylases KDM4C and KDM6A: towards understanding selectivity of inhibitors targeting oncogenic histone demethylases. *FEBS Lett.* 12: 1951-1956.

Kunderfranco P, Mello-Grand M, Cangemi R, Pellini S, Mensah A, Albertini V *et al.* (2010). ETS transcription factors control transcription of EZH2 and epigenetic silencing of the tumor suppressor gene Nkx3.1 in prostate cancer. *PLoS One* 5: e10547.

Lee BH, Yegnasubramanian S, Lin X and Nelson WG. (2005). Procainamide is a specific inhibitor of DNA methyltransferase 1. *J.Biol.Chem.* 49: 40749-40756.

Lee MG, Wynder C, Schmidt DM, McCafferty DG and Shiekhattar R. (2006). Histone H3 lysine 4 demethylation is a target of nonselective antidepressive medications. *Chem.Biol.* 6: 563-567.

Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS *et al.* (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc.Natl.Acad.Sci.U.S.A.* 24: 11733-11737.

Leonhardt H, Page AW, Weier HU and Bestor TH. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* 5: 865-873.

Li J, Bench AJ, Vassiliou GS, Fourouclas N, Ferguson-Smith AC and Green AR. (2004). Imprinting of the human L3MBTL gene, a polycomb family member located in a region of chromosome 20 deleted in human myeloid malignancies. *Proc.Natl.Acad.Sci.U.S.A.* 19: 7341-7346.

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J *et al.* (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 7271: 315-322.

Liu L, Kron KJ, Pethe VV, Demetrashvili N, Nesbitt ME, Trachtenberg J *et al.* (2011). Association of tissue promoter methylation levels of APC, TGFbeta2, HOXD3 and RASSF1A with prostate cancer progression. *Int.J.Cancer* 10: 2454-2462.

Liu T, Liu PY and Marshall GM. (2009). The critical role of the class III histone deacetylase SIRT1 in cancer. *Cancer Res.* 5: 1702-1705.

Lodygin D, Diebold J and Hermeking H. (2004). Prostate cancer is characterized by epigenetic silencing of 14-3-3sigma expression. *Oncogene* 56: 9034-9041.

Loenarz C, Ge W, Coleman ML, Rose NR, Cooper CD, Klose RJ *et al.* (2010). PHF8, a gene associated with cleft lip/palate and mental retardation, encodes for an Nepsilon-dimethyl lysine demethylase. *Hum.Mol. Genet.* 2: 217-222.

Lohse B, Kristensen JL, Kristensen LH, Agger K, Helin K, Gajhede M *et al.* (2011). Inhibitors of histone demethylases. *Bioorg.Med.Chem.* 12: 3625-3636.

Lohse B, Nielsen AL, Kristensen JB, Helgstrand C, Cloos PA, Olsen L *et al.* (2011). Targeting histone lysine demethylases by truncating the histone 3 tail to obtain selective substrate-based inhibitors. *Angew.Chem.Int. Ed Engl.* 39: 9100-9103.

Lopez-Serra L, Ballestar E, Fraga MF, Alaminos M, Setien F and Esteller M. (2006). A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. *Cancer Res.* 17: 8342-8346.

Majumder S, Liu Y, Ford OH,3rd, Mohler JL and Whang YE. (2006). Involvement of arginine methyltransferase CARM1 in androgen receptor function and prostate cancer cell viability. *Prostate* 12: 1292-1301.

Marrocco DL, Tilley WD, Bianco-Miotto T, Evdokiou A, Scher HI, Rifkind RA *et al.* (2007). Suberoylanilide hydroxamic acid (vorinostat) represses androgen receptor expression and acts synergistically with an androgen receptor antagonist to inhibit prostate cancer cell proliferation. *Mol.Cancer.Ther.* 1: 51-60.

Maruyama R, Toyooka S, Toyooka KO, Virmani AK, Zochbauer-Muller S, Farinas AJ *et al.* (2002). Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. *Clin. Cancer Res.* 2: 514-519.

Mehra R, Tomlins SA, Shen R, Nadeem O, Wang L, Wei JT *et al.* (2007). Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer. *Mod.Pathol.* 5: 538-544.

Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A *et al.* (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 7205: 766-770.

Meister G and Tuschl T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* 7006: 343-349.

Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH *et al.* (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 7057: 436-439.

Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK *et al.* (2009). DZNep is a global histone methylation inhibitor that reactivates developmental

genes not silenced by DNA methylation. *Mol.Cancer. Ther.* 6: 1579-1588.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL *et al.* (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc.Natl.Acad.Sci.U.S.A.* 30: 10513-10518.

Miteva MA, Lee WH, Montes MO and Villoutreix BO. (2005). Fast structure-based virtual ligand screening combining FRED, DOCK, and Surflex. *J.Med.Chem.* 19: 6012-6022.

Molife LR, Attard G, Fong PC, Karavasilis V, Reid AH, Patterson S *et al.* (2010). Phase II, two-stage, single-arm trial of the histone deacetylase inhibitor (HDACi) romidepsin in metastatic castration-resistant prostate cancer (CRPC). *Ann.Oncol.* 1: 109-113.

Morey Kinney SR, Smiraglia DJ, James SR, Moser MT, Foster BA and Karpf AR. (2008). Stage-specific alterations of DNA methyltransferase expression, DNA hypermethylation, and DNA hypomethylation during prostate cancer progression in the transgenic adenocarcinoma of mouse prostate model. *Mol.Cancer. Res.* 8: 1365-1374.

Mostaghel EA, Marck BT, Plymate SR, Vessella RL, Balk S, Matsumoto AM *et al.* (2011). Resistance to CYP17A1 inhibition with abiraterone in castration-resistant prostate cancer: induction of steroidogenesis and androgen receptor splice variants. *Clin.Cancer Res.* 18: 5913-5925.

Mujtaba S, Zeng L and Zhou MM. (2007). Structure and acetyl-lysine recognition of the bromodomain. *Oncogene* 37: 5521-5527.

Mulholland DJ, Tran LM, Li Y, Cai H, Morim A, Wang S *et al.* (2011). Cell autonomous role of PTEN in regulating castration-resistant prostate cancer growth. *Cancer.Cell.* 6: 792-804.

Musselman CA and Kutateladze TG. (2009). PHD fingers: epigenetic effectors and potential drug targets. *Mol.Interv.* 6: 314-323.

Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G *et al.* (2006). A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 7089: 106-110.

Nguyen HC, Xie W, Yang M, Hsieh CL, Drouin S, Lee GS *et al.* (2012). Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. *Prostate*

Nielsen AL, Kristensen LH, Stephansen KB, Kristensen JB, Helgstrand C, Lees M *et al.* (2012). Identification of catechols as histone-lysine demethylase inhibitors. *FEBS Lett.* 8: 1190-1194.

Nilsson S, Franzen L, Parker C, Tyrrell C, Blom R, Tennvall J *et al.* (2007). Bone-targeted radium-223 in symptomatic, hormone-refractory prostate cancer: a randomised, multicentre, placebo-controlled phase II study. *Lancet Oncol.* 7: 587-594.

Nilsson S, Strang P, Aksnes AK, Franzen L, Olivier P, Pecking A *et al.* (2012). A randomized, dose-response, multicenter phase II study of radium-223 chloride for the palliation of painful bone metastases in patients with castration-resistant prostate cancer. *Eur.J.Cancer* 5: 678-686.

O'Connor OA, Heaney ML, Schwartz L, Richardson S, Willim R, MacGregor-Cortelli B *et al.* (2006). Clinical experience with intravenous and oral formulations of the novel histone deacetylase inhibitor suberoylanilide hydroxamic acid in patients with advanced hematologic malignancies. *J.Clin.Oncol.* 1: 166-173.

Ogasawara D, Suzuki T, Mino K, Ueda R, Khan MN, Matsubara T *et al.* (2011). Synthesis and biological activity of optically active NCL-1, a lysine-specific demethylase 1 selective inhibitor. *Bioorg.Med.Chem.* 12: 3702-3708.

Ogishima T, Shiina H, Breault JE, Tabatabai L, Bassett WW, Enokida H *et al.* (2005). Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer. *Clin.Cancer Res.* 3: 1028-1036.

Ostling P, Leivonen SK, Aakula A, Kohonen P, Makela R, Hagman Z *et al.* (2011). Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells. *Cancer Res.* 5: 1956-1967.

Park JY, Zheng W, Kim D, Cheng JQ, Kumar N, Ahmad N *et al.* (2007). Candidate tumor suppressor gene SLC5A8 is frequently down-regulated by promoter hypermethylation in prostate tumor. *Cancer Detect. Prev.* 5: 359-365.

Patra SK, Patra A, Zhao H, Carroll P and Dahiya R. (2003). Methyl-CpG-DNA binding proteins in human prostate cancer: expression of CXXC sequence containing MBD1 and repression of MBD2 and MeCP2. *Biochem. Biophys.Res.Commun.* 4: 759-766.

Patra SK, Patra A, Zhao H and Dahiya R. (2002). DNA methyltransferase and demethylase in human prostate cancer. *Mol.Carcinog.* 3: 163-171.

Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR *et al.* (2010). How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat.Rev.Drug Discov.* 3: 203-214.

Perry AS, Foley R, Woodson K and Lawler M. (2006). The emerging roles of DNA methylation in the clinical management of prostate cancer. *Endocr.Relat.Cancer* 2: 357-377.

Perry AS, Watson RW, Lawler M and Hollywood D. (2010). The epigenome as a therapeutic target in prostate cancer. *Nat.Rev.Urol.* 12: 668-680.

Petrylak DP. (2011). Current clinical trials in castrate-resistant prostate cancer. *Curr.Urol.Rep.* 3: 173-179.

Piekarz RL, Frye R, Prince HM, Kirschbaum MH, Zain J, Allen SL *et al.* (2011). Phase 2 trial of romidepsin

in patients with peripheral T-cell lymphoma. *Blood* 22: 5827-5834.

Pili R, Salumbides B, Zhao M, Altiok S, Qian D, Zwiebel J *et al.* (2012). Phase I study of the histone deacetylase inhibitor entinostat in combination with 13-cis retinoic acid in patients with solid tumours. *Br.J.Cancer* 1: 77-84.

Qi HH, Sarkissian M, Hu GQ, Wang Z, Bhattacharjee A, Gordon DB *et al.* (2010). Histone H4K20/H3K9 demethylase PHF8 regulates zebrafish brain and craniofacial development. *Nature* 7305: 503-507.

Rana TM. (2007). Illuminating the silence: understanding the structure and function of small RNAs. *Nat.Rev.Mol. Cell Biol.* 1: 23-36.

Rantala JK, Makela R, Aaltola AR, Laasola P, Mpindi JP, Nees M *et al.* (2011). A cell spot microarray method for production of high density siRNA transfection microarrays. *BMC Genomics* 162.

Rathkopf D, Wong BY, Ross RW, Anand A, Tanaka E, Woo MM *et al.* (2010). A phase I study of oral panobinostat alone and in combination with docetaxel in patients with castration-resistant prostate cancer. *Cancer Chemother.Pharmacol.* 1: 181-189.

Reibenwein J, Pils D, Horak P, Tomicek B, Goldner G, Worel N *et al.* (2007). Promoter hypermethylation of GSTP1, AR, and 14-3-3sigma in serum of prostate cancer patients and its clinical relevance. *Prostate* 4: 427-432.

Ren G, Baritaki S, Marathe H, Feng J, Park S, Beach S *et al.* (2012). Polycomb Protein EZH2 Regulates Tumor Invasion via the Transcriptional Repression of the Metastasis Suppressor RKIP in Breast and Prostate Cancer. *Cancer Res.* 12: 3091-3104.

Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH *et al.* (2009). miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. *Cancer Res.* 18: 7165-7169.

Rodriguez-Paredes M and Esteller M. (2011). Cancer epigenetics reaches mainstream oncology. *Nat.Med.* 3: 330-339.

Rokhlin OW, Glover RB, Guseva NV, Taghiyev AF, Kohlgraf KG and Cohen MB. (2006). Mechanisms of cell death induced by histone deacetylase inhibitors in androgen receptor-positive prostate cancer cells. *Mol. Cancer.Res.* 2: 113-123.

Rose NR, McDonough MA, King ON, Kawamura A and Schofield CJ. (2011). Inhibition of 2-oxoglutarate dependent oxygenases. *Chem.Soc.Rev.* 8: 4364-4397.

Rosenfeld MG, Lunyak VV and Glass CK. (2006). Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* 11: 1405-1428.

Roth SY, Denu JM and Allis CD. (2001). Histone acetyltransferases. *Annu.Rev.Biochem.* 81-120.

Saad F. (2002). Zoledronic acid significantly reduces pathologic fractures in patients with advanced-stage prostate cancer metastatic to bone. *Clin.Prostate Cancer.* 3: 145-152.

Sabnis GJ, Goloubeva O, Chumsri S, Nguyen N, Sukumar S and Brodie AM. (2011). Functional activation of the estrogen receptor-alpha and aromatase by the HDAC inhibitor entinostat sensitizes ER-negative tumors to letrozole. *Cancer Res.* 5: 1893-1903.

Sams-Dodd F. (2005). Target-based drug discovery: is something wrong? *Drug Discov.Today* 2: 139-147.

Santer FR, Hoschele PP, Oh SJ, Erb HH, Bouchal J, Cavarretta IT *et al.* (2011). Inhibition of the acetyltransferases p300 and CBP reveals a targetable function for p300 in the survival and invasion pathways of prostate cancer cell lines. *Mol.Cancer.Ther.* 9: 1644-1655.

Saramaki OR, Tammela TL, Martikainen PM, Vessella RL and Visakorpi T. (2006). The gene for polycomb group protein enhancer of zeste homolog 2 (EZH2) is amplified in late-stage prostate cancer. *Genes Chromosomes Cancer* 7: 639-645.

Scher HI, Beer TM, Higano CS, Anand A, Taplin ME, Efstathiou E *et al.* (2010). Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study. *Lancet* 9724: 1437-1446.

Schwer B and Verdin E. (2008). Conserved metabolic regulatory functions of sirtuins. *Cell.Metab.* 2: 104-112.

Seligson DB, Horvath S, McBrian MA, Mah V, Yu H, Tze S *et al.* (2009). Global levels of histone modifications predict prognosis in different cancers. *Am.J.Pathol.* 5: 1619-1628.

Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M *et al.* (2005). Global histone modification patterns predict risk of prostate cancer recurrence. *Nature* 7046: 1262-1266.

Shang Y, Myers M and Brown M. (2002). Formation of the androgen receptor transcription complex. *Mol.Cell* 3: 601-610.

Shi X, Kachirskaia I, Walter KL, Kuo JH, Lake A, Davrazou F *et al.* (2007). Proteome-wide analysis in Saccharomyces cerevisiae identifies several PHD fingers as novel direct and selective binding modules of histone H3 methylated at either lysine 4 or lysine 36. *J.Biol.Chem.* 4: 2450-2455.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA *et al.* (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 7: 941-953.

Shin S and Janknecht R. (2007). Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. *Biochem.Biophys.Res.Commun.* 3: 742-746.

Shukeir N, Pakneshan P, Chen G, Szyf M and Rabbani SA. (2006). Alteration of the methylation status of tumor-promoting genes decreases prostate cancer cell invasiveness and tumorigenesis in vitro and in vivo. *Cancer Res.* 18: 9202-9210.

Simard EP, Ward EM, Siegel R and Jemal A. (2012). Cancers with increasing incidence trends in the United States: 1999 through 2008. *CA Cancer.J.Clin.*

Soriano AO, Yang H, Faderl S, Estrov Z, Giles F, Ravandi F *et al.* (2007). Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. *Blood* 7: 2302-2308.

Stein R, Gruenbaum Y, Pollack Y, Razin A and Cedar H. (1982). Clonal inheritance of the pattern of DNA methylation in mouse cells. *Proc.Natl.Acad.Sci.U.S.A.* 1: 61-65.

Steinhoff C and Schulz WA. (2003). Transcriptional regulation of the human LINE-1 retrotransposon L1.2B. *Mol.Genet.Genomics* 5: 394-402.

Steinkamp MP, O'Mahony OA, Brogley M, Rehman H, Lapensee EW, Dhanasekaran S *et al.* (2009). Treatment-dependent androgen receptor mutations in prostate cancer exploit multiple mechanisms to evade therapy. *Cancer Res.* 10: 4434-4442.

Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO and Trapman J. (2002). Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. *Int.J.Cancer* 3: 309-317.

Stratmann A and Haendler B. (2012). Histone demethylation and steroid receptor function in cancer. *Mol.Cell.Endocrinol.* 1: 12-20.

Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B *et al.* (2008). TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. *Oncogene* 40: 5348-5353.

Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA *et al.* (2010). Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *J.Clin. Invest.* 8: 2715-2730.

Sun T, Wang Q, Balk S, Brown M, Lee GS and Kantoff P. (2009). The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines. *Cancer Res.* 8: 3356-3363.

Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E *et al.* (2007). Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer. Cell.* 6: 498-512.

Swanton C, Nicke B, Marani M, Kelly G and Downward J. (2007). Initiation of high frequency multi-drug resistance following kinase targeting by siRNAs. *Cell. Cycle* 16: 2001-2004.

Swinney DC and Anthony J. (2011). How were new medicines discovered? *Nat.Rev.Drug Discov.* 7: 507-519.

Takai D and Jones PA. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl.Acad.Sci.U.S.A.* 6: 3740-3745.

Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN *et al.* (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N.Engl.J.Med.* 15: 1502-1512.

Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS *et al.* (2010). Integrative genomic profiling of human prostate cancer. *Cancer.Cell.* 1: 11-22.

Thalhammer A, Mecinovic J, Loenarz C, Tumber A, Rose NR, Heightman TD *et al.* (2011). Inhibition of the histone demethylase JMJD2E by 3-substituted pyridine 2,4-dicarboxylates. *Org.Biomol.Chem.* 1: 127-135.

Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE *et al.* (2008). Role of the TMPRSS2-ERG gene fusion in prostate cancer. *Neoplasia* 2: 177-188.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW *et al.* (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 5748: 644-648.

Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V *et al.* (2009). Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 5928: 787-790.

Tsai MC, Wang JK and Chang HY. (2010). Tumor suppression by the histone demethylase UTX. *Cell. Cycle* 11: 2043-2044.

Tuck-Muller CM, Narayan A, Tsien F, Smeets DF, Sawyer J, Fiala ES *et al.* (2000). DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. *Cytogenet.Cell Genet.* 1-2: 121-128.

Turner NC, Lord CJ, Iorns E, Brough R, Swift S, Elliott R *et al.* (2008). A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor. *EMBO J.* 9: 1368-1377.

Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B *et al.* (2008). Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science* 5908: 1695-1699.

Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG *et al.* (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 6907: 624-629.

Villoutreix BO, Eudes R and Miteva MA. (2009). Structure-based virtual ligand screening: recent success stories. *Comb.Chem.High Throughput Screen.* 10: 1000-1016.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C *et al.* (1995). In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat.Genet.* 4: 401-406.

Waltering KK, Helenius MA, Sahu B, Manni V, Linja MJ, Janne OA *et al.* (2009). Increased expression of androgen receptor sensitizes prostate cancer cells to low levels of androgens. *Cancer Res.* 20: 8141-8149.

Waltering KK, Urbanucci A and Visakorpi T. (2012). Androgen receptor (AR) aberrations in castration-resistant prostate cancer. *Mol.Cell.Endocrinol.*

Wang Q, Williamson M, Bott S, Brookman-Amissah N, Freeman A, Nariculam J *et al.* (2007). Hypomethylation of WNT5A, CRIP1 and S100P in prostate cancer. *Oncogene* 45: 6560-6565.

Weichert W, Roske A, Gekeler V, Beckers T, Stephan C, Jung K *et al.* (2008). Histone deacetylases 1, 2 and 3 are highly expressed in prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical prostatectomy. *Br.J.Cancer* 3: 604-610.

Welsbie DS, Xu J, Chen Y, Borsu L, Scher HI, Rosen N *et al.* (2009). Histone deacetylases are required for androgen receptor function in hormone-sensitive and castrate-resistant prostate cancer. *Cancer Res.* 3: 958-966.

Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL *et al.* (1998). Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc.Natl.Acad.Sci.U.S.A.* 9: 5246-5250.

Whitaker HC, Hanrahan S, Totty N, Gamble SC, Waxman J, Cato AC *et al.* (2004). Androgen receptor is targeted to distinct subcellular compartments in response to different therapeutic antiandrogens. *Clin.Cancer Res.* 21: 7392-7401.

Whitehurst AW, Bodemann BO, Cardenas J, Ferguson D, Girard L, Peyton M *et al.* (2007). Synthetic lethal screen identification of chemosensitizer loci in cancer cells. *Nature* 7137: 815-819.

Wikber J, Eklund M, Willighagen E, Spjuth O, Lapin M, Engkvist O *et al.* Introduction to Pharmaceutical Bioinformatics. *Oakleaf Academics Publishing House, Stockholm 2010*

Wissmann M, Yin N, Muller JM, Greschik H, Fodor BD, Jenuwein T *et al.* (2007). Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. *Nat.Cell Biol.* 3: 347-353.

Wolf SS, Patchev VK and Obendorf M. (2007). A novel variant of the putative demethylase gene, s-JMJD1C, is a coactivator of the AR. *Arch.Biochem.Biophys.* 1: 56-66.

Xiang Y, Zhu Z, Han G, Ye X, Xu B, Peng Z *et al.* (2007). JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. *Proc.Natl.Acad.Sci.U.S.A.* 49: 19226-19231.

Yamane K, Toumazou C, Tsukada Y, Erdjument-Bromage H, Tempst P, Wong J *et al.* (2006). JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell* 3: 483-495.

Yang XJ and Seto E. (2008). The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat.Rev.Mol.Cell Biol.* 3: 206-218.

Yang XJ and Seto E. (2003). Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. *Curr.Opin.Genet.Dev.* 2: 143-153.

Yang Y, Tse AK, Li P, Ma Q, Xiang S, Nicosia SV *et al.* (2011). Inhibition of androgen receptor activity by histone deacetylase 4 through receptor SUMOylation. *Oncogene* 19: 2207-2218.

Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z *et al.* (2008). DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. *Cancer Res.* 21: 8954-8967.

Yu J, Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G *et al.* (2007). A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. *Cancer Res.* 22: 10657-10663.

Zhang X, Morrissey C, Sun S, Ketchandji M, Nelson PS, True LD *et al.* (2011). Androgen receptor variants occur frequently in castration resistant prostate cancer metastases. *PLoS One* 11: e27970.

Zhang Y, Kwon S, Yamaguchi T, Cubizolles F, Rousseaux S, Kneissel M *et al.* (2008). Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. *Mol.Cell.Biol.* 5: 1688-1701.

Zhao JC, Yu J, Runkle C, Wu L, Hu M, Wu D *et al.* (2012). Cooperation between Polycomb and androgen receptor during oncogenic transformation. *Genome Res.* 2: 322-331.

Zhou HJ, Yan J, Luo W, Ayala G, Lin SH, Erdem H *et al.* (2005). SRC-3 is required for prostate cancer cell proliferation and survival. *Cancer Res.* 17: 7976-7983.

Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA *et al.* (2011). RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 7370: 524-528.