View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by ScholarShip

TMEFF2 is an epigenetic modulator that promotes androgen independent growth in castration-resistant prostate cancer cells

by

Joshua Corbin

August, 2014

Director of Thesis: Maria Ruiz-Echevarria, Ph.D.

Major Department: Oncology

While the ability to detect PCa has improved significantly due to PSA screenings, the survival rate for men diagnosed with PCa has remained stagnant, and the disease remains the second leading cause of cancer related deaths in men. Most patients initially respond to androgen deprivation treatment; however, a significant percentage of patients relapse with currently untreatable castration resistant prostate cancer (CRPC), during which the PCa cells develop the ability to grow in androgen depleted conditions. The androgen receptor (AR) plays a vital role in prostate development and homeostasis, and the deregulation of AR drives PCa tumorigenesis and progression to CRPC. Delineating molecular mechanisms that contribute to AR activity and/or PCa cell growth in androgen-depleted conditions may aid in the development of future CRPC therapies

Epigenetic alterations play a critical role in differentiation during development, and aberrations in epigenetic regulation are associated with tumorigenesis and cancer progression. Two types of epigenetic modifications, DNA methylation and the methylation of multiple histone lysines, play significant roles in prostate cancer (PCa). Many histone methyltransferases (HMT) and demethylases (HDM), including the JMJD2 family of histone demethyalses, act as coregulators of AR, and many of these enzymes are implicated in CRPC. Because of this, HDMSs and HMTs have proven as attractive targets for therapeutic intervention.

We have been studying TMEFF2, a protein that is regulated transcriptionally and translationally by the AR, and is overexpressed in PCa and CRPC suggesting a role in this disease. Data presented here demonstrate that TMEFF2 modulates JMJD2 controlled methyl histone marks and increases growth in androgen depleted conditions in CRPC cells. In correlation with its effect on histone methylation and growth, TMEFF2 overexpression increases resistance to the anti-growth effects of the pan-jumonji demethylase inhibitor, JIB-04, suggesting that TMEFF2 modulates growth, at least in part, by increasing jumonji demethylase activity. Additionally, TMEFF2 positively regulates PSA expression without altering AR levels in CRPC cells, indicating that TMEFF2 is a novel activator of AR. All together this data suggests a model in which TMEFF2, by modulating the activity of AR and JMJD2 enzymes, increases CRPC cell growth. Because CRPC remains to be a significant obstacle in the successful treatment of metastatic PCa, the results presented have the potential to be of therapeutic value.

TMEFF2 is an epigenetic modulator that promotes androgen independent growth in castration-resistant prostate cancer cells

A thesis in partial fulfillment of the requirements for the degree:

Master's in Biomedical Science

Department of Oncology at East Carolina University

By

Joshua Corbin

August, 2014

TMEFF2 is an epigenetic modulator that promotes androgen independent growth in castration-resistant prostate cancer cells

Master's Thesis

Joshua Moses Corbin

Approved by:

Thesis Director: ____

Maria Ruiz-Echevarria, Ph.D.

Committee Member: ____

Li Yang, Ph.D.

Committee Member: ____

Yan-Hua Chen, Ph.D.

Committee Member: ____

David Terrian, Ph.D.

Program Director: ____

Richard Franklin, Ph.D.

Dean of Graduate School: ____

Paul Gemperline, Ph.D.

© Joshua Corbin, 2014

Acknowledgements

There are many people, to whom I owe significant gratitude for aiding me in my journey as a master's student and development as a scientist. First I would like to thank my mentor, Maria Ruiz-Echevarria, for guiding me through my studies, and for the endless support she provided. I would also like to give thanks to my thesis committee members, Li Yang, Yan-Hua Chen and David Terrian. Thank you for the commitment you displayed and for the advice you gave during the development of my thesis. I also owe a significant amount of thanks for the assistance given by my fellow lab members, Tom Green, Greg Tipton, and Xiaofei Chen. Additionally, I would like to thank my parents, David and Connie Corbin, and my siblings, Chris, Justin and Danielle. Thank you for the love, support, and kindness you have shown me my entire life. Finally, I would like to thank all of my professors, family and friends who have played a role in my life, and who have helped me become a better person and scientist.

Table of Contents

Title Page……i

Signature Page………ii

Copyright Page……iii

Acknowledgements ………iv

Table of contents ………v

List of figures and tables ……………………………………………………………………………………………………vi

Chapter 1: Introduction …………………………………………………………………………………………………… 1-21

Epigenetics Overview ……………………………………………………………………………………………… 1-5

One Carbon Metabolism and its role in epigenetic regulation ……………………………………………… 6-7

Epigenetics in Cancer ……………………………………………………………………………………………… 8-12

The Roles of Histone Methyltransferases and Demethylases in Prostate Cancer ………………………… 13-16

Targeting Epigenetic Enzymes for Cancer Therapy ……………………………………………………………… 17-18

Delineating the Role of TMEFF2 in Prostate Cancer …………………………………………………………… 19-21

Chapter 2: Materials and Methods …………………………………………………………………………………… 22-25

Cell culture ……… 22

Protein extraction and western blot analysis …………………………………………………………………… 22-23

Growth analysis ……………………………………………………………………………………………………… 23-24

Statistical analysis …………………………………………………………………………………………………… 25

Chapter 3: Results …………………………………………………………………………………………………… 26-38

TMEFF2 modulates JMJD2 controlled methyl histone marks in androgen depleted conditions ……… 26-29

TMEFF2 increases androgen independent growth and AR activity …………………………………………… 30-35

TMEFF2 and androgens confer resistance to the anti-growth effects of JIB-04 …………………………… 36-38

Chapter 4: Discussion ……………………………………………………………………………………………… 39-43

References ……… 44-62

List of Figures and Tables

Figure 1. One carbon metabolism schematic ……………………………………………………………………………………. 7

Table 1. Panel of methyl histone marks implicated in prostate cancer ……………………………………………….. 21

Figure 2. Knocking-down TMEFF2 increases JMJD2 regulated
methyl histone marks in the absence of DHT………………………………………………………………………. 28-29

Figure 3. TMEFF2 overexpression increases androgen independent
growth in C4-2B cells…………………………………………………………………………………………………….. 32-33

Figure 4. TMEFF2 increases growth independent of AR in the absence of DHT,
and increases androgen- regulated growth in presence of DHT
when AR levels are depleted…………………………………………………………………………………………. 33-34

Figure 5. TMEFF2 positively regulates PSA expression
without modulating AR protein levels…………………………………………………………………………….... 35

Figure 6. TMEFF2 and androgens confer
resistance to the anti-growth effects of JIB-04……………………………………………………………….. 37-38

Figure 7. A proposed model for the role of TMEFF2 in CRPC growth,
through modulating JMJD2 and AR activity……………………………………………………………………… 43

Chapter 1: Introduction

Epigenetics Overview

During mitosis, the parental cell's DNA is replicated in a precise process with high fidelity and limited error. Because every cell that makes up an organism is a result of repeated cell division that can be traced back to a single cell, each cell has an almost identical genome, and variances that occur in DNA base sequence within an individual's lifetime are only a result of error during DNA replication. Despite having the same genome, different cell types have drastically different phenotypes and carry out different functions that contribute to a physiologically functional organism. The phenotype and function of a specific cell type is determined by the genes that are expressed, and it is therefore vital that cells maintain gene expression signatures through cell division. In a sense, a cell must have "memory" of its specific cell type. During development, pluripotent embryonic stem cells (ESCs) differentiate and commit to specific cell lineages and eventually, after multiple cycles of growth and differentiation, give rise to all of the different cell types that make up an organism. The process of differentiation is accompanied by alterations in gene expression in a manner that is lineage specific, and the newly acquired gene expression signature of the differentiated cell must be maintained through cell division or until the cell differentiates once again. Many of the mechanisms that contribute to cellular memory and gene expression changes during development are regulated by differential packaging of the DNA in the nucleus.

Each human cell contains approximately two meters of DNA, which is tightly packed into a nucleus with a diameter of roughly five to ten micrometers (1). To solve this spatial problem, DNA is both highly organized and tightly packaged in the form of chromatin. The primary structure of chromatin involves the interaction between the DNA and core histone proteins (1). The four core histone proteins (H2A, H2B, H3, and H4) form an octameric complex, which includes two of each

(1).Because of their high arginine and lysine content, histone proteins carry a positive charge, which gives the octameric histone complex a high affinity for negatively charged DNA. The DNA is wrapped around the core histone octameric complex to form a nucleosome, the primary structure of chromatin. Nucleosomes are connected by what is called linker DNA, and are further packaged to form more complex structures, such as chromatin fibers, and other chromatin-associated proteins and remodeling factors aid in the dynamics of the packaging process. The majority of a cell's DNA does not contain genes; therefore these sections of the genome are tightly packed in a form of chromatin called constitutive heterochromatin (2). Regions of the chromatin that contain genes are called euchromatin, and in these regions chromatin compaction must be a dynamic process. In regions of actively transcribed genes, the euchromatin forms an open structure which allows for transcription factor access; while euchromatic regions of transcriptionally repressed genes are tightly condensed into facultative heterochromatin, which resembles constitutive heterochromatin in structure (2). In this manner, the dynamic capacity of euchromatin structure controls gene transcription by affecting transcription factor and RNA polymerase accessibility to promoter and enhancer elements. The mechanisms that regulate gene expression via chromatin structure modulation are studied in a field called epigenetics.

Epigenetics is the study of heritable alterations in phenotype and/or gene expression that are not a result of changes in DNA base sequence. DNA methylation and histone post-translational modifications are two main mechanisms of epigenetic regulation, and will be briefly discussed below. Both modifications contribute to chromatin-remodeling and chromatin condensation and/or decondensation; therefore, both modifications can influence gene expression. Importantly, the regulated organization of chromatin is inherited through cell division via epigenetic mechanisms; for this reason, epigenetic regulation provides mechanisms through which the cell can transmit a blueprint for its cell type-specific gene expression signature to future cell generations (3,4,5). Not only does

epigenetic regulation contribute to the rigidity of cell memory, but it also has plasticity, which provides a cell with a certain level of adaptability to alter gene expression in response to environmental stimuli, and during differentiation in development (5).The relevance of epigenetic mechanisms in regulating cellular homeostasis is underscored by the fact that epigenetic aberrations have been linked to many different disease states, such as, cancer, diabetes, and Alzheimer's (6,7,8).

DNA methylation occurs on cytosine nucleotides which are located next to a guanine nucleotide on the 3' end. These motifs are called CpG sites. Interestingly, CpG sites are globally under-represented across the genome when compared to other dinucleotides (9,10). This under-representation is thought to be a result of spontaneous deamination events that occur on methylcytosines, which results in a conversion of the methylcytosine to thymine, or a C to T transition. However, clusters of CpG sites, or CpG islands, occur within the promoter regions of roughly 60% of the genes across the genome (11), and the methylation of these CpG sites can influence the expression of associated genes by 1) acting as a steric block for transcription factor binding (12,13,14), 2) recruiting chromatin remodeling factors that can then condense the chromatin in regions proximal to gene promoters (15,16). CpG methylation is catalyzed by the DNA methyltransferases DNMT1, DNMT3a and DNMT3b. DNMT1 is a maintenance methyltransferase, in that it only has the ability to methylate hemi-methylated DNA, and primarily functions to maintain DNA methylation patterns through cell division(17); while DNMT3a and DNMT3b are both de novo methyltransferases (17,18). Although DNA methylation is a stable mark, it is reversible via a hydroxylation reaction catalyzed by the TET family of DNA demethylases (19). Both DNMT's and TET demethylases are involved in development and are dysregulated in many cancers, and hypomethylating agents, azacitidine and decitabine, are FDA approved drugs for treating myelodysplastic syndromes (20).

Core histones are globular proteins that have terminal peptide tails that radiate out from the central nucleosome (21). These histone tails contain multiple amino acids which serve as sites for a

multitude of post-translational modifications (PTMs), including methylation, acetylation, phosphorylation, ubiquitynation, and sumoylation (22). Both lysine acetylation and methylation are prominent gene expression regulatory mechanisms, and will be discussed further.

Histone lysine acetylation is by catalyzed histone acetyltransferases (HATs) (23). Upon the addition of the acetyl mark, the positive charge of the lysine is neutralized. It is hypothesized that this loss of charge reduces the affinity of the histone tail for the DNA, and thus contributes to chromatin decondensation. In support of this hypothesis, it has been long understood that acetyl lysine marks occur near the promoter regions of actively transcribed genes (22,24). Histone deacetylation is carried out by histone deacetylases (HDACs) (25). Because the removal of the acetyl group restores the positive charge on the lysine, HDACs act as transcriptional repressors (25).Multiple HDAC enzymes have been found to be overexpressed in a variety of cancers, and there are currently two FDA approved HDAC inhibitors for cancer therapy, vorinostat and depsipetide (26).

Histone lysine methylation is catalyzed by histone methyltransferases (HMTs) (27). Unlike acetylation, methylation does not alter the charge of the lysine. Histone lysine residues can be monomethylated (me1), dimethylated (me2), or trimethylated (me3), and depending on the target lysine, the degree of methylation can have a differential impact on gene expression (28,29). Most of the currently understood histone methyl marks occur on H3 and H4. Of the trimethylated lysine marks, histone 3 lysine 9 trimethylation (H3K9me3), H3K27me3, and H4K20me3 are usually transcriptionally repressive marks, while H3K4me3, H3K36me3, and H3K79me3 are usually transcriptionally active marks (29). Although the charge of lysine is not affected by the methylation state, there is evidence that methyl histone marks may act as targets for reader proteins, which bind to the methyl histone mark and contribute to the modulation of the chromatin state (30). Multiple protein domains have been found to have histone methyl lysine binding capabilities, including, PHD fingers, chromodomains and tudor domains (30). Heterchromatin protein 1 (HP1) is a well characterized example of a histone reader

protein that modulates chromatin structure. HP1 binds to H3K9me3 via its chromodomain, and this recognition has been shown to be critical for heterochromatin formation and contributes to DNA methylation (31,32).

Histone methyl lysines are demethylated in a reaction catalyzed by two classes of histone demethylases (HDMs), the Jumonji C (JMJC)- domain containing family and the LSD family (33). The LSD family utilizes a FAD-dependent oxidase reaction mechanism, during which FAD acts as an oxidizing agent and the target methyl group is released in the form of formaldehyde in the presence of oxygen (34). Because the LSD family reaction mechanism requires a free electron pair on the nitrogen of the target lysine, LSD enzymes are only capable of demethylating me1 or me2 lysines (33). The JMJC-domain containing, or jumonji demethylases, are characterized by a C-terminal jumonji domain, which contains the catalytic center for these enzymes (35). The jumonji demethylases utilize iron and α-ketoglutarate as cofactors. In the presence of oxygen, the jumonji demethylases hydroxylate the target methyl group, which then results in the spontaneous release of formaldehyde (36,37). The jumonji demethylases can theoretically demethylate me3, me2 or me1 lysine residues; however, each jumonji demethylase may have preferential activity towards a particular level of methylation (33).

Because histone methylation can activate or repress transcription depending on the target lysine and degree of methylation, HMTs and HDMs can act as either transcriptional activators or repressors in a manner that is dependent on their catalytic activity. Importantly, HMTs and HDMs have been shown to play key roles in gene expression regulation during development, and aberrations in their expression and/or activity have been observed in many different cancer types (12,33).

One Carbon Metabolism and its Role in Epigenetic Regulation

One carbon metabolism can be separated into three separate cycles or shunts: the folate cycle, the methionine cycle, and the transsulfuration pathway (see figure 1 for one carbon metabolism schematic). The folate cycle contributes to pyrimidine and purine synthesis, as well as methionine regeneration in the methionine cycle. Methyl THF is a cofactor for methionine synthase, which catalyzes the methyl transfer from methyl THF to homocysteine to form methionine. Methionine is then converted to S-adenosylmethionine (SAM) in an ATP-dependent reaction catalyzed by methionine adenosine transferase. SAM is the universal methyl donor for methyltranserase reactions involved in DNA methylation, histone methylation, as well as the methylation of other proteins. Upon donating a methyl group in methyltransferase reactions, SAM is converted to S-adenosylhomocysteine (SAH), which is then converted back to homocysteine in a reaction catalyzed by SAH hydrolase. Because SAM is required as a methyl donor in DNMT and HMT reactions, alterations in one carbon metabolism flux can influence epigenetic modifications and therefore the expression of genes regulated by DNA and/or histone methylation (38). One example that provides evidence that epigenetic alterations can be caused by disruptions in one carbon metabolism is seen in studies conducted with methylene tetrahydrofolate reductase (MTHFR) deficient mice. MTHFR is an enzyme within the folate cycle that catalyzes the formation of methyl THF from methylene THF. Offspring of MTHFR deficient/wild type crosses inherit epigenetic alterations that result in developmental disorders that are inherited through multiple generations (39).

Homocystiene can also be converted into cystathionine in a reaction catalyzed by cystathionine β synthase (CBS), and this reaction is the first and committed step of the transsulfuration shunt of one carbon metabolism. The transsulfuration pathway is responsible for the synthesis of glutathione, a required metabolite for glutathione peroxidase and glutathione transferase enzymes, which are responsible for radical oxygen species (ROS) and toxin neutralization, respectively (40). It has been

demonstrated that increases in ROS can contribute to HIF-1α stabilization by oxidizing iron in the active site of HIF-1α specific prolyl hydroxylases, thereby inhibiting their activity (41).On a similar note, increases in ROS have been demonstrated to contribute to histone hypermethylation (42), suggesting that ROS can also inhibit jumonji histone demethylases, which are also iron dependent hydroxylases. Therefore, changes in one carbon flux that influence entrance into the transsulfuration pathway may alter the activity of histone demethylases; however, the net effect of ROS on demethylation is complicated, as HIF-1α, which is stabilized by increases in ROS, increases the expression of multiple hydroxylases, including many of the jumonji demethylases (43). This method of regulation is thought to be a compensatory mechanism, so that, when iron-dependent hydroxylase activity is low from increases in ROS or decreases in oxygen, the expression of these enzymes is increased. Furthermore, it has been shown that methyl cytosines are sensitive to hydroxylation caused by hydroxyl radicals, which can lead to a loss in DNA methylation (44). Therefore, although mechanistically complicated, fluxes in one carbon metabolism can influence epigenetic regulation by altering methylation and demethylation reactions.

Figure 1

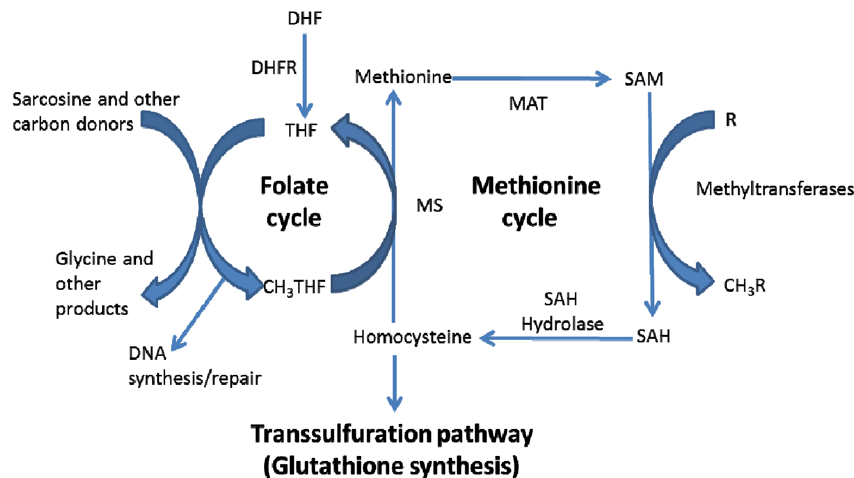


Figure 1. One carbon metabolism schematic. Above is a simplified schematic of one carbon metabolism; showing the relationship between the folate cycle, the methionine cycle, and the transsulfuration pathway.

Epigenetics in Cancer

Cancer has long been only considered a disease of the genome, during which, mutations in the DNA base sequence occur within tumor suppressor genes and oncogenes that drive the transformation of normal cells into cancer cells. Two different types of mutations occur during cancer development, loss of function and gain of function mutations. Loss of function mutations occur within tumor suppressor genes and lead to the suppressed function of the coded proteins. Tumor suppressor genes include proteins that apply brakes to the cell cycle, suppress invasion, contribute to apoptosis, participate in DNA repair, and control cell metabolism. Conversely, gain of function mutations occur within oncogenes and contribute to the aberrant activation of the coded proteins which promote tumor growth and development. During oncogenesis and cancer progression, genetic mutations continue to accumulate, and mutations that contribute to a growth advantage are naturally selected for through cell division as the tumor grows and evolves. While considering cancer a disease of the genome is accurate, this definition of cancer is incomplete at best; cancer should also be defined as a disease of the epigenome. Epigenetic aberrations in cancer lead to decreased expression of tumor suppressor genes and increased expression of proto-oncogenes during tumorigenesis and cancer progression (12). Because epigenetic modifications, such as DNA methylation and histone PTM's, are inherited through cell division, epigenetic alterations that contribute to a selective growth advantage are transmitted to future generations of cancer cells through clonal expansion.

Aberrant DNA methylation is a common occurrence in cancer. Both promoter specific hypermethylation and global hypomethylation are prevalent epigenetic characteristics of cancer cells (45). In cancer cells, promoter specific hypermethylation often occurs at tumor suppressor genes, which represses their expression. The tumor suppressor, Rb, was the first gene found to be silenced via DNA hypermethylation in cancer (46). Since that finding, a multitude of tumor suppressor genes, including, BRCA1, p16, PTEN, GSTP1, and MGMT, have been discovered to be hypermethylated in many cancers

(47-50). All three DNMTs have been found to be over-expressed in many different cancer types, which may contribute to promoter hypermethylation and gene silencing (51,52,53).Finally, HDAC enzymes are also overexpressed in cancer and contribute to DNA-methylation induced gene silencing in cancer cells (54). Through their interaction with CpG binding proteins, HDACs are recruited to hypermethylated promoters, where they further repress transcription by deacetylating proximal histones (15,16).

Global hypomethylation involves losses of DNA methylation at repetitive sequences, which can lead to transposable element activation, and increased genomic and chromosomal instability (55,56). This instability can contribute to an increased mutation rate and chromosomal aberrations, such as, deletions and translocations in cancer cells. Hypomethylation occurring at the promoter of oncogenes can also result in aberrant increases in oncogene expression (57,58,59). The causation of genomic hypomethylation is probably multi-factorial, and has yet to be delineated. One intriguing correlation links one carbon metabolism to DNA hypomethylation and cancer risk. Although somewhat controversial, multiple studies have found that decreased folate intake, which is an important cofactor for one carbon transfer reactions in one carbon metabolism, correlates with DNA hypomethylation and increased cancer risk, especially in colon cancer (60,61,62). Because folate plays a key role in the methionine cycle and S-adenosylmethionine (SAM) regeneration, which is the universal methyl donor for methyltransferase reactions, it is possible that folate deficiency may have a causational role in the development of global hypomethylation in some cancers. Furthermore, polymorphisms in MTHFR, which reduce its activity, correlate with increased cancer risk in a number of cancers, and this risk may be exacerbated by low dietary folate intake (63,64).

Histone methylation also plays an important role in cancer development and progression. Multiple cancer types have been found to exhibit a multitude of aberrations in histone methylation, and many HMTs and HDMs have altered expression in cancer cells in comparison to non-transformed cells (65). By acting as transcriptional activators and repressors, HMTs and HDMs alter the gene expression

and phenotype of cancer cells. For example, the H3K27me3 methyltransferase, EZH2, has been shown to be overexpressed in many cancers, including, prostate, breast, lung, bladder, and hepatocellular carcinoma (66-69). Moreover, a gain of function heterozygous missense mutation at Y641 of EZH2 has been detected in lymphoma and myeloid neoplasms (70). This mutation decreases the H3K27 monomethylation, but increases the H3K27 trimethylation activity of EZH2 (71). In many cancer cell types, EZH2 increases migration, invasion and proliferation, and targets of H3K27me3 mediated silencing include the tumor suppressor genes, E cadherin, p21, and Runx3 (72-76).

As an additional example of HMT deregulation in cancer, the mixed-lineage leukemia (MLL) family of H3K4 methyltransferases are commonly translocated and amplified in various leukemias (77,78).These genetic alterations to the MLL genes result in increased expression of many MLL target genes, including, HoxA7 and HoxA9, which are required for MLL-mediated transformation (79). Additionally, Ash2, which is an essential member of MLL complexes, has elevated protein levels in many cancers, and has been shown to be required for Ha-Ras-induced transformation of mouse embryonic fibroblasts (80).

HDMs are also deregulated in many cancers. LSD1 is overexpressed in neuroblastoma, prostate and breast cancer (81,82,83), and its expression correlates with poor prognosis and reduced differentiation in neuroblastoma tumors (81). Importantly, LSD1 can act as a gene activator or repressor dependent on its H3K9me1/2 or H3K4me1/2 demethylase activity, respectively (84,85)

The H3K4me2/3 specific- JARID family of jumonji demethylases have important roles as transcriptional repressors in many cancers (65,86,87). JARID1A has been found to contribute to cancer cell proliferation through the repression of the cyclin dependent kinase inhibitors, p16, p21 and p27 in gastric and cervical cancers (86). Knocking-down JARID1B in breast cancer cells induced senescence and resulted in the derepression of the tumor suppressor genes, BRCA1, HoxA5, and Let-7E (87). Seemingly

counterintuitive to role of the JARID family as H3K4 demethylases, JARID1B has been demonstrated to interact with the AR and activate the transcription of AR responsive genes (88).

The JMJD2 family of jumonji demethylases are overexpressed in many cancers (65,89). Members of this family possess H3K9me2/3 and H3K36me2/3 demethylase activity, and can act as transcriptional activators or repressors. In prostate and breast cancer, JMJD2 demethylases interact with the androgen receptor (AR) and estrogen receptor (ER) and are recruited to AR/ER responsive genes, where they act as transcriptional coactivators in a manner that is dependent on their H3K9me2/3 demethylase activity (90-94). Some of the members of the JMJD2 family are discussed briefly below.

JMJD2A is overexpressed in breast, prostate, colon, and bladder cancer, and has been shown to promote cancer cell proliferation (90,92,95,96). In fact, knocking-down JMJD2A inhibits proliferation in ER-positive and ER-negative breast cancer cell lines, as well as colon cancer cell lines (97,98,99).Because JMJD2A overexpression inhibits HP1 recruitment to heterochromatin, it is hypothesized that JMJD2A may increase cancer cell proliferation by antagonizing HP1 and promoting an open chromatin state during S-phase of the cell cycle, which would potentially aid in accelerated DNA replication (100).

JMJD2B is overexpressed in both prostate and breast cancer (91,93). Unlike JMJD2A, modulating JMJD2B expression in breast cancer cells only alters the proliferation of ER-positive breast cancer cells, while the proliferation of ER-negative breast cancer cells remains unaffected (91,101). JMJD2B may also have a unique role in colorectal cancer and hypoxic response. While JMJD2 genes are all transcriptionally regulated by HIF-1α, as are many oxygen-dependent hydroxylases, the expression of JMJD2B is increased more substantially than the other JMJD2 members (102). It has also been demonstrated that by removing the H3K9me2/3 repressive mark from the promoters of hypoxia-responsive genes, JMJD2B is critical for the increased expression of HIF-1α-regulated genes in response to hypoxia in colorectal cancer cells (102).

JMJD2C is overexpressed in prostate cancer, and the gene is amplified in esophageal carcinoma, B-cell lymphoma, Hodgkin lymphoma and breast cancer, which further supports its role as an oncogene (94, 103-105). Similar to the role of JMJD2B in colorectal cancer, JMJD2C has been shown to be a coactivator of HIF-1α in breast cancer (106). Furthermore, overexpressing JMJD2C led to mammosphere formation in nontransformed immortalized breast epithelial cells (105). Because mammosphere formation is a phenotype of cancer stem cells, and JMJD2C has been implicated in Oct4-mediated stem cell maintenance (107), these findings suggest that JMJD2C has a direct influence on tumorigenesis and cancer stem cells.

The Roles of Histone Methyltransferases and Demethylases in Prostate Cancer

Prostate cancer (PCa) is the most frequently diagnosed and the second leading cause of cancer related deaths in men (108). While most patients initially respond to androgen deprivation treatment, a significant percentage of patients relapse with currently untreatable castration resistant prostate cancer (CRPC) (109). During the progression to castration resistance (also referred to as androgen independence), PCa cells develop the ability to grow in androgen depleted conditions. The AR plays a vital role in prostate development and homeostasis, and the deregulation of the AR drives PCa tumorigenesis and progression (110). Delineating molecular mechanisms that contribute to AR activity may provide further insight into how aberrations in androgen signaling occur during the advancement of PCa, and may lead to the discovery of targets with prognostic and therapeutic value.

AR is a steroid receptor that normally resides in the cytoplasm when not bound to its ligand. Upon interaction with androgens or androgen-like compounds, AR undergoes a conformational change, homodimerizes and translocates to the nucleus where it binds to androgen responsive elements (AREs) and regulates the transcription of target genes (110,111). AR recruits coregulators which modulate its transcriptional activity. Multiple HMTs and HDMs have been found to be overexpressed in prostate cancer, and these enzymes interact with AR and influence its transcriptional activity (112,113). Importantly, the modulation of AR coregulators is linked to sustained AR activity in androgen-depleted conditions, which contributes to the growth of CRPC cells (114), and the roles of HMTs and HDMs in CRPC are beginning to be brought to light. Upon being recruited to AR binding sites, histone modifying enzymes, including EZH2, NSD2, LSD1, and JMJD2 demethylases act as co-repressors and co-activators of the AR in a manner that is dependent on their methylation and demethylation activity (82,92-94,115-117). The roles of specific HMTs and HDMs on AR activity and the development of CRPC are briefly discussed below.

The HMT EZH2 is overexpressed in PCa (66). Importantly, EZH2 has been shown to repress multiple tumor suppressor genes via H3K27me3, and increase cellular proliferation, migration and invasion (72). Interestingly, while it has been demonstrated that EZH2 can interact with AR and act as a transcriptional corepressor of certain AR target genes (116), a study carried out by Xu et al suggests that EZH2 changes roles in CRPC cells, and becomes a coactivator of AR (115). Upon being phosphorylated at serine 21 (S21), EZH2 methylates AR, and the EZH2/AR complex then activates the transcription of non-canonical AR targets in CRPC cells (115). The phosphorylation of EZH2 at S21 is catalyzed by AKT, and inhibits the association of EZH2 with PRC2, thus abolishing the H3K27 methyltransferase activity of EZH2 (115). Interestingly, AKT activity is higher in CRPC and H3K27me3 levels, although higher in PCa in comparison to benign cells, are reduced in CRPC (115,118). Furthermore, EZH2 levels and S21 phosphorylated EZH2 have higher expression in CRPC, and the overexpression of EZH2 is sufficient to induce androgen independent (AI) growth in androgen dependent (AD) LNCaP PCa cells (115). Because of the coregulatory role of EZH2 on AR in CRPC, EZH2 inhibition should receive attention for future CRPC treatment possibilities.

The precise catalytic activity of NSD2 has been elusive, as different studies have demonstrated that NSD2 possesses H3K36, H3K4, H3K27, and H4K20 methyltransferase activity (117,119-121). Regardless, NSD2 is overexpressed in PCa and acts as a transcriptional activator of AR in a manner that is dependent on its methyltransferase activity (117). In addition to being a transcriptional target of NF- κB signaling itself, NSD2 has been shown to be critical for the expression of NF- κB regulated genes in CRPC cells (122). Interestingly, the regulatory role of NSD2 on NF- κB target genes correlated with increases in activating marks, H3K36me2 and H3K36me3, at their respective promoters (122). Phenotypically, knocking-down NSD2 in CRPC cells decreased proliferation, increased apoptosis, and decreased tumor growth in mouse xenographs (122).While more research needs to be carried out on the roles of NSD2 in

PCa, present research indicates that NSD2 is involved in androgen and NF- κB signaling, which are both central to PCa development and progression.

The HDM LSD1 was the first histone methyl modifying enzyme found to interact with AR and influence its transcriptional activity (85). Upon interacting with the AR, LSD1 can act as a coactivator or corepressor depending on the histone methyl mark targeted, H3K9me2/1 or H3K4me2/1 (85,123). Interestingly, AR, in partnership with LSD1, represses transcription of the AR gene itself when AR is ligand bound (123). Therefore, it is hypothesized that during androgen deprivation treatment, the lack of ligand causes derepression of AR transcription, contributing to increased AR expression and CRPC. LSD1 overexpression correlates with PCa relapse (82), and preliminary data suggests that inhibiting LSD1 decreases the proliferation of CRPC cells (124). Therefore, LSD1 may be a viable therapeutic target for the treatment of CRPC.

While LSD1 has H3K9me2 and H3K9me1 demethylating capabilities in complex with AR, LSD1 does not have the ability to demethylate H3K9me3. However, as previously mentioned, AR interacts with JMJD2A, JMJD2B and JMJD2C, all of which have H3K9me3 demethylation activity and are overexpressed in PCa (92,93,94). In correlation with JMJD2 overexpression, H3K9me2 and H3K9me3 are both decreased with PCa progression (125). It has been demonstrated that AR can form a complex with LSD1 and JMJD2C, which cooperate for the sequential demethylation of H3K9me3, H3K9me2 and H3K9me1, thus facilitating an open chromatin state to allow for efficient AR binding and transcription activation of AR responsive genes (94). Although JMJD2A, 2B, and 2C all target the same histone marks for demethylation, the three enzymes do not seem to be redundant in their effect on AR. When the enzymes are overexpressed in PCa cells, only JMJD2C overexpression increases the PSA induction in response to androgens (94). Similarly, knocking-down JMJD2C in PCa cells decreases PSA induction in response to androgens (94); however, knocking-down JMJD2A in PCa cells reduces basal PSA levels, while androgen-induced PSA levels remain similar to the control cells (92). JMJD2B not only effects AR

activity, but also increases AR stability through deubiquitination of AR (93). Interestingly, JMJD2B is also an androgen responsive gene, thus forming a positive feedback loop with androgen signaling (93). Of particular interest in CRPC, JMJD2C expression is elevated in CRPC in comparison to AD PCa (126). Additionally, depleting JMJD2C levels in CRPC cells reduces both proliferation and the expression of genes known to be AR-regulated in CRPC (127). Therefore, while the inhibition of JMJD2A, 2B and 2C may all have therapeutic potential, the inhibition of JMJD2C may be of particular importance for CRPC treatment.

Targeting Epigenetic Enzymes for Cancer Therapy

Epigenetic enzymes are attractive targets for cancer therapy, because unlike DNA mutations, epigenetic marks are reversible. Therefore, investigating epigenetic alterations that contribute to cancer progression may have therapeutic value in cancer treatment. The DNMT inhibitor and hypomethylating agent, 5-azacytidine (5-AzaC), is often used in combination with other chemotherapeutic drugs in cancer therapy. While 5-AzaC derepresses many tumor suppressor genes that are silenced by promoter hypermethylation in cancer cells (128), the cytotoxicity of 5-AzaC is largely due to the DNA damage-induced apoptosis that occurs in cancer cells as a result of further increased hypomethylation (129). It has been found that the combination treatment of 5-AzaC and TSA or vorinostat, HDAC inhibitors, synergistically induces apoptosis in many cancer cell types (130), and the combinatory treatment of 5-AzaC and vorinostat is currently being investigated in clinical trials (131).

HMTs and HDMs are also attractive targets for drug development and cancer therapy. Delineating the oncogenic role of EZH2 has led to the development of the EZH2 inhibitors, DZNeP and GSK126, which are currently being investigated for use in cancer therapeutics (132,133,134). Importantly, these inhibitors have been demonstrated to derepress various tumor suppressor genes which are targets of H3K27me3 mediated repression, as well as, to inhibit cancer cell proliferation and invasion (132,133,134).

The oncogenic roles of various jumonji demethylases has led to an emphasis being placed on the development of jumonji demethylase inhibitors. Various inhibitors have been developed, including, α-ketoglutarate mimics and iron chelators (135); however, these inhibitors have likely side effects, due to nonspecific inhibition of other important hydroxylases. Additionally, while numerous in vitro inhibitors have been developed, the development of cell permeable inhibitors with in vivo activity specific to jumonji demethylases has proved to be a challenge. Recently, a pan-jumonji demethylase inhibitor, JIB-04, was discovered using an unbiased locus derepression assay (136). Importantly, JIB-04 did not inhibit

other hydroxylases tested, and selectively killed cancer cells in comparison to normal cells (136). Furthermore, because JIB-04 works in the sub-micromolar range, and was shown to inhibit tumor growth and increase survival in a mouse breast cancer model (136), it is possible that JIB-04 may be useful therapeutically.

Because epigenetic modifications are reversible, and various epigenetic enzymes drive gene expression signatures that support malignant phenotypes of cancer cells, targeting epigenetic enzymes has proved, and will likely continue to prove, to be a fruitful endeavor for cancer therapy.

Delineating the Role of TMEFF2 in Prostate Cancer

The transmembrane protein with epidermal-like growth factor domain and two follistatin domains (TMEFF2) is a glycoprotein that is expressed embryonically, and in the adult brain and prostate (137,138,139). A role for TMEFF2 in PCa was suggested by studies which found that TMEFF2 expression increases during PCa progression and androgen independence (140). The overexpression of TMEFF2 can potentially be explained by findings demonstrating that TMEFF2 expression is increased by the AR at the level of transcription and translation (141,142). Although overexpressed in PCa as an androgen responsive gene, the TMEFF2 promoter is highly methylated in many other cancer types, and multiple findings suggest that TMEFF2 phenotypically acts as a tumor suppressor (141, 143-149). Our lab has shown that TMEFF2 inhibits migration and invasion in PCa cells, and that this phenotype of TMEFF2 may be, at least in part, a result of its ability to modulate one carbon metabolism (143,144). We found that TMEFF2 interacts with sarcosine dehydrogenase (SARDH), an enzyme that catalyzes the reaction converting sarcosine to glycine while donating a methyl group to tetrahydrofolate (THF) to form 5,10 methylene THF (143). Importantly, sarcosine levels has been demonstrated to have prognostic potential, as sarcosine increases with PCa progression, and sarcosine addition increases invasion in both PCa and benign prostate cells (150). Our lab has demonstrated that TMEFF2 overexpression, in nontransformed prostate epithelial cells, resulted in decreased sarcosine levels and a reduction in sarcosine induced invasion (143). While knocking-down TMEFF2 increased invasion of AI 22RV1 PCa cells, the TMEFF2 knockdown cells displayed a significantly greater loss in invasion in response to methotrexate treatment when compared to control cells (144). Because methotrexate is a dihydrofolate reductase inhibitor which decreases THF generation from dihydrofolate (DHF), these results suggest that TMEFF2 modulates one carbon flux in prostate cancer cells (see figure 1 for one carbon metabolism schematic).

Based on its role in one carbon metabolism, we hypothesized that TMEFF2 influences epigenetic regulation in PCa cells. In order to test this hypothesis, we studied the effect of altering

TMEFF2 expression on the level of specific methyl histone marks. All of the methyl histone marks selected for this study have particular importance in PCa, either because the marks themselves are modulated during PCa progression, or because the expression of the enzymes that regulate the methyl marks are altered in PCa (see Table 1 for a list of selected methyl histone marks, and the enzymes that regulate the marks). Because of the importance of studying androgen independence for the progression of PCa therapy, we chose to use the C4-2B CRPC cell line for these studies. The C4-2B cell line is an AI derivative of the AD LNCaP cell line. Importantly, it has been demonstrated that the LNCaP/C4-2B model system shares similarities with the clinical progression from AD to AI PCa (151). In this study we demonstrate that knocking-down TMEFF2 with shRNA in C4-2B cells increases the level of H3K9me3, H3K9me2, and H3K36me3 in androgen depleted conditions, while the addition of dihydrotestosterone (DHT) abolishes this effect. Importantly, we also demonstrate that TMEFF2 expression levels correlate with changes in AI growth and resistance to the antigrowth effects of the pan-jumonji demethylase inhibitor, JIB-04. Because H3K9me3, H3K9me2 and H3K36me3 are methyl marks targeted by the JMJD2 family of demethylases, these results indicate that TMEFF2 may work through one or multiple JMJD2 enzymes to regulate histone methylation in C4-2B cells. Taken together our data demonstrate that TMEFF2 is a novel regulator of AI growth and epigenetic regulation in CRPC cells.

Table 1

Mark	Effect on Transcription	Methyltransferases	Demethylases
H3K9me3	Repression	SUV39H1/2, SETDB1/2	JMJD2A/B/C
H3K9me2	Repression	G9a, GLP, SUV39H1/2, SETDB1/2	LSD1/2, JMJD2A/B/C
H3K9me1	Both	G9, GLP	LSD1/2, JMJD1A/B/C
H3K36me3	Activation	SETD2	JMJD2A/B/C
H3K4me3	Activation	MLL, MLL2/3/4/5, SETD1A/B, SMYD3	JARID1A/B/C/D
H3K27me3	Repression	EZH2	JMJD3, UTX

Table 1. Panel of methyl histone marks implicated in prostate cancer. Above is a table showing the histone methyl marks selected for this study, the transcriptional effect of methyl marks, and the HMTs and HDMs that regulate the specific marks. Histone methyl marks that are dysregulated in PCa are colored in blue, and HMTs and HDMs that are overexpressed in PCa are colored red. References for methyl histone marks deregulated in PCa: H3K9me3 and H3K9me2 (125), H3K27me3 (115,118), H3K4me3 (125). References for HMTs and HDMs overexpressed in PCa: JMJD2A (92), JMJD2B (93), JMJD2C (94), LSD1 (82), JARID1B (88), JARID1C (152), EZH2 (66), JMJD3 (153).

Chapter 2: Material and Methods

Cell culture

The C4-2B cell line was purchased from ATCC, and maintained in RPMI media supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/mL streptomycin, and Amphotericin B (LifeTechnologies). TMEFF2 was stably knocked-down in C4-2B cells using retroviral transduction with the pLKO.1 vector (Open biosystems) containing TMEFF2 targeted shRNA, with the pLKO.1 vector containing non-target shRNA being used for the scrambled control. TMEFF2 was stably over-expressed in the C4-2B from a CMV promoter within the pLenti vector (Invitrogen), which was transduced into C4-2B cells via lenti viral transduction; empty pLenti vector was transduced into C4-2B cells as a control. For DHT treatments, C4-2B cells were grown in 10% CSS RPMI for 24 hours, at which time, cells were treated with 10% CSS RPMI with either 10 nM DHT or .0001% EtOH for –DHT vehicle control. Cells were then grown in 10 nM DHT or .0001% EtOH for 24 hours before being harvested.

Protein extraction and western blot analysis

For protein extraction, cultured cells were trypsinized, pelleted, and washed twice in PBS. Pelleted cells were then either lysed in Pierce RIPA buffer with protease inhibitor cocktail (Sigma) for whole cell lysate, or Triton extraction buffer (TEB) containing .5% Triton X 100, 2 mM PMSF, .02% sodium azide in PBS for histone acid extraction. RIPA lysate was then centrifuged at 14,000xg for 15 minutes at 4°C, and supernatant containing protein was then aliquoted and stored at -80°C. ABCAM histone acid extraction protocol was used for histone extraction. Briefly, after lysis with TEB, nuclei were pelleted via centrifugation at 6,500xg for 10 minutes at 4°C. Nuclei pellets were then washed in TEB, and pelleted again using the same centrifugation conditions. Pelleted nuclei were then lysed in .2 N HCl, and

incubated overnight at 4°C. Lysates were then centrifuged at 6,500xg for 10 minutes at 4°C, supernatant containing histones was then aliquoted and stored at -80°C.

For western blot analysis, protein lysates (20 ug whole cell lyate, 10 ug acid extract) were separated in 4% to 20% gradient gels, and transferred to nitrocellulose membranes (Biorad). Membranes were blocked in TBS-T (Tris buffered saline with .01% Tween 20) with 5% NFDM for 1hour at room temperature. Membranes were incubated in the following primary antibodies in 5% NFDM TBS-T either overnight at 4°C or 1 hour at room temperature: H3K27me3 (Millipore 07-449, 1:7000), H3K9me3 (Millipore 07-442, 1:500), H3K9me2 (Millipore 07-441, 1:500), H3K9me1 (Abcam 9045, 1:6000), H3K4me3 (Millipore 07-473, 1:2500), H3K36me3 (Abcam 9050, 1:2000), H4 (Abcam 10158, 1:5000), JMJD2B (Bethyl labs A301-478A, 1:2000), JMJD2A (Bethyl labs A300-861A, 1:2000), JMJD2C (Bethyl labs A300-885A, 1:2000), AR (Santa Cruz 441, 1:1000), TMEFF2 (SDIX, 1:1000), Calnexin (Abcam 22595, 1:2000). Goat anti-rabbit hrp-conjugated secondary antibodies were used at 1:10,000 in .5% NFDM TBS-T (Santa Cruz Biotechnologies). All washes were conducted in TBS-T, and SuperSignal West Pico Chemiliminescent Substrate was used for protein detection (Thermo Scientific). Image J program was used for western blot quantification.

Growth analysis

For MTT assays, cells grown in 96 well plates were incubated for 3 hours in 100 µl of phenol red free RPMI, supplemented with MTT (.5mg/ml; Sigma) and 1% FBS. Following 3 hour incubation, 250µl of DMSO per well was added to solubilize crystals formed from MTT treatment. Absorbance was then read at 550 nm wavelength.

For analyzing C4-2B cell growth in the presence and absence of DHT, cells were hormone starved in 10% CSS RPMI for 24 hours, before being trypsinized and seeded in 96 well plates at a density of 8,000 cells per well. After allowing 24 hours for cell attachment in 10% CSS RPMI, one plate of cells

was subjected to MTT assay to establish a time zero, while cells on remaining plates were treated with 10 nM DHT or .0001% EtOH vehicle control, and allowed to grow for 72 hours, before being analyzed by MTT assay. Growth was analyzed as the change in absorbance obtained from the untreated time zero plate to the absorbance obtained from plate 72 hours after treatment.

For analyzing C4-2B cell growth in the presence and absence of DHT and in the presence and absence of AR siRNA, cells were hormone starved and seeded in 96 well plates as previously described. After allowing 24 hours for attachment in 10% CSS RPMI, cells were transfected with siRNA either targeting AR (Thermo Scientific Dharmacon L-003400-00) or with non-target control siRNA (Thermo Scientific Dharmacon D-001206-14-05). The Thermo Scientific DharmaFECT protocol was followed for the transfections, using 25nM siRNA and .1% DharmFECT reagent #3. Transfections were allowed to proceed for 48 hours, to allow for efficient AR knockdown before subsequent treatments. After 48 hour transfection, one plate was subjected to MTT assay to provide a time zero reading before DHT treatment. In remaining plates, the transfection media was replaced with 10% CSS RPMI media, supplemented with either 10 nM DHT or .0001% EtOH vehicle control. Cells were then grown for 72 hours, before being evaluated by MTT assay. Growth was analyzed as the change in absorbance obtained from the untreated time zero plate to the absorbance obtained from plate 72 hours after treatment. For analyzing AR knockdown efficiency, cells were seeded in 6 well plates, and AR knock-down and DHT treatments were carried out as described for growth analysis. Cells were harvested for protein extraction 24 hours after DHT treatment, and AR knock-down efficiency was analyzed by western blot.

For analyzing C4-2B cell growth in response to JIB-04 (prepared in DMSO) in the presence and absence of DHT, cells were hormone starved and seeded in 96 well plates as previously described. After allowing 24 hours for attachment in 10% CSS RPMI, one plate was subjected to MTT assay to provide a time zero growth reading, and remaining cells were split into four different treatments: JIB-04(Sigma

SML0808) and 10 nM DHT, .0025% DMSO (JIB-04 vehicle) and 10 nM DHT, JIB-04 and .0001% EtOH (DHT vehicle), or .0025% DMSO (JIB-04 vehicle) and .0001% EtOH (DHT vehicle). JIB-04 concentrations ranged from .05 µM to .5 µM, and all concentrations of JIB-04 contained a total of .0025% DMSO. Cells were then grown for 72 hours, before being analyzed by MTT assay. Growth was analyzed as the change in absorbance obtained from the untreated time zero plate to the absorbance obtained from plate 72 hours after treatment.

Statistical analysis

Data are presented as mean -/+ SD. Differences were analyzed using paired t-tests. Two-tail paired t-tests were used unless otherwise indicated. $p<.05$ was considered significant, while $p<.1$ was considered trending towards significance in some data presented.

Chapter 3: Results

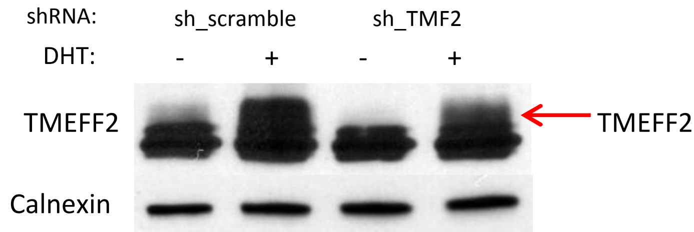
TMEFF2 modulates JMJD2 controlled methyl histone marks in androgen depleted conditions

We have previously demonstrated that TMEFF2 is a modulator of one carbon metabolism in PCa cells (). Because changes in one carbon metabolism can alter the activity of DNA and histone methyltransferases and demethylases, we hypothesized that TMEFF2 regulates epigenetic processes in PCa cells. To begin testing this hypothesis, we determined the effect of silencing TMEFF2 on specific methyl histone marks. TMEFF2 was stably knocked-down in C4-2B cells using shRNA (C4-2B sh_TMF2) (fig 2A), and the levels of selected methyl histone marks were measured via western blot analysis. Because HMTs and HDMs are involved in androgen signaling, we also treated the cells with 10nM DHT, or with EtOH as vehicle control. We investigated a panel of six methyl histone marks based on their relevance in PCa; four of the marks are deregulated in PCa, and all six marks are regulated by enzymes that are overexpressed in PCa (table 1). In androgen depleted conditions (-DHT), TMEFF2 silencing resulted in an approximate two-fold increase in H3K9me3 and H3K9me2 levels ($p<0.05$), and an approximate 50% increase in H3K36me3 ($p=0.08$), with respect to the sh_scramble negative control (C4-2B sh_scramble) (fig 2B & 2C); however, in each case, the addition of DHT abolished or reduced the effect of silencing TMEFF2 on the levels of these methyl histone marks. In fact, DHT treatment decreased H3K9me3 levels in C4-2B sh_TMF2 cells to the level of C4-2B sh_scrambled - which displayed similar levels of H3K9me3 in the presence and absence of DHT. While DHT addition did not result in significant changes in H3K9me2 levels, sh_scrambled cells demonstrated an approximate 50% increase in H3K9me2 with DHT addition ($p=0.06$), thus contributing to the lack of difference seen in H3K9me2 between sh_scrambled and sh_TMF2 cells in the presence of DHT. Importantly, neither H3K4me3 nor H3K27me3 displayed change in the presence or absence of DHT in response to TMEFF2 KD, indicating specificity in the methyl histone marks modulated by TMEFF2. Interestingly, the histone methyl marks

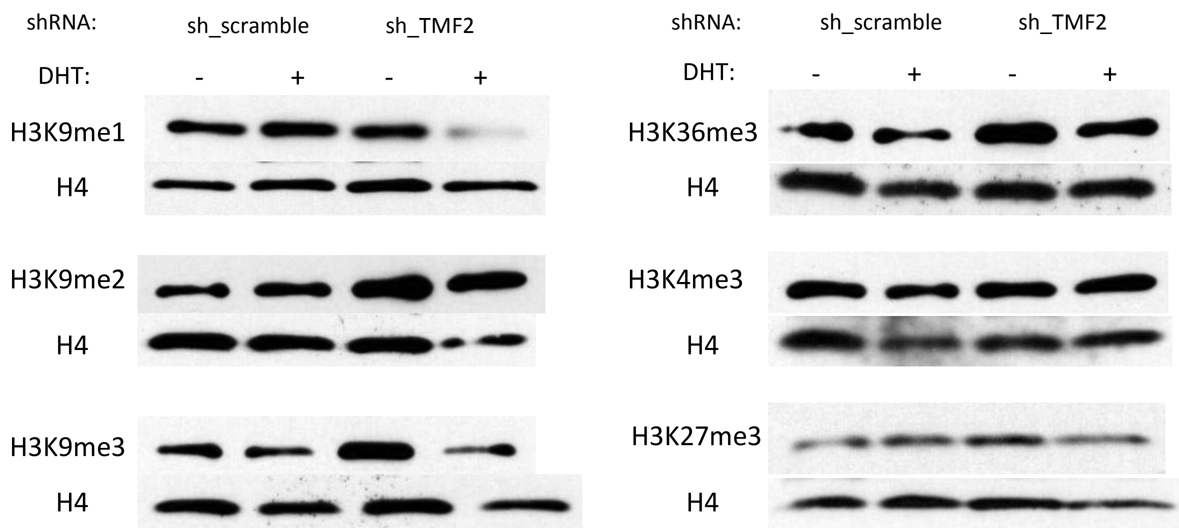
that presented increases in response to TMEFF2 KD in androgen depleted conditions, H3K9me3, H3K9me2, and H3K36me3, are all methyl marks regulated by the JMJD2 family of demethylases, suggesting that TMEFF2 may work through one or multiple JMJD2 demethylases to modulate histone methylation.

We then tested whether TMEFF2 modulates the expression of any of the three JMJD2 demethylases known to be overexpressed in PCa, JMJD2A, JMJD2B and JMJD2C, which could account for the increase in methyl mark observed in response to TMEFF2 silencing (fig 2D,2E). Surprisingly, while not statistically significant, both JMJD2B and JMJD2C showed trends toward increased protein expression in sh_TMF2 cells grown in the absence of DHT, when compared to the sh_scrambled control. JMJD2A expression was not changed significantly by TMEFF2 silencing or DHT addition. JMJD2B protein expression was androgen responsive, as previously demonstrated (93), and had equal expression in sh_scrambled and sh_TMF2 cells in the presence of DHT. All together these results indicate that while JMJD2 regulated methyl histone marks are elevated in sh_TMF2 cells grown in the absence of DHT, the expression levels of the JMJD2 demethylases do not correlate with this observation, suggesting that TMEFF2 may affect the JMJD2 demethylase activity.

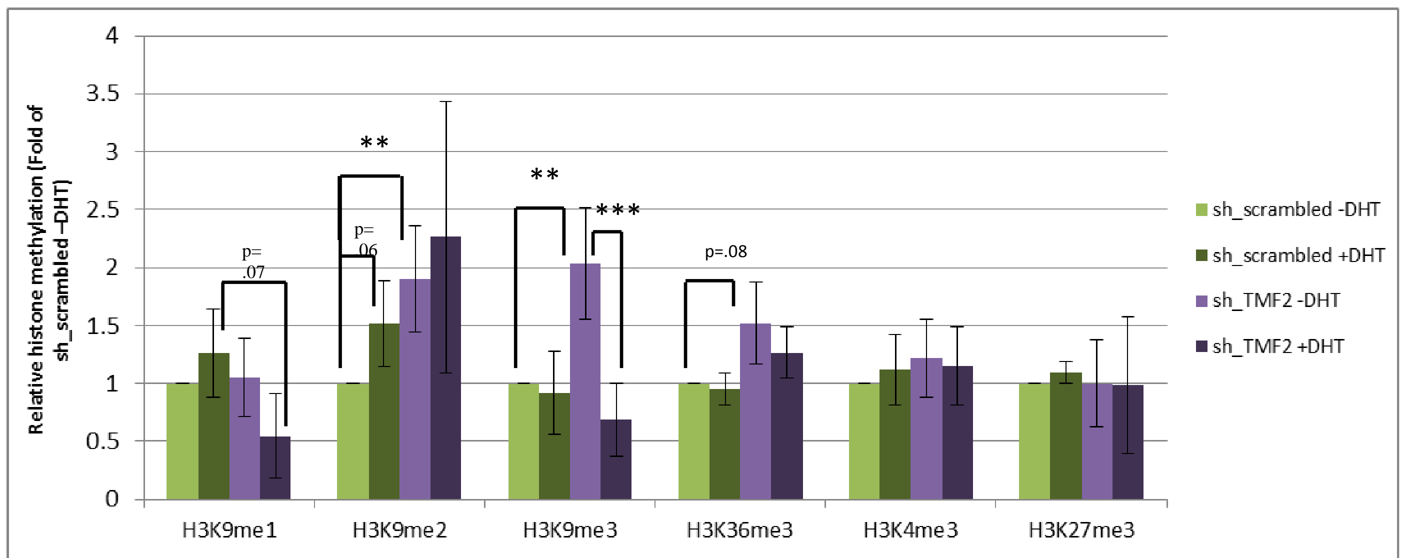
2A



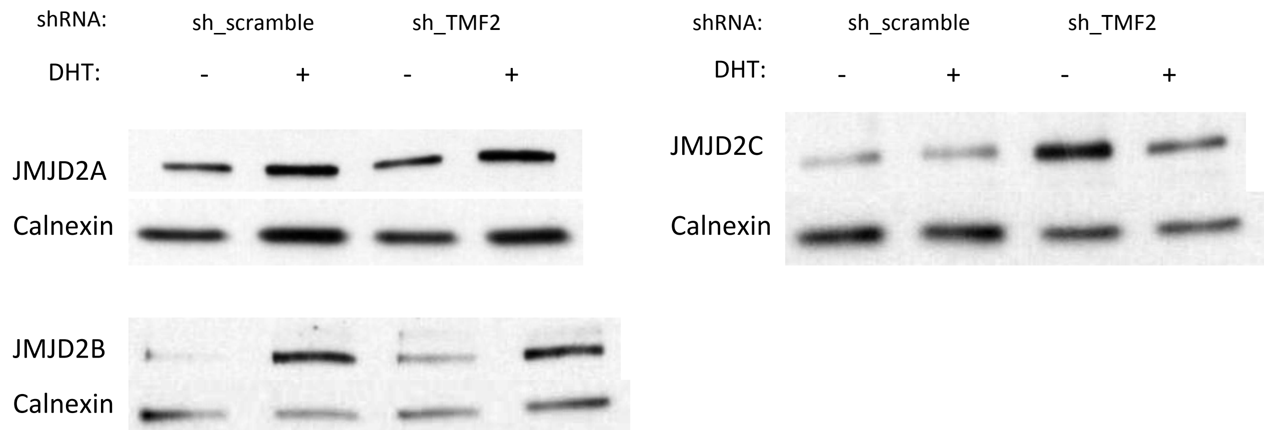
2B



2C



2D



2E

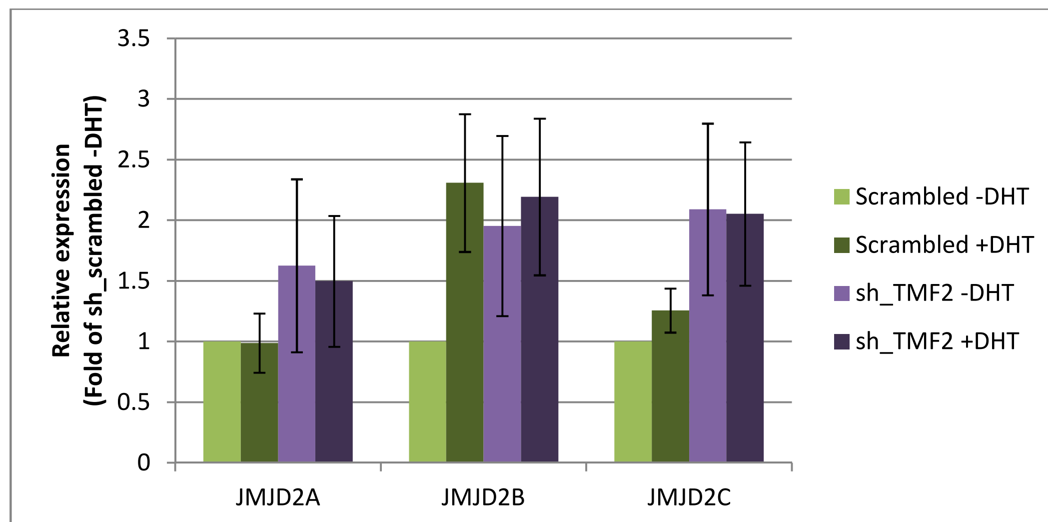


Figure 2. Knocking-down TMEFF2 increases JMJD2 regulated methyl histone marks in the absence of DHT. A: Western blot analysis of whole cell lysate demonstrating TMEFF2 knockdown in C4-2B sh_TMF2 cells in the presence and absence of DHT. The red arrow indicates the TMEFF2 band. Calnexin was used as a loading control. **B:** Representative methyl histone western blots from histone acid extracts, demonstrating the methylation of specific histone lysine residues in C4-2B sh_scrambled and C4-2B sh_TMF2 cells grown in the presence and absence of DHT. H4 was used as a loading control. **C:** Graphical representation of the average levels of methyl histone marks in C4-2B sh_scrambled and C4-2B sh_TMF2 cells grown in the presence and absence of DHT, as quantified from western blot analysis. Averages and statistics were calculated from 3 or 4 independent repeats. * indicates $p<0.1$, ** $p<0.05$, *** $p<0.02$. **D:** Representative western blots from whole cell lysate, demonstrating JMJD2A, JMJD2B, and JMJD2C expression in C4-2B sh_scrambled and C4-2B sh_TMF2 cells grown in the presence and absence of DHT. Calnexin was used as a loading control. **E:** Graphical representation of the average levels of JMJD2A, JMJD2B, and JMJD2C protein expression in C4-2B sh_scrambled and C4-2B sh_TMF2 cells grown in the presence and absence of DHT, as quantified from western blot analysis. Averages and statistics were calculated from 3 independent repeats.

TMEFF2 increases androgen independent growth and AR activity

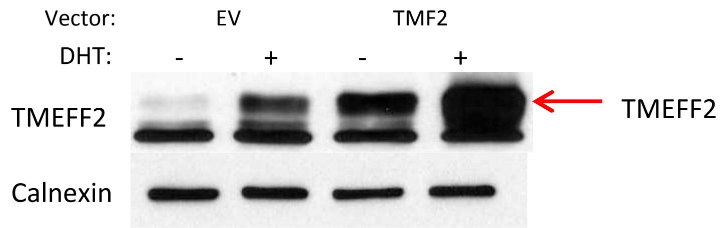
The results presented indicate that TMEFF2 modulates histone methyl marks levels by affecting the activity of JMJD2 demethylases. Since the JMJD2 enzymes increase growth in many cancer cell types, we hypothesized that TMEFF2 can modulate growth in C4-2B cells by influencing JMJD2 activity. To begin testing this hypothesis, we used MTT analysis to measure C4-2B cell growth in response to both, silencing and overexpressing TMEFF2, in the presence and absence of DHT (fig 3B & 3C). TMEFF2 overexpression was achieved by stably transducing C4-2B cells with a pLenti vector, which expressed TMEFF2 from a CMV promoter (fig 3A). The results indicated that DHT addition significantly increased growth in all C4-2B cells tested; indicating that while the C4-2B cell line has the ability to grow in androgen depleted conditions, C4-2B cell growth is responsive to androgens (fig 3B & 3C). Neither silencing nor overexpressing TMEFF2 significantly affected androgen induced growth in C4-2B cells. Conversely, in the absence of DHT, TMEFF2 overexpressing cells (C4-2B TMF2), but not TMEFF2 silenced cells, displayed a significant increase in growth in comparison to the empty vector control cells (C4-2B EV).These results demonstrate that TMEFF2 overexpression increases androgen independent growth in C4-2B cells.

Since ligand-independent AR activity is an important mechanism for androgen independent growth in many AR positive CRPC cell lines, we wanted to determine whether the increase in androgen independent growth conferred by TMEFF2 overexpression in C4-2B cells is dependent on AR. To investigate the role of AR in TMEFF2 mediated growth, we transiently knocked-down AR with siRNA in C4-2B EV and C4-2B TMF2 cells and measured cell growth in the presence and absence of androgens (fig 4A). Western blot analysis demonstrates that AR KD efficiency was approximately 90% at the level of protein expression, and was similar in both C4-2B EV and C4-2B TMF2 cells (fig 4B). Furthermore, AR KD dramatically reduced TMEFF2 expression in the presence of DHT in C4-2B EV and C4-2B TMF2 cells (fig 4B). As expected, AR KD completely abolished DHT-induced growth in C4-2B EV cells to the level

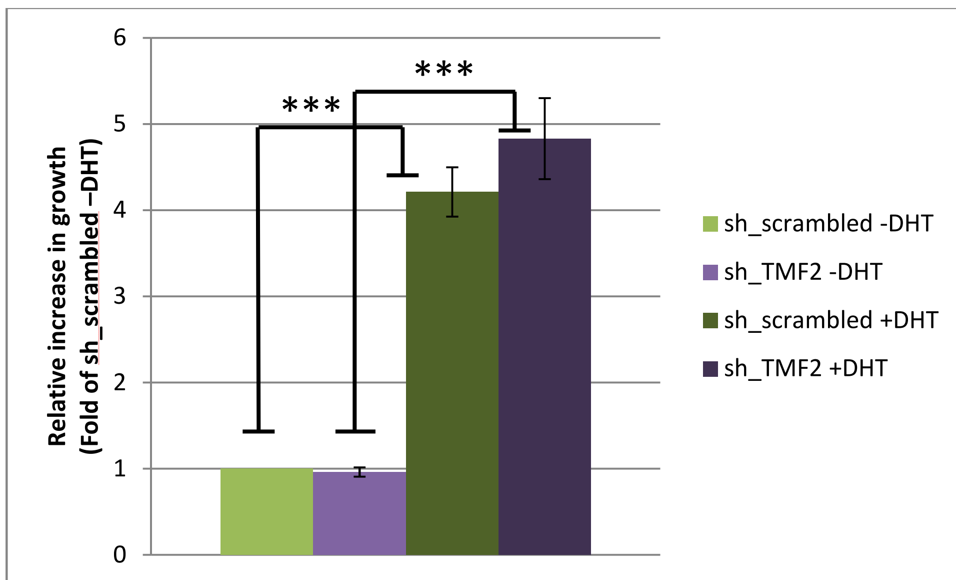
observed in the absence of DHT. Significantly, however, TMEFF2 overexpressing cells demonstrated a 30% increase in DHT-induced growth even when the AR was KD. Moreover, AR KD did not abolish the increase in androgen independent growth conferred by TMEFF2 overexpression. These results indicate that TMEFF2 regulates growth independent of AR in the absence of androgens and also increases androgen-induced growth when AR levels are depleted; thus indicating that TMEFF2 modulates growth both dependently and independently of AR in the presence and absence of ligand, respectively. Importantly, while TMEFF2 expression from the CMV promoter in C4-2B TMF2 cells is responsive to androgens (fig 3A), AR KD inhibited TMEFF2 induction in response to DHT (fig 4B); therefore, the increase in growth in the AR KD C4-2B TMF2 cells grown in the presence of DHT is not a result of increased TMEFF2 expression. Furthermore, because AR protein expression is not increased by TMEFF2 overexpression (fig 4B), these results indicate that TMEFF2 may be an effector of AR activity.

In order to test whether TMEFF2 modulates AR activity, we measured PSA protein levels in C4-2B EV/TMF2 and C4-2B sh_scrambled/sh_TMF2 cells grown in the presence and absence of DHT (fig 5). While silencing TMEFF2 decreased PSA levels in the absence and presence of DHT, overexpressing TMEFF2 only increased the induction of PSA with the addition of DHT. Importantly, because neither silencing nor overexpressing TMEFF2 altered AR protein expression in the presence or absence of DHT, these data indicate that TMEFF2 is a coactivator of AR mediated transcription.

3A



3B



3C

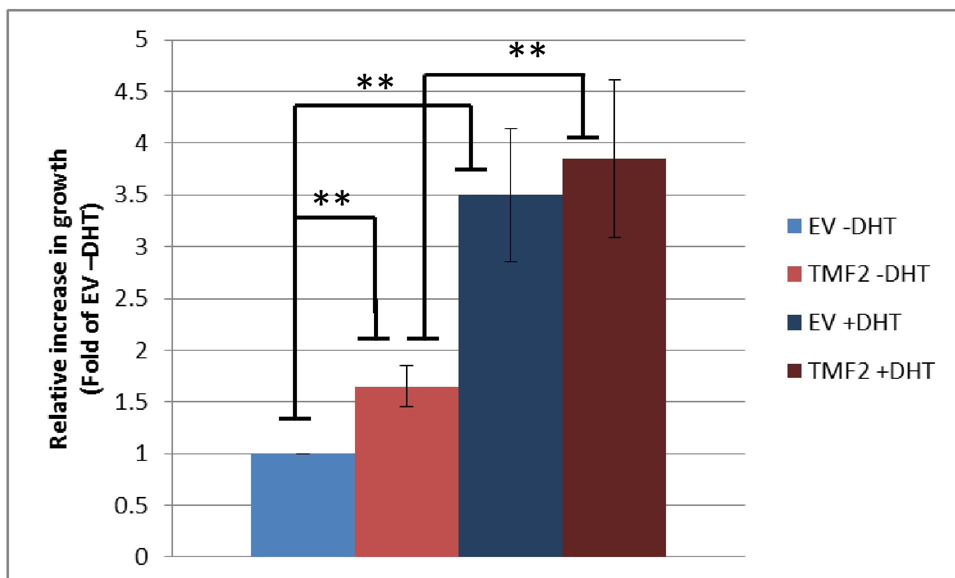
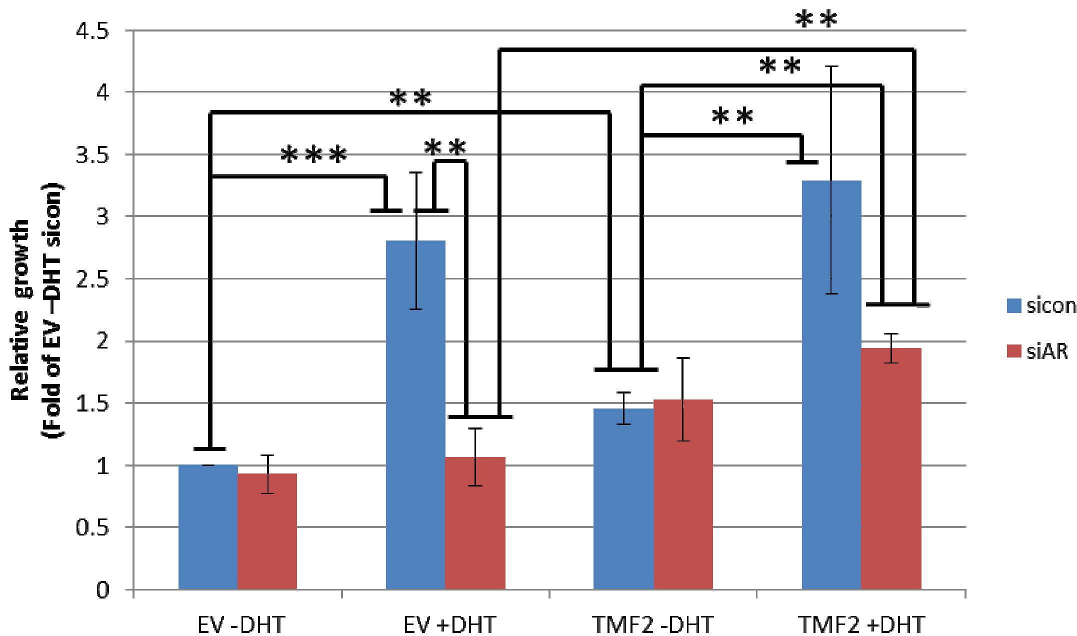


Figure 3. TMEFF2 overexpression increases androgen independent growth in C4-2B cells. A: Western blot analysis of whole cell lysate demonstrating TMEFF2 overexpression in C4-2B TMF2 cells and induction in the presence of DHT. The red arrow indicates the TMEFF2 band. Calnexin was used as a loading control. **B:** Cell growth of C4-2B sh_scrambled and C4-2B sh_TMF2 cells grown in the presence and absence of DHT. Growth was calculated relative to the growth of C4-2B sh_scrambled –DHT. n=3, with each repeat consisting of an average of 6 replicates for each condition; *** indicates p<0.02. **C:** Cell growth of C4-2B EV and C4-2B TMF2 cells grown in the presence and absence of DHT. Growth was calculated relative to the growth of C4-2B EV –DHT. n=3, with each repeat consisting of an average of 6 replicates for each condition; ** indicates p<0.05.

4A



4B

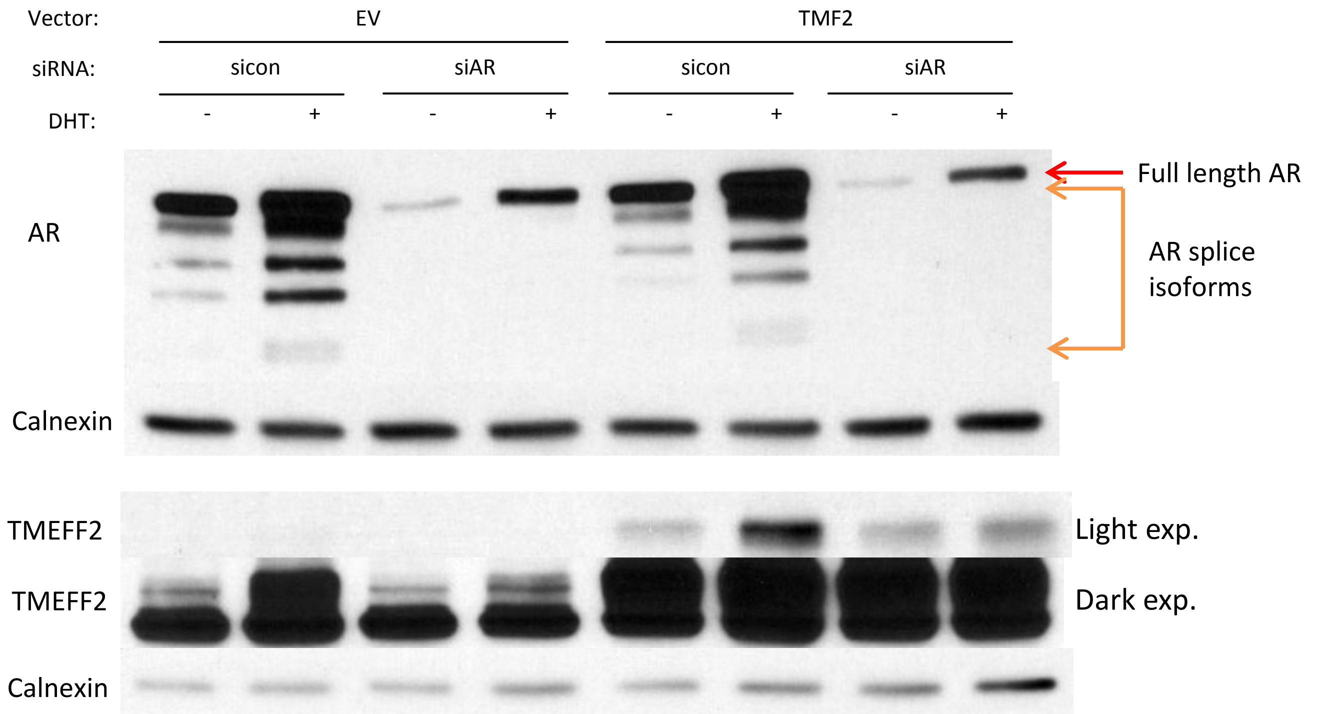
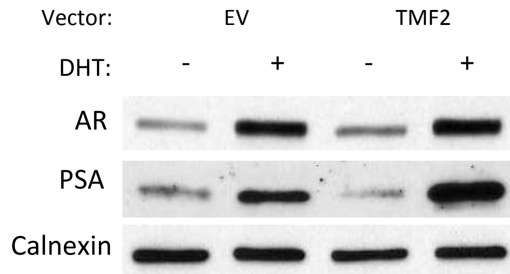


Figure 4. TMEFF2 increases growth independent of AR in the absence of DHT, and increases androgen-regulated growth in presence of DHT when AR levels are depleted. A: Cell growth of C4-2B EV and C4-2B TMF2 cells grown in the presence and absence of DHT, and the presence and absence of AR KD. siAR designates cell growth in the presence of AR siRNA, while sicon designates cell growth in the presence of the non-target siRNA control. Growth was calculated as fold of EV –DHT sicon. n=3, with each repeat consisting of an average of 6 replicates for each condition; ** indicates $p<0.05$, *** indicates $p<0.02$. One tail t-tests were used for statistical comparisons between sicon –DHT and sicon +DHT, while two-tail t-tests were used for other statistical comparisons. **B:** Western blot analysis demonstrating AR KD efficiency and TMEFF2 expression in response to AR KD in the presence and absence of DHT. Red arrow indicates full length AR, while remaining bands designated within the orange bracket indicate splice isoforms of AR.

5A



5B

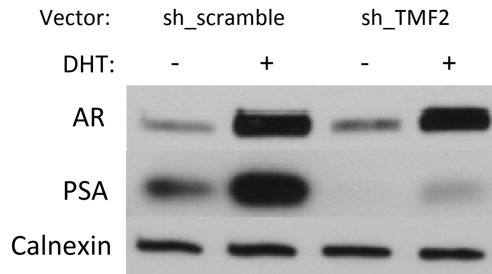


Figure 5. TMEFF2 positively regulates PSA expression without modulating AR protein levels. A: Representative western blots from whole cell lysate, demonstrating AR and PSA expression in C4-2B sh_scrambled and C4-2B sh_TMF2 cells grown in the presence and absence of DHT. Calnexin was used as a loading control. **B:** Representative western blots from whole cell lysate, demonstrating AR and PSA expression in C4-2B EV and C4-2B TMF2 cells grown in the presence and absence of DHT. Calnexin was used as a loading control.

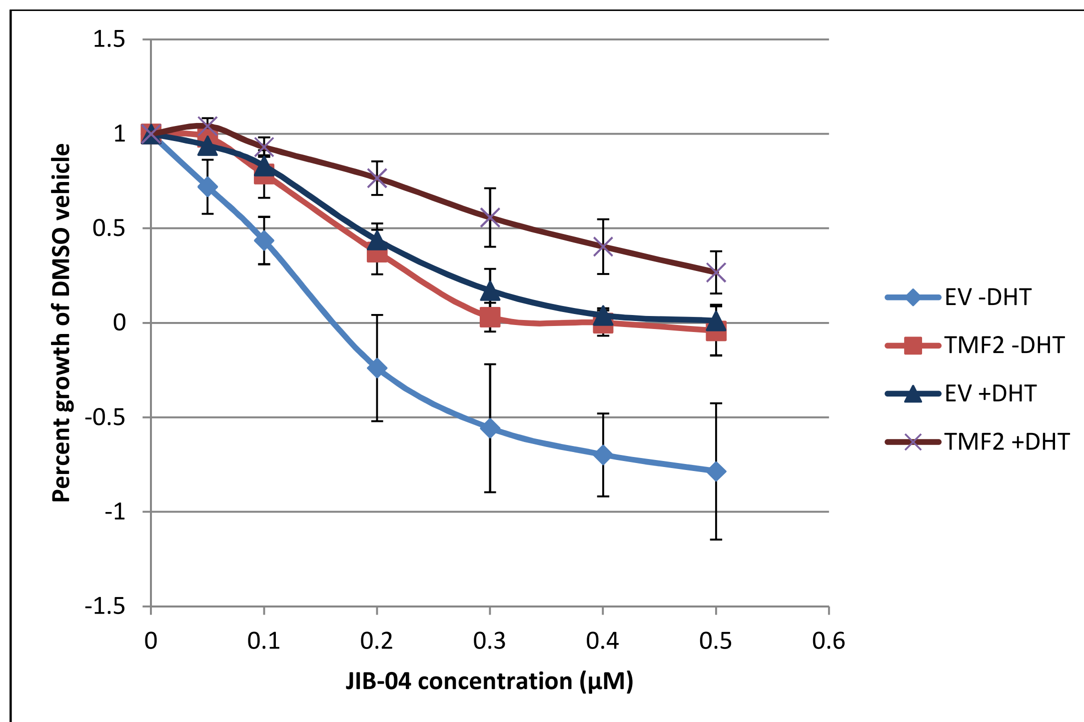
TMEFF2 and androgens confer resistance to the anti-growth effects of JIB-04

Since TMEFF2 decreases JMJD2 controlled methyl histone marks and increases growth in androgen depleted conditions, we wanted to determine whether these two effects of TMEFF2 are mechanistically linked and therefore whether TMEFF2 modulates androgen independent growth by influencing JMJD2 activity. In order to address this, we measured the effect of TMEFF2 on growth in response to JIB-04, a pan-jumonji demethylase inhibitor. TMEFF2 overexpressing or EV control cells were grown in the presence or absence of JIB-04 and DHT and the effect on growth was determined using an MTT assay. In order to measure growth sensitivity to JIB-04, growth was measured relative to the DMSO vehicle control for each of the four conditions, C4-2B EV -/+DHT and C4-2B TMF2 -/+DHT. While JIB-04 addition negatively affected growth in all cell lines/conditions tested, the addition of DHT improved relative growth in response to JIB-04, in both EV and TMEFF2 overexpressing cells (fig 6A). Furthermore, C4-2B TMF2 cells demonstrated increased relative growth when compared to C4-2B EV cells in response to JIB-04, in both the presence and absence of DHT. These results indicate that androgens and/or TMEFF2 confer resistance to the anti-growth effect of JIB-04. Interestingly, C4-2B TMF2 cells grown in the absence of DHT showed almost identical relative growth to C4-2B EV cells grown in the presence of DHT, at each JIB-04 concentration measured. This result suggests that overexpressing TMEFF2 and increasing androgen signaling have a similar effect on jumonji demethylase activity.

In order to statistically analyze the growth inhibitory effect of JIB-04, the GI_{50}, or the drug concentration that results in 50% growth inhibition, was calculated for C4-2B EV and C4-2B TMF2 grown in the presence and absence of DHT (fig 6B). The addition of DHT significantly increased the GI_{50} from .1 µM to .19 µM for the C4-2B EV cells and from .18 µM to .35 µM for the C4-2B TMF2 cells. Importantly, because in the absence of JIB-04, C4-2B EV cells have increased growth in the presence of DHT when compared to C4-2B TMF2 cells grown in the absence of DHT (fig 3C), the increased resistance to JIB-04

demonstrated by the TMEFF2 overexpressing cells cannot be solely accounted for by differences in basal growth. Additionally, when grown in the presence of DHT, C4-2B TMF2 cells demonstrated increased resistance to JIB-04, compared C4-2B EV cells (fig 6A & 6B), while the growth of C4-2B TMF2 was similar to C4-2B EV in the presence of DHT (fig 3C); these results further indicate that TMEFF2 confers resistance to JIB-04 independent of modulating basal growth. These results demonstrate that both TMEFF2 and androgens confer resistance to JIB-04, at least partially, independent of basal cell growth; thus indicating that both TMEFF2 and androgens may increase jumonji demethylase activity in C4-2B cells. Because TMEFF2 and androgen signaling are coregulated, it is difficult to suggest whether a synergistic effect is present when TMEFF2 is overexpressed and androgens are added. Therefore, it is difficult to speculate whether TMEFF2 and androgen signaling act on the jumonji demethylases through a similar or independent mechanism.

6A



6B

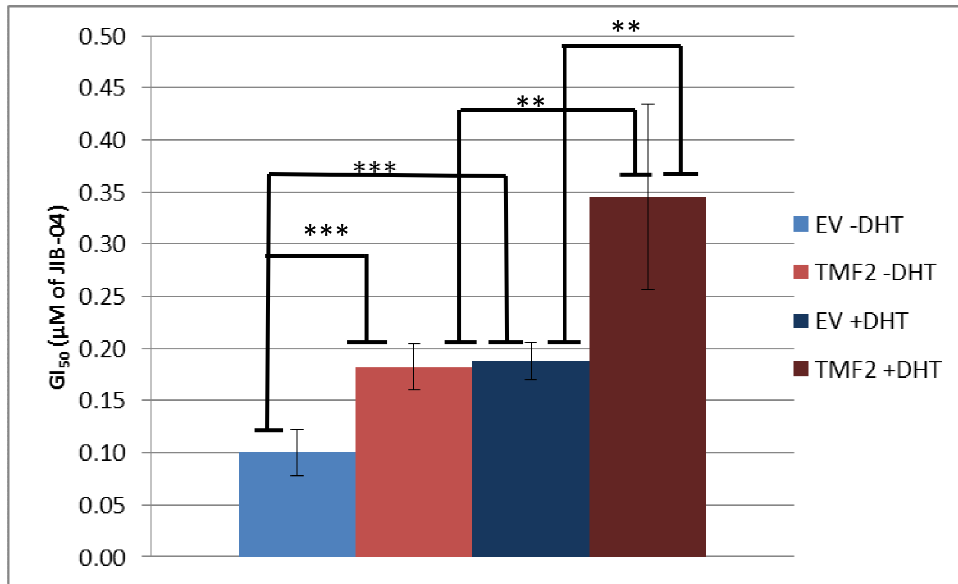


Figure 6. TMEFF2 and androgens confer resistance to the anti-growth effects of JIB-04. A: Cell growth at each concentration of JIB-04 is presented as percent growth of cells grown in the presence of the DMSO vehicle, for C4-2B EV and C4-2B TMF2 grown in the presence and absence of androgens. The growth inhibition curve was constructed using the average percent growth of four independent repeats, with each repeat consisting of four replicates at each concentration and condition. **B:** Graphical representation of the average GI_{50} for JIB-04 in C4-2B EV and C4-2B TMF2 grown in the presence and absence of DHT. The GI_{50}'s were calculated from the growth inhibition curve generated from each individual repeat. n=4; ** indicates $p<0.05$, *** $p<0.02$.

Chapter 4: Discussion

We have previously demonstrated that TMEFF2 modulates one carbon metabolism, and suppresses migration and invasion in PCa cells (143,144). Because alterations in flux within one carbon metabolism can affect epigenetic processes by potentially modifying the activity of methyltransferases and/or demethylases, we investigated the ability of TMEFF2 to influence PCa cell epigenetics. In this study we demonstrated that silencing TMEFF2 increases H3K9me3, H3K9me2 and H3K36me3 histone levels in androgen independent C4-2B PCa cells grown in androgen depleted conditions (fig 2B & 2C). The increase is possibly a result of decreased activity of one or more JMJD2 enzymes (since the JMJD2 family has H3K9me2/3 and H3K36me2/3 demethylase activity) through silencing TMEFF2. Interestingly, the addition of DHT abrogated the effect of silencing TMEFF2 on these histone methyl marks, which can be potentially explained by a few different mechanisms: 1) TMEFF2 may work partially through AR to modulate JMJD2 activity, and the addition of DHT and androgen signaling may compensate for the silencing of TMEFF2 in C4-2B sh_TMF2 cells. In fact, we have also demonstrated that TMEFF2 increases AR activity (fig 5A & 5B). 2) Since JMJD2B is an androgen responsive gene, it is possible that with the addition of DHT, JMJD2B expression rises to a sufficient level to compensate for the increase in JMJD2 controlled marks caused by silencing TMEFF2. However, this mechanism is unlikely, as silencing TMEFF2 results in a tendency towards increased basal expression of JMJD2B and JMJD2C in the absence of DHT (fig 2D,2E), while JMJD2 controlled marks are elevated with respect to the sh_scrambled control. 3) Because TMEFF2 is androgen responsive at the level of transcription and translation, TMEFF2 protein levels may rise enough to abolish the effect of silencing TMEFF2 when C4-2B sh_TMF2 cells are grown in the presence of DHT. However, TMEFF2 levels rise significantly in sh_scrambled cells with addition of DHT, while JMJD2 controlled methyl histone marks are not significantly modulated by DHT in these cells (fig 2A,2B,2C). Therefore, interplay between AR and TMEFF2 in modulating JMJD2 controlled methyl histone marks appears to be the most favorable potential mechanism.

While silencing TMEFF2 increased JMJD2 controlled marks in androgen depleted conditions, JMJD2B and JMJD2C protein levels demonstrated trends toward higher expression in C4-2B sh_TMF2 cells when compared to their levels in C4-2B sh_scrambled cells grown in the absence of DHT (fig 2D,2E). These results indicate that TMEFF2 may increase JMJD2 activity. Importantly, HIF-1α has been demonstrated to increase the transcription of certain JMJD2 genes under conditions of compromised JMJD2 activity, such as, hypoxia or oxidative stress. Therefore, by modulating one carbon metabolism and potentially the cellular redox state, silencing TMEFF2 could simultaneously decrease the activity and increase the expression of JMJD2 enzymes; however, the net effect of such regulation on global histone methylation is extremely complicated and difficult to predict. Nevertheless, because TMEFF2 decreases JMJD2 controlled methyl histone marks in androgen depleted conditions, our data indicate that TMEFF2 is a novel epigenetic modulator in CRPC.

In correlation with the role of JMJD2 enzymes as positive growth regulators in PCa cells, we demonstrated that TMEFF2 overexpression increases androgen independent growth in C4-2B cells (fig 3C). Importantly, TMEFF2 overexpression increased C4-2B resistance to the anti-growth activity of JIB-04, a pan-jumonji demethylase inhibitor, in the presence and absence of DHT(fig 6A & 6B); thus suggesting that the effect of TMEFF2 on methyl histone marks and cell growth are somewhat linked. Along with being an effector of JMJD2 regulated methyl histone marks and growth in androgen depleted conditions, we demonstrated that TMEFF2 also increases androgen induced growth in C4-2B cells when AR levels are depleted (fig 4A). Since this result indicates that TMEFF2 modulates AR activity, we tested the effect of both silencing and overexpressing TMEFF2 on PSA levels in C4-2B cells grown in the presence and absence of DHT (fig 5A & 5B). In both cases PSA induction in response to DHT positively correlated with TMEFF2 expression. Furthermore, silencing TMEFF2 drastically reduced PSA levels in androgen depleted conditions, while overexpressing TMEFF2 did not alter PSA expression in the absence of DHT. Taken together, our results indicate that TMEFF2 promotes androgen independent growth, and

increases JMJD2 and AR activity in C4-2B cells, suggesting that TMEFF2 has an oncogenic role in CRPC, mediated by its effect on AR and JMJD2 activity.

Several studies have demonstrated that TMEFF2 acts as a tumor suppressor in gastric cancer (149), and the TMEFF2 promoter is hypermethylated in multiple cancers (146,147). In fact, our lab has demonstrated that TMEFF2 inhibits migration and invasion in PCa cells (143,144,148). However, the role of TMEFF2 in PCa has been perplexing, as TMEFF2 expression has been found to increase during PCa progression and in CRPC (140), which suggests an oncogenic role of TMEFF2 in PCa. It is possible that the biological function of TMEFF2 is regulated, so that it initially signals as a tumor suppressor, while its oncogenic roles are dependent on more advanced stages of PCa. In correlation with increased expression in CRPC, we demonstrate in this study that TMEFF2 is a novel modulator of androgen independent growth in C4-2B cells. Importantly, during the progression to CRPC, PCa cells obtain means to maintain AR activity in androgen depleted conditions and/or become more reliant on other pathways for growth (114). The data presented here indicate that while TMEFF2 influences AR activity, it may increase androgen independent growth through increasing JMJD2 activity, which in turn can increase cell growth dependently and independently of the AR, as JMJD2 enzymes are known activators of AR. Therefore we propose that not only does AR regulate TMEFF2 expression, but that the role of TMEFF2 in PCa involves both its influence on JMJD2 and AR activity, and thereby CRPC growth (see fig 7 for proposed model).

Importantly, it has been demonstrated that the extracellular ectodomain of TMEFF2 can be cleaved via an ADAM17/γsecretase mechanism, and our lab and other studies have shown that the soluble ectodomain has a pro-growth function (143,154). It is therefore possible that by expressing a high amount of proteases, C4-2B cells may cleave TMEFF2 in an efficient manner, so that the oncogenic soluble ectodomain is increased significantly when TMEFF2 is overexpressed in this cell line. Importantly, protease cascades have been shown to increase with the progression of PCa in the TRAMP

mouse PCa model (155). It is also possible that DHT addition stimulates cleavage of TMEFF2 and ectodomain release, as it has been observed for other transmembrane proteins (156), contributing to its growth promoting effect. Indeed, it is possible that the TMEFF2 soluble ectodomain is required for both the epigenetic and growth phenotypes observed in the C4-2B cell line. In future experiments, the soluble ectodomain will be overexpressed in C4-2B cells, and it can be determined how the growth effect of TMEFF2 ectodomain overexpression compares to full-length TMEFF2 overexpression in C4-2B cells. Additionally, TMEFF2 cleavage in cell media can be quantified via western blot analysis.

Importantly, because TMEFF2 increases PCa growth in androgen depleted conditions and when AR protein levels are depleted, it is possible that TMEFF2 overexpression may contribute to PCa resistance to anti-androgen or potential AR silencing treatments. Because developed resistance to anti-androgens is a key process for CRPC relapse, TMEFF2 overexpression may contribute to progression of PCa to CRPC. Furthermore, its potential role with AR and JMJD2 demethylases may make TMEFF2 relevant in future CRPC therapeutics.

Future experiments will be directed at further delineating the molecular mechanisms through which TMEFF2 increases CRPC growth. We will be conducting Chip-seq analysis with AR and H3K9me3 in C4-2B sh_scrambled and sh_TMF2 cells grown in the presence and absence of DHT. Importantly, the Chip-seq analysis will be partnered with microarray analysis, so that targets of TMEFF2 mediated through its effect on AR and/or H3K9me3 can be identified. Furthermore, microarray analysis with C4-2B EV and C4-2B TMF2 cells grown in the presence and absence of androgens, will provide potential targets of TMEFF2 that may be involved in its pro-growth phenotype. Additionally, the experiments mentioned above will be carried out in LNCaP cells, in which TMEFF2 is silenced by shRNA. These experiments will aid in determining whether TMEFF2 changes roles during the progression of PCa from AD to CRPC, and its therapeutic potential alone or in combination with anti-androgens or other AR-targeted treatments.

Figure 7

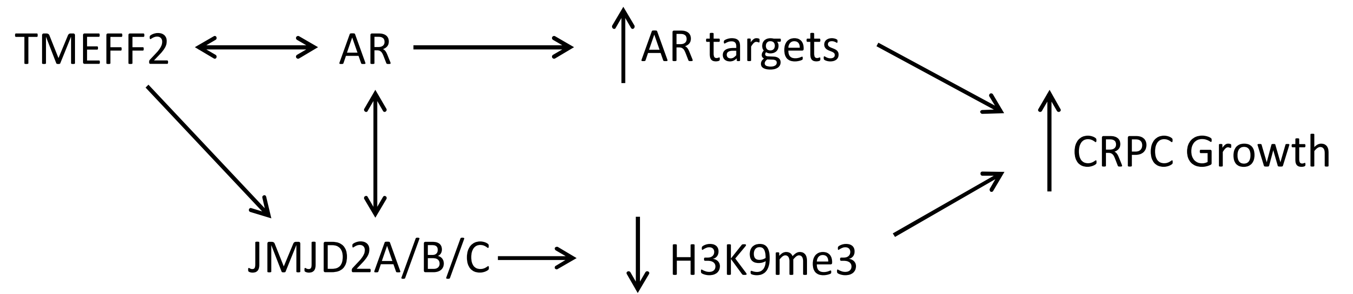


Figure 7. A proposed model for the role of TMEFF2 in CRPC growth, through modulating JMJD2 and AR activity.

References

1. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Chromosomal DNA and Its Packaging in the Chromatin Fiber.
2. Arrighi F.E., Hsu T.C. (1971) Localization of heterochromatin in human chromosomes. Cytogenetics 10:81–86.
3. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev. 16(1):6-21.
4. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genetics 3(9):662-73.
5. Reik W (2002) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447(7143):425-32.
6. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet. 3(6):415-28.
7. Veerappan CS, Sleiman S, Coppola G (2013) Epigenetics of Alzheimer's disease and frontotemporal dementia. Neurotherapeutics 10(4):709-21.
8. Pinney SE, Simmons RA (2010) Epigenetic mechanisms in the development of type 2 diabetes. Trends Endocrinol Metab. 21(4):223-9.
9. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res. 8(7): 1499–1504.
10. Venter JC et al (2001) The sequence of the human genome. Science 291(5507):1304-51.
11. Wang Y, Leung FC (2004) An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics 20(7):1170-7.
12. Shikhar Sharma, Theresa KK and Peter AJ (2009) Epigenetics in cancer. Carcinogenesis 31(1): 27–36

13. Prendergast GC, Ziff EB (1991) Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science 251(4990):186-9.

14. Watt F, Molloy PL (1988) Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev. 2(9):1136-43.

15. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet. 19(2):187-91.

16. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393(6683):386-9.

17. Kim GD, Ni J, Kelesoglu N, Roberts RJ, Pradhan S. (2002) Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. EMBO J. 21: 4183–4195.

18. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99(3):247-57.

19. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930-5.

20. Choi J, Ritchey J, Prior JL, Holt M, Shannon WD, Deych E, Piwnica-Worms DR, DiPersio JF (2010) In vivo administration of hypomethylating agents mitigate graft-versus-host disease without sacrificing graft-versus-leukemia. Blood 116(1):129-39.

21. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389(6648):251-60.

22. Kouzarides T (2007) Chromatin modifications and their function. Cell 128(4):693-705.

23. Roth SY, Denu JM, Allis CD (2001) Histone acetyltransferases. Annu Rev Biochem. 70:81-120.

24. Hebbes TR, Thorne AW, Crane-Robinson C (1980) A direct link between core histone acetylation and transcriptionally active chromatin. EMBO J. 7(5):1395-402.

25. De Ruijter AJ, Van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J. 370(3):737-49.

26. Kim HJ, Bae SC (2011) Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res. 3(2):166-79.

27. Trievel RC (2004) Structure and function of histone methyltransferases. Crit Rev Eukaryot Gene Expr. 14(3):147-69.

28. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129(4):823-37.

29. Martin C, Zhang Y (2005) The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol. 6(11):838-49.

30. Yun M, Wu J, Workman JL, Li B (2011) Readers of histone modifications. Cell Res. 21(4):564-78.

31. Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, Kouzarides T (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410(6824):120-4.

32. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410(6824):116-20.

33. Højfeldt JW, Agger K, Helin K (2013) Histone lysine demethylases as targets for anticancer therapy. Nat Rev Drug Discov. 12(12):917-30.

34. Forneris F, Binda C, Vanoni MA, Mattevi A, Battaglioli E (2005) Histone demethylation catalysed by LSD1 is a flavin-dependent oxidative process. FEBS Lett. 579(10):2203-7.

35. Kooistra SM, Helin K (2012) Molecular mechanisms and potential functions of histone demethylases. Nat Rev Mol Cell Biol. 13(5):297-311.

36. Hausinger RP (2004) FeII/alpha-ketoglutarate-dependent hydroxylases and related enzymes. Crit Rev Biochem Mol Biol. 39(1):21-68.

37. McDonough MA, Loenarz C, Chowdhury R, Clifton IJ, Schofield CJ (2010) Structural studies on human 2-oxoglutarate dependent oxygenases. Curr Opin Struct Biol. 20(6):659-72.

38. Teperino R, Schoonjans K, Auwerx J (2010) Histone methyl transferases and demethylases; can they link metabolism and transcription?. Cell Metab. 12(4):321-7.

39. Padmanabhan N, Jia D, Geary-Joo C, Wu X, Ferguson-Smith AC, Fung E, Bieda MC, Snyder FF, Gravel RA, Cross JC, Watson ED (2013) Mutation in folate metabolism causes epigenetic instability and transgenerational effects on development. Cell 155(1):81-93.

40. Hayes JD, McLellan LI (1999) Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res. 31(4):273-300.

41. Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC (2007) Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. Mol Cell Biol. 27(3):912-25.

42. Sullivan LB, Martinez-Garcia E, Nguyen H, Mullen AR, Dufour E, Sudarshan S, Licht JD, Deberardinis RJ, Chandel NS (2013) The proto-oncometabolite fumarate binds glutathione to amplify ROS-dependent signaling. Mol Cell 51(2):236-48.

43. Pollard PJ, Loenarz C, Mole DR, McDonough MA, Gleadle JM, Schofield CJ, Ratcliffe PJ (2008) Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J. 416(3):387-94.

44. Madugundu GS, Cadet J, Wagner JR (2014) Hydroxyl-radical-induced oxidation of 5-methylcytosine in isolated and cellular DNA. Nucleic Acids Res. 42(11):7450-60.

45. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400-13.

46. Greger V, Passarge E, Höpping W, Messmer E, Horsthemke B (1989) Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet. 83(2):155-8.

47. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB, Herman JG (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst. 92(7):564-9.

48. Shim YH, Kang GH, Ro JY (2000) Correlation of p16 hypermethylation with p16 protein loss in sporadic gastric carcinomas. Lab Invest. 80(5):689-95.

49. García JM, Silva J, Peña C, Garcia V, Rodríguez R, Cruz MA, Cantos B, Provencio M, España P, Bonilla F (2004) Promoter methylation of the PTEN gene is a common molecular change in breast cancer. Genes Chromosomes Cancer 41(2):117-24.

50. Singal R, van Wert J, Bashambu M (2001) Cytosine methylation represses glutathione S-transferase P1 (GSTP1) gene expression in human prostate cancer cells. Cancer Res. 61(12):4820-6.

51. K D Robertson, E Uzvolgyi, G Liang, C Talmadge, J Sumegi, F A Gonzales, and P A Jones (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res. 27(11): 2291–2298.

52. Qu Y, Mu G, Wu Y, Dai X, Zhou F, Xu X, Wang Y, Wei F (2010) Overexpression of DNA methyltransferases 1, 3a, and 3b significantly correlates with retinoblastoma tumorigenesis. Am J Clin Pathol. 134(5):826-34.

53. Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, Sasaki H (2001) Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood 97(5):1172-9.

54. Glozak MA, Seto E (2007) Histone deacetylases and cancer. Oncogene 26(37):5420-32.

55. Rodriguez J, Frigola J, Vendrell E, Risques RA, Fraga MF, Morales C, Moreno V, Esteller M, Capellà G, Ribas M, Peinado MA (2006) Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. Cancer Res. 66(17):8462-9468.

56. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300(5618):455.

57. Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, Feinberg AP (1993) Relaxation of imprinted genes in human cancer. Nature 362(6422):747-9.

58. Ogawa O, Eccles MR, Szeto J, McNoe LA, Yun K, Maw MA, Smith PJ, Reeve AE (1993) Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. Nature 362(6422):749-51.

59. Cui H, Cruz-Correa M, Giardiello FM, Hutcheon DF, Kafonek DR, Brandenburg S, Wu Y, He X, Powe NR, Feinberg AP (2003) Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. Science 299(5613):1753-5.

60. Kim Y (2004) Folate and DNA methylation: a mechanistic link between folate deficiency and colorectal cancer?. Cancer Epidemiol Biomarkers Prev. 13(4):511-9.

61. Piyathilake CJ, Johanning GL, Macaluso M, Whiteside M, Oelschlager DK, Heimburger DC, Grizzle WE (2000) Localized folate and vitamin B-12 deficiency in squamous cell lung cancer is associated with global DNA hypomethylation. Nutr Cancer 37(1):99-107.

62. Ericson U, Sonestedt E, Gullberg B, Olsson H, Wirfält E (2007) High folate intake is associated with lower breast cancer incidence in postmenopausal women in the Malmö Diet and Cancer cohort. Am J Clin Nutr 86:434–43.

63. Larsson SC, Giovannucci E, Wolk A (2006) Folate intake, MTHFR polymorphisms, and risk of esophageal, gastric, and pancreatic cancer: a meta-analysis. Gastroenterology 131(4):1271-83.

64. Chen J, Gammon MD, Chan W, Palomeque C, Wetmur JG, Kabat GC, Teitelbaum SL, Britton JA, Terry MB, Neugut AI, Santella RM (2005) One-carbon metabolism, MTHFR polymorphisms, and risk of breast cancer. Cancer Res. 65(4):1606-14.

65. Varier RA, Timmers HT (2011) Histone lysine methylation and demethylation pathways in cancer. Biochim Biophys Acta. 1815(1):75-89.

66. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419(6907):624-9.

67. Kleer CG1, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA, Chinnaiyan AM (2003) EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A 100(20):11606-11.

68. Arisan S, Buyuktuncer ED, Palavan-Unsal N, Caşkurlu T, Cakir OO, Ergenekon E (2005) Increased expression of EZH2, a polycomb group protein, in bladder carcinoma. Urol Int. 75(3):252-7.

69. Sudo T, Utsunomiya T, Mimori K, Nagahara H, Ogawa K, Inoue H, Wakiyama S, Fujita H, Shirouzu K, Mori M (2005) Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br J Cancer 92(9):1754-8.

70. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, Paul JE, Boyle M, Woolcock BW, Kuchenbauer F, Yap D, Humphries RK, Griffith OL, Shah S, Zhu H, Kimbara M, Shashkin P,

Charlot JF, Tcherpakov M, Corbett R, Tam A, Varhol R, Smailus D, Moksa M, Zhao Y, Delaney A, Qian H, Birol I, Schein J, Moore R, Holt R, Horsman DE, Connors JM, Jones S, Aparicio S, Hirst M, Gascoyne RD, Marra MA (2010) Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet. 42(2):181-5.

71. Yap DB, Chu J, Berg T, Schapira M, Cheng SW, Moradian A, Morin RD, Mungall AJ, Meissner B, Boyle M, Marquez VE, Marra MA, Gascoyne RD, Humphries RK, Arrowsmith CH, Morin GB, Aparicio SA (2011) Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. Blood 117(8):2451-9.

72. Bryant RJ, Cross NA, Eaton CL, Hamdy FC, Cunliffe VT (2007) EZH2 promotes proliferation and invasiveness of prostate cancer cells. Prostate 67(5):547-56.

73. Eskander RN, Ji T, Huynh B, Wardeh R, Randall LM, Hoang B (2013) Inhibition of enhancer of zeste homolog 2 (EZH2) expression is associated with decreased tumor cell proliferation, migration, and invasion in endometrial cancer cell lines. Int J Gynecol Cancer 23(6):997-1005.

74. Cao Q, Yu J, Dhanasekaran SM, Kim JH, Mani RS, Tomlins SA, Mehra R, Laxman B, Cao X, Yu J, Kleer CG, Varambally S, Chinnaiyan AM (2008) Repression of E-cadherin by the polycomb group protein EZH2 in cancer. Oncogene 27(58):7274-84.

75. Fan T, Jiang S, Chung N, Alikhan A, Ni C, Lee CC, Hornyak TJ (2011) EZH2-dependent suppression of a cellular senescence phenotype in melanoma cells by inhibition of p21/CDKN1A expression. Mol Cancer Res. 9(4):418-29.

76. Fujii S, Ito K, Ito Y, Ochiai A (2008) Enhancer of zeste homologue 2 (EZH2) down-regulates RUNX3 by increasing histone H3 methylation. J Biol Chem 283(25):17324-32.

77. Hess JL (2004) MLL: a histone methyltransferase disrupted in leukemia. Trends Mol Med. 10(10):500-7.

78. Krivtsov AV, Armstrong SA (2007) MLL translocations, histone modifications and leukaemia stem-cell development. Nat Rev Cancer 7(11):823-33.

79. Ayton PM, Cleary ML (2003) Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. Genes Dev. 17(18):2298-307.

80. Lüscher-Firzlaff J, Gawlista I, Vervoorts J, Kapelle K, Braunschweig T, Walsemann G, Rodgarkia-Schamberger C, Schuchlautz H, Dreschers S, Kremmer E, Lilischkis R, Cerni C, Wellmann A, Lüscher B (2008) The human trithorax protein hASH2 functions as an oncoprotein. Cancer Res. 68(3):749-58.

81. Schulte JH, Lim S, Schramm A, Friedrichs N, Koster J, Versteeg R, Ora I, Pajtler K, Klein-Hitpass L, Kuhfittig-Kulle S, Metzger E, Schüle R, Eggert A, Buettner R, Kirfel J (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res. 69(5):2065-71.

82. Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R, Solleder G, Bastian PJ, Ellinger J, Metzger E, Schüle R, Buettner R (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. 66(23):11341-7.

83. Lim S, Janzer A, Becker A, Zimmer A, Schüle R, Buettner R, Kirfel J (2010) Carcinogenesis 31(3):512-20.

84. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119(7):941-53.

85. Metzger E, Wissmann M, Yin N, Müller JM, Schneider R, Peters AH, Günther T, Buettner R, Schüle R (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437(7057):436-9.

86. Zeng J, Ge Z, Wang L, Li Q, Wang N, Björkholm M, Jia J, Xu D (2010) The histone demethylase RBP2 Is overexpressed in gastric cancer and its inhibition triggers senescence of cancer cells. Gastroenterology 138(3):981-92.

87. Yamane K, Tateishi K, Klose RJ, Fang J, Fabrizio LA, Erdjument-Bromage H, Taylor-Papadimitriou J, Tempst P, Zhang Y (2007) PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. Mol Cell 25(6):801-12.

88. Xiang Y, Zhu Z, Han G, Ye X, Xu B, Peng Z, Ma Y, Yu Y, Lin H, Chen AP, Chen CD (2007) JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. Proc Natl Acad Sci U S A. 104(49):19226-31.

89. Berry WL, Janknecht R (2013) KDM4/JMJD2 histone demethylases: epigenetic regulators in cancer cells. Cancer Res. 73(10):2936-42.

90. Berry WL, Shin S, Lightfoot SA, Janknecht R (2012) Oncogenic features of the JMJD2A histone demethylase in breast cancer. Int J Oncol. 41(5):1701-6.

91. Kawazu M, Saso K, Tong KI, McQuire T, Goto K, Son DO, Wakeham A, Miyagishi M, Mak TW, Okada H (2011) Histone demethylase JMJD2B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PLoS One 6(3):e17830.

92. Shin S, Janknecht R (2007) Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. Biochem Biophys Res Commun. 359(3):742-6.

93. Coffey K, Rogerson L, Ryan-Munden C, Alkharaif D, Stockley J, Heer R, Sahadevan K, O'Neill D, Jones D, Darby S, Staller P, Mantilla A, Gaughan L, Robson CN (2014) The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover. Nucleic Acids Res. 41(8):4433-46.

94. Wissmann M, Yin N, Müller JM, Greschik H, Fodor BD, Jenuwein T, Vogler C, Schneider R, Günther T, Buettner R, Metzger E, Schüle R (2007) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol. 9(3):347-53.

95. Kim TD, Fuchs JR, Schwartz E, Abdelhamid D, Etter J, Berry WL, Li C, Ihnat MA, Li PK, Janknecht R (2014) Pro-growth role of the JMJD2C histone demethylase in HCT-116 colon cancer cells and identification of curcuminoids as JMJD2 inhibitors. Am J Transl Res. 6(3):236-47.

96. Kogure M, Takawa M, Cho HS, Toyokawa G, Hayashi K, Tsunoda T, Kobayashi T, Daigo Y, Sugiyama M, Atomi Y, Nakamura Y, Hamamoto R (2013) Deregulation of the histone demethylase JMJD2A is involved in human carcinogenesis through regulation of the G(1)/S transition. Cancer Lett. 336(1):76-84.

97. Li BX, Zhang MC, Luo CL, Yang P, Li H, Xu HM, Xu HF, Shen YW, Xue AM, Zhao ZQ (2011) Effects of RNA interference-mediated gene silencing of JMJD2A on human breast cancer cell line MDA-MB-231 in vitro. J Exp Clin Cancer Res. 30:90.

98. Li BX, Luo CL, Li H, Yang P, Zhang MC, Xu HM, Xu HF, Shen YW, Xue AM, Zhao ZQ (2012) Effects of siRNA-mediated knockdown of jumonji domain containing 2A on proliferation, migration and invasion of the human breast cancer cell line MCF-7. Exp Ther Med. 4(4):755-761.

99. Kim TD, Shin S, Berry WL, Oh S, Janknecht R (2012) The JMJD2A demethylase regulates apoptosis and proliferation in colon cancer cells. J Cell Biochem. 113(4):1368-76.

100. Klose RJ, Yamane K, Bae Y, Zhang D, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y (2006) The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature 442(7100):312-6.

101. Yang J, Jubb AM, Pike L, Buffa FM, Turley H, Baban D, Leek R, Gatter KC, Ragoussis J, Harris AL (2010) The histone demethylase JMJD2B is regulated by estrogen receptor alpha and hypoxia, and is a key mediator of estrogen induced growth. Cancer Res. 70(16):6456-66.

102. Fu L, Chen L, Yang J, Ye T, Chen Y, Fang J (2012) HIF-1α-induced histone demethylase JMJD2B contributes to the malignant phenotype of colorectal cancer cells via an epigenetic mechanism. Carcinogenesis 33(9):1664-73.

103. Yang ZQ, Imoto I, Fukuda Y, Pimkhaokham A, Shimada Y, Imamura M, Sugano S, Nakamura Y, Inazawa J (2000) Identification of a novel gene, GASC1, within an amplicon at 9p23-24 frequently detected in esophageal cancer cell lines. Cancer Res. 60(17):4735-9.

104. Rui L, Emre NC, Kruhlak MJ, Chung HJ, Steidl C, Slack G, Wright GW, Lenz G, Ngo VN, Shaffer AL, Xu W, Zhao H, Yang Y, Lamy L, Davis RE, Xiao W, Powell J, Maloney D, Thomas CJ, Möller P, Rosenwald A, Ott G, Muller-Hermelink HK, Savage K, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Weisenburger DD, Chan WC, Gascoyne RD, Levens D, Staudt LM (2010) Cooperative epigenetic modulation by cancer amplicon genes. Cancer Cell 18(6):590-605.

105. Liu G, Bollig-Fischer A, Kreike B, van de Vijver MJ, Abrams J, Ethier SP, Yang ZQ (2009) Genomic amplification and oncogenic properties of the GASC1 histone demethylase gene in breast cancer. Oncogene 28(50):4491-500.

106. Luo W, Chang R, Zhong J, Pandey A, Semenza GL (2012) Histone demethylase JMJD2C is a coactivator for hypoxia-inducible factor 1 that is required for breast cancer progression. Proc Natl Acad Sci U S A 109(49):E3367-76.

107. Loh YH, Zhang W, Chen X, George J, Ng HH (2007) Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev. 21(20):2545-57.

108. Kilpeläinen TP, Tammela TL, Malila N, Hakama M, Santti H, Määttänen L, Stenman UH, Kujala P, Auvinen A (2013) Prostate cancer mortality in the Finnish randomized screening trial. J Natl Cancer Inst. 105(10):719-25.

109. Scher HI, Heller G (2000) Clinical states in prostate cancer: toward a dynamic model of disease progression. Urology 55(3):323-7.

110. Heinlein CA, Chang C (2004) Androgen receptor in prostate cancer. Endocr Rev. 25(2):276-308.

111. McPhaul MJ (2003) Factors that mediate and modulate androgen action. J Investig Dermatol Symp Proc. 8(1):1-5.

112. Crea F, Sun L, Mai A, Chiang YT, Farrar WL, Danesi R, Helgason CD (2012) The emerging role of histone lysine demethylases in prostate cancer. Mol Cancer 11:52.

113. Heemers HV, Tindall DJ (2007) Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 28(7):778-808.

114. Saraon P, Jarvi K, Diamandis EP (2011) Molecular alterations during progression of prostate cancer to androgen independence. Clin Chem. 57(10):1366-75.

115. Xu K, Wu ZJ, Groner AC, He HH, Cai C, Lis RT, Wu X, Stack EC, Loda M, Liu T, Xu H, Cato L, Thornton JE, Gregory RI, Morrissey C, Vessella RL, Montironi R, Magi-Galluzzi C, Kantoff PW, Balk SP, Liu XS, Brown M (2012) EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. Science 338(6113):1465-9.

116. Wu L, Runkle C, Jin HJ, Yu J, Li J, Yang X, Kuzel T, Lee C, Yu J (2014) CCN3/NOV gene expression in human prostate cancer is directly suppressed by the androgen receptor. Oncogene 33(4):504-13.

117. Kang HB1, Choi Y, Lee JM, Choi KC, Kim HC, Yoo JY, Lee YH, Yoon HG (2009) The histone methyltransferase, NSD2, enhances androgen receptor-mediated transcription. FEBS Lett. 583(12):1880-6.

118. Ellinger J, Kahl P, von der Gathen J, Heukamp LC, Gütgemann I, Walter B, Hofstädter F, Bastian PJ, von Ruecker A, Müller SC, Rogenhofer S (2012) Global histone H3K27 methylation levels are different in localized and metastatic prostate cancer. Cancer Invest. 30(2):92-7.

119. Nimura K, Ura K, Shiratori H, Ikawa M, Okabe M, Schwartz RJ, Kaneda Y (2009) A histone H3 lysine 36 trimethyltransferase links Nkx2-5 to Wolf-Hirschhorn syndrome. Nature 460(7252):287-91.

120. Marango J, Shimoyama M, Nishio H, Meyer JA, Min DJ, Sirulnik A, Martinez-Martinez Y, Chesi M, Bergsagel PL, Zhou MM, Waxman S, Leibovitch BA, Walsh MJ, Licht JD (2008) The MMSET protein is a histone methyltransferase with characteristics of a transcriptional corepressor. Blood 111(6):3145-54.

121. Kim JY, Kee HJ, Choe NW, Kim SM, Eom GH, Baek HJ, Kook H, Kook H, Seo SB (2008) Multiple-myeloma-related WHSC1/MMSET isoform RE-IIBP is a histone methyltransferase with transcriptional repression activity. Mol Cell Biol. 28(6):2023-34.

122. Yang P, Guo L, Duan ZJ, Tepper CG, Xue L, Chen X, Kung HJ, Gao AC, Zou JX, Chen HW (2012) Histone methyltransferase NSD2/MMSET mediates constitutive NF-κB signaling for cancer cell proliferation, survival, and tumor growth via a feed-forward loop. Mol Cell Biol. 32(15):3121-31.

123. Cai C, He HH, Chen S, Coleman I, Wang H, Fang Z, Chen S, Nelson PS, Liu XS, Brown M, Balk SP (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 20(4):457-71.

124. Emily Theisen, Jared Bearss, Venkataswamy Sorna, David Bearss, Sunil Sharma. Targeted inhibition of LSD1 in castration-resistant prostate cancer. [abstract]. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2013;73(8 Suppl):Abstract nr 1003. doi:10.1158/1538-7445.AM2013-1003

125. Ellinger J, Kahl P, von der Gathen J, Rogenhofer S, Heukamp LC, Gütgemann I, Walter B, Hofstädter F, Büttner R, Müller SC, Bastian PJ, von Ruecker A (2010) Global levels of histone modifications predict prostate cancer recurrence. Prostate 70(1):61-9.

126. Suikki HE, Kujala PM, Tammela TL, van Weerden WM, Vessella RL, Visakorpi T (2010) Genetic alterations and changes in expression of histone demethylases in prostate cancer. Prostate 70(8):889-98.

127. Lihong Zhang, Jinling Hou, Andreana Holowatyj, Zeng-Quan Yang. The role of histone demethylase GASC1 in promoting prostate cancer progression. [abstract]. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2013;73(8 Suppl):Abstract nr 2966. doi:10.1158/1538-7445.AM2013-2966

128. Su Y, Xu H, Xu Y, Yu J, Xian Y, Luo Q (2012) Azacytidine inhibits the proliferation of human promyelocytic leukemia cells (HL60) by demethylation of MGMT, DAPK and p16 genes. Hematology 17(1):41-6.

129. Kiziltepe T, Hideshima T, Catley L, Raje N, Yasui H, Shiraishi N, Okawa Y, Ikeda H, Vallet S, Pozzi S, Ishitsuka K, Ocio EM, Chauhan D, Anderson KC (2007) 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Mol Cancer Ther. 6(6):1718-27.

130. Zhu WG, Otterson GA (2003) The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. Curr Med Chem Anticancer Agents 3(3):187-99.

131. Silverman LR, Verma A, Odchimar-Reissing R, Feldman EJ, Navada SC, Demakos EP, Baer MR, Najfeld V, Sparano JA, Piekarz R (2013) A Phase II Trial Of Epigenetic Modulators Vorinostat In

Combination With Azacitidine (azaC) In Patients With The Myelodysplastic Syndrome (MDS): Initial Results Of Study 6898 Of The New York Cancer Consortium. Blood 122(21).

132. Crea F, Hurt EM, Mathews LA, Cabarcas SM, Sun L, Marquez VE, Danesi R, Farrar WL (2011) Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer. Mol Cancer 10:40.

133. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, Liu Y, Graves AP, Della Pietra A 3rd, Diaz E, LaFrance LV, Mellinger M, Duquenne C, Tian X, Kruger RG, McHugh CF, Brandt M, Miller WH, Dhanak D, Verma SK, Tummino PJ, Creasy CL (2012) EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature 492(7427):108-12.

134. Crea F, Fornaro L, Bocci G, Sun L, Farrar WL, Falcone A, Danesi R (2012) EZH2 inhibition: targeting the crossroad of tumor invasion and angiogenesis. Cancer Metastasis Rev. 31(3-4):753-61.

135. Labbé RM, Holowatyj A, Yang ZQ (2013) Histone lysine demethylase (KDM) subfamily 4: structures, functions and therapeutic potential. Am J Transl Res. 6(1):1-15.

136. Wang L, Chang J, Varghese D, Dellinger M, Kumar S, Best AM, Ruiz J, Bruick R, Peña-Llopis S, Xu J, Babinski DJ, Frantz DE, Brekken RA, Quinn AM, Simeonov A, Easmon J, Martinez ED (2013) A small molecule modulates Jumonji histone demethylase activity and selectively inhibits cancer growth. Nat Commun. 4:2035.

137. Heanue TA, Pachnis V (2006) Expression profiling the developing mammalian enteric nervous system identifies marker and candidate Hirschsprung disease genes. Proc Natl Acad Sci U S A. 103(18):6919-24.

138. Xiao-Yan Zhao, Schneider D, Biroc S, Parry R, Alicke B, Toy P, Xuan J, Sakamoto C, Wada K, Schulze M, Müller-Tiemann B, Parr G, Dinter H (2005) Targeting tomoregulin for radioimmunotherapy of prostate cancer. Cancer Research 65(7):2846-53.

139. Afar DE, Bhaskar V, Ibsen E, Breinberg D, Henshall SM, Kench JG, Drobnjak M, Powers R, Wong M, Evangelista F, O'Hara C, Powers D, DuBridge RB, Caras I,Winter R, Anderson T, Solvason N, Stricker PD, Cordon-Cardo C, Scher HI, Grygiel JJ, Sutherland RL, Murray R, Ramakrishnan V, Law DA (2004) Preclinical validation of anti-TMEFF2-auristatin E-conjugated antibodies in the treatment of prostate cancer. Mol Cancer Ther 3(8):921-32.

140. Glynne-Jones E, Harper ME, Seery LT, James R, Anglin I, Morgan HE, Taylor KM, Gee JM, Nicholson RI (2001) TENB2, a proteoglycan identified in prostate cancer that is associated with disease progression and androgen independence. Int J Cancer 94(2):178-84.

141. Gery S, Sawyers CL, Agus DB, Said JW, Koeffler HP (2002) TMEFF2 is an androgen-regulated gene exhibiting antiproliferative effects in prostate cancer cells. Oncogene 21: 4739–4746.

142. Overcash RF, Chappell VA, Green T, Geyer CB, Asch AS, Ruiz-Echevarría MJ (2013) Androgen signaling promotes translation of TMEFF2 in prostate cancer cells via phosphorylation of the α subunit of the translation initiation factor 2. PLoS One 8(2).

143. Chen X, Overcash R, Green T, Hoffman D, Asch AS, et al. (2011) The tumor suppressor activity of the transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) correlates with its ability to modulate sarcosine levels. J Biol Chem 286: 16091–16100

144. Green T, Chen X, Ryan S, Asch A, and Ruiz-Echevarria MJ (2013) TMEFF2 and SARDH cooperate to modulate one carbon metabolism and invasion of prostate cancer cells. Prostate 73(14):1561-75.

145. Elahi A, Zhang L, Yeatman TJ, Gery S, Sebti S, Shibata D (2008) HPP1-mediated tumor suppression requires activation of STAT1 pathways. Int J Cancer 122(7):1567-72.

146. Zhao BJ, Tan SN, Cui Y, Sun DG, Ma X (2008) Aberrant promoter methylation of the TPEF gene in esophageal squamous cell carcinoma. Dis Esophagus 21(7):582-8.

147. Brücher BL, Geddert H, Langner C, Höfler H, Fink U, Siewert JR, Sarbia M (2006) Hypermethylation of hMLH1, HPP1, p14(ARF), p16(INK4A) and APC in primary adenocarcinomas of the small bowel. nt J Cancer 119(6):1298-302.

148. Chen X, Corbin JM, Tipton GJ, Yang LV, Asch AS, Ruiz-Echevarría MJ (2014) The TMEFF2 tumor suppressor modulates integrin expression, RhoA activation and migration of prostate cancer cells. Biochim Biophys Acta. 1843(6):1216-24.

149. Sun T, Du W, Xiong H, Yu YN, Weng Y, Ren L, Zhao H, Wang Y, Chen Y, Xu J, Xiang Y, Qin W, Cao W, Zou W, Chen H, Hong J, Fang J (2014) TMEFF2 deregulation contributes to gastric carcinogenesis and indicates poor survival outcome. Clin Cancer Res. pii: clincanres.0315.2014.

150. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, Nyati MK, Ahsan A, Kalyana-Sundaram S, Han B, Cao X, Byun J, Omenn GS, Ghosh D, Pennathur S, Alexander DC, Berger A, Shuster JR, Wei JT, Varambally S, Beecher C, Chinnaiyan AM (2009) Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature 457(7231):910-4.

151. Thalmann GN1, Sikes RA, Wu TT, Degeorges A, Chang SM, Ozen M, Pathak S, Chung LW (2000) LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. Prostate 44(2):91-103.

152. Kim TD, Shin S, Janknecht R (2008) Repression of Smad3 activity by histone demethylase SMCX/JARID1C. Biochem Biophys Res Commun. 366(2):563-7.

153. Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD (2007) JMJD3 is a histone H3K27 demethylase. Cell Res. 17(10):850-7.

154. Ali N, Knaüper V (2007) Phorbol ester-induced shedding of the prostate cancer marker transmembrane protein with epidermal growth factor and two follistatin motifs 2 is mediated by the disintegrin and metalloproteinase-17. J Biol Chem. 282(52):37378-88.

155. Bok RA, Hansell EJ, Nguyen TP, Greenberg NM, McKerrow JH, Shuman MA (2003) Patterns of protease production during prostate cancer progression: proteomic evidence for cascades in a transgenic model. Prostate Cancer Prostatic Dis. 6(4):272-80.

156. Kiyomiya K, Lee MS, Tseng IC, Zuo H, Barndt RJ, Johnson MD, Dickson RB, Lin CY (2006) Matriptase activation and shedding with HAI-1 is induced by steroid sex hormones in human prostate cancer cells, but not in breast cancer cells. Am J Physiol Cell Physiol. 291(1):C40-9.