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DNA Hypermethylation in Prostate Cancer

ACADEMIC DISSERTATION
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YHTEENVETO

Eturauhassyövän syntyyn ja syövän etenemiseen liittyvät merkittävät molekyylitason muutokset tunnetaan vain osittain. Useita eturauhassyövässä toistuvia geneettisiä ja epigeneettisiä muutoksia on löydetty, mutta niiden avulla voidaan selittää vain osa syövistä. Siksi onkin oletettavaa, että vielä tunnistamattomilla syöpä- ja kasvurajoitegeeneillä olisi merkittävä rooli eturauhassyövässä. Viimeisen vuosikymmenen aikana erityisesti epigeneettisiä muutoksia kuvaavien tutkimusten määrä on kasvanut valtavasti, ja geenien ilmentymisen säätelyyn vaikuttavien kromatiinitason muutosten ymmärtäminen on lisääntynyt merkittävästi. Tässä väitöskirjassa pyrittiin löytämään geenejä, joiden ilmentyminen eturauhassyöpäsolulinjoissa ja kliinisessä eturauhassyövässä on hiljennetty epigeneettisesti. Lisäksi eturauhassyöpätutkimuksessa paljon käytetystä *in vivo*-mallista (TRAMP, transgenic adenocarcinoma of mouse prostate) johdettu solulinja, TRAMP-C2, karakterisoitiin sekä geneettisesti että epigeneettisesti.

TRAMP-C2-solulinjasta löytyi vain harvoja DNA kopiolukumuutoksia, ja nämä vastasivat vain muutamaa usein kliinisistä eturauhassyövistä löydettyä ihmisen vastaavilla kromosomialueilla sijaitsevaa muutosta. Eturauhassyövässä yleisten 8q11.21 ja 8q24.13-q24-3 -alueiden monistumien ja 6q14.1-14-3 ja 10q26.11-q26.3 -alueiden häviämien löytyminen myös TRAMP-C2-solulinjasta vahvisti näiden alueiden jo ennestään tunnettua merkitystä eturauhassyövässä. TRAMP-C2-solulinjan geenien kopioluku ja geenien ilmentyminen korreloivat merkittävästi.

Epigeneettisen karakterisoinnin tuloksena tunnistettiin clusterin-geeni (*Clu*), joka ilmentyi erittäin matalasti ja joka oli metyloitunut TRAMP-C2 soluissa. Tutkituissa ihmisen eturauhassyöpäsolulinjoissa *CLU* ilmentyi vähiten LNCaP-solulinjassa, jossa geenin promoottorialue oli merkittävästi metyloitunut. Kliinisissä kasvainnäytteissä *CLU*-geenin ilmentyminen oli merkittävästi vähäisempää syövässä verrattuna eturauhasen hyvänlaatuiseen liikakasvuun. Nämä tulokset yhdessä aiemman tiedon kanssa clusterinin tehtävästä eturauhassyövässä tukevat ajatusta, jonka mukaan *CLU* olisi kasvurajoitegeeni eturauhassyövässä ja sen ilmentymistä säädeltäisiin epigeneettisesti.

Dual specificity phosphatase 1 (*DUSP1*)- ja serum/glucocorticoid regulated kinase 1 (*SGK1*)-geenien ilmentyminen kasvoi merkittävästi PC-3 eturauhassyöpäsolulinjassa, kun soluja altistettiin kemikaaleille, jotka estävät epigeneettisiä modifikaatioita. Kummankaan geenin promoottorialueelta ei kuitenkaan löydetty metylaatiota näissä soluissa. Molempien geenien ilmentyminen väheni merkittävästi sekä lähetti-RNA- että proteiinitasolla verrattaessa syöpänäytteitä eturauhasen hyvänlaatuiseen liikakasvuun. Julkaisemattomien tulosten perusteella *DUSP1*- tai *SGK1*-geenin yli-ilmentäminen ei kuitenkaan muuttanut PC-3-solujen ilmiasua, joten näiden geenien mahdollinen tehtävä kasvurajoitegeeninä eturauhassyövässä ei ole täysin selvä.

Proteiineja koodaavien geenien lisäksi myös mikro-RNA-geenien ilmentymistä voidaan säädellä epigeneettisesti. *miR-193b*-geenin ilmentymisen osoitettiin olevan hiljennetty metylaation avulla 22Rv1-eturauhassyöpäsolulinjassa, ja myös kliinisissä syöpänäytteissä *miR-193b*:n ilmentyminen oli merkittävästi vähäisempää kuin kasvaimen viereisessä normaalikudoksessa. Kun *miR-193b*:n toimintaa kasvurajoitetekijänä tutkittiin 22rv1-soluissa, sen yli-ilmeneminen johti solukasvun hidastumiseen, vähensi aktiivisesti jakautuvien solujen määrää solusyklissä ja heikensi näiden solujen kykyä kasvaa ilman pohjaan kiinnittymistä. Näiden tulosten perusteella *miR-193b* toimii epigeneettisesti säädeltynä kasvurajoitegeeninä eturauhassyövässä.

Tässä väitöskirjatyössä tunnistettiin useita geenejä, joiden ilmentyminen kasvoi eri eturauhassyöpämalleissa, kun näiden solulinjojen epigeneettisiä hiljentämismekanismeja estettiin kemiallisesti. Näistä geeneistä joidenkin ilmentyminen oli myös merkittävästi vähäisempää kliinisissä kasvainnäytteissä kuin hyvänlaatuisessa eturauhaskudoksessa. *CLU*- ja *miR-193b*-geenien osoitettiin olevan myös metyloituneita näissä eturauhassyöpämalleissa. Jo olemassa oleva tieto yhdessä tässä työssä esitettyjen tulosten kanssa tukee ajatusta *CLU*- ja *miR-193b*-geenien toiminnasta kasvurajoitegeeneinä eturauhassyövässä.

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LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following communications, which are referred to in the text by their Roman numerals. In addition, unpublished data is included:

I Rauhala HE, Porkka KP, Tolonen TT, Martikainen PM, Tammela TL, Visakorpi T. Dual-specificity phosphatase 1 and serum/glucocorticoid-regulated kinase are downregulated in prostate cancer. Int J Cancer 2005;117:738-45.

II Rauhala HE, Porkka KP, Saramäki OR, Tammela TL, Visakorpi T. Clusterin is epigenetically regulated in prostate cancer. Int J Cancer 2008;123:1601-9.

III Rauhala HE, Jalava SE, Isotalo J, Bracken H, Tammela TL, Oja H, Visakorpi T. *miR-193b* is an epigenetically regulated putative tumor suppressor in prostate cancer. 2009. Submitted for publication.

ABBREVIATIONS

5azadC	5-aza-2'-deoxycytidine
ABCB1	ATP-binding cassette, sub-family B, member 1
aCGH	array comparative genomic hybridization
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
AR	androgen receptor
ASF1	antisilencing function 1
BPH	benign prostate hyperplasia
CAF-1	chromatin assembly factor 1
CBP	CREB binding protein
CDH1	cadherin 1
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CENP-A	centromere protein A
CGI	CpG island
ChIP	chromatin immunoprecipitation
CIMP	CpG island methylator phenotype
CLU	clusterin
CpG	cytidine-guanidine dinucleotide
CREB	cAMP responsive element binding protein 1
CRPC	castration-resistant prostate cancer
DCN	decorin
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	double-stranded breakage
DUSP1	dual specificity phosphatase 1
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)
ERK	extra-cellular regulated kinase
ES cell	embryonic stem cell
EZH2	enhancer of zeste homolog 2
FDA	Food and Drug Administration
GFP	green fluorescent protein
GSTP1	glutathione S-transferase pi 1
H1/2A/2B/3/4	histone 1 /2A /2B /3 /4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HGPIN	high-grade prostatic intraepithelial neoplasia
HMT	histone methyltransferase
HNPCC	hereditary non-polyposis colorectal cancer
HOX	homeobox

HP1	heterochromatin protein 1
IGF2	insulin-like growth factor 2
IHC	immunohistochemistry
Kac	lysine acetylation
Kme	lysine methylation
LINE	long interspersed nuclear element
LOH	loss of heterozygosity
LSD1	lysine K-specific demethylase
MAGE	melanoma antigen gene
MAPK	mitogen-activated protein kinase
MBD1	methyl CpG-binding domain protein 1
meC	methylated cytidine
MECP2	methylated CpG binding protein 2
Min	multiple intestinal neoplasia
miRNA	micro-RNA
MLH1	mutL homologue 1
mRNA	messenger RNA
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
PCa	prostate cancer
PcG	polycomb group
PCR	polymerase chain reaction
PRC	polycomb repressive complex
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RASSF1	Ras association (RalGDS/AF-6) domain family member 1
RITS	RNA-induced transcriptional silencing
RNA	ribonucleic acid
SAM	s-adenosylmethionine
SGK1	serum/glucocorticoid regulated kinase 1
siRNA	small interfering RNA
SIRT	sirtuin
SSH	suppression subtractive hybridization
S/Tph	serine/threonine phosphorylation
TBP	TATA-binding protein
TERT	telomerase reverse transcriptase
TMPRSS2	transmembrane protease, serine 2
TP53	tumor protein 53
TRAMP	transgenic adenocarcinoma of mouse prostate
TSA	trichostatin A
TSG	tumor suppressor gene
TSS	transcription start site
TURP	transurethral resections of prostate
UTR	untranslated region

ABSTRACT

The molecular alterations initially leading to prostate cancer and subsequently allowing the cancer to progress to a castration-resistant, lethal disease are not comprehensively understood. While several recurrent genetic and epigenetic aberrations have been described, it is reasonable to assume that more genes with tumor suppressor or oncogenic properties in prostate cancer have yet to be found. Over the last decade, the number of studies on cancer epigenetics has exploded, and our understanding of the highly complex nature of gene expression regulation through various chromatin modifications has expanded. The present study aimed to identify genes that are epigenetically silenced in prostate cancer cell lines and in clinical prostate cancer, and to genetically and epigenetically characterize the TRAMP-C2 cell line (derived from a widely used *in vivo* prostate cancer model, transgenic adenocarcinoma of mouse prostate, TRAMP).

A relatively low number of genomic aberrations were found in the TRAMP-C2 cell line, and these alterations matched only a few homologous human regions commonly found to be lost or gained in clinical prostate cancers. However, the presence of 8q11.21 and 8q24.13-q24.3 gains and 6q14.1-q14.3 and 10q26.11-q26.3 losses in the mouse model confirms the significance of these regions to prostate cancer. The TRAMP-C2 gene copy number and gene expression levels were highly correlated.

From the epigenetic profiling of TRAMP-C2, clusterin (*Clu*) was identified as a methylated and lowly expressed gene in these cells. Of the studied human prostate cancer cell lines, LNCaP had the lowest expression levels with marked methylation at the *CLU* promoter. In clinical samples of prostate cancer and benign prostate hyperplasia (BPH), *CLU* expression was significantly lower in cancer compared to BPH samples. Together with the known data on clusterin, our results support the idea that clusterin is a tumor suppressor gene in prostate cancer with possible epigenetic modifications involved in its regulation.

In the PC-3 prostate cancer cell line, dual specificity phosphatase 1 (*DUSP1*) and serum/glucocorticoid regulated kinase 1 (*SGK1*) were identified as genes with increased expression when these cells were treated with pharmacological agents reversing epigenetic silencing modifications. While no methylation was detected at the promoters of these genes, their expression was significantly decreased in clinical cancer samples compared to BPH, both at the mRNA and at protein levels. According to our unpublished data, *DUSP1* or *SGK1* overexpression in PC-3 cells did not significantly alter the phenotype of these cells; thus, the tumor suppressor role of these genes remains uncertain.

In addition to protein coding genes, epigenetic regulation of micro-RNAs (miRNAs) in prostate cancer was addressed. *miR-193b* was shown to be silenced through methylation in the 22Rv1 prostate cancer cell line, and moreover, *miR-193b* expression was decreased significantly in cancer samples as compared to normal

tissue adjacent to tumor tissue. Our functional studies on 22Rv1 cells with transient overexpression of *miR-193b* clearly demonstrated the tumor suppressor properties of this miRNA, as these cells had a lower proliferation rate, significantly reduced ability to grow anchorage-independently, and fewer cells entering the S-phase of the cell cycle than in the control cells. All of these data indicate that *miR-193b* is an epigenetically silenced tumor suppressor gene in prostate cancer.

In conclusion, in the work presented here, several genes were identified with increased expression in various prostate cancer models when treated with pharmacological agents reversing epigenetic modifications. Some of these genes had decreased expression levels in cancerous compared to benign prostate tissues, and two of them (*i.e. CLU* and *miR-193b*) were also methylated in the prostate cancer models. Data from the literature and our functional studies further support the tumor suppressor role of these two genes in prostate cancer.

INTRODUCTION

Cancer is caused by the accumulation of genetic and epigenetic changes into cells that render them self-sufficient on growth signals, allow them to replicate limitlessly and to invade into neighboring tissues (reviewed by Hanahan and Weinberg, 2000). These acquired or inherited changes target two major classes of genes involved in cancer, tumor suppressor genes and oncogenes. Tumor suppressors act normally as a safeguard mechanism of the cells to help retain genomic integrity. Their altered or lost function allows genomic abnormalities to persist and run cellular transformation. Oncogenes are present in normal cells as proto-oncogenes with important function *e.g.* in signal transduction and cell cycle regulation. Activating mutations or increased expression of these proto-oncogenes can turn them into oncogenes that excessively promote cellular growth and survival. Accumulation of several of these genetic and epigenetic changes eventually leads to the transformation of these cells into cancer cells.

Prostate cancer is the most common malignancy among men in Western countries. Its etiology is not fully understood, but established risk factors include family history, androgens, race and age (reviewed in Grönberg *et al.* 2003). Age is one of the most notable risk factors because prostate cancer incidence increases with age, similar to many other cancer types. Hereditary factors are known to significantly affect the risk of prostate cancer, with as much as 42% of the risk of prostate cancer suggested to be explained by hereditary components (Lichtenstein *et al.* 2000). However, no single high-penetrant predisposing gene has been identified, concordant with the highly heterogeneous nature of prostate cancer. Race is also known to contribute to prostate cancer risk, with men of African ancestry having a higher risk of developing prostate cancer than Caucasians, and their disease is generally associating with poorer prognosis (Reddy *et al.* 2003, Evans *et al.* 2008). Additionally, already almost 70 years ago, prostate cells were shown to be dependent on androgen stimulus for their growth and differentiation, and androgen withdrawal was demonstrated as an effective treatment for prostate cancer (Huggins and Hodges 1941). More recently, finasteride, an inhibitor of 5α-reductase type 2, (an enzyme involved in androgen metabolism) has been shown to be effective in prostate cancer prevention, decreasing the risk of prostate cancer by almost 30% (Redman *et al.* 2008). Finally, dietary factors, such as increased intake of animal fats and depletion of vitamin D, have been associated with prostate cancer; however, their role is still speculative (Grönberg *et al.* 2003).

In 2007, 4189 new prostate cancer cases were diagnosed in Finland (~32% of all diagnosed male cancers in 2007), while prostate cancer deaths accounted for less than 14% of all male cancer deaths (Finnish Cancer Registry, www.syoparekisteri.fi). As the mean age of onset for prostate cancer is high (72 years), and in most cases prostate cancer progresses fairly slowly, most of the patients die from other causes than the cancer itself. However, approximately one

third of prostate cancer patients eventually die from the cancer. Currently, prostatectomy and radical radiation therapy are the only curative treatments for the disease, and they are only applicable if the cancer is detected early enough. For advanced prostate cancers, no curative treatment options are available. The first results on prostate cancer mortality from a randomized prostate-specific antigen (PSA) screening trial were published very recently (Schröder *et al.* 2009). This multicenter cohort study showed that while PSA testing can significantly decrease prostate cancer mortality (by 20%), over-diagnosis is still a large problem. To prevent one prostate cancer death, 1410 men would have to be screened, and an additional 48 men would have to be treated. These results clearly demonstrate the need for novel, specific biomarkers to be used in the clinical management of prostate cancer.

Therefore, the major task in prostate cancer research is to find markers suitable 1) for diagnostic use to efficiently and accurately detect prostate cancer, 2) for prognostic use to identify patients with cancer that can be left untreated or should be treated, and 3) for predictive use to identify which patients are most likely to benefit from each available treatment. In addition to finding markers, it is believed that a molecular understanding of prostate cancer will offer new treatment options for the castration-resistant and metastatic diseases.

As the traditional genetic approaches based on mutational, gene copy number and expression analyses to decipher prostate cancer have failed to fully uncover prostate cancer tumorigenesis, additional mechanisms must be present. One such possibility is an epigenetic mechanism. Indeed, over the past few years, several genes have been identified that are epigenetically modified, and the components of the epigenetic pathway itself are altered in prostate cancer, thus contributing to prostate cancer. Additionally, some of these epigenetic marks have shown potential as diagnostic and prognostic markers in early studies. Therefore, epigenetic information on prostate cancer may prove to be very useful in understanding the molecular essence of the disease, as well as to offer novel diagnostic, prognostic and predictive tools for clinicians.

REVIEW OF THE LITERATURE

1. Epigenetics

The term epigenetics has been defined in several ways over the years. Currently, the most widely used definition phrases epigenetics as “the heritable changes in phenotype or gene expression not caused by the alterations in the underlying DNA sequence” (Probst *et al.* 2009). The word epigenetics originates from the Greek *epi* meaning above, on top; thus epigenetics means ‘on top of genetics’ (*i.e.*, the additional information provided with the DNA sequence itself). The early use of the term epigenetics combined genetics with epigenesis, referring to the developmental processes in embryogenesis where totipotent cells are programmed to differentiate into all different cell types regardless of their identical DNA content (Waddington 1942). Both of these definitions, although markedly different, ultimately contain the main idea of epigenetics: how genetically identical cells can behave differentially in time and space. Heritability stands for epigenetic modifications being inherited mitotically by daughter cells and/or meiotically by the next generations. However, quite recently, Bird (2007) suggested that the definition of epigenetics should comprise all chromatin and DNA modifications regardless of their heritability as in “the structural adaptation of chromosomal regions so as to register, signal and perpetuate altered activity states”. In this way, transient chromatin marks would also be accounted as epigenetic.

1.1 Chromatin

Chromatin is the higher-order structure found in the nucleus of eukaryotic cells and in the nucleoid of prokaryotic cells containing the genetic material of the cell. It serves two main functions: 1) it allows a huge amount of genetic information to be packed into an extremely condensed format to fit into the cell nucleus and more over, to be duplicated and split in a regulated manner during mitosis or meiosis; and 2) it provides information about gene expression regulation, DNA repair and replication in addition to the core information dictated by the DNA sequence itself.

The basic chromatin unit is a nucleosome, a hetero-octamer of core histone proteins with DNA tightly wrapped around it. One histone-octamer contains two H3-H4 histone dimers facing each other with two H2A-H2B histone dimers on either side (Luger *et al.* 1997). The arrangement of nucleosomes is best described as “beads on a string”, referring to the organized setting of nucleosomes along the DNA. In the structural hierarchy of chromatin, this is followed by a so-called 30 nm fiber in which the chain of nucleosomes folds into a condensed helix. The H1 histone binds DNA at both ends of the nucleosome, marking DNA entry and exit

points, and is particularly important in stabilizing the 30 nm fiber (Thoma *et al.* 1979, Belikov and Karpov 1998). Further compaction is achieved as different chromatin scaffold proteins bind the chromatin. In its most compact format DNA can be seen during cell division, when the condensed metaphase chromosomes can be visualized in their characteristic X-shape. The packaging of DNA is graphically illustrated in Figure 1.

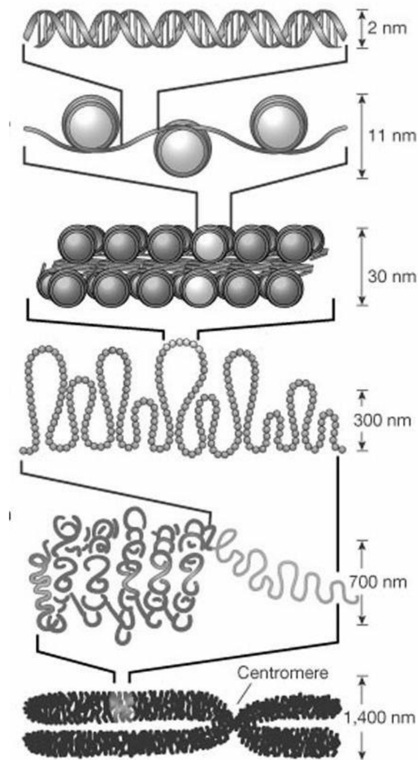

Figure 1*. Schematic representation of DNA packaging and chromatin structure. (Adapted by permission from Macmillan Publishers Ltd: Nature. Felsenfeld G, Groudine M, Controlling the double helix. 421:448-53, copyright (2003)).*

Based on the functional activity of chromatin, it can be roughly divided into two stages: the more loosely packed euchromatin, descriptive of the actively transcribed genomic regions; and the tightly packed heterochromatin, descriptive of centromeric and untranscribed chromosome regions (reviewed in Jenuwein and Allis 2001). Euchromatin resides mainly in the "beads on a string" conformation that can be further opened and depleted of nucleosomes upon transcriptional activation. Heterochromatin can be constitutive in areas where long-term silencing is needed, including regions harboring genes whose expression is specific for a certain developmental process or at gene-poor areas with a high content of repetitive sequences (*e.g.,* centromeres and telomeres, which are needed for maintaining chromosomal integrity during cell division). Heterochromatin protein 1 (HP1) binding to chromatin is fundamental to the proper packaging of chromatin, and it decorates constitutive heterochromatin widely (Wreggett *et al.* 1994). Facultative heterochromatin, in turn, describes areas of chromatin used differentially in a time- and space-dependent manner, *i.e.*, being able to adapt heterochromatin structures

and functions in cells where the chromosomal area is inactive while being actively transcribed in another setting with a euchromatin structure. X-inactivation, a process of silencing one of the two copies of the X-chromosome in females to achieve dosage compensation, is the most prominent example of facultative heterochromatin (Heard *et al.* 2001, Plath *et al.* 2003).

1.2 Histones

At the heart of chromatin structure are histones with their N-terminal tails protruding outside of the core complex of a nucleosome. In the early days of chromatin research, histones were regarded merely as inactive structural components of chromatin with the sole purpose of providing a scaffold for DNA to wrap around. However, it is now clearly evident that histones play as fundamental part in transcriptional regulation as does DNA itself, and they are crucially important in various other nuclear DNA-dependent functions (*e.g.*, DNA replication, repair, recombination and chromosome segregation). A clear demonstration of the central role of histones comes from conservation studies showing that all four core histones (H2A, H2B, H3 and H4) are highly conserved between eukaryotic species, and the ancestors of these proteins can be traced to archaea (Malik and Henikoff 2003).

The histone octamer is formed by the local structural function of histones, *i.e.*, the interactions between the core domains of the histones. Recent evidence has shown that histone tails do not have a specific function in the structural organization of the core nucleosomes, but instead they have a role in dictating the higher-order chromatin structure (Wang and Hayes 2008, Kan *et al.* 2009). Histone N-terminal tails are rich in basic amino acids, such as lysine and arginine, offering a base for a distinct set of modifications to take place on these residues. The importance of these tail modifications to cellular function was clearly demonstrated by a mutational analysis that changed the H4 tail lysine residues to arginines, leading to a lethal phenotype in yeast (Megee *et al.* 1990). The majority of the histone modifications are found on the H3 and H4 tails, but the short H2A and H2B tails can also be modified. The wide spectrum of post-translational modifications (PTMs) include lysine acetylation (Kac), lysine and arginine methylation (mono-, di- and trimethylation, K/Rme1,2,3), serine and threonine phosphorylation (S/Tph), ADP-ribosylation, lysine ubiquitination (Kub), and lysine sumoylation (Ksu). Some of these modifications are depicted in Figure 2. These PTMs, in turn, offer or block landing places for a variety of effector proteins to bind and further enhance the original message encoded by the tail modifications. For example, nearly all histone acetyltransferase (HAT) coactivators, and several of the HATs themselves, contain a bromodomain that is needed for their binding to the acetylated histone tails (Candau *et al.* 1996, Dhalluin *et al.* 1999). Similarly, chromodomain-containing effectors, such as Polycomb (Pc) and HP1, associate with methylated lysine residues potentiating their message (Lachner *et al.* 2001, Bannister *et al.* 2001, Fischle *et al.* 2003).

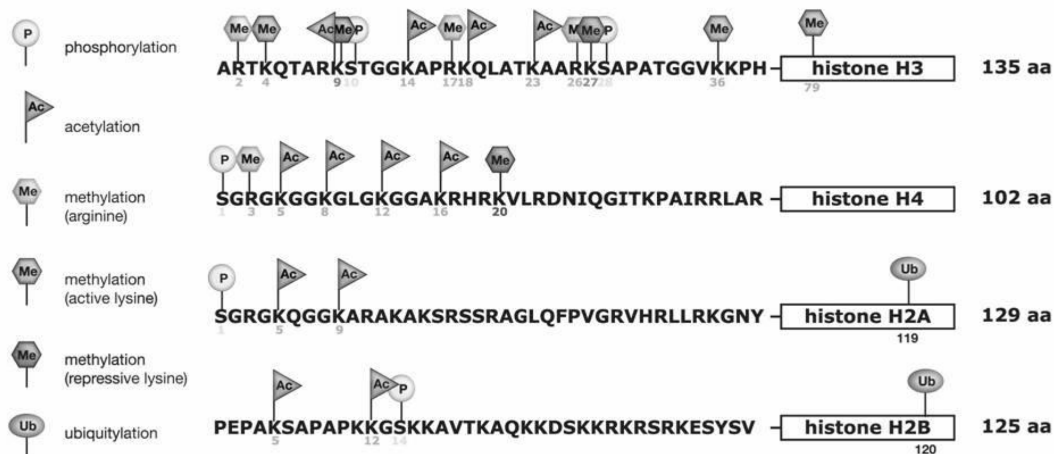

Figure 2. *Common sites for histone modifications. (Reprinted by permission from Cold Spring Harbor Laboratory Press: Allis, Jenuwein, Reinberg, 2007).*

1.2.1 Histone acetylation, methylation and phosphorylation

Lysine acetylation is by far the most well known and best-studied post-translational histone modification. In its widest definition, histone acetylation is associated with active transcription and euchromatin, whereas deacetylated histones mark condensed transcriptionally silenced heterochromatin. Acetylation removes the positive charge from lysine residues, thus diminishing the interaction between the histone tails and the negatively charged DNA backbone, making the DNA more available and accessible for active transcription (Sealy and Chalkey 1978, Wang *et al.* 2001c). However, apart from weakening the nucleosome-DNA interactions, the likely more important role of acetylation (and indeed other histone PTMs as well) is the recruitment of other non-histone proteins to the chromatin.

H3 can be acetylated at several lysine residues, *e.g.*, K9, K14, K18 and K23. H3K9ac and H3K14ac are among the most common marks of transcriptionally active chromatin in most species, while the most common acetylation sites in H4 (H4K5 and H4K12) are acetylated in newly synthesized histones and target these histones for nucleosomal assembly (Sobel *et al.* 1995). Other lysine residues commonly acetylated on H4 are K8 and K16 signaling active transcription (Clarke *et al.* 1993, O'Neill and Turner 1995, Allis *et al.* 2007).

In contrast to acetylation, where the presence of the mark normally dictates the output in terms of transcriptional activation or inactivation, the effect of histone methylation is more complex. First, both lysine and arginine residues can be methylated. Second, not only does the mark itself matter, but also its extent, as the methylation can be mono-, di- or trivalent. Third, the same mark, *e.g.*, H3K4 dimethylation (H3K4me2) or H3K9 trimethylation (H3K9me3), can signal both activation and repression depending on the context (Santos-Rosa *et al.* 2002, Vakoc *et al.* 2005). Histone methylation mainly takes place at H3 and H4, however, some extent of methylation is also observed on H1 (Byvoet *et al.* 1986). H3 can be

methylated at several lysine and arginine residues (*e.g.*, K4, K9, R17, K27, K36 and K79), but H4 is mainly methylated on R3 and K20 (Allfrey and Mirsky 1964, DeLange *et al.* 1970, Sarnow *et al.* 1981, Schurter *et al.* 2001, Wang *et al.* 2001a, Strahl *et al.* 2002).

H3K4me3 is a well-established mark of the 5' end of active genes (Schneider *et al.* 2004). A recent study (combining chromatin immunoprecipitation (ChIP) with third generation sequencing system) to assess histone methylation at the genome-wide level showed that as H3K4 mono-, di- and trimethylation all associate with active genes, H3K4me3 peaks closest to the transcription start site (TSS), while mono- and dimethylation seem to mark active genes further downstream from the TSS and associate more with the median than the highest expressing genes (Barski *et al.* 2007). H3K36me3 is a mark shown to associate with transcriptional elongation, as it is observed throughout the length of active genes (Bannister *et al.* 2005, Vakoc *et al.* 2006, Mikkelsen *et al.* 2007). Indeed, Mikkelsen *et al.* (2007) showed that the combination of H3K4me3 and H3K36me3 marks allows the study of alternative promoter usage, with H3K4me3 indicating alternative TSSs along the H3K36me3-marked transcript.

Trimethylation of H3K27 is probably the most prominent mark of repressed chromatin and is found, for example, at the inactivated X chromosomes in females (Cao *et al.* 2002, Czermin *et al.* 2002, Plath *et al.* 2003). H3K9me3 is a descriptive modification of pericentromeric transcriptionally inert heterochromatin (Sewalt *et al.* 2002), and is needed for the induction of another pericentric silencer mark, H4K20me3, known to associate also with aging (Sarg *et al.* 2002, Schotta *et al.* 2004). All of these trimethylation marks (H3K9me3, H3K27me3 and H4K20me3) are significantly enriched across a variety of different repetitive sequences, thus marking them for silencing (Martens *et al.* 2005). However, in its monovalently methylated form, H3K27 methylation associates with active genes (Vakoc *et al.* 2006, Barski *et al.* 2007). In addition to H3K27me1, H3K9 and H4K20 are also associated with active genes when monovalently methylated (Vakoc *et al.* 2006, Barski *et al.* 2007). H3K9 methylation is also needed for HP1 recruitment through its chromodomain; the binding HP1 to the core nucleosome enhances the packaging of chromatin and serves importantly in heterochromatin formation (Bannister *et al.* 2001, Lachner *et al.* 2001, Nakayama *et al.* 2001).

Recently, promoters containing opposing histone marks, *i.e.*, H3K4me3 and H3K27me3, were identified in mouse embryonic stem (ES) cells (Azuara *et al.* 2006, Bernstein *et al.* 2006). The explanation for the somewhat contradictory coexistence of these modifications was offered by "bivalent chromatin", in which both activating and repressing modifications are present. The repressive marks tend to keep this type of chromatin in a silenced state (Mikkelsen *et al.* 2007), but upon removal of the repressive marks, transcription is rapidly initiated. Thus, these bivalent promoters are often said to be poised for transcription as their activity does not need additional activation marks to be brought on, merely the removal of the repressive modifications. These bivalent promoters are particularly found in ES cells where they regulate the expression of important developmental genes, and they have been suggested to provide a novel mechanism for maintaining pluripotency (Bernstein *et al.* 2006, Mikkelsen *et al.* 2007). In addition to developmental genes,

imprinted regions can also be marked by opposing modifications. H3K204me3 and H3K9me3 are often found at methylated imprinted alleles, whereas H3K4 methylation marks the opposing unmethylated allele (Delaval *et al.* 2007). In a genome-wide scan of histone modifications by Mikkelsen *et al.* (2007), bivalent marking with H3K9me3 and H3K4me3 was shown to be a common signature of imprinting control regions in ES cells. Recently, Rodriguez *et al.* (2008) demonstrated how these bivalent promoters appear to carry the memory of epigenetic silencing. They found that promoters with bivalent marks and low expression were frequently methylated in colon cancer cells. Further, while 5-aza-2'-deoxycytidine (5azadC) treatment was able to reverse DNA methylation, the histone marks persisted and were able to re-establish DNA methylation at these promoters after the treatment.

Histone tail residues can also be phosphorylated. Phosphorylation takes place at either serine or threonine residues *via* specific enzymes (*i.e.*, serine/threonine kinases). Histone tail serine phosphorylation, as a prominent mark of condensed mitotic chromatin, was first observed more than 30 years ago (Gurley *et al.* 1973, Paulson and Taylor 1982). One common phosphorylation mark in mitosis is H3S10ph, which associates with heterochromatin, but also contradictorily with transcriptional activity (Barrat *et al.* 1994, Hendzel *et al.* 1997, Thomson *et al.* 1999). A hypothesis on binary methyl/phosphoryl switches suggests that as there are no known effectors binding directly to phosphorylated histone tail residues, the effect of phosphorylation may be routed through inhibition of effector molecules binding to the adjacent methylated or acetylated lysines (Fischle *et al.* 2003b). This is indeed the case with H3S10ph, which causes HP1 dissociation from heterochromatic H3K9me3 (Fischle *et al.* 2005), thus providing a dynamic control for HP1 binding to heterochromatin that does not involve the reversal of the more stable methylation marks. H3S10ph was further shown to also depend on the acetylation status of H3, with HDAC3-mediated deacetylation of H3K9 being necessary for Aurora B kinase to phosphorylate H3S10 (Li *et al.* 2006).

1.2.2 Histone variants

In addition to the four core histones (H2A, H2B, H3 and H4), several variants, mainly of H2A and H3, have been identified. The core histones are derived from multicopy, intronless genes, whereas the variant histones result mainly from single-copy genes residing apart from the core histone gene cluster. This has enabled the evolutionary conservation of the core histones with the concurrent introduction of genetic variation to specific histone functions. Many variants display only single residue changes compared to the parental histone, while others have significantly differing structures. For most of the histone variants, a specific function and location in chromatin has been assigned.

Probably the most-studied histone variant is H2A.Z, which accounts for 5-10% of H2A in a cell and takes part in several cellular functions (*e.g.*, heterochromatin maintenance and transcription) (Palmer *et al.* 1980, Leach *et al.* 2000, Rangasamy *et al.* 2003, Sarcinella *et al.* 2007). Much like the parental histones, the histone

variants also undergo post-translational modifications. In fact, the inactivated X-chromosome, an example of facultative heterochromatin, is decorated with ubiquitinated H2A.Z (Sarcinella *et al.* 2007). In yeast, H2A.Z has also been shown to mark the boundaries of heterochromatin and euchromatin, preventing the spread of heterochromatin to euchromatin (Meneghini *et al.* 2003). The role of H2A.Z in transcriptional activity is controversial, as studies from yeast and higher eukaryotes give contrasting results; even reports within species describe this histone variant at both transcriptionally active and silent chromatin (Adam *et al.* 2001, Guillemette *et al.* 2005). One possible explanation was offered by Millar *et al.* (2006) who showed that Htz1, the yeast homologue of human H2A.Z, was specifically acetylated at K14 when bound to active chromatin at promoters, whereas telomerically distributed Htz1 was devoid of this acetylation, suggesting the acetylation-dependent assembly of H2A.Z to promoters upon gene activation.

Another H2A variant, H2A.X, functions specifically in DNA double-strand break repair (Rogakou *et al.* 1998). It differs from the core histone H2A by its variant C-terminus, and this C-terminus becomes phosphorylated upon H2A.X recruitment to DNA double-stranded break (DSB) sites. Lack of H2A.X in mice increases their genetic instability (Celeste *et al.* 2002). In addition to DSB repair, H2A.X is involved in other processes, such as meiotic recombination and apoptosis (Rogakou *et al.* 2000, Hunter *et al.* 2001).

A common H3 variant, H3.3, differs from the core histone H3 only by few amino acids (Franklin and Zweidler, 1977). H3 is loaded onto chromatin upon replication, and H3.3 rapidly replaces it at regions of actively transcribed chromatin (Ahmad and Henikoff, 2002, Wirbelauer *et al.* 2005). As expected from its localization, H3.3 is highly enriched with lysine tail modifications indicative of active transcription (Kac and H3K4me3) and is deficient of repressive marks, such as H3K9me2 (McKittrick *et al.* 2004). H3.3 occupancy has also been shown to exclude the linker histone H1 from the chromatin, thus contributing to the maintenance of the open chromatin state (Braunschweig *et al.* 2009). Moreover, it was recently shown that H3.3/H2A.Z double variant –containing nucleosomes can occupy the previously "nucleosome-free"-described regions, for example, at the promoters of active genes, and their relative instability allows them to be quickly replaced by transcription factors upon gene activation (Jin *et al.* 2009).

Unlike several other histone variants, centromere protein A (CENP-A) varies markedly from H3, with only ~63% homology between their C-terminal domains and an individual N-terminus (Sullivan *et al.* 1994). As evident from its name, CENP-A is specifically found at the centromeres, and rather surprisingly, it is the C-terminal domain (with homology to H3) that confers the centromere targeting property (Sullivan *et al.* 1994). CENP-A is essential in the recruitment of components needed for kinetochore formation and chromosome segregation, the two key functions of centromeres (Howman *et al.* 2000). Recently, RNA interference (RNAi)-directed heterochromatin flanking the centromeres was demonstrated to be crucial in CENP-A recruitment to centromeres in yeast, but not needed for the maintenance of this centromeric chromatin state once established (Folco *et al.* 2008).

1.2.3 Histone code

All of these histone modifications were originally thought to function by altering histone-DNA interactions and thereby affecting the chromatin structure. However, it has become evident that even more than just affecting DNA-histone interactions, these modifications work in concert to recruit chromatin remodelers and transcription factors to delineate what the chromatin has to tell. The term "histone code" was first introduced in 2000 by Strahl and Allis as a generalized term to be used when referring to these different combinations of histone tail modifications, as read by various effector proteins, and leading to distinct read-outs. Ever since, the essence of the histone code (and the mere existence of such a thing) has been debated. Largely, it has been argued that because histone tails display a large variety of different modifications, in different combinations and in different cellular contexts, it is an overstatement to say that "a histone code" exists. Hake *et al.* (2004) reintroduced the term histone "language", which likely more accurately describes the complex nature of histone tail modifications, leaving enough space for plasticity in the definition.

1.3 DNA methylation

DNA methylation is the second type of epigenetic modification, in addition to histone modifications. This chemical modification is found at the position 5 of the cytosine carbon ring (5meC), most often at CpG dinucleotides but also at CNG repeats. The first human methylome resolved at a base pair resolution showed striking differences in the methylation patterns between ES and differentiated cells (Lister *et al.* 2009). While in the fetal fibroblasts methylation took place only at the CpG dinucleotides, in ES cells almost 25% of all methylation was observed at non-CpG context. The non-CpG methylation was lost during differentiation, but restored again in induced pluripotent stem cells, suggesting that the non-CpG methylation might have an important role in the establishment and maintenance of the pluripotency.

Approximately 70% of mammalian genome CpGs are methylated, but neither the CpG distribution nor the CpG methylation is random (McClelland and Ivarie 1982, Cooper *et al.* 1987, Costello *et al.* 2000). The Human Genome Project (HUGO) identified roughly 50,000 CpG islands (CGI) in the human genome, of which, approximately 60% reside in non-repeat areas of the genome (Lander *et al.* 2001) and cover ~70% of gene promoters (Saxonov *et al.* 2006). An earlier study of CGIs estimated them to account for ~15% of all CpGs in the genome (Antequera and Bird 1993). Thus, the majority of CpGs resides outside of the CGIs and is methylated, in contrast to the mainly unmethylated CGIs (Song *et al.* 2005). A widely used definition for CGI is a genomic region of >500 bp in length, with CG content >55%, and observed CpG/expected CpG > 0.65 (Takai and Jones 2002). As compared to an early, less stringent definition by Gardiner-Garden and Frommer (1987), this new

definition efficiently excludes most Alu sequences, concentrating on promoter-associated CGIs.

Most of the human genome is depleted of CpG dinucleotides due to the intrinsic property of 5meC to undergo spontaneous deamination into thymine (T), as first evidenced in 1978 by the finding of mutational hotspots at 5meC in the *Escherichia coli lacI* gene (Coulondre *et al.* 1978). This spontaneous deamination is a property of unmethylated cytosine as well, but it results in the transition of cytosine to uracil, which is then excised from the DNA by uracil-DNA glycosylase as a DNA-repair mechanism (Lindahl *et al.* 1977, Duncan and Miller 1980). The depletion of CpGs from the genome is accompanied by a concurrent increase in TpGs and CpAs, the transition products of 5meC (McClelland and Ivarie 1982). This deamination and the consequent 5meC→T transition is a global genomic phenomenon, whereas promoter-specific CGIs, mainly unmethylated, remain "safe".

The non-CGI CpG methylation of the genome marks genomic repeats, parasitic regions and retrotransposons, which are in general relatively CG-rich (*e.g.*, endogenous retroviruses and Alu elements) (Yoder *et al.* 1997, Meneveri *et al.* 1993). Repeat elements are prone to undergo homologous recombination, consequences of which can be quite deleterious in humans (Rouyer *et al.* 1987, Puget *et al.* 1997). Thus, these chromosomal regions are preferably embedded in heterochromatin to ensure the integrity of the genome (Taruscio *et al.* 1991, Yoder *et al.* 1997, Junakovic *et al.* 1998). Indeed, there are several lines of evidence supporting the role of DNA methylation as a "genomic safeguard". In yeast, it has been shown that methylation of a certain meiotic recombination hot-spot directly reduces the recombination frequency by several hundred-fold (Maloisel and Rossignol 1998). Moreover, murine ES cells lacking DNA methyltransferase 1 (DNMT1), the gene responsible for DNA methylation maintenance, have a ten-fold higher mutation rate than wild type cells (Chen *et al.* 1998). In this same methylation-impaired model, Walsh *et al.* (1998) also showed a significant increase in the intracisternal A particle (IAP) retrovirus transcript levels that were not expressed in normal ES cells. It was suggested that this resulted from the demethylation of flanking LTR (long terminal repeat) sequences that are known to be methylated in mouse somatic cells (Feenstra *et al.* 1986).

Unmethylated CGIs, in turn, are descriptive of the promoter and 5' untranslated regions (5'UTR) of genes, carry histone PTMs defining active transcription (*e.g.*, H3 and H4 acetylation) and lack linker histone H1 (Gardiner-Garden and Frommer, 1987, Tazi and Bird 1990). Some CGIs have also been shown to be partial nucleosome-deserts, even though the nucleosome distribution between CGI and non-CGI genomic regions is generally found equal (Tazi and Bird 1990). When methylated, these CGIs are tightly linked to transcriptional silencing. The key event in methylated CGI-induced gene silencing is the binding of methyl-CpG binding protein 2 (MECP2) to the methylated CpGs (Nan *et al.* 1996). MECP2 has two major domains, one for meCpG binding and another for recruitment of transcriptional corepressors, such as histone deacetylases (HDACs) (Nan *et al.* 1996, Nan *et al.*1997, Jones *et al.* 1998). Binding of chromatin modifiers to MECP2 and to other methyl CpG-binding proteins, such as UHRF1, further inactivates the chromatin by introducing additional silencing modifications or by purely physically

blocking the proper assembly of transcriptional machinery on the chromatin (Kaludov and Wolffe 2000, Bostick *et al.* 2007).

1.4 Epigenetic machinery

The variety of different epigenetic modifications described above are brought about by a distinct set of enzymes that specifically function by either placing or removing these chemical modifications on and from their targets on DNA or histone tails. Apart from the actual assemblers of DNA methylation and histone modifications themselves (*e.g.*, DNMTs, HATs and HDACs), a great number of other proteins/complexes also exists, whose functions are needed for proper epigenetic regulation (Figure 3). For example, chromatin-remodeling complexes are responsible for the displacement and replacement of nucleosomes during transcription and replication, whereas various effector proteins serve as a bridge between the primary modification and additional modifiers, which need to be recruited to fully establish and maintain a given epigenetic state.

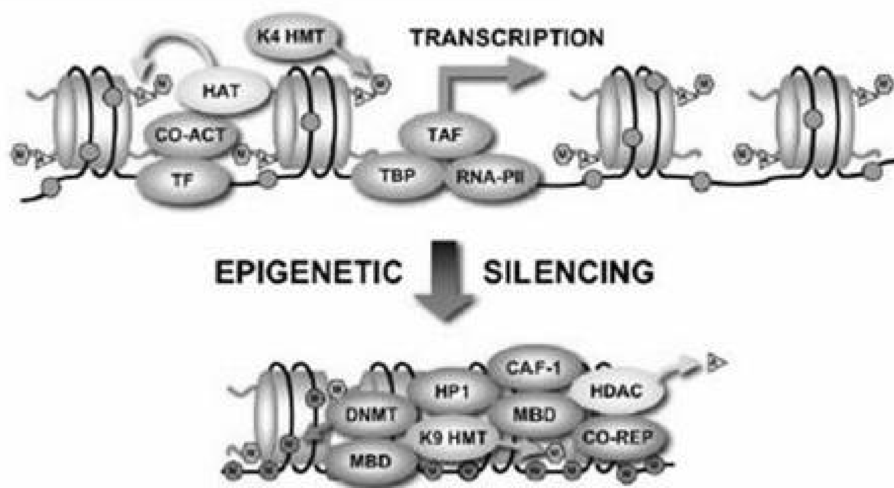

***Figure 3**. Schematic presentation of some chromatin modifications and modifying enzymes. (K4 HMT, K4 histone methyltransferase; HAT, histone acetyltransferase; CO-ACT, co-activator; TF, transcription factor; TAF, TBP-associated factor; TBP, TATA-binding protein; RNA-PII, RNA polymerase II; K9 HMT, K9 histone methyltransferase; DNMT, DNA methyltransferase; MBD, methyl-CpG binding domain protein; HP1, heterochromatin protein 1; CAF-1, chromatin assembly factor 1; HDAC, histone deacetylase; CO-REP, co-repressor; histone acetylation, triangles; histone methylation, hexagons; CpGs, circles, "m" denoting methylation). (Laird, Cancer Epigenetics, Human Molecular Genetics, 2005, 14, Review Issue 1, R65-R76, by permission of Oxford University Press).*

1.4.1 Initiation and spread of epigenetic modifications

Histone acetylation is brought about by specific enzymes, HATs or more recently renamed as K-acetyltransferases (KATs, Allis *et al.* 2007). First KATs, Hat1 and HAT A, were cloned in the mid-1990s from yeast and tetrahymena (Kleff *et al.* 1995, Brownell *et al.* 1996), and today there are close to 20 enzymes with known acetyltransferase activity in humans alone (Allis *et al.* 2007). Most HATs have been identified as histone acetyltransferases, but were later shown to also acetylate other proteins. For example, all core histones, TP53 (tumor protein 53), C-MYC (v-myc myelocytomatosis viral oncogene homolog) and HDAC6 are among the more than 70 targets of p300/CBP [p300/(cAMP responsive element binding protein 1)-binding protein] (Ogryzko *et al.* 1996, Gu and Roeder 1997, Zhang *et al.* 2005). The counteracting histone deacetylation results from the action of another group of specific enzymes, histone deacetylases (HDACs). The two groups of enzymes work in an orchestrated manner to offer a highly balanced acetylation pattern on chromatin corresponding to its activation status.

HATs can be divided into two general groups based on their location. A-HATs are nuclear and primarily function in larger complexes carrying out specific transcription-related acetylation processes (Garcea and Alberts 1980, Grant and Berger 1999). Conversely, B-HATs are cytosolic, accounting for the acetylation of *de novo*-synthesized histones prior to their nuclear localization and nucleosomal assembly (Garcea and Alberts 1980, Allis *et al.* 1985, Kleff *et al.* 1995). A-HATs are further subcategorized, based on sequence similarities, into the GNAT (Gcn5-related N-acetyltransferase)-, MYST- and p300/CBP (CREB-binding protein)-families. The differences in the catalytic domains between these families confer substrate-specificity, thus making them active at different histones and chromatin remodeling-requiring cellular functions. A MYST-family member, MYST2 (also called HBO1), functions in replication by directly interacting with ORC (origin recognition complex) and acetylates H4K5 and K12, whose acetylation is deposition-related (Sobel *et al.* 1995, Iizuka and Stillman 1999). On the contrary, GCN5 and PCAF (p300/CBP associated factor), two GNAT-family members, acetylate H3K9 and K14, marks of actively transcribed chromatin (Kuo *et al.* 1996, Zhang *et al.* 1998a, Schiltz *et al.* 1999), while TIP60 acetylates H4K8 and K16, also associated with transcriptional activity (Kimura and Hirokoshi 1998). GNAT- and p300/CBP-family members function as general transcription factors and also acetylate several non-histone proteins (Sterner and Berger 2000).

Histone deacetylases can be divided into two groups: “classical” HDACs are Zn^{2+}-dependent, and yeast Sir2 (Silent information regulator 2) homologues (sirtuins or SIRTs) are NAD^{+}–dependent (de Ruijter *et al.* 2003, Haigis and Guarente 2006). Classical HDACs and most sirtuins function either in the nucleus or the cytoplasm; however SIRT3, SIRT4 and SIRT5 are mitochondrial (de Ruijter *et al.* 2003, Michishita *et al.* 2005, Haigis and Guarente 2006). Another way to classify HDACs is based on their homology to yeast HDACs (Gregoretti *et al.* 2004). Group I, II and IV HDACs are the classical HDACs, while group III encompasses the sirtuins. Most classical HDACs are present as large complexes, such as the Sin3 and NuRD complexes, which contain several co-repressors harboring chromatin

remodeling activities and the ability to bind other transcription factors and methylated DNA (Alland *et al.* 1997, Kim *et al.* 1999, Wade *et al.* 1999, Zhang *et al.* 1999, Fuks *et al.* 2000, Humphrey *et al.* 2001). HDAC1 and HDAC2 are often found in the same complexes; however, they can also function independently, allowing additional levels of functional variation (Zhang *et al.* 1998b, Nicolas *et al.* 2007, Trivedi *et al.* 2007).

Histone methyltransferases (HMTs) catalyze the methylation of lysine and arginine residues in H3 and H4 tails (Cao *et al.* 2002). Even though histone methylation has been known to take place for more than 40 years (Murray 1963), it was only recently that the first HMTs were actually identified (Rea *et al.* 2000). Human SUV39H1 is a homologue of *Drosophila Melanogaster* Su(var)3-9, which was originally identified as a heterochromatin-associated protein (Tschiersch *et al.* 1994). The SU(VAR) group of proteins was initially characterized in *D. melanogaster* as suppressors of position effect variegation (PEV). The heterochromatin-associated SUV39H1 specifically methylates H3K9, consistent with the role of this modification in silencing (Rea *et al.* 2000). HP1 binds to both the modified histone, H3K9me3, and the histone-modifier, SUV39H1, and is an important factor in the assembly and spreading mechanisms of heterochromatin formation (Bannister *et al.* 2001, Lachner *et al.* 2001, Yamamoto and Sonoda 2003). A common nominator for the HMTs is their SET domain (Su(var)3-9, Enhancer of zeste, Trithorax), which is commonly found in a large number of proteins in different organisms. There are >60 proteins with SET domains in humans and >2700 in all sequenced organisms (SMART database; Schultz *et al.* 2000). Enhancer of zeste homolog 2 (EZH2), part of polycomb repressive complex 2 (PRC2), is the histone methyltransferase targeting H3K27 methylation to chromatin in need of silencing (Cao *et al.* 2002). Moreover, EZH2 has been shown to specifically interact with DNMTs, recruiting them to repressive chromatin, thus representing a direct link between these two key regulators of silenced chromatin structure (Viré *et al.* 2006). In contrast to the histone tail lysine and arginine methyltransferases, the *Saccharomyces cerevisiae* Dot1 HMT (human homologue = DOTL1) does not contain a SET domain and methylates a specific H3 core lysine residue, K79 (van Leeuwen *et al.* 2002). Methylation of this residue is required for proper telomeric silencing, as evidenced by a mutational analysis of H3K79 that abolished the binding of additional silencing proteins, Sir2p and Sir3p, to the chromatin.

The role of histone methylation as the only true epigenetic histone PTM was long under debate, as there were no known histone demethylases (HDMs), thus supporting the idea that once established, histone methylation marks could not be removed and would therefore serve as a base for “epigenetic memory”. In 2004 however, the first lysine demethylase, lysine K-specific demethylase 1 (LSD1), was characterized, and a whole demethylase family (Jumonji) has since been identified (Shi *et al.* 2004, Lee *et al.* 2005, Tsukada *et al.* 2006, Christensen *et al.* 2007, Klose *et al.* 2007). Similar to HATs, HDACs and HMTs, HDMs also have clear substrate-specificities, preferring demethylation of certain lysine residues either in the mono-, di- or trimethylated states.

DNA methylation patterns in cells are established and maintained by the methyltransferase activity of specific enzymes, DNA methyltransferases (DNMTs). DNMTs catalyze a reaction where S-adenosylmethionine (SAM) serves as a methyl donor for the 5' position of the cytosine pyrimidine ring. DNMT1 is responsible for the maintenance methylation of already-methylated CpGs and functions mainly during DNA replication, using hemimethylated DNA as a template (Pradhan *et al.* 1999). A DNMT1 isoform, DNMT1o, is expressed in oocytes and is needed for the correct establishment of methylation at imprinted genes (Mertineit *et al.* 1998, Howell *et al.* 2001). DMNT3A and DNMT3B, in turn, are *de novo* DNA methyltransferases responsible for the introduction of methyl groups to unmethylated DNA during early embryonic development (Okano *et al.* 1999). DNMT3L (DNMT3-like), a regulatory protein lacking the actual DNA methyltransferase catalytic activity, is expressed during gametogenesis, where it is needed for proper maternal imprinting (Aapola *et al.* 2000, Bourc'his *et al.* 2001). Some genes also undergo age-related CpG methylation, implying that DNA methylation as a critical regulator in the aging process (Ono *et al.* 1993, Issa *et al.* 2001, Oakes *et al.* 2003, So *et al.* 2006). Lopatina *et al.* (2002) studied the activities of individual DNMTs during aging and concluded that DNMT1 activity decreases markedly during aging, consistent with the decrease in global genomic hypomethylation; whereas the activity of *de novo* DNA methyltransferase, DNMT3b, was significantly increased in aging and was thus linked to the observed increased regional CpG hypermethylation. Another study demonstrating target specificities for DNMTs showed that after 5azadC-treatment inhibiting DNMT activity in bladder cancer cells, CGIs were remethylated only in replicating cells, whereas gene-poor CpGs and repetitive elements were remethylated also in non-dividing cells (Velicescu *et al.* 2002). The methylation activity at the repetitive elements was assigned to DNMT3a, which was the only DNMT expressed in these cells, while replication was needed for the remethylation of promoter associated CGIs. This is in line with the finding that DNMT1 co-localizes with proliferating cell nuclear antigen (PCNA) at replication forks, but DNMT3a is preferably found at heterochromatic areas together with HP1 and MeCP2 (Bachman *et al.* 2001). Except for methylation at repeat regions, centromeres or at imprinted loci, DNA methyltransferases are thought to possess only minimal sequence-specificity in targeting their activity. However, there are reports indicating that DNMT1 binds to specific transcription factors and hence is targeted in a sequence-specific manner. For example, retinoblastoma (RB) tumor suppressor and E2F1 transcription factor (silenced by RB-binding) were shown to form a complex with DNMT1, with DNMT1 participating in RB-mediated silencing of E2F1 target genes (Robertson *et al.* 2000). This repressive complex also contained HDAC1, in agreement with the cooperative silencing of DNA methylation and histone deacetylation.

The mechanism of DNA demethylation is less understood than the reversal of histone modifications. DNA demethylation can be achieved through passive demethylation during replication by inhibition of DNMT1 activity in mitotic cells. In non-mitotic cells, DNA demethylation can only be accomplished through an active process. In 1999, Bhattacharya *et al.* identified the first mammalian DNA demethylase, which was then demonstrated to specifically demethylate 5meC at

CpG dinucleotides (Ramchandani *et al.* 1999). Sequence comparisons showed that this DNA demethylase was identical to the previously described murine methyl-CpG binding domain protein 2 (MBD2) (Hendrich and Bird 1998). The apparent discrepancy of MBD2 in both DNA demethylation and DNA methylation-mediated silencing has been explained, at least in part, by the sequence-specificity of MBD2 DNA demethylase activity (Detich *et al.* 2002). In addition to the activity of DNA demethylase, 5meCs can be removed from the genome by the activity of thymine DNA-glycosylase (TDG, also known as 5-methyl-cytosine DNA glycosylase, 5-MCDG), after which the DNA repair system replaces the abasic site with an unmethylated cytosine (Vairapandi *et al.* 1993, Jost *et al.* 1995). In addition, a G/T mismatch repair enzyme, G/T mismatch DNA glycosylase (also known as methyl-CpG binding domain protein 4, MBD4), has been shown to possess this 5-MCDG activity at hemimethylated DNA (Zhu *et al.* 2000). Recently, protein kinase C (PKC)-mediated phosphorylation of MBD4 was shown to increase its glycosylase activity on methylated DNA (Kim *et al.* 2009). MBD4 can also function together with 5meC deaminase by replacing the 5meC deamination product T with unmethylated C through its G/T mismatch repair activity (Morgan *et al.* 2004). In fact, this 5meC deamination coupled with G/T mismatch repair is thought to be the main form of active DNA demethylation in humans (Zhu *et al.* 2009). Additionally, growth arrest and DNA-damage-inducible alpha (GADD45A) has been shown to mediate DNA demethylation by promoting DNA repair machinery recruitment (Barreto *et al.* 2007). Recently, it was also shown that the *de novo* DNMTs, DNMT3a and DNMT3b, can actively deaminate 5meC, thus contributing to the DNA demethylation process (Métivier *et al.* 2008). The involvement of the same enzymes in opposing actions on cyclical DNA methylation at active promoters expands our understanding of epigenetic marks and their maintenance during the cell cycle and differentiation.

1.4.2 Maintenance and inheritance of epigenetic modifications

A key determinant of epigenetic modification is its heritability, primarily from a mother cell to a daughter cell, but also from one generation to another. Apart from the actual DNA sequence, DNA methylation and chromatin modifications also need to be effectively and accurately copied and transferred. The mechanisms by which this occurs have been established in quite some detail for DNA methylation, whereas the mode of inheritance of various histone marks remains less comprehensive. Various proteins associated with the replication fork have shed light on these events, both ensuring conservation of information stored in the form of epigenetic marks, as well as enabling the plasticity needed for any developmental switches to take place.

DNA replication occurs during the S-phase of the cell cycle and is by nature a semiconservative process, where the parental strands serve as templates for the newly synthesized DNA strands (Watson and Crick 1953). As DNA is replicated, PCNA molecules are loaded onto both the leading and lagging DNA strands of the

replication fork (Prelich and Stillman 1988). PCNA then further recruits general factors such as histone modifiers (*e.g.*, HDACs and HMTs; Milutinovic *et al.* 2002, Huen *et al.* 2008) and chromatin remodelers (*e.g.*, Williams syndrome transcription factor (WSTF) - imitation switch-type nucleosome-remodeling factor (SNF2H); Poot *et al.* 2004). If the replicated DNA is methylated, UHRF1, a protein with high affinity for hemimethylated DNA, binds to the post-replicative hemimethylated DNA, and together with PCNA attracts DNMT1 to the newly synthesized DNA strand (Hermann *et al.* 2004, Bostick *et al.* 2007, Sharif *et al.* 2007). DNMT1 then methylates the unmethylated CpGs of the daughter strand using the parental strand CpG methylation as a template (Gruenbaum *et al.* 1982). The role of UHRF1 in targeting DNMT1 specifically to hemimethylated DNA ensures the proper maintenance of DNA methylation upon DNA replication.

In contrast to the rather intelligible mechanism of DNA methylation conservation, the inheritance of the wide variety of histone marks (in a process involving disassembly and reassembly of nucleosomes upon replication) is a more complicated and less comprehensively understood phenomenon. Before the histone modifiers can enforce the actual tail modifications, the nucleosomal structure needs to be reassembled on the replicated DNA. Histone deposition onto DNA involves the coordinated assembly of H3-H4 tetramers with two H2A-H2B dimers to form the histone octamers. Upon chromatin disassembly and resynthesis, histone dimers complex with various histone chaperones, such as antisilencing function 1 (ASF1) and chromatin assembly factor 1 (CAF-1), which in turn mediate the reassembly of the histones on the newly synthesized DNA (Mello *et al.* 2002, Hoek and Stillman 2003, Natsume *et al.* 2007). In the case of loading newly synthesized histones onto chromatin, a maturation step is needed to preserve the parental histone modifications over the marks of newly synthesized histones, such as H4K5 and K12 acetylation (Sobel *et al.* 1995). PCNA, at the replication fork, recruits CAF-1, which is needed for histone deposition, and HDAC1, which in turn deacetylates the new histones upon nucleosome assembly (Shibahara and Stillman 1999, Zhang *et al.* 2000, Milutinovic *et al.* 2002). Some histone modifiers, like HDAC1 and histone methyltransferase G9a, can also bind the DNMT1 already present at the newly replicated DNA and thus indirectly use DNA methylation to guiding the correct modifications of histone tails (Fuks *et al.* 2000, Esteve *et al.* 2006). Similarly, methyl CpG-binding domain protein 1 (MBD1), present at methylated DNA, recruits histone methyltransferase SETDB1 to CAF-1, thus coupling histone deposition and histone methylation (Hoek and Stillman 2003, Sarraf and Stancheva 2004).

If the assembly of old and new histones takes place in a random fashion, the correct histone modifications are thought to be guided to new histones by a neighboring effect, where the old histones spread their modification information. This type of model is particularly suitable for heterochromatin maintenance, where large chromatin areas become similarly marked. Another possibility models DNA methylation maintenance. Here, parental histones are distributed in a semi-conservative manner onto both DNA strands where they are complemented with new histones to form histone octamers. These new histones are then modified by mimicking the old histones as a template. In an asymmetric histone assembly model

old and new histones are distributed onto separate DNA strands. This type of model would require additional inter-strand crosstalk to maintain parental histone marks on both strands (reviewed in Probst *et al.* 2009.)

1.5 Epigenetics in development, X-inactivation and imprinting

Epigenetic modifications can force long-term silencing or activation on gene expression and therefore are essential in guiding developmental processes and maintaining differentiated cell types. A well-studied example of epigenetic regulation in development comes from the homeobox (HOX) genes, first identified in *D. melanogaster*. The expression of HOX genes is essential in the development of the anterior-posterior axis and is controlled by the Polycomb Group (PcG) and trithorax Group (trxG) proteins (reviewed in Schwartz and Pirrotta 2007). PcGs function as repressors of chromatin and trxGs antagonize their function by activating chromatin structure. PcGs and trxGs do not themselves initiate the transcriptional process at HOX genes but are needed for maintenance of the established epigenetic state throughout development. PcG proteins are conserved in evolution and are also needed in mammals for proper cell lineage specification and stem cell maintenance. PcG proteins form multi-protein complexes, polycomb repressive complex 1 (PRC1) and PRC2 (Shao *et al.* 1999, Saurin *et al.* 2001). The catalytically active subunit of PRC2, EZH2, possesses histone methyltransferase activity and is needed for depositing the H3K27 trimethylation mark, descriptive of silenced chromatin (Cao *et al.* 2002). In addition to histone marks, DNA methylation is also involved in the regulation of gene expression restricted to certain developmental phases and places. One group of genes under CpG methylation directed germ line-specific expression pattern is the cancer/testis antigen family. These genes are unmethylated and expressed in testis, and silenced by DNA methylation in somatic tissues (de Smet *et al.* 1999).

Another key event where epigenetics plays an important role is X-chromosome inactivation (XCI) in females, which provides dosage compensation to adjust the gene dosage of X-linked genes. XCI occurs in *cis* via the X-inactivation center that produces inactive X (X_i) specific transcript (*Xist*), which then coats the X-chromosome to be inactivated (Brown *et al.* 1991a, Brown *et al.* 1991b, Clemson *et al.* 1996). *Xist* expression on an active X chromosome (X_a) is silenced by DNA methylation, while the transcribed *Xist* locus in X_i is unmethylated (Norris *et al.* 1994). Accumulation of specific histone modifications is also needed for full X-inactivation. H4 hypoacetylation, H3K27me3, H3K9me3, lack of H3K4me3 and recruitment of a X_i-preferring histone variant, macroH2A1, are all XCI-linked histone modifications (Jeppesen *et al.* 1993, Gilbert *et al.* 1999, Costanzi and Pehrson 1998, Heard *et al.* 2001, Boggs *et al.* 2002, Plath *et al.* 2003). Apart from guiding the expression of *Xist*, DNA methylation seems to have role in maintaining XCI by CGI methylation (Norris *et al.* 1991, Sado *et al.* 2000, Hellman and Chess

2007). However, *Xist* expression is not required for XCI maintenance once X_i is established (Brown *et al.* 1994).

Another genomic process involving DNA methylation is genomic imprinting. In imprinting, either the maternally or paternally inherited copies of certain autosomal genes (<1% of all genes; Wilkinson *et al.* 2007) become silenced through a DNA methylation-mediated process resulting in parent-of-origin specific monoallelic expression. In mammals, imprinting occurs during gametogenesis and embryonic development. Primordial germ cells are first demethylated early in gametogenesis, and the imprints are replaced later during the maturation of gametocytes, with oocytes gaining their imprints after birth. At fertilization, imprints are again erased from both sperm and oocytes and re-established upon implantation. At both stages, the de- and remethylation steps provide a mechanism to remove acquired epigenetic changes while maintaining the original imprints (reviewed in Reik *et al.* 2001). The imprinted loci are generally clusters of genes including several protein-coding genes and a non-coding RNA (ncRNA) gene, and the expression of these clusters is controlled in *cis* through imprinting control regions (ICRs) (Edwards and Ferguson-Smith 2007). These ICRs are CpG-rich in sequence and carry the imprinting information in the form of parent-of-origin specific methylation (Kikyo *et al.* 1997). An example of imprinted loci is the mouse *Igf2r/Air* cluster that is maternally imprinted by methylation of ICR, and as a result, *Igf2r* is only expressed from this imprinted allele (Barlow *et al.* 1991, Stöger *et al.* 1993). The ncRNA of the cluster, *Air*, is expressed from the unmethylated paternal allele where the ICR works as its promoter, and it is required for the silencing of the other genes in the cluster in *cis* (Lyle *et al.* 2000, Sleutels *et al.* 2002). The role of histone modifications in imprinting is less clear, but the PcG protein EED has been shown to be important for the maintenance of imprinting (Wang *et al.* 2001b, Mager *et al.* 2003).

1.6 Non-coding RNAs and epigenetic silencing

RNA interference (RNAi) was first identified in *Caenorhabditis elegans* as means to silence gene expression by targeting mRNAs with short double-stranded RNA (dsRNA) molecules (Fire *et al.* 1998). This discovery, that just a few dsRNA molecules are effective in inducing post-transcriptional RNA interference, led to the suggestion of an endogenous mechanism to augment the signal. Indeed, an RNA-induced transcriptional silencing complex (RITS) and several enzymes (*e.g.*, Dicer and Argonaute-2) have since been identified (Hammond *et al.* 2000, Bernstein *et al.* 2001, Hammond *et al.* 2001). Although the RNAi mechanism for silencing was identified in yeast in the context of exogenous small RNA molecules (small interfering RNAs, siRNAs), it was quickly demonstrated to also occur in higher organisms, including humans (Elbashir *et al.* 2001b, Morris *et al.* 2004). The first micro-RNAs (miRNAs) were identified in 1993 (Lee *et al.* 1993, Wightman *et al.* 1993), but the full scope of the phenomenon was only understood much later. Cells can produce the necessary small RNA molecules from repetitive regions in their genomes as dsRNAs or as longer primary miRNAs from miRNA genes, which are

then processed by the enzymes Drosha (miRNAs) and Dicer (siRNAs and miRNAs) to produce the final ~22 nt long siRNAs/miRNAs, both of which can then be loaded into the RITS complex (Lagos-Quintana *et al.* 2001, Hutvagner and Zamore 2002). Whereas siRNAs are generally fully complementary to their target mRNAs and induce their degradation by the RITS complex, miRNAs have varying complementarity to their target sequences, and therefore, miRNA-induced silencing can result from either mRNA translation inhibition or target mRNA degradation (Elbashir *et al.* 2001b, Brennecke *et al.* 2005). It is estimated that as many as 30% of human genes may be regulated through miRNAs, and even more importantly, one miRNA can target several mRNAs (Bartel *et al.* 2004, Lim *et al.* 2005).

While the first reports on RNAi demonstrated it as a regulatory mechanism to silence individual transcripts, its more global role in heterochromatin formation and maintenance was soon discovered (Hall *et al.* 2002, Volpe *et al.* 2002). In yeast, components of the RNAi machinery have been shown to be necessary for the proper centromere homologous repeat (c*enH*)-induced establishment of heterochromatin at an ectopic site (Hall *et al.* 2002). Genome-wide studies of the distribution of RNAi components showed strikingly overlapping occupancy of RNAi factors with H3K9me3 and Swi6 (*Schizosaccharomyces pombe* homologue of human HP1) at heterochromatic regions (Noma *et al.* 2004, Cam *et al.* 2005), and the presence of these RNAi components was further shown to depend on the Clr4 methyltransferase (the yeast homologue of the mammalian SUV39H1), as the disruption of its enzymatic activity led to a concomitant loss of H3K9me3 and RNAi components from heterochromatin (Cam *et al.* 2005).

In *S. pombe*, one strand of centromeric repeats is constitutively transcribed at low levels by RNA polymerase II (Volpe *et al.* 2002). dsRNA is produced by also transcribing the opposing strand or by RNA-dependent RNA polymerase 1 (Rdp1) and is then converted to siRNA by the action of RNAi (Makeyev and Bamford 2002, Kato *et al.* 2005). These primary siRNAs are then targeted back to the repetitive regions, either by the RITS complex or by association with other heterochromatin components, such as Clr4 and H3K9 methylation. This leads to the formation of a self-reinforcing loop and amplification of the silencing signal as secondary siRNAs are produced from the heterochromatin and additional heterochromatin associated factors are recruited (Sugiyama *et al.* 2005). In addition to the general establishment of heterochromatin at repetitive sequences, RNAi has been shown to be necessary for the specific recruitment of CENP-A to centromeric heterochromatin (Folco *et al.* 2008).

In human cells, there is evidence of promoter-associated RNAs mediating siRNA-dependent gene silencing (Han *et al.* 2007). The elongation factor 1 alpha (*EF1A*) promoter was shown to produce sense-orientation RNAs helping to recruit antisense siRNAs, and further induce silencing with specific repressive chromatin marks (*e.g.*, H3K27me3, K3K9me2 and DNMT3a) present at the silenced promoter. If this is found to be a more general phenomenon, the promoter-targeted siRNAs may represent a novel mechanism to target gene silencing through epigenetic control.

Additionally, miRNAs can control transcription through epigenetic mechanisms in mammalians. Kim *et al.* (2008) showed that *miR-320* recruits components of

epigenetic silencing, such as AGO1 (Argounaute-1), EZH2, and H3K27me3, to the promoter of the *POLR3D* gene, which harbors *miR-320* in its antisense orientation. Similarly, *Hoxd4* expression was epigenetically silenced in breast cancer cell lines by *miR-10a*-targeted recruitment of DICER, AGO1 and H3K27me3 to the *Hoxd4* promoter (Tan *et al.* 2009). *miR-10a* expression was also needed to guide *de novo* methylation at the *Hoxd4* promoter, which is essential for the silencing. These observations demonstrate RNAi-mediated epigenetic silencing in the regulation of gene transcription.

2. Epigenetics and cancer

In normal cells, epigenetics has an important role in several key processes depicted above. Over the past decade, interest in epigenetics has also dramatically increased in relation to cancer research. One notable reason for this was the completion of the Human Genome Project and the realization that knowing the entire human genomic sequence does not produce and understanding of what goes wrong in cells when they are transformed. Additional sequencing approaches to find mutations and rearrangements specifically in cancer genomes are ongoing; however, there are also ongoing international efforts to establish the human epigenome (The Cancer Genome Project, http://www.sanger.ac.uk/genetics/CGP and Human Epigenome Project, http://www.epigenome.org/index.php). Overall, the focus is shifting from the mere DNA sequence to understanding how gene expression changes are brought about. An ever growing number of reports show how the usage of the genome is altered in cancer. This alteration is achieved through changes in epigenetic patterns, *i.e.*, the changes in DNA methylation and histone modifications and the expression or functional changes in the enzymes and co-activators/repressors in charge of these epigenetic modifications.

Cancer is by nature a genetic disease in which the cumulative changes in the normal structure and function of the genome eventually lead to the transformation of the cell. In the famous paper "The hallmarks of cancer", Hanahan and Weinberg (2000) described six characteristics that are shared by all cancers, albeit through differing mechanisms but resulting in a similar end-point, such as self-sufficiency in growth signals. For example, amplification and overexpression of epidermal growth factor receptor (EGFR) or expression of a mutant ligand-independent EGFR ultimately offer the cells a means to propagate the growth signal without the need for normal external stimuli (Grandis and Sok 2004). In addition to the "traditional" genetic changes, such as mutations and copy number alterations, the role of epigenetics in tumorigenesis has been widely recognized, and epigenetics is currently one of the "hot" topics in cancer research. In general, cancer epigenetics entail reduced overall methylation of the genome, increased CGI methylation and aberrant histone modifications.

2.1 Aberrant histone modifications in cancer

In regard to cancer epigenetics, DNA methylation has been studied extensively for years, whereas reports on aberrations in histones, their modifications, and modifiers, have only begun to appear recently. Some commonly observed modifications in normal and cancer cells are illustrated in Figure 4. Much effort has been expended to identify tumor suppressor genes (TSGs) undergoing transcriptional silencing during tumorigenesis through epigenetic changes, namely DNA methylation and histone deacetylation. One commonly used approach in the hunt for these genes has been the inhibition of HDAC activity together with DNA demethylation, combined with the identification of the resulting re-expression of genes (Cameron *et al.* 1999). Several TSGs have indeed been identified that undergo this type of epigenetic silencing in various cancer types. Often both histone modifications and DNA methylation work synergistically in the silencing of epigenetically targeted genes, but they can also work independently of each other. Cyclin-dependent kinase inhibitor 1A (*CDKN1A*, a key mediator of the p53-dependent cell cycle arrest signal) silencing in bladder cancer is an example of epigenetic silencing through histone deacetylation without promoter DNA methylation (Richon *et al.* 2000).

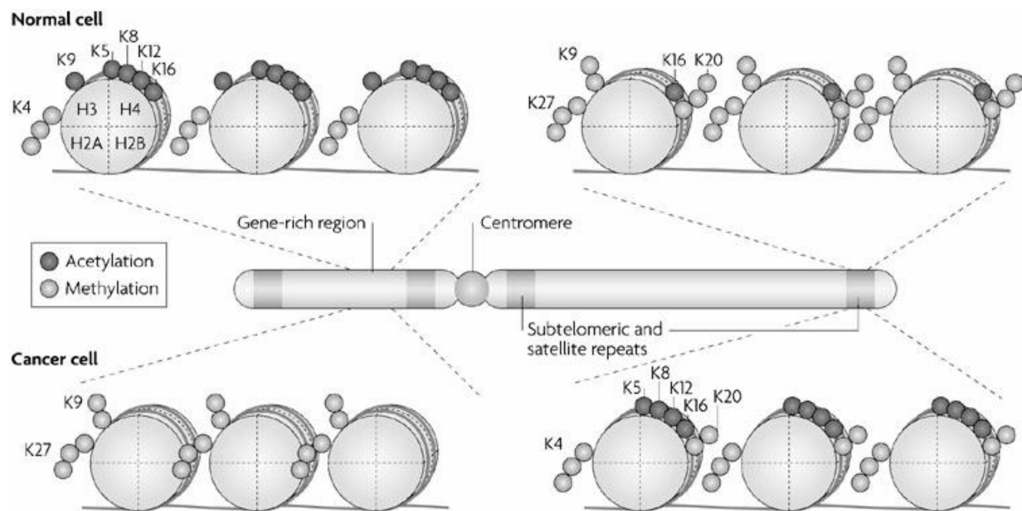

***Figure 4**. Typical histone modifications in normal and cancer cells. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Esteller, Copyright, 2007.)*

Losses of H4K16 acetylation and H4K20 trimethylation at genomic repetitive sequences have been shown to occur commonly in human cancer cells, and the loss of these histone marks is associated with global DNA hypomethylation (Fraga *et al.* 2005). Tryndyak *et al.* (2006) and Pogribny *et al.* (2006) further linked the loss of H4K20me3 in breast cancer cell lines and in a rat model of hepatocellular carcinoma

to decreased levels of the histone methyltransferase SUV4-20H2, an H4K20-specific histone methyltransferase (Schotta *et al.* 2004).

Certain histone modifications are enriched in cancer. For example, a recent report demonstrated how H3K27me3, the common chromatin silencing modification, is enriched at promoters in prostate cancer (Kondo *et al.* 2008). This H3K27me3-mediated silencing was shown to be independent of DNA methylation, as the enrichment was observed mainly at promoters showing relatively low CpG methylation. The H3K27me3 enrichment in prostate cancer is in line with the fact that EZH2 is known to be overexpressed and amplified in various cancers, including prostate cancer (Varambally *et al.* 2002, Kleer *et al.* 2003, Arisan *et al.* 2005, Saramäki *et al.* 2006b). The main mechanism for the overexpression of EZH2 in prostate cancer, however, is likely the loss of *miR-101* expression, which was shown to target EZH2 and to have an inversely correlated expression pattern with EZH2 (Varambally *et al.* 2008). In addition to prostate cancer, high EZH2 expression is associated with aggressive tumors in breast and endometrial cancers and in melanoma (Kleer *et al.* 2003, Bachmann *et al.* 2006). EZH2 has additionally been linked to cell cycle regulation, as it is a down-stream target of the Rb tumor suppressor pathway (Müller *et al.* 2001). An example of PRC-repressed TSG is DOC-2/DAB2 interactive protein (*DAB2IP*), whose expression is downregulated through EZH2-mediated H3K27me3 in prostate cancer cells (Chen *et al.* 2005). In addition to histone methylation-mediated repression, *DAB2IP* silencing involves DNA methylation (Chen *et al.* 2003), and this methylation has also been observed in breast and gastric cancers (Dote *et al.* 2004, Dote *et al.* 2005).

Another repressive histone mark, H3K9 methylation, also associates with DNA methylation-mediated gene silencing (Fahrner *et al.* 2002). The HMT responsible for H3K9 methylation, PRDM2 (PR domain containing 2) (Kim *et al.* 2003), is silenced, deleted or mutated in various cancers (Chadwick *et al.* 2000, Du *et al.* 2001, Carling *et al.* 2003, Kim *et al.* 2003). In addition, a large number of translocations involving several of the HMTs have been described to occur in leukemias (reviewed in Fog *et al.* 2007).

HATs and HDACs can also be aberrantly expressed in cancer, thus leading to altered histone modification patterns, resulting in altered target gene expression. For example, the p300/CBP HAT is associated with cancer through mutations causing decreased enzymatic activity and translocations causing altered transcriptional activation patterns (Muraoka *et al.* 1996, Chaffanet *et al.* 2000, Iyer *et al.* 2004, Roelfsema *et al.* 2005). Genetic alterations of *HDAC*s appear very rarely, and no mutations have been found thus far in *HDAC1* or *HDAC2* genes (Özdag *et al.* 2006). Rather, the contribution of HDACs to tumorigenesis comes from their altered expression patterns, altered recruitment to chromatin, or altered recruitment of additional chromatin remodeling components (Halkidou *et al.* 2004, Zhu *et al.* 2004). A fusion gene, PML-RARα (promyelocytic leukemia - retinoic acid receptor alpha) causing acute promyeloid leukemia (APL), is a representative example of the improper recruitment of HDACs resulting in tumorigenesis. RARα recruits HDAC1-containing silencing complexes to promoters (Nagy *et al.* 1997). PML-RARα is insensitive to retinoic acid-induced transcriptional activation involving dissociation of HDAC1. Thus, retinoic acid (RA) target genes become constitutively

silenced, resulting in a myeloid differentiation blockage, which is the eventual underlying cause of APL (Minucci and Petucci 2006). In addition to HDAC1, PML-RARα also recruits other epigenetic silencers to RA-target promoters, such as DNMT1, DNMT3a and MBD1 (Di Croce *et al.* 2002, Villa *et al.* 2006). Another fusion resulting in an inappropriate recruitment of chromatin modifiers is the MYST3–CREB fusion found in acute myeloid leukemias (Borrow *et al.* 1996). MYST3 (also called MOZ) is a HAT, and the fusion protein contains the acetyltransferase domain from MYST3 and the almost intact CREB protein, allowing improper targeting of HAT-activity, thus possibly contributing to malignant transformation.

HDACs 1, 2 and 3 are all overexpressed in prostate cancer, and high levels of HDAC2 are associated with poor prognosis (Weichert *et al.* 2008). Androgen receptor (AR) has been shown to directly interact with HDAC1, and the acetylation and deacetylation of AR itself was suggested to be important for its transcriptional activity (Gaughan *et al.* 2002). A recent report showed that HDAC1 overexpression results from HDAC1-targeting *miR-449* downregulation in prostate cancer (Noonan *et al.* 2009). Additionally, HDAC1 overexpression and its target gene silencing are associated with *ERG*-fusion positive cancers (Iljin *et al.* 2006). Pharmacological inhibition of HDAC activity was efficient in blocking androgen-dependent *ERG* fusion gene expression *in vitro*, and when combined with anti-androgen treatment, resulted in retention of AR in the cytoplasm causing silencing of AR signaling (Björkman *et al.* 2008). However, silencing of any single *HDAC* by shRNAs was ineffective at inhibiting *TMPRSS2* (transmembrane protease, serine 2) activation (the most common fusion partner of *ERG*), suggesting that *ERG* expression regulation through HDACs is redundant, while some other AR target genes are more strictly regulated by specific HDACs (*e.g.*, PSA by HDAC1 and HDAC3) (Welsbie *et al.* 2009). These reports also demonstrated HDAC activity to be necessary for AR transcriptional activation, independent of AR levels and irrespective of the fusion status, with HDAC inhibition resulting in aberrant AR complexing with RNA pol II and AR co-activators, thus rendering AR transcriptionally inactive. These results suggest that HDAC inhibition may be beneficial, in general, for the treatment of castration-resistant prostate cancers, where traditional androgen ablation has failed. Further, *ERG* fusion-positive prostate cancers may be a specific subgroup most likely to benefit from the therapeutic approaches combining androgen blockage and epigenetic targeting.

Histone demethylases have also been implicated in cancer. One HDM (lysine (K)-specific demethylase 4C, KDM4C; also known as gene amplified in squamous cell carcinoma 1, GASC1) was first identified as a gene amplified in esophageal squamous cell carcinoma (Yang *et al.* 2000) and was only later found to harbor histone demethylase activity (Cloos *et al.* 2006). GASC1 is also overexpressed in prostate cancer. LSD1, another HDM, and GASC1 act together as co-regulators of AR by demethylating repressive marks on chromatin to potentiate AR transactivation (Metzger *et al.* 2005, Wissmann *et al.* 2007). Consistent with the role of LSD1 in AR transactivation, LSD1 expression is increased significantly in prostate cancer and correlates with relapse during follow-up (Kahl *et al.* 2006). The substrate specificity of LSD1 may be specified through the interacting transcription

factor because the AR-associated LSD1 demethylates H3K9me2 and H3K9me1, whereas it was originally discovered to demethylate H3K4me2 and H3K4me1 (Shi *et al.* 2004). Recently, LSD1 was shown to be involved in telomerase reverse transcriptase (TERT) silencing, both in normal and malignant cells, and this was mediated through specific H3K4 demethylation at the *TERT* promoter, together with HDAC-activity (Zhu *et al.* 2008). Apart from being implicated in prostate cancer, LSD1 has also been shown to be upregulated in neuroblastoma, and its expression is correlated with poor outcome. Quite recently, *UTX*, a H3K27-specific HDM, was shown to carry inactivating somatic mutations in several cancer types, including leukemias, colon and breast cancers (van Haaften *et al.* 2009). Reintroduction of *UTX* into *UTX*-null cell lines significantly increased the cell doubling times, indicating that UTX has a role in controlling proliferation of these cells. These mutations clearly link genetic abnormalities in cancer to epigenetic-level deregulation.

Abnormal histone phosphorylation has also been linked to cancer. Aurora-B kinase, which mediates the H3S10 phosphorylation essential in mitosis, is overexpressed in several cancers (including prostate) and correlates with poor clinical outcome (Giet and Glover 2001, Katayama *et al.* 1999, Chieffi *et al.* 2006, Gautschi *et al.* 2008). Increased H3S10ph increases abnormal chromosomal numbers, suggesting a role in carcinogenesis by mediating aneuploidy (Ota *et al.* 2002). In addition to being phosphorylated during mitosis, H3S10ph associates with transcriptional activation of certain proto-oncogenes, such as *c-jun* and *c-fos* (Clayton *et al.* 2000). Similarly, H3S10ph is needed for MYC-dependent transcriptional activation and oncogenic transformation of HEK 293 cells, and the phosphorylation is mediated by the PIM-1 serine/threonine kinase (Zippo *et al.* 2007).

There are also few reports on histone variants and cancer. Lack of H2A.X, the DSB repair-associated variant, has been shown to induce lymphomas and solid tumors in mice in a dose-dependent manner (Bassing *et al.* 2003). Another histone variant, H2A.Z, is a transcriptional target of MYC, and is estradiol-induced in breast cancer with significant association to lymph node metastasis and decreased breast cancer survival (Hua *et al.* 2008).

2.2 DNA methylation changes in cancer

DNA methylation patterns are altered in cancer, both through decreased and increased methylation, as graphically described in Figure 5. Global hypomethylation of the genome leads to abnormal expression of repetitive sequences and thus to increased genomic instability. The relative instability of meCpG contributes to cancer *via* its increased mutation rates. DNA hypermethylation, in part, is associated mainly with silencing TSGs and thereby contributing to tumorigenesis. Finally, the most recently described targets of DNA methylation in cancer are miRNAs, several of whose expression seems to be regulated through DNA methylation-mediated silencing in various cancers.

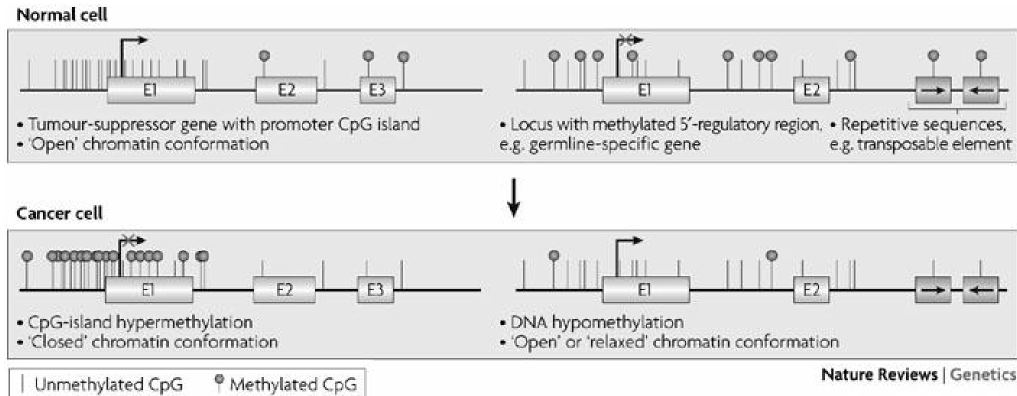

Figure 5*. DNA methylation patterns in normal and cancer cells. (Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Esteller, Copyright, 2007.)*

2.2.1 DNA hypomethylation

There are several possible causes for the decreased levels of methylation observed in cancer, and it is likely that different cancers exploit different routes to establish a hypomethylated state. At least three hypotheses have been presented that could lead to hypomethylation: 1) deregulation of DNA methylation through changes in the overall chromatin organization, 2) uncoupling of the DNA methylation machinery from the replication machinery as a consequence of a disturbed cell cycle, and 3) depletion of the methyl donor, SAM, from the cells (reviewed in Hoffmann and Schultz 2005).

The first real evidence of a link between cancer and epigenetics dates back to 1983, when several reports showed decreased DNA methylation levels, both globally at repetitive sequences and gene-associated, when comparing cancer cells to normal cells (Diala *et al.* 1983, Feinberg and Vogelstein 1983a, Gama-Sosa *et al.* 1983). Since then, the level of hypomethylation has been shown to correlate with the disease progression in several cancer types and to be an independent marker of poor prognosis in ovarian cancer (Fraga *et al.* 2004, Widschwendter *et al.* 2004). Hypomethylation mainly results from the loss of methylation at normally methylated genomic repeat elements (Yoder *et al.* 1997, Florl *et al.* 1999), and the resulting genomic instability can be viewed as one of the hallmarks of cancer. Additionally, DNA hypomethylation in cancer cells can be observed in specific gene-poor regions of the genome (Weber *et al.* 2005). Rodriguez *et al.* (2006) demonstrated that DNA hypomethylation correlates with cumulative DNA damage in colon cancer, and this DNA hypomethylation-related instability was chromosomal by nature, not associating preferentially with any specific genomic aberration.

DNA hypomethylation is also associated with loss of imprinting (LOI), and in certain cases, this has been shown to contribute to tumor formation. For example, LOI at *IGF2* (insulin-like growth factor) is common in the hereditary Beckwith-

Wiedemann syndrome, which is characterized by macroglossia, gigantism, and an increased risk of cancer (reviewed in Steenman *et al.* 2000, Delaval *et al.* 2006). The LOI at *IGF2* is also linked to the development of Wilm's tumor and to increased risk of colorectal cancer (Cui *et al.* 2003, Steenman *et al.* 2000). The role of *IGF2* in tumorigenesis was demonstrated as early as 1994, when Christofori *et al.* showed that *IGF2* expression potentiated SV40-induced transformation of pancreatic cells by providing the needed growth signal. In prostate cancer the LOI at *IGF2* is associated with aging, and becomes more pronounced during malignant growth (Fu *et al.* 2008).

DNA hypomethylation is also observed in the gene-specific context. Cancer-testis genes are a heterogeneous group of immunogenic proteins (CT antigens) that are normally expressed solely in male germ cells, while being silenced by DNA methylation in somatic tissues. However, some members of these CT antigen families, such as melanoma antigen (MAGE) genes, become demethylated and expressed in various cancers (de Smet *et al.* 1996, de Smet *et al.* 1999, Jungbluth *et al.* 2000). In addition, other genes, such as HRAS, S100 calcium binding protein A4 (S100A4) and synuclein gamma (SNCG), are demethylated, and thus aberrantly expressed, in various cancers including colon, breast, pancreatic, prostate and gastric cancers (Feinberg and Vogelstein 1983b, Ji *et al.* 1997, Nakamura and Takenaga 1998, Rosty *et al.* 2002, Lu *et al.* 2001, Liu *et al.* 2005). Moreover, SNCG has been shown to be a prognostic marker for poor clinical outcome in several cancers including breast, colon and pancreatic cancers (Wu *et al.* 2007, Ye *et al.* 2008, Hibi *et al.* 2009), and its inhibition or downregulation *in vitro* increases the sensitivity of breast cancer cells to antimicrotubule drugs, such as paclitaxel (Zhou *et al.* 2006, Singh *et al.* 2007).

In $Apc^{Min/+}$ mice, *Dnmt1* disruption leads to DNA hypomethylation with a concurrent increase in microadenomas associated with LOH at *Apc*, and a significant decrease in macroscopic intestinal tumors (Yamada *et al.* 2005). This supports the general idea of global DNA hypomethylation being an early event in carcinogenesis, making the genome more susceptible for further, even more detrimental changes to take place, while gene-specific hypomethylation occurs later, affecting more precise mechanisms in carcinogenesis and metastatic spread.

In prostate cancer, hypomethylation of repetitive LINE-1 (long interspersed nuclear element) sequences, which are heavily methylated in normal tissues, is found in approximately half of the cases (Santourlidis *et al.* 1999, Florl *et al.* 2004). In contrast to the general idea of global DNA hypomethylation being an early event in carcinogenesis, the opposite seems to hold true for prostate cancer. Florl *et al.* (2004) showed that CpG hypermethylation of specific genes is an earlier event than LINE-1 hypomethylation, which takes place rather late during tumor progression. Similarly, it was shown that global 5meC content only decreases significantly in metastatic prostate cancer, and LINE-1 hypomethylation also becomes more pronounced in metastatic cancer, even though it can be detected at lower levels earlier in primary lesions (Yegnasubramanian *et al.* 2008). This is evidenced by the heterogeneity of LINE hypomethylation found at different metastatic sites within the same patients. However, no studies have shown that the hypomethylated repetitive regions are actually expressed in prostate cancer. In addition to global

hypomethylation, the cancer-associated re-expression of normally methylation-silenced cancer testis antigens (such as *MAGE* genes) was found to take place rather late in prostate cancer progression (Yegnasubramanian *et al.* 2008). LINE-1 and ALU hypomethylation also associates with several clinical parameters of prostate cancer, like preoperative PSA and Gleason score, and preoperative PSA and tumor stage, respectively (Cho *et al.* 2007). Aside from the clinicopathological parameters, chromosomal aberrations, namely loss of 8p and gain of 8q, are also associated with LINE-1 hypomethylation (Schulz *et al.* 2002).

2.2.2 DNA hypermethylation

In addition to DNA hypomethylation, aberrant CGI hypermethylation has proven to be one of the key mechanisms of cells for silencing TSGs during tumorigenesis and the progression of cancer. The first TSGs identified to undergo transcriptional silencing through DNA methylation were the calcitonin (*CALCA*) and *RB* genes (Baylin *et al.* 1986, Greger *et al.* 1989). Since then, several known tumor suppressors, such as *VHL* (von Hippel-Lindau), *CDKN2A*, *MLH1* (the human homologue of MutL *E. coli*), and *BRCA1* (breast cancer 1, early onset), have been shown to undergo methylation-mediated downregulation in cancer (Herman *et al.* 1994, Merlo *et al.* 1995, Kane *et al.* 1997, Esteller *et al.* 2000a). In addition to finding methylation as the second hit of Knudson's "two hit hypothesis" of previously described TSGs (Grady *et al.* 2000), approaches based on finding methylated genes have enabled the identification of novel TSGs with previously unknown functions in cancer. Tumor suppressors identified through this type of epigenetic profiling include transcription factor gene *ID4* in leukemia (Yu *et al.* 2005), retinoic acid synthesis gene *ALDH1A2* in prostate cancer (Kim *et al.* 2005), and the insulin-like growth factor binding protein 3 (*IGFBP3*) gene in skin cancer (Fraga *et al.* 2004). Table 1 lists some hypermethylated genes in human cancers.

Promoter hypermethylation-mediated TSG silencing can affect various cellular processes, such as cell cycle, DNA repair, cell-to-cell and cell-to-matrix interactions, apoptosis and angiogenesis, all events that can contribute to tumorigenesis (Herman and Baylin 2003, Esteller 2007). The fact that methylation targets several DNA repair-associated genes, such as *MLH1* and *BRCA1*, directly links epigenetic changes with further genetic abnormalities. *Vice versa*, it has been shown that Myc can recruit Dnmt3b to the *Cdkn1a* promoter, and *de novo* methylation is needed for this Myc-mediated gene silencing, thus the primary genetic aberration is targeting further epigenetic silencing (Brenner *et al.* 2005). In addition to DNA methylation, *MLH1* repression in colorectal cancer cells was shown to associate with H3K9me3, demonstrating the cooperation of two epigenetic pathways (Fahrner *et al.* 2002). In colon cancer, DNA hypermethylation of *MLH1* can be observed in the very early abnormalities of the crypt, and these changes differ according to the molecular type of the disease (*i.e.*, sporadic and familial cancers) (Chan *et al.* 2002a). The DNA methylation patterns observed in colon cancer can be used for efficient subclassification of these tumors based on the

presence of the CpG island methylator phenotype (CIMP) (reviewed in chapter 2.2.3).

***Table 1**. Examples of hypermethylated genes in human cancers.*

Gene	Function	Cancer
APC	Inhibitor of β-catenin	Prostate, colon (Florl *et al.* 2004, Lee *et al.* 2004, Ellinger *et al.* 2008)
BRCA1	DNA repair, transcription	Breast, ovary (Esteller *et al.* 2000a)
CDH1	E-cadherin, cell adhesion	Prostate, breast, colon, gastric, other (Graziano *et al.* 2004, Lee *et al.* 2004)
CDKN2A	Cyclin-dependent kinase inhibitor	Glioma, lung, others (Merlo *et al.* 1995, Lee *et al.* 2004)
CDKN2B	Cyclin-dependent kinase inhibitor	Acute myeloid leukemia, Myelodysplastic syndrome (Quesnel *et al.* 1998, Aggerholm *et al.* 1999)
DAPK1	Pro-apoptotic	Colon (Lee *et al.* 2004)
GSTP1	Conjugation to glutathione	Prostate (Lee *et al.* 1994)
HIC1	Transcription factor	Colon (Ahuja *et al.* 1997)
ID4	Transcription factor	Leukemia (Yu *et al.* 2005)
IGFBP3	Growth-factor binding protein	Skin (Fraga *et al.* 2004)
MED15	Transcription factor	Prostate (Cho *et al.* 2007)
MGMT	DNA repair of O6-alkyl-guanine	Glioma, colon (Lee *et al.* 2004)
MLH1	DNA mismatch repair	Colon (Kane *et al.* 1997)
PTGS2	Cyclo-oxygenase-2	Prostate (Ellinger *et al.* 2008)
RARB	Retinoic acid receptor beta	Prostate (Nakayama *et al.* 2001b)
RASSF1	Ras effector homologue	Prostate, breast, various others (Dammann *et al.* 2001, Donninger *et al.* 2007)
RB	Cell cycle inhibitor	Retinoblastoma
THBS1	Thrombospondin 1, anti-angiogenic	Colon, glioma (Ahuja *et al.* 1997, Li *et al.* 1999)
VHL	Ubiquitin ligase component	Kidney (Herman *et al.* 1994)

Profiling multiple cancer types for CGI methylation, at several TSG promoters known to undergo methylation in cancer, has shown the existence of cancer-specific "hypermethylomes" (Costello *et al.* 2000, Esteller *et al.* 2001a). This type of DNA hypermethylation signature can be found both in sporadic and hereditary cancers (Esteller *et al.* 2001b). One genome-wide study on CGI methylation, using colon and prostate cancer cell lines, showed that most genes undergoing *de novo* methylation in cancer are actually already transcriptionally silenced in normal cells, and thus the methylation does not provide additional gene silencing (Keshet *et al.* 2006). Therefore, it was suggested that the *de novo* methylation of genes in cancer takes place in an instructive manner, *i.e.*, the *cis*-acting DNA sequence motifs and *trans*-acting chromatin modifiers recruit DNMTs, resulting in DNA hypermethylation. Contradictorily, a more recent study by Kondo *et al.* (2008) demonstrated that, in prostate cancer cells, EZH2-mediated H3K27 methylation is mostly present at promoters with no or low DNA methylation, suggesting a DNA methylation-independent mechanism for EZH2-targeted TSG silencing. The authors speculated that the apparent discrepancy between these results could actually reflect tissue- and cancer-specific differences related to the activation of specific silencing pathways. The co-operative silencing function of EZH2 and DNMTs would indeed take place during X-inactivation (Plath *et al.* 2003), and silencing of certain cancer types, like colon cancers with the methylator phenotype, whereas they would function independently in other cancers, such as prostate cancer. These observations of the non-coexistence of DNA methylation and H3K27 methylation marks in prostate cancer were confirmed by Gal-Yam *et al.* (2008). The authors presented a model where the repressive mechanisms exist in parallel to reprogram the cancer

epigenome. They showed that developmental genes, which are normally silenced through PRC, undergo epigenetic switching in cancer where H3K27me3 is replaced by DNA methylation, keeping the gene silenced, and possibly reducing epigenetic plasticity by locking the genes into a more stable silencing mode. This is the case for most constitutively silenced genes, whereas *de novo* repression was associated with the two other modes, ^{5me}C reprogramming and PRC reprogramming. In ^{5me}C reprogramming, no marked changes are observed for Polycomb marks, but DNA methylation is increased; while in PRC reprogramming, only Polycomb marks are increased, and DNA methylation levels stay unaltered.

In prostate cancer, there are a number of genes identified that undergo extensive cancer-specific methylation. Methylation of certain genes, namely glutathione S-transferase pi 1 (*GSTP1*), adenomatous polyposis coli (*APC*), Ras association (RalGDS/AF-6) domain family member 1 (*RASSF1*), and retinoic acid receptor beta (*RARB*), is found in 70-95% of prostate cancers, and the methylation profile of these genes is capable of identifying malignant from benign samples with high specificity (Lee *et al.* 1994, Nakayama *et al.* 2001b, Florl *et al.* 2004, Jerónimo *et al.* 2004a, Cho *et al.* 2007). *GSTP1*, *RASSF1* and *RARB* are also methylated in an age-dependent manner, suggesting that methylation may precede and predispose cells to tumor formation (Kwabi-Addo *et al.* 2007). The age-associated methylation, however, remains significantly lower, with full-blown tumors clearly distinguishable from benign tissues by the extent of methylation. The methylation in normal cells appears patchy, especially with *RASSF1*, whereas tumor-associated methylation is more continuous throughout the CGIs (Florl *et al.* 2004). The fact that these genes are so highly methylated in cancer suggests their methylation to be a rather early event in carcinogenesis. Indeed, increased methylation of these genes can be readily detected in the well-established early lesions of prostate cancer, high-grade prostatic intraepithelial neoplasia (HGPIN) (Jerónimo *et al.* 2004b). The methylation of *GSTP1* and *RARB*, as well as the hypomethylation at LINE-1, were demonstrated to associate with elevated levels of the EZH2 histone methyltransferase (Hoffmann *et al.* 2007). Other genes undergoing cancer-specific promoter CGI methylation in prostate cancer include B-cell CLL/lymphoma 2 (*BCL2*), mediator complex subunit 15 (*MED15*, also known as *TIG1*), ATP-binding cassette, sub-family B, member 1 (*ABCB1*, also known as *MDR1*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*, also known as *COX2*), and the methylation of these genes, alone or in pairs, is associated with either the tumor stage or the Gleason score in prostate cancer samples (Cho *et al.* 2007, Ellinger *et al.* 2008).

Hereditary non-polyposis colorectal cancer (HNPCC) is caused by genetic mutations in the DNA mismatch repair (MMR) system genes, *MLH1* and *MSH2* (mutS homologue 2) (reviewed in Lynch *et al.* 2008). Over the past several years, reports have demonstrated the existence of heritable epimutations, *i.e.*, hereditary changes in methylation patterns of patients with HNPCC, affecting MMR genes. The first report showed soma-wide methylation of *MLH1* in an HNPCC-criteria meeting patient with no genetic mutations in *MLH1* or *MSH2*, and methylation was also observed in spermatozoa, indicating the possibility of transmission to offspring (Suter *et al.* 2004). Another report demonstrated *MLH1* epimutation in an HNPCC

patient and mother, suggesting inheritance (Morak *et al.* 2008). Additionally, two individuals with no germline mutations were identified who carry *MLH1* epimutations, and it was suggested that these epimutations are more likely to occur in HNPCC patients with no or weak family history of HNPCC (Gylling *et al.* 2009). Cross-generation epimutations have also been found in *MSH2* (Chan *et al.* 2006), suggesting that inherited epigenetic changes can predispose one to certain hereditary malignancies, including HNPCC.

2.2.3 Alterations in DNA methylation pathway in cancer

Overexpression of all DNA methyltransferases is a common event in several human cancer types (de Marzo *et al.* 1999, Robertson *et al.* 1999). In many cases, this results from the defective degradation of the enzymes at the end of the S-phase (Agoston *et al.* 2005, Agoston *et al.* 2007). Early studies of the role of DNMTs in tumorigenesis showed that NIH 3T3 mouse fibroblasts can be transformed in culture by overexpressing exogenous *Dnmt1* (Wu *et al.* 1993). Similarly, pharmacological inhibition of Dnmt1 activity in *Dnmt1*-heterozygous APC^{min} mice resulted in a reduced number of tumors in the intestinal track (Laird *et al.* 1995).

Genetic disruption of *DNMT1* by homologous recombination in colon cancer cells surprisingly caused only a 20% decrease in overall genomic methylation levels, even though DNMT1 activity was markedly diminished (Rhee *et al.* 2000). While certain repeat sequences became unmethylated, CGIs (*e.g.*, the *CDKN2A* promoter) remained fully methylated. Even less of an effect on global methylation was achieved by disruption of *DNMT3b* (Rhee *et al.* 2002). However, eliminating both of these DNMTs almost fully removed the DNA methylation, rendering the genome vastly unmethylated at repetitive, gene-poor and promoter associated CpGs (Rhee *et al.* 2002). These results support the cooperative and complementary role of the DNMTs in establishing and maintaining DNA methylation. They also give important evidence of the role of methylation-induced TSG silencing in cancer, as the double knock-out cells with marginal DNMT activity also showed significant growth suppression, supposedly due to the reactivation of TSGs (*e.g.*, *CDKN2A*) expression. Using the same *DNMT1*-null model, Karpf and Matsui (2005) showed that *DNMT1*-depletion leads to genomic instability.

As in most other cancers, the expression of DNMTs is also increased in prostate cancer (Patra *et al.* 2002, Morey *et al.* 2006). Using a transgenic adenocarcinoma of mouse prostate (TRAMP) model, Morey *et al.* (2008) showed that the increase in DNMT expression appears to be an early event in prostate cancer and is not associated with increased cell proliferation, as cyclin A-adjusted DNMT expression did not increase in poorly-differentiated tumors but only in HGPIN and well-differentiated carcinomas. Further, increased DNMT expression did not significantly correlate with either promoter CGI hypermethylation or global hypomethylation, suggesting that at least in the TRAMP model of prostate cancer, the amount of DNMTs is not the key factor in cancer-specific methylation events. Another methylation related factor shown to be important in prostate cancer cells is the methyl-CpG-binding protein MECP2. In prostate cancer cells, its expression was

shown to promote growth without the need for androgen stimulation, while shRNA-induced gene silencing lead to growth retardation both in normal and cancerous prostate cells (Bernard *et al.* 2006).

2.2.4 CpG island methylator genotype CIMP

More than ten years ago researchers found a specific subgroup of sporadic colon cancers with high cancer-specific methylation at cyclin-dependent kinase inhibitor 2A (*CDKN2A*, encoding INK4A), thrombosponsin 1 (*THBS1*), *IGF2*, hypermethylated in cancer-1 (*HIC1*), and *MLH1*, and this methylation was specifically observed in cancers characterized by microsatellite instability (MSI) (Ahuja *et al.* 1997, Kane *et al.* 1997 Kuismanen *et al.* 1999, Kuismanen *et al.* 2000). Soon after, this phenomenon of aberrant cancer-associated high-frequency gene methylation was termed the CpG island methylator phenotype, or CIMP (Toyota *et al.* 1999). In general terms, CIMP can be regarded as the frequent methylation of several tumor-related genes present in a highly concordant manner in a subgroup of cancers. The colorectal CIMP+ cancers have 3-5–fold elevated methylation frequency, and they account for ~80% of MSI+ cancers. Toyota *et al.* (1999) suggested that sporadic colorectal cancers could actually be divided into four subgroups based on the presence of MSI and CIMP (*i.e.*, CIMP+MSI+, CIMP+MSI-, CIMP-MSI+ and CIMP-MSI-). Further genetic characterization of CIMP has shown that CIMP+ cancers with MSI resulting from *MLH1* methylation also very frequently carry *BRAF* mutations and have fairly favorable prognosis (Toyota *et al.* 1999, Kambara *et al.* 2004, Shen *et al.* 2007, Ogino *et al.* 2009). The CIMP+MSI- cancers are in part characterized by frequent *KRAS* mutations and have poorer clinical outcome than CIMP+MSI+ cancers (Toyota *et al.* 2000, Lee *et al.* 2008). On the other hand, CIMP+ tumors only rarely carry *TP53* mutations, which are commonly found in CIMP- tumors lacking *KRAS* and *BRAF* mutations (Toyota *et al.* 2000, Samowitz *et al.* 2005, Shen *et al.* 2007). In HNPCC, no *BRAF* mutations are found, whereas *KRAS* is frequently mutated, with the most common mutation type being the same commonly found also in MSI+ sporadic cancers with no *MLH1* methylation (CIMP-MSI+) (Oliveira *et al.* 2004). These results imply that HNPCC and sporadic colorectal cancers, depending on their MSI status and *MLH1* methylation, may target distinct RAS-ERK-MAPK pathway kinases with preferential mutation types.

Additionally, CIMP is more commonly observed in older patients, in females and in tumors localizing to the proximal colon (Samowitz *et al.* 2005). Goel *et al.* (2007) showed that as CIMP often associates with MSI, it has an inverse correlation to chromosomal instability (CIN), which is frequently demonstrated by loss of heterozygosity (LOH) at several tumor suppressor loci. This suggests that CIN and CIMP are two independent mechanisms by which genetic and epigenetic instability might be achieved in colorectal cancers. Similar results were obtained in a study were CIN was defined by both copy number changes and LOH (Cheng *et al.* 2008). Additionally, two very recent papers demonstrated that $CIMP^{+}MSI^{+}$ tumors associate with overexpression of DNMT3B (the *de novo* DNA methyltransferase)

and SIRT1 (an HDAC), offering mechanistic evidence for the increased CGI methylation and gene silencing observed in these cancers (Nosho *et al.* 2009a, Nosho *et al.* 2009b).

Apart from being largely studied in colon cancer, CIMP has also been observed in other cancer types as well. In gastric cancer, CIMP is associated with tumor MSI, and patients with CIMP$^+$ tumors seem to have longer median survival than those with CIMP$^-$ tumors (An *et al.* 2005). However, CIMP status is not an independent predictor of overall survival in gastric cancer. In neuroblastomas and in a subgroup of childhood acute lymphoblastic leukemias (ALL) characterized by t(12;21) translocation, the CIMP phenotype significantly associates with poor prognosis (Abe *et al.* 2005, Roman-Gomez *et al.* 2006).

2.3 Epigenetics and miRNAs in cancer

Since the discovery of miRNAs in 2001, numerous reports have implicated them in various diseases, including cancer. Expression profiling of miRNAs can be used to accurately identify tumor subgroups and predict clinical outcome, even more so than with the previously used mRNA expression profiling (Lu *et al.* 2005). Similarly to protein coding genes in cancer, miRNA genes have been shown to harbor mutations, undergo gene copy number changes, and become aberrantly regulated and expressed (Calin *et al.* 2004 Calin *et al.* 2005, Zhang *et al.* 2006, Porkka *et al.* 2007). One of the first miRNAs shown to have oncogenic properties was the *miR-17-92* cluster, which is overexpressed in cancers (He *et al.* 2005). Another miRNA, *let-7*, targets the *RAS* oncogene and is commonly downregulated in cancer, thus contributing to tumorigenesis by reverting *RAS* silencing (Johnson *et al.* 2005). Recently, tens of miRNAs have been shown to have either oncogenic or tumor suppressor functions.

Apart from genomic alterations, epigenetic changes also affect miRNAs in cancer. miRNAs are known to be both epigenetically regulated themselves and to target components of the epigenetic machinery. Several tumor suppressor property-possessing miRNAs, such as *miR-34a* targeting E2F transcription factor 3 (*E2F3*) and *BCL-2*, *miR-124a* targeting cyclin dependent kinase 6 (*CDK6*), and *miR-127* targeting *BCL-6*, have been shown to be epigenetically silenced through DNA methylation in various cancers (Saito *et al.* 2006, Lujambio *et al.* 2007, Lodygin *et al.* 2008). In contrast, *let-7a-3* was shown to be normally methylated and to become hypomethylated and expressed in lung adenocarcinoma cell lines (Brueckner *et al.* 2007). Another study found increased methylation of *let-7a*-3 in ovarian cancers, with this methylation associating with reduced mortality (Lu *et al.* 2007). The slightly contrasting *let-7* data most likely reflects the varying expression and functional patterns of the *let-7* family members.

In oral cancer and normal cell lines, expression profiling and pharmacological reversal of epigenetic modification led to the identification of four miRNAs (*miR-34b, miR-137*, *miR-193a* and *miR-203*), which were all methylated in a cancer-specific manner (Kozaki *et al.* 2008). It was further shown that expression of *miR-137* and *miR-193a* leads to downregulation of their potential target genes, *CDK6*

and *E2F6*, respectively. *miR-34b/c* has also been implicated in several other cancers (*i.e.*, leukemia, melanoma, colon cancer, and head and neck cancer), where its expression is silenced through aberrant CpG methylation (Lujambio *et al.* 2008, Toyota *et al.* 2008, Roman-Gomez *et al.* 2009). Moreover, Lujambio *et al.* (2008) suggested that the use of DNA methylation profiles of miRNAs as prognostic markers for identifying cancers likely to have metastatic potential.

In prostate cancer, there are a few reports on miRNAs being regulated through methylation. Lodygin *et al.* (2008) showed that *mir-34a* is methylated in ~80% of primary prostate cancers (and to a lower extent in melanoma), and in cancer cell lines of multiple origins. Re-expression of *miR-34a* leads to cell cycle arrest, at least partly through targeting *CDK6*. DNA methylation-mediated silencing has also been observed for *miR-9* in leukemia, breast and colon cancers (Lehmann *et al.* 2008, E *et al.* 2009, Roman-Gomez *et al.* 2009). In ovarian cancer, *miR-9* expression was also decreased significantly and shown to be a potent marker for recurrent ovarian cancer together with *miR-223* (Laios *et al.* 2008). *miR-126* resides intronically in the EGF-like domain, multiple 7 (*EGFL7*) gene. This gene can be transcribed using three different promoters. One of them is embedded in a CGI, and treatment with DNA demethylating agent and HDAC inhibitor induces both *EGFL7* and *miR-126* expression in human cancer cell lines (Saito *et al.* 2009). It was further shown that *miR-126* expression is decreased in samples of prostate and bladder cancer, and the silencing of expression in the cancer cell lines does not result from DNA methylation but more likely from histone deacetylation.

While most studies thus far have described DNA methylation-mediated silencing of miRNAs, aberrant histone modifications in miRNA regulation have also been reported. Ke *et al.* (2009) performed a genome-wide analysis of active (H3K4me3) and repressive (H3K27me3) histone marks in prostate cancer and normal cell lines. They found that the expression status of differentially expressed miRNAs between the PC-3 prostate cancer cell line and the EP156T normal prostate epithelial cell line highly correlated with the histone marks at the miRNA genes. Lowly expressed miRNAs were enriched with H3K27me3 and highly expressed miRNAs with H3K4me3. Among the miRNAs showing strong histone modifications mediated expression regulation were *miR-205* and *miR-200b*, both of which have been shown to target transcription factors ZEB1 (zinc finger E-box binding homeobox 1) and SIP1 (survival of motor neuron protein interacting protein 1) (Gregory *et al.* 2008), through which they could potentially contribute to carcinogenesis. Roman-Gomez *et al.* (2009) identified epigenetically silenced miRNAs in ALL cell lines through ChIP (chromatin immunoprecipitation) -on-chip. Nine miRNAs (*miR-9, -10b, -34b, -34c, -124a, -132, -196b, -203* and *-212,* 13 loci) were enriched for repressive H3K9me2 with concomitant low levels of active H3K4me3 marks at their promoters. Methylation-specific PCR (MSP) showed that all of these miRNAs were also methylated at their promoter CGIs. In a large set of ALL samples, 65% harbored methylation of at least one of these miRNAs, and the methylation profile was an independent prognostic factor for predicting disease-free survival and overall survival associating with poor prognosis.

In addition to miRNAs being targeted by epigenetic modifications, miRNAs can target regulators of epigenetic pathways. *de novo* DNMTs, DNMT3A and

DNMT3B, are known to be direct targets of *miR-29* family miRNAs, which are commonly downregulated in lung cancers (Fabbri *et al.* 2007). Restoration of *miR-29* expression decreases DNMT3A and DNMT3B levels in lung cancer cells with concurrent reactivation of known TSGs. HDAC1 expression is elevated in several cancers (Marks *et al.* 2001), and Noonan *et al.* (2009) recently showed that HDAC1 is targeted by *miR-449a* in prostate cancer. Another histone modifier with increased expression in prostate cancer, EZH2, is targeted by *miR-101* (Varambally *et al.* 2008). Both of these miRNAs are downregulated in prostate cancer, and targeted re-expression results in cell cycle arrest, suggesting them as potential targets for reversing epigenetic aberrations in prostate cancer. In glioma cells, *miR-128* was shown to directly target the BMI1 polycomb ring finger oncogene (*BMI1*), a component of the PRC1 repressor complex (Godlewski *et al.* 2008). *miR-128* expression was significantly reduced in gliomas compared to normal brain cells, and this coincided with a marked increase in BMI1 expression. Downregulation of BMI1 through re-expression of *mir-128* resulted in reduction of H3K27me3, together with recently described BMI1-loss associated self-renewal of neuronal stem cells with a concomitant increase in *CDKN1A* and a reduction in *AKT* expression (Guo *et al.* 2007, Fasano *et al.* 2007).

Not only miRNAs, but also the components of the machinery producing them, are of importance to epigenetics. DICER1, the enzyme processing both siRNAs and miRNAs in their precursor forms upon maturation and loading to RITS complex, is essential for the maintenance of proper telomeric DNA methylation in mouse ES cells (Benetti *et al.* 2008). *Dicer1* depletion results in a similar phenotype as seen with the depletion of DNMTs, with DNA methylation defects at subtelomeric regions, increased telomeric recombination and elongated telomeres. The link between Dicer1 and DNMTs was further shown as *miR-290*-regulated Rb proteins are capable of silencing DNMT expression. Similarly, Ting *et al.* (2008) showed that DICER is needed for proper maintenance of CGI methylation-mediated gene silencing in colon cancer cells. In prostate cancer, DICER is upregulated, and the upregulation correlates with tumor grade and stage (Chiosea *et al.* 2006). The demonstrated deregulation of the miRNA machinery could explain, in part, the common deregulation of miRNAs themselves in cancer. These findings importantly show alternative ways to target epigenetic modifications apart from the actual DNMTs and histone modifiers themselves.

2.4 Epigenetics in cancer management and treatment

Due to the fact that many epigenetic changes occur early in cancer, they have great potential for use in diagnostic settings. Methylated TSGs as cancer biomarkers especially hold promise for early detection of several cancers. These epigenetic changes can also be used as prognostic factors, as certain modifications may be indicative of further cancerous behavior or the aggressiveness of the disease. The reversible nature of DNA methylation and histone modifications also makes them highly attractive targets for cancer therapeutics. There are currently dozens of

ongoing clinical trials to study the effects of various inhibitors targeting epigenetic modifications, both on their own and in combination with traditional chemotherapeutic drugs (reviewed in Mai and Altucci 2009).

2.4.1 Epigenetic cancer diagnosis and prognosis

For a biomarker to be valuable in clinical diagnostic use, it must not only perform with high sensitivity (*i.e.*, detect accurately all the patients with the disease) and specificity (*i.e.*, exclude accurately the subjects without the disease from the diagnosis) but also be detectable in a sample obtained non-invasively (*e.g.*, sputum, stool, urine or blood). The methylation-based biomarker analyses are thought to be especially feasible since detached tumor cells or free-floating DNA is readily found in bodily fluids.

One of the most promising epigenetic biomarkers is the DNA methylation of *GSTP1* in prostate cancer. Because this gene is methylated in ~90% of prostate cancers, and the methylation appears in early lesions (HGPIN) while remaining unmethylated in normal prostate and benign prostate hyperplasia, it can function as a highly specific marker for prostate cancer (Lee *et al.* 1994, Jerónimo *et al.* 2004b). A recent paper by Yegnasubramanian *et al.* (2006) introduced an assay combining methylated DNA immunoprecipitation with methylation sensitive restriction enzymes (COMPARE-MS) to identify *GSTP1* methylation in prostate cancer. Their assay reached 99.2% sensitivity with 100% specificity. These highly cancer/non-cancer discriminative results with *GSTP1* have mostly been obtained using tissue samples. Several studies have investigated the potential of also detecting *GSPT1* methylation in urine, plasma and serum samples with contradictory results. The first reports on *GSTP1* methylation detection from urine samples showed that only ~30% of the methylated cancers were detected (Cairns *et al.* 2001). Hoque *et al.* (2005) reported *GSTP1* methylation detectable in 48% of urine samples from patients with methylation-positive primary tumors, with no methylation observed in age-matched controls. They also showed that the combinatory analysis of methylation status of four genes, *GSTP1*, *CDKN2A* (both *p16* and *ARF*) and *ABCB1*, was able to detect prostate cancer with 87% sensitivity at 100% specificity. Bastian *et al.* (2008), in turn, studied methylation of TSGs in serum samples from prostate cancer patients. While *GSTP1* methylation was detected in <20% of cancers, *ABCB1* methylation was detectable in ~40% of primary tumors and in ~90% of metastases. Another recent study compared the efficacy of prostate cancer detection based on methylation markers in urine and plasma samples (Payne *et al.* 2009). They found urine to be more sensitive and specific for prostate cancer detection than plasma samples. In their analysis, *GSTP1* methylation was detected with 63% sensitivity at 95% specificity when young asymptomatic males were used as controls. In their panel of tested markers (*GSTP1*, *RASSF2*, *TFAP2E* and *HIST1H4K*), combinations of several genes did not improve the detection rate for prostate cancers. Additionally, the detection of *GSTP1* methylation after digital rectal examination (DRE) was shown to be concordant with methylation detected after prostate needle

biopsy, suggesting post-DRE urine samples as suitable for epigenetic analysis as post biopsy samples (Rogers *et al.* 2006). In a study of two large cohorts, methylation of *APC* associated significantly with increased risk of prostate-cancer specific mortality, while the methylation of *GSTP1* had no prognostic value (Richiardi *et al.* 2009). These results imply that some methylated genes, such as *GSTP1*, might be useful for the diagnostic purposes and others, such as *APC*, are more indicative of the aggressiveness of the disease.

Other genes studied for their potential use as diagnostic tools in various cancer types include *CDKN2A*, *CDKN2B*, *RASSF1*, *APC*, E-cadherin (*CDH1*) and death-associated protein kinase 1 (*DAPK1*) (reviewed in Grønbaek *et al.* 2007). For example, *CDH1* methylation in gastric cancers is seen in more than half of the patients (Graziano *et al.* 2004). Muretto *et al.* (2008) demonstrated how endogastric capsules used for obtaining gastric juice can be used to detect *CDH1* methylation in gastric cancer patients and to differentiate them from controls. Moreover, this may also be a feasible way to assess the risk of hereditary diffuse gastric cancer in families with predisposing germline mutations in *CDH1*, as hypermethylation of the other allele of *CDH1* has been shown be a common way to produce ”the second hit” (Grady *et al.* 2000). *CDH1* and *DAPK1* might also be useful in the detection of bladder cancer, as their methylation was detected in 63% and 58% of urine samples from cancer patients, respectively, but not in normal urine controls (Chan *et al.* 2002b). In breast cancer, *RASSF1* methylation is readily observed in 65% of the patients (Dulaimi *et al.* 2004). *RASSF1* methylation appears as an early change in breast cancer with the progressive nature of increased methylation in more aggressive tumors (Dammann *et al.* 2001, Yan *et al.* 2003). Methylation analysis using serum samples showed a high sensitivity of 88%, suggesting the potential of *RASSF1* methylation analysis from serum samples in early detection of breast cancers (Shukla *et al.* 2006). A fairly recent study on breast cancer identified a large number of cancer-specifically methylated genes that were highly specific with sensitivity varying between 34 and 90% (Ordway *et al.* 2007). Their most specific (100%) and sensitive (90%) marker for breast marker was methylation of the growth hormone secretagogue receptor (*GHSR*) gene. While many of these genes represent high cancer-specific methylation as detected from tumor tissues, their clinical usefulness as biomarkers detectable in non-invasively obtained samples is not as fully established and requires further validation.

The prognostic use of methylation markers has also been studied. A prime example of a methylated TSG with prognostic significance is O-6-methylguanine-DNA methyltransferase (*MGMT*) in gliomas. MGMT is a DNA repair enzyme responsible for reversing DNA alkylation, a phenomenon leading to cross-linking of DNA and thus resulting in cell death. The expression of *MGMT* is regulated by methylation in glioma cells (Costello *et al.* 1994), and ~30% of gliomas are negative for *MGMT* expression (Silber *et al.* 1998). These *MGMT* expression-negative gliomas are clinically responsive to treatment with alkylating agents, and the methylation status of *MGMT* is an independent and strong positive prognostic factor for glioma (Esteller *et al.* 2000b). Moreover, the extent of methylation is prognostically significant in patients treated with chemotherapy and alkylating

agents, with high methylation of *MGMT* associated with longer progression-free survival and overall survival (Dunn *et al.* 2009).

The detection of aberrant histone modifications for diagnostic, prognostic or predictive purposes in cancer has not been studied as extensively as DNA methylation. However, within the past several years, reports on the applicability of these modifications have also started to emerge. The first such report showed H3K18ac, H3K4me2 and H4R3me2 to be useful marks in classifying low-grade prostate cancers into prognostically different subgroups (Seligson *et al.* 2005). While these histone modifications were not able to provide information on recurrence-free time on the sample set as a whole, primary classification based on clinical grading allowed identification of a group of low-grade patients with decreased H3K18ac and H3K4me2 levels significantly associating with shorter progression-free survival. The decreased levels of H3K18ac and H3K4me2 were more recently shown to also associate with poor prognosis of lung and kidney cancers (Seligson *et al.* 2009). In these three different cancer types, low levels of H3K18ac and H3K4me2 were prognostic factor for short disease-free survival independent from classical clinicopathological variables. Additionally, low levels of H3K9me2 were associated with poor prognosis of prostate and kidney cancers. Furthermore, this loss of histone modifications in aggressive cancers was shown to correlate with decreased levels of histone modifications at genomic repetitive elements while the overall promoter specific histone marking remained unchanged (in line with the known function of de-repressed repetitive elements in chromosomal instability and carcinogenesis).

In contrast to prostate, lung and kidney cancers, low levels of H3K18ac and H3K27me3 in squamous cell carcinoma of esophagus were associated with better survival, especially in the early stage cancers (Tzao *et al.* 2009). Wei *et al.* (2008), in turn, reported that low H3K27me3 levels associate with poor prognosis of breast, ovarian and pancreatic cancers. These results are in contrast with previous findings showing high EZH2 (the H3K27 methyltransferase) expression to associate with aggressive breast cancer and being an independent prognostic factor for breast cancer recurrence and death (Kleer *et al.* 2003). Wei *et al.* (2008) did not find any correlation between H3K27me3 and EZH2 expression. One possible explanation for these rather perplexing results is the lately described EZH2 overexpression-driven formation of the PRC4 complex (Kuzmichev *et al.* 2005). This complex has slightly differing histone methylation target preferences as compared to the PRC2 complex; it preferentially methylates H1 instead of H3.

In addition to the histone modifications themselves, some histone modifying enzymes also exhibit potential prognostic value. In prostate cancer, high EZH2 expression associates with poor prognosis (Varambally *et al.* 2002, Laitinen *et al.* 2008), while overexpression of LSD1, another HMT, correlates with poor clinical outcome in neuroblastomas (Schulte *et al.* 2009). The association of LSD1 expression with prostate cancer prognosis, however, is controversial, as there is data both for and against such association (Kahl *et al.* 2006, personal communication, Suikki H and Visakorpi T). hMOF, a histone acetyltransferase, is downregulated in primary breast cancers and medulloblastomas (Pfister *et al.* 2008). hMOF specifically acetylates H4K16, and the loss of this acetylation mark is common in

various cancers (Fraga *et al.* 2005, Taipale *et al.* 2005). This observed low hMOF expression, as well as the decreased levels of H4K16ac, were independent prognostic factors for poor survival in medulloblastomas. The polycomb repression signature consisting of 14 directly PcG-downregulated genes in prostate cancer metastasis was established by modeling several prostate cancer microarray data sets and genome-wide mapping of H3K27me3 (Yu *et al.* 2007). This signature was able to classify prostate and breast cancer patients with significantly different clinical outcomes, with PcG repression associated with poor prognosis. Notably, they also demonstrated that the PcG repression signature in cancer is markedly similar to H3K27me3 targets in stem cells (Lee *et al.* 2006), thus functionally linking stem cells, cancer metastasis and poor prognosis.

2.4.2 Epigenetic cancer therapy

Epigenetic alterations are highly attractive targets for clinical interventions due to their reversible nature. The aim of epigenetic therapies is to convert the epigenome of rapidly dividing tumor cells closer to a "normal" state (*e.g.*, reactivate methylation-silenced TSGs through inhibition of DNMTs). The more slowly growing or static normal cells remain less affected by these therapeutic compounds. The different strategies using epigenetic therapy for cancer are summarized in Figure 6.

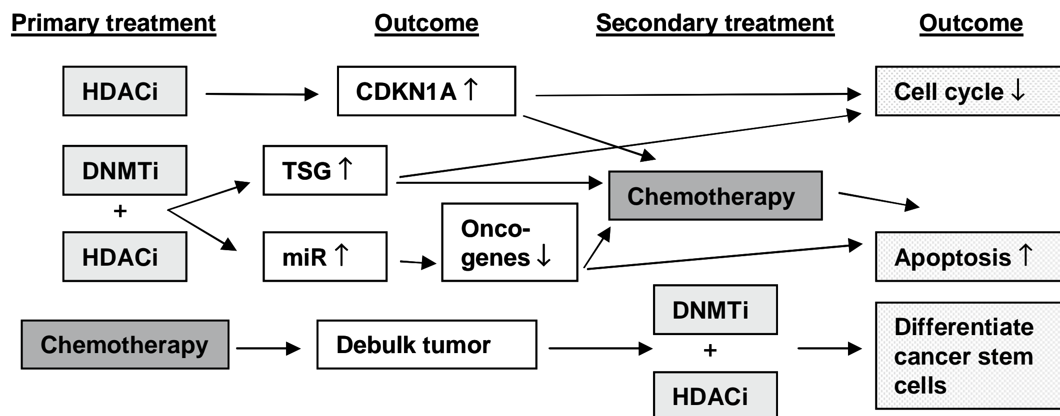

Figure 6. *Strategies for epigenetic therapy in cancer. (HDACi, HDAC inhibitor; DNMTi, DNMT inhibitor). (Modified from Grønbaek et al. 2007).*

Two widely studied nucleoside analogues for DNA methylation inhibition are 5-azacytidine (5azaC) and 5-aza-2'-deoxycytidine (5azadC), clinically known as azacitadine (Vidaza®) and decitabine (Dacogen®), respectively. Both of these compounds are approved by the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS), a hematopoietic malignancy with a high risk of transformation into AML. The chemical structures of 5azaC and 5azadC differ from cytidine and deoxycytidine, respectively, only by the replacement of the

fifth carbon of the pyrimidine ring with a nitrogen. When these nucleoside analogues are incorporated into DNA, they trap DNMTs by covalently binding them (Michalowsky *et al.* 1987), resulting in the eventual depletion of DNMTs from the cells and leading to DNA demethylation during the subsequent replication cycles as DNA methylation is not copied to the daughter cells (Jones and Taylor, 1980, Stresemann *et al.* 2008).

Due to their mechanism-of-action, DNMT inhibitors are more effective when administered over a period of time. Indeed, it has been shown that for hematopoietic malignancies, extended (10 d) low-dose administration is more effective than higher doses or prolonged (20 d) administration (Issa *et al.* 2004). Moreover, not only do MDS patients treated with decitabine responded to the treatment, but for high risk MDS patients, decitabine treatment is an independent favorable prognostic factor for survival, with significant decreases in mortality rates when compared to patients undergoing intensive chemotherapy (Kantarjian *et al.* 2006, Kantarjian *et al.* 2007). In addition to being used alone, DNMT inhibitors have also been tested for their clinical applicability as an adjuvant therapy together with more traditional treatment options, like radio- and chemotherapy. Several reports demonstrate increased or restored sensitivity to primary therapy after decitabine treatment (Plumb *et al.* 2000, Appleton *et al.* 2007, Oki *et al.* 2007, De Schutter *et al.* 2009).

The major drawbacks of these first-generation DNMT inhibitors are their relative instability *in vivo* and their hematological toxicities. Novel DNMT inhibitors, such as Zebularine, are currently being developed and tested, and it is hoped that they will overcome these problems (Holleran *et al.* 2005). Additionally, several non-nucleoside-analogue small-molecules, such as procaine, have been tested for their potential as DNA methylation inhibitors (Villar-Garea *et al.* 2003, reviewed in Mai and Altuzzi 2009). The mechanism of DNA demethylation for all of these non-nucleoside compounds is not known, but procaine is thought to function by binding to CpG-rich sequences and thereby preventing DNMT action on those genomic regions (Villar-Garea *et al.* 2003). Regardless of their potential in evading the problems encountered with azacitadine and decitabine, these small-molecule compounds appear less effective in the actual DNA demethylation (Chuang *et al.* 2005), thus, novel compounds and additional studies are needed.

In addition to silencing TSGs, promoter methylation maintains the silenced state of several pro-invasive and pro-metastatic genes (Ateeq *et al.* 2008). These genes are just as likely to become reactivated upon DNMT inhibition, and the net result of such a therapy may actually promote rather than slow the cancer progression. The delicate balance between targeted demethylation of cancer-specifically hypermethylated CGIs and more global DNA hypomethylation remains one of the hurdles to tackle when targeting the DNA methylation machinery for anti-cancer therapy.

HDAC inhibitors are another group of compounds targeting epigenetic modifications that are widely assessed in clinical trials. HDAC inhibitors affect cancer cells through induction of apoptosis and cell cycle arrest and by inhibiting angiogenesis (reviewed in Johnstone and Licht 2003). Similar to DNMT inhibitors, HDAC inhibitors can reactivate the expression of hypoacetylation-silenced TSGs. For example, suberoylanilide hydroxamic acid (SAHA), a general HDAC inhibitor,

has been shown to specifically induce *CDKN1A* expression with a concurrent increase in acetylation of *CDKN1A*-associated histones (Richon *et al.* 2000). However, the *CDKN1A*-induced cell cycle arrest is dependent on a functional ATM (ataxia telangiectasia mutated) pathway, through which the HDAC inhibition-induced *CDKN1A* functions (Ju and Muller 2003).

As the HDACs can be divided into four classes, the HDAC inhibitors can also be categorized according to their specificities in targeting one or several HDAC classes. For example, the widely studied trichostatin A (TSA) and SAHA are general HDAC inhibitors targeting all HDAC classes (except class III, *i.e.*, SIRTs), while depsipeptide specifically inhibits class I HDACs (reviewed in Grønbaek *et al.* 2007). SAHA, like other hydroxyamic acid-type HDAC inhibitors, inhibits HDACs by binding the Zn ion in the catalytic pocket of the enzyme, thus rendering the enzyme catalytically inactive. SAHA is currently the only FDA approved HDAC inhibitor, being approved for the treatment of cutaneous T-cell lymphoma (CTCL). Early trials with SAHA showed an overall response rate of ~30% in CTCL patients, with non-responding patients also benefiting from reduced pruritus (Olsen *et al.* 2007). SAHA is currently in clinical trials for the treatment of various solid tumors. Results from phase II trials with breast cancer and recurrent glioblastoma multiforme suggest no significant function for SAHA alone, but combinatorial therapies might prove useful in further trials (Luu *et al.* 2008, Galanis *et al.* 2009).

A large number of other HDAC inhibitors are also undergoing clinical trials, both alone and in combination with chemo- and radiotherapy (Karagiannis and El-Osta 2006). *In vitro* studies have shown how HDAC inhibitors sensitize tumor cells to chemotherapeutic compounds and radiotherapy, as well as overcome drug resistance (Maiso *et al.* 2006, Kim *et al.* 2006, Sonnemann *et al.* 2006). Additionally, these agents are tested in combination with DNMT inhibitors, as the synergistic inhibition of these epigenetic silencing mechanisms is known to be more effective for the induction of certain target genes (Cameron *et al.* 1999). Response to this type of therapy has been seen in AML (Gore *et al.* 2006). While the effect of HDAC inhibitors has been studied *in vitro* in prostate cancer cell lines with some promising results (Kim *et al.* 2007, Sonnemann *et al.* 2007), no reports on HDAC inhibition in prostate cancer clinical trials are available.

Similar to DNMT inhibition, HDAC inhibition can also reactivate unwanted gene targets. As recently demonstrated *in vitro* by Hauswald *et al.* (2009), treatment of AML cells with various HDAC inhibitors resulted in a drug resistance phenotype that was even broader than the "classic multidrug resistance phenotype", which is characterized by the expression of the multidrug resistance 1 (*MDR1*) gene, encoding a unidirectional drug-efflux pump (Shen *et al.* 1986). These results raise an important concern for the potential adverse effects that the use of these agents as anticancer therapeutics may have.

AIMS OF THE STUDY

The aim of this study was to identify epigenetically silenced genes in different prostate cancer cell line models and subsequently evaluate their clinical significance in tumor samples. The specific aims were:

1. To characterize gene copy number and gene expression changes in the TRAMP-C2 transgenic mouse prostate cancer cell line, and compare them to human prostate cancer.
2. To identify epigenetically silenced genes in the TRAMP-C2 cell line, and further study them in human prostate cancer.
3. To identify epigenetically silenced genes in the PC-3 human prostate cancer cell line, and evaluate their clinical significance in benign and malignant prostate samples.
4. To identify epigenetically silenced micro-RNAs (miRNAs) in human prostate cancer cell lines, and evaluate their clinical significance in benign and malignant prostate samples.

MATERIALS AND METHODS

1. Cell lines (I, II, III)

Human prostate cancer cell lines (PC-3, DU145, LNCaP, and 22Rv1), the TRAMP-C2 murine prostate cancer cell line and the HEK293T/17 human kidney cell line were obtained from the American Type Cell Collection (Manassas, VA, USA). Human prostate cancer cell lines (LAPC-4 and VCaP) and the hTERT-transformed human normal prostate epithelial cell line (EP156T; Kogan *et al.* 2006) were kindly made available by Dr. Charles Sawyers (MSKCC, New York, NY, USA), Dr. Jack Schalken (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands) and Dr. Olli Kallioniemi (Institute for Molecular Medicine Finland FIMM, Helsinki, Finland). Primary prostate epithelial cells, PrECs, were obtained from Lonza (Lonza Walkersville Inc., Walkersville, MD, USA). All cells were grown under the conditions recommended by the provider.

2. DNA demethylation and histone deacetylation inhibition treatments (I, II, III)

All human prostate cancer cell lines and primary prostate epithelial cells were treated with 1 µM 5-aza-2'deoxycytidine (5azadC; Sigma Aldrich, St Louis, MO, USA) for 72 h, starting 24 h after seeding the cells. When indicated, 300 nM trichostatin A (TSA, Sigma Aldrich) was added for the last 24 h. For TSA treatment alone, the cells were first grown for 72 h and then exposed to 300 nM TSA for 24 h. In study I, additional concentrations of 500 nM 5azadC and 1 µM TSA were used. TRAMP-C2 cells were treated with 50 nM 5azadC for 48 h starting 48 h after seeding. When indicated, 100 nM TSA was added for the last 24 h. TRAMP-C2 cells treated with TSA alone were first grown for 48 h and then treated with 100 nM TSA for 24 h. After the treatments with 5azadC and/or TSA, the cells were harvested in Trizol Reagent and total RNA was extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

3. Clinical tissue samples (I, II, III)

Freshly frozen prostate tumor specimens representing benign prostate hyperplasia (BPH, I: n = 9; II: n = 9; and III: n = 4), untreated prostate cancer (untreated PCa, I: n = 30; II: n = 29; and III: n = 5), and castration-resistant PCa (CRPC, I: n = 12; II:

n = 11; and III: n = 4) carcinomas were obtained from Tampere University Hospital (Tampere, Finland). The BPH samples were obtained from prostatectomy specimens from cancer patients and were histologically verified not to contain any cancerous cells. Samples from castration-resistant prostate cancers (CRPC) were obtained from transurethral resections of the prostate (TURP) from patients experiencing urethral obstruction despite ongoing hormonal therapy. The specimens were histologically examined for the presence of tumor cells using H&E staining. Only samples containing >60% cancerous or hyperplastic epithelial cells were selected for the analyses. The time from the beginning of hormonal therapy to progression (TURP) varied from 15 to 60 months. The use of clinical tumor material was approved by the Ethical Committee of Tampere University Hospital.

4. Immunohistochemistry (IHC) (I)

Protein expression was established using standard immunohistochemical techniques. Polyclonal rabbit antibodies were used against dual-specificity phosphatase 1, DUSP1 (M-3786, Sigma-Aldrich) and serum/glucocorticoid regulated kinase 1, SGK1 (#3272, Cell Signaling Technology, Beverly, MA, USA). For improved staining, high-temperature antigen retrieval in autoclave was used. The primary antibody was visualized using anti-rabbit antibody conjugated to horseradish peroxidase with diaminobenzidine as a chromogen (PowerVision+ Detection System, ImmunoVision, Springdale, AR, USA). DUSP1 and SGK1 expressions were classified into two groups (no or weak staining, 0 or 1+, and moderate or high staining, 2+ or 3+).

5. Microarrays (I, II, III)

Various microarray platforms were used to study gene copy number (II), gene expression (I, II) and miRNA expression (III). The array data were submitted to the ArrayExpress database using MIAMExpress (II, III) (accession numbers E-MEXP-1609, E-MEXP-1610, E-MEXP-1611, E-MEXP-2313, and E-MEXP-2319).

5.1 cDNA microarrays (I, II)

A human cDNA microarray consisting of 432 cDNA clones from Suppression Subtractive Hybridization (SSH) between the PC-3 prostate cancer cell line and a BPH sample was used to screen for epigenetically regulated genes in prostate cancer (I). The construction of this library is described in Porkka *et al.* (2001). Briefly, a PCR-Select cDNA Subtraction Kit (BD BioSciences, Palo Alto, CA, USA) was

used to create an SSH-cDNA library with cDNA clones present more abundantly in the BPH sample than in the PC-3 cell line. This library was TOPO TA-cloned, amplified and spotted in triplicate onto poly-L-lysine-coated glass slides at the Finnish DNA Microarray Center (Turku, Finland).

Mou15K-1 murine cDNA slides for the gene copy number and gene expression analyses of the TRAMP-C2 cell line were obtained from the Finnish DNA Microarray Center (II). These arrays contained ~15,000 mouse cDNA clones spotted in duplicate. All cDNA microarrays were pre-treated with succinic-anhydride according to the manufacturer's protocol.

5.1.1 Expression analysis (I, II)

SSH-cDNA arrays were used to study gene expression in the PC-3 prostate cancer cell line after relieving the epigenetic modifications by treating the cells with 5azadC and/or TSA (I). Total RNA (40 µg) from the treated and untreated PC-3 cells was oligo d(T)18-primed, Cy5- and Cy3-labeled, respectively, and co-hybridized overnight at 65°C onto the cDNA slides. After hybridization, slides were washed and scanned a using ScanArray 4000 scanner (GSI Lumonics, Billerica, MA, USA). QuantArray software (Packard BioScience, BioChip Technology LCC, Billerica, MA, USA) was used to quantitate signal intensities. Cy5 and Cy3 signals were normalized to the mean signals of one subarray. Increased expression after 5azadC and/or TSA treatments was defined as the median Cy5/Cy3 ratio of the background subtracted signals of three replicates >2. These clones were amplified using adaptor-specific NES1 and NESR1 primers (BD BioSciences), the PCR products purified and sequenced with a BigDye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

Mou15K-1 cDNA microarrays were used for TRAMP-C2 gene expression profiling (II). TRAMP-C2 gene expression *per se* was studied using normal mouse total RNA (Clontech, Mountainview, CA, USA) as a reference. For epigenetic profiling after 5azadC and/or TSA treatments, untreated TRAMP-C2 total RNA was used as a reference. Sample preparation and hybridization were carried out as above. After washes, slides were scanned using an Agilent DNA Microarray Scanner BA (Agilent Technologies, Santa Clara, CA, USA), and signal quantification was done with Feature Extraction Software Version 7.5.1 (Agilent Technologies). Before analysis, low-quality data points (*i.e.*, low signal-to-noise ratio, saturated signal intensity or non-uniform signal) were omitted. Non-background subtracted signals were normalized using the LOWESS method, and Cy5/Cy3 ratios were calculated. For epigenetic profiling, a Cy5/Cy3 ratio >2 was defined as a treatment response.

5.1.2 Array comparative genomic hybridization (aCGH) (II)

For TRAMP-C2 gene copy number analysis, TRAMP-C2 genomic DNA was hybridized against normal mouse whole blood cell DNA (Clontech) as a reference. In brief, 6 µg of *Alu*I- and *Rsa*I-digested DNAs were Cy5/Cy3-labeled using a BioPrime Labeling Kit (Invitrogen), as the test and reference samples, respectively, and co-hybridized onto Mou15K-1 slides overnight at 65°C. After washes, image scanning, signal quantification, and data normalization were performed as with the TRAMP-C2 expression data.

Visualization of the gene copy number data and identification of chromosomal areas of gains and losses was done as described by Saramäki *et al.* (2006a). First, the chromosomal locations of genes present on the Mou15K-1 array were retrieved from the UCSC Genome Browser databases (http://hgdownload.cse.ucsc.edu/downloads.html). Second, GraphPadPrism4 software (GraphPad Software, San Diego, USA) was utilized to create chromosome-specific Lowess curves of normalized and $\log_2$-transformed hybridization signals. To define areas as gained or lost, at least four adjacent clones needed to show $\log_2$ ratios above or below the set cut-off, a mean $\log_2$ ratio ±0.5*SD. In addition, regions where five of six adjacent clones showed $\log_2$ ratios beyond the cut-off value were treated as gained or lost. Human chromosomal regions corresponding to regions of loss and gain in the TRAMP-C2 genome where retrieved online from Ensembl Mouse Synteny View (http://www.ensembl.org/Mus_musculus/syntenyview) based on the homology of the two genomes.

5.2 miRNA microarrays (III)

miRNA microarrays for miRNA expression profiling were purchased from Agilent Technologies. v1 arrays containing 470 human miRNAs and 64 viral miRNAs were used to study miRNA expression in the cell lines, and v2 arrays containing 723 human miRNAs and 76 viral miRNAs were used for the clinical samples.

Total RNAs extracted with Trizol reagent were labeled and hybridized using Agilent's miRNA microarray system. Briefly, 100 ng of total RNA was dephosphorylated and 3'-end-labeled with pCp-Cy3 using Agilent's miRNA labeling and hybridization kit. Purified, labeled miRNAs were hybridized onto miRNA microarrays overnight at 55°C. After hybridization slides were washed and scanned with Agilent's DNA Microarray Scanner BA. Signal intensities were quantified using Feature Extraction 9.5.1 software.

Data analysis was done with an R statistical computing environment and GeneSpring GX 7.3.1 (Agilent Technologies). miRNA expression values were normalized by the quantile normalization method. Increased expression after 5azadC+TSA treatments was defined as a treated/untreated ratio >1.5. Significant expression of miRNA in a treated cell line was defined as being among the 30% of the highest expressed miRNAs, and low expression in untreated cell lines was defined as being among the lowest 70% of miRNAs. In clinical samples, the

percentages were 40% and 50%, respectively. Additional validation for differential expression between the treatments was calculated using Mann-Whitney U-tests with the 20 replicate measurements for each miRNA on the array (mock treatment or TSA treatment alone < 5azadC+TSA treatment). Prior to testing, all feature and background non-uniformity and population outliers were replaced with the mean value of each probe. miRNA was considered differentially expressed if the associated p-value was <0.05.

6. Real-time quantitative RT-PCR (I, II, III)

Real-time quantitative RT-PCR (qRT-PCR) was used to verify microarray gene expression data and to investigate the expression of selected genes in additional samples.

6.1 mRNA expression with qRT-PCR (I, II)

Total RNA was reversed transcribed into cDNA using either oligo d(T)18 or random primers. Light Cycler® Fast-Start DNA Master SYBR Green I and HybProbe kits (Roche Applied Science, F. Hoffman-La Roche Ltd, Basel, Switzerland) and a QuantiTech SYBR® Green RT-PCR kit (Qiagen) were used for qPCR with a Light Cycler apparatus (Roche) according to the manufacturer's instructions. Expression values were normalized to TATA-box binding protein (TBP) expression. Relative expression was calculated from the TBP-normalized values by dividing each expression value with the expression value of its control sample. Primers for qPCR were designed to anneal to different exons or to expand exon-intron boundaries to eliminate amplification from possible genomic DNA contamination.

6.2 miRNA expression by qRT-PCR (III)

miRNA expression was assessed with Applied Biosystems' TaqMan microRNA assays *via* a slightly modified protocol. Briefly, 100 ng of total RNA from each sample was reverse transcribed with a *miR-193b*-specific primer together with an RNU48-specific primer, using SuperScriptIII® reverse transcriptase (Invitrogen). qPCR detection was done with a miRNA-specific fluorescent probe (Applied Biosystems) using a LightCycler® FastStart DNA Master HybProbe kit (Roche). *miR-193b* expression was normalized to RNU48 expression.

7. Fluorescent in situ hybridization (FISH) (II)

TRAMP-C2 aCGH data was validated for one gained (16qC4) and one lost (7qD1) region using fluorescent *in situ* hybridization (FISH). A probe for an unaltered region (9qA2) was used as a reference. Mouse genomic BAC-clones for these regions (RP23-247E2 for 16qC4, RP23-70H15 for 7qD1 and RP23-149D5 for 9qA2) were purchased from ResGen (Invitrogen). Probes for gain and loss were labeled with digoxigenin-dUTP (Roche Applied Biosciences) by nick translation. The reference probe was nick-labeled with Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA). TRAMP-C2 interphase slides were prepared using standard techniques and dual color FISH was performed according to previously published guidelines (Hyytinen *et al.* 1994). After stringent washes, slides were stained with anti-digoxigenin-FITC (Roche Applied Biosciences) and counterstained with an antifade solution (Vectashield, Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI). Fluorescent signals were scored from non-overlapping nuclei using an Olympus BX50 epifluorescence microscope (Tokyo, Japan).

8. Methylation analysis (I, II, III)

Genomic DNA was studied for CGI methylation at the promoter/5'UTR of selected genes using genomic bisulphite sequencing.

8.1 CpG island prediction (I, II, III)

Promoter and 5'UTR regions of genes with increased expression after 5azadC and/or TSA treatments were assessed for the presence of CGIs using the National Center for Biotechnology Information (NCBI) Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=search&term=). CpG-rich sequences were retrieved from the database, and the MethPrimer program (http://www.urogene.org//methprimer/) was used both to predict CGIs in the sequence and to design primers for genomic bisulphite sequencing (Li and Dahiya 2002). The definition of a CGI used for the primer design was "a region of DNA greater than 200 bp, with GC content above 0.5 and an observed/expected CpG (Obs/Exp) ratio above 0.6".

For epigenetic profiling of miRNAs, the area chosen for bisulphite sequencing depended on the genomic location of each miRNA. For intergenic miRNAs (*i.e.*, miRNAs located between protein-coding genes), an area 5' to the mature miRNA sequence was studied for the presence of CGIs. For intronic miRNAs (*i.e.*, miRNAs located in the introns of protein-coding genes), the promoter/5'UTR region of the hosting gene was studied for the presence of CGIs. When available,

known/predicted pri-miR-sequences were used to define the promoter/5'UTR regions for miRNAs (Saini *et al.* 2008).

8.2 Genomic bisulphite sequencing (I, II, III)

Genomic DNA was extracted from all samples using standard proteinase K, phenol-chloroform extraction procedures. Bisulphite modification was performed as described in Aapola *et al.* (2001) (I, II), by using a CpGenome™ DNA Modification Kit (Chemicon® International, Temecula, CA, USA) (I) or by using an EZ DNA Methylation Gold kit (Zymo Research Corp., Orange, CA, USA) (III). Bisulphite-modified CGI amplicons were amplified with Amplitaq Gold Taq Polymerase (Applied Biosystems) (I, II) or using a GC2 Advantage kit (Clontech) (II, III). PCR products were purified and TOPO TA-cloned (Invitrogen). Several colonies were picked, grown and PCR amplified. These PCR products were purified using MultiScreen PCR96 Filter Plates (Millipore, Billerica, MA, USA) and sequenced with a BigDye Terminator v3.1 Cycler Sequencing kit. Sequences were analyzed for CpG methylation using SeqMan software (DNASTAR Inc, Madison, WI, USA) and visualized using CpGviewer (Carr *et al.* 2007).

9. Lentivirus-mediated gene overexpression

Lentiviruses were used for gene transfer to create a PC-3 cell line with stable overexpression of *DUSP1* or *SGK1*. The cDNA clones for *DUSP1* and *SGK1* genes were obtained from Invitrogen. The coding regions were cloned into a lentiviral plasmid WPI (kindly provided by Dr. Jarmo Wahlfors, University of Tampere, Finland), and the clonings were verified by sequence analysis. pWPI with no insert was used as a control.

VSVg pseudotyped lentiviral particles were produced in HEK 293T/17 cells by calcium phosphate precipitation technique. pWPI-DUSP1 or pWPI-SGK1 were co-transfected with packaging plasmids pCMV 8.9 and pVSV-G (System Biosciences, Mountain View, CA) (Lois *et al.* 2002). pWPI contains green fluorescence protein (GFP) as a reporter gene. Titers were measured by transducing PC-3 cells using a serial dilution of viruses and analyzing the percentage of GFP positive cells by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) 72 hr after the transduction. PC-3 cells were then transduced with the multiplicity of infection (MOI) 10. Several individual infected (GFP+) PC-3 cells were isolated by cloning rings, and these positive cells were propagated and pooled to obtain transgenic *DUSP1*- or *SGK1*-overexpressing PC-3 cell lines.

10. pre- and anti-miR transfections (III)

Transient transfections of *pre-mir-193b* were used to study the functional significance of *hsa-miR-193b* in prostate cancer cells. *pre-miR-193b* ssRNA oligonucleotides (Applied Biosystems) were transfected, at a 5 nM concentration, into 22Rv1 cells using INTERFERin transfection reagent (Polyplus-transfection SA, Illkirch, France). Transfections with scrambled pre-miR oligonucleotides were used as controls. *miR-193b* expression was studied at 4 days post-transfection using TaqMan microRNA assays (Applied Biosystems).

11. Functional studies on cell lines

Changes in the cellular phenotype were assessed in PC-3 cells stably overexpressing *DUSP1* or *SGK1* through lentivirus-mediated gene transfer and in 22Rv1 cells transiently overexpressing *pre-miR-193b*.

11.1 Proliferation assay (III)

DUSP1- or *SGK1*-overexpressing and control PC-3 cells were trypsinized and seeded at low density. Proliferation was determined 1, 4 and 6 days after seeding by calculating the cells using CoulterCounter (BeckmanCoulter, Inc., Fullerton, CA, USA). Relative growth was calculated by normalizing the absorbance of each day to day 1.

pre-miR-193b- and scrambled control-transfected cells were trypsinized and seeded at low density 1 day post transfection. Proliferation of the cells was assayed 1, 3, 5 and 7 days after reseeding (2, 4, 6 and 8 days after transfection) using alamarBlue® Cell Viability Reagent (Invitrogen). Relative growth was calculated by normalizing the measured absorbance of each day to day 1.

11.2 Cell cycle analysis (III)

Cell cycle analysis was performed according to published guidelines (Krishan *et al.* 1975). Briefly, pre-miR-transfected cells were collected 4 days after transfection and stained using hypotonic staining buffer containing propidium iodine. The cell cycle was analyzed with a flow cytometer (COULTER® EPICS XL-MCL, Beckman Coulter Inc), and the proportion of cells in each phase of the cell cycle was determined using ModFitLT 3.2 software (Verity Software House, Topsham, ME, USA).

11.3 Soft agar assay (III)

The ability of cells to grow anchorage-independently can be assessed using soft agar colony formation assays. Single cell suspensions of stably *DUSP1*-overexpressing PC-3 cells or *pre-miR-193b*-transfected 22Rv1 cells in a medium containing 0.35% low gelling agarose were casted on top of a 0.5% agarose bottom layer. Cells were allowed to grow at 37°C for two weeks, and the colony formation was assessed microscopically. pWPI-transduced PC-3 cells or pre-miR-scrambled-transfected 22Rv1 cells were used as controls.

12. Statistical analysis (I, II, III)

The association of gene expression with sample type and the association of gene expression with gene copy number were calculated using non-parametric Kruskall-Wallis tests (I, II). The association of protein expression with sample type, clinical stage, Gleason score, and prostate specific antigen (PSA) levels was calculated with Fisher's exact and $\chi 2$ tests and variance analysis (I). Mann-Whitney U –tests were used to identify differentially expressed miRNAs between treatment levels (mock treatment/TSA treatment alone < 5azadC+TSA treatment) and to determine statistical differences in *miR-193b* expression between normal prostate and prostate cancer samples (III). Unpaired t-tests or Mann-Whitney U-tests were used to determine statistical differences between scrambled-pre-miR- and *pre-miR-193b*-transfected samples in various functional assays (III).

RESULTS

1. Genomic characterization of the TRAMP-C2 cell line (II)

TRAMP-C2, a cell line derived from a transgenic adenocarcinoma of the mouse prostate, was characterized at the genomic level by analyzing gene copy number using aCGH. In general, the TRAMP-C2 cell line had relatively few genomic alterations: five areas of loss and six areas of gain (Table 2). The human cytogenetic regions corresponding to TRAMP-C2 deletions and amplifications did not significantly match the most commonly altered genomic regions in human prostate cancers. Among the few matches were gains of 8q11.21 and 8q24.13-q24.3 and losses of 6q14.1-q14.3 and 10q26.11-q26.3, all commonly found in human prostate cancer. Copy number changes were verified using FISH at two loci, a gain at 16q (showing 4-6 16qC4 locus-specific signals) and a loss at 7q (showing 2-3 7qD1 locus-specific signals), both with 2-3 reference signals (9qA2).

Table 2*. Chromosomal copy number changes in the TRAMP-C2 cell line and the corresponding human cytogenetic regions with the regions commonly altered in prostate cancer in bold.*

Chromosome	Cytogenetic region in mouse	Corresponding cytogenetic region in humans
Losses		
7	A3-F3	1q44; 11p14.3-p15.4; 11q13.4-q14.3; 15q11.2-q13.3; 15q25.1-q26.3
7	F4-F5	10q26.11-q26.3; 16p11.2
9	E3.1-E3.4	3q24; 6q14.1-q14.3; 15q24.3-q25.1
9	F3-F4	3p22.1-p22.3; 3p24.1
10	C2-C3	12q21.32-q23.2
Gains		
8	A2-A3	8p23.1-p23.3; 16q22.1
8	B3.3-C4	4q31.21-q32.3; 8p21.3-p22; 19p12-p13.13
8	C5-E2	1q42.13; 16q12.2-q24.3
15	D2-F1	8q24.13-q24.3; 22q12q.3-q13.33
15	F3	12q13.12-q13.2
16	B1-C4	3p11.2-p12.3; 3q11.2-q21.2; 3q27.1-q29; 8q11.21; 21q21.2-q22.3; 22q11.21-q11.22

Gene expression in TRAMP-C2 was analyzed, with normal mouse total RNA as a reference using the same array platform as for aCGH. Gene expression was highly associated with gene copy number ($p < 0.001$), with amplified genes having, on average, higher expression and deleted genes lower expression than genes with normal copy number.

2. Screens to identify epigenetically regulated genes in different prostate cancer models (I, II, III)

Chemical inhibition of key enzymes of epigenetic pathways (DNMTs and HDACs) offers a simple, albeit indirect, way to study epigenetic modifications in cultured cells. Here, mRNA expression and miRNA expression analyses were performed on several prostate cancer cell lines, both human and murine, after treating these cell lines with the DNMT inhibitor 5-aza-2'-deoxycytidine (5azadC) and/or the HDAC inhibitor trichostatin A (TSA) to relieve epigenetic modifications. Array-based expression analyses were performed to reveal genes with increased expression after the treatments (*i.e.*, potential epigenetically silenced TSGs).

2.1 Epigenetically regulated genes in the TRAMP-C2 cell line (II)

The search for epigenetically silenced TSGs in the TRAMP-C2 cell line was narrowed down by focusing on 5azadC and TSA treatment-upregulated genes with decreased expression in TRAMP-C2 compared to normal mouse, loss in TRAMP-C2 at the genomic region harboring the gene, or previous evidence of the gene being epigenetically regulated in prostate cancer. Forty-three genes had >2-fold increased expression after the treatments compared to the untreated cells and were less expressed in the TRAMP-C2 cell line than in normal mouse (expression ratio <0.5) (Table 3). Of these, four genes were located at areas of loss in TRAMP-C2, and human homologues of seven genes are located on commonly deleted areas in human prostate cancers. Additionally, seven genes have previously been shown to respond to 5azadC and TSA treatments in human prostate cancer cell lines, and four genes have been shown to be methylated in human prostate cancer cell lines or in clinical prostate cancers (Li *et al.* 2000, Kim *et al.* 2005, Lodygin *et al.* 2005, Leiblich *et al.* 2006).

Clusterin (*Clu*) and decorin (*Dcn*) were the two most highly expressed genes after the treatments, had significantly lower expression in TRAMP-C2 than in normal mouse, and were located on chromosomally deleted regions either in TRAMP-C2 (*Dcn*) or in clinical prostate cancers (*CLU*). However, *DCN* has previously been shown to be expressed mainly in stromal cells. Thus, it is not a likely target for cancer-specific epigenetic silencing, and was therefore discarded from additional studies (Banerjee *et al.* 2003).

***Table 3**. Candidate genes for epigenetic silencing in the TRAMP-C2 cell line. Bolded chromosomal locations mark areas of loss in TRAMP-C2 or commonly lost regions in human prostate cancers. ↑ and M denote genes previously shown to be upregulated by 5azadC+TSA treatments in prostate cancer cell lines and genes previously shown to be methylated either in prostate cancer cell lines or in clinical prostate cancer samples, respectively.*

Gene	Gene symbol	Mouse chr	Human chr region	Previous epigenetic knowledge
Decorin	*Dcn*	**10**	12q21.33	
Clusterin	*Clu*	14	**8p21-p12**	↑
Selenoprotein P, plasma, 1	*Sepp1*	15	5q31	
Lipopolysaccharide binding protein	*Lbp*	2	20q11.23-q12	
Hexosaminidase A	*Hexa*	9	15q23-q24	
Regulator of G-protein signaling 2	*Rgs2*	1	1q31	
Integral membrane protein 2B	*Itm2b*	14	**13q14.3**	
Forkhead box O1	*Foxo1*	3	**13q14.1**	
Epoxide hydrolase 1, microsomal	*Ephx1*	1	1q42.1	
Alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB)	*Naglu*	11	17q21	
Matrix gamma-carboxyglutamate (gla) protein	*Mgp*	6	**12p13.1-p12.3**	↑
DnaJ (Hsp40) homolog, subfamily B, member 9	*Dnajb9*	12	14q24.2-q24.3	
Nucleobindin 2	*Nucb2*	**7**	11p15.1-p14	
Myosin, light polypeptide 9, regulatory	*Myl9*	2	20q11.23	
Estrogen receptor 1 (alpha)	*Esr1*	10	6q25.1	M
Microsomal glutathione S-transferase 1	*Mgst1*	6	**12p12.3-p12.1**	
Histocompatibility 13	*H13*	2	6p21.3	
Leukotriene B4 12-hydroxydehydrogenase	*Ltb4dh*	4	9q31.3	
Glutathione S-transferase, mu 2	*Gstm2*	3	1p13.3	↑
Inhibitor of DNA binding 2	*Id2*	12	2p25	
Beta-2 microglobulin	*B2m*	2	15q21-q22.2	
Neighbor of Brca1 gene 1	*Nbr1*	11	17q21.31	
Glutathione peroxidase 3	*Gpx3*	11	**5q23**	↑ M
Lysosomal-associated protein transmembrane 5	*Laptm5*	4	1p34	
Elongation factor RNA polymerase II-like 3	*Ell3*	2	15q15.3	
N-myc downstream regulated 2	*Ndrg2*	14	14q11.2	
SH3 domain binding glutamic acid-rich protein-like 3	*Sh3bgrl3*	4	1p35-p34.3	
RAB11a, member RAS oncogene family	*Rab11a*	9	15q21.3-	
PTEN induced putative kinase 1	*Pink1*	1	1p36	
BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3	*Bnip3*	**7**	10q26.3	
ATP synthase, H+ transporting, V1 subunit D	*Atp6v1d*	12	14q23-q24.2	
Glutathione S-transferase, mu 5	*Gstm5*	3	1p13.3	↑
Nedd4 family interacting protein 1	*Ndfip1*	18	5q31.3	
Glutathione S-transferase, mu 6	*Gstm6*	3		
Lactate dehydrogenase 2, B chain	*Ldhb*	6	**12p12.2-p12.1**	M
X-box binding protein 1	*Xbp1*	11	22q12	
Bone morphogenetic protein 15	*Bmp15*	X	Xp11.2	
Glutathione S-transferase, mu 1	*Gstm1*	3	1p13.3	↑ M
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	*Atp5l*	9	11q23.3	
Argininosuccinate lyase	*Asl*	5	7cen-q11.2	
Cystatin C	*Cst3*	2	20p11.21	
Eukaryotic translation initiation factor 3, subunit 5 (epsilon)	*Eif3s5*	**7**	11p15.4	
Interferon gamma inducible protein 30	*Ifi30*	8	19p13.1	↑

Clusterin (*Clu*) expression was highly increased with 5azadC and TSA, significantly lower in TRAMP-C2 than in normal mouse, and the human homologue *CLU* is located on 8p21-p12, an area commonly deleted in human prostate cancer. Additionally, *CLU* expression has previously been shown to increase in response to 5azadC in human prostate cancer cell lines (Kim *et al.* 2005). Microarray results were verified using qRT-PCR, and methylation analysis at the *Clu* promoter was carried out by bisulphite sequencing. This analysis showed 64% methylation at a CGI ~400 bp downstream of TSS of *Clu* in the TRAMP-C2 cell line.

Expression of *CLU* was also studied in four human prostate cancer cell lines (LNCaP, LAPC-4, PC-3 and DU145) by qRT-PCR (Figure 7A). LNCaP had the lowest expression, and bisulphite sequencing revealed 50% and 23% methylation at two CGIs surrounding the TSS of *CLU*. The other three cell lines with higher expression levels exhibited markedly lower levels of CGI methylation, ranging from 0.9 to 9.5% at the first CGI and from zero to 0.8% at the second CGI (Figure 7A). *CLU* expression was also significantly decreased in human prostate carcinoma samples compared to BPH samples (p = 0.0095), as analyzed by qRT-PCR (Figure 7B).

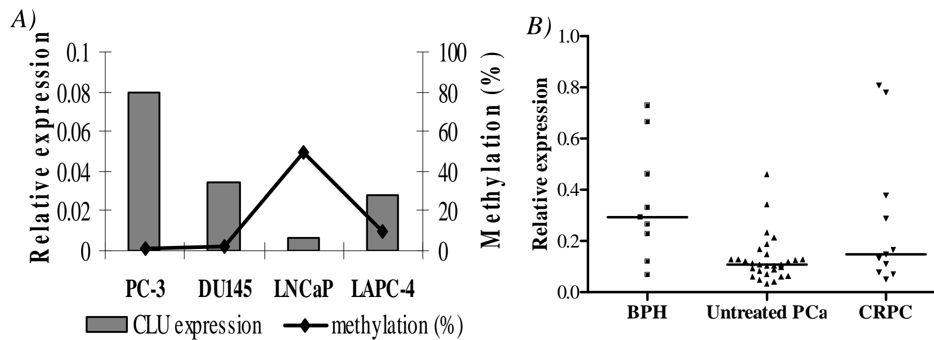

Figure 7. *Clusterin expression and methylation levels in human prostate cancer cell lines (A), and expression in different clinical sample groups (B).*

2.2 Epigenetically regulated genes in the PC-3 cell line (I)

Gene expression profiling in the PC-3 prostate cancer cell line after 5azadC and TSA treatments was done using custom cDNA microarrays. These arrays contained a cDNA library of clones with decreased expression in the PC-3 prostate cancer cell line compared with BPH (Porkka *et al.* 2001). Of the 432 different SSH clones on the array, 17 had at least 2-fold increased expression after the treatments. Sequencing revealed these 17 clones to represent 11 genes (Table 4). Dual-specificity phosphatase 1 (*DUSP1*), serum/glucocorticoid-regulated kinase (*SGK1*) and spermidine/spermine N1-acetyltransferase (*SAT*) were chosen for further studies based on their known functions.

***Table 4**. Genes upregulated by 5azadC and TSA treatment in the PC-3 cell line.*

Gene	Gene symbol	Chr location
Spermidine/spermine N1-acetyltransferase	*SAT*	Xp22.1
Dual specificity phosphatase 1	*DUSP1*	5q34
Serum/glucocorticoid regulated kinase	*SGK1*	6q23
Haplotype A mitochondrion		mtDNA
Beta-2-microtubulin	*BM2*	15q21-q22.2
Ribosomal protein S27 (metallopanstimulin 1)	*RPS27*	1q21
Haplotype U5 mitochondrion		mtDNA
Thymosin, beta 4	*TMSB4X*	Xq21.3-q22
Mitochondrial cytochrome b		mtDNA
Heat shock protein 90, alpha	*HSPCA*	14q32.33
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	*NFKBIA*	14q13

DUSP1, *SGK1* and *SAT* mRNA expression in cell lines after the treatments was verified using qRT-PCR. The expression of these genes was then assessed in clinical samples using qRT-PCR and immunohistochemistry (Table 5). *DUSP1* mRNA expression was significantly reduced in castration-resistant samples compared to untreated carcinoma and BPH samples (p = 0.0001), while the protein levels decreased both with untreated and castration-resistant carcinomas compared to BPH samples ($p < 0.0001$). DUSP1 staining was uniform and high in normal prostate epithelial cells with the highest intensity in basal cells. BPH samples all showed varying levels of expression, whereas 92% of carcinoma samples were negative or only weakly positive. Similar to *DUSP1*, *SGK1* mRNA expression was significantly lower in castration-resistant prostate cancers compared to untreated prostate cancer and BPH samples (p = 0.0331). All BPH samples were positive for SGK1 protein expression with high nuclear staining, while ~50% of carcinoma samples were negative or only weakly expressed SGK1 (p = 0.0021). However, DUSP1 or SGK1 protein expression was not associated with clinical stage, Gleason score or PSA levels of untreated cancers. mRNA expression of *SAT* did not vary between the sample groups. Bisulphite sequencing analysis of *DUSP1* and *SGK1* at their promoter/5'UTR region-associated CGIs revealed no methylation at these loci in the PC-3 cell line.

DUSP1- and *SGK1*-overexpression after transducing PC-3 cells with lentiviruses was assessed by qRT-PCR. *DUSP1* expression was >300-fold higher in PC-3 *DUSP1*-transduced cells than in control cells. *SGK1*-transduced PC-3 cells overexpressed *SGK1* ~10-fold more than the control cells. No measurable difference was observed between the control and *DUSP1*- or *SGK1*-overexpressing cells in proliferation or colony formation ability.

Table 5*. DUSP1 and SGK1 mRNA and protein expression in clinical prostate cancer samples.*

mRNA	***DUSP1*** BPH (n = 9)	untreated PCa (n = 30)	CRPC (n = 12)	***SGK1*** BPH (n = 9)	untreated PCa (n = 30)	CRPC (n = 12)
relative expression, median (range)	63 (9-330)	70 (0.1-397)	3 (1.6-61)	3.6 (0.6-26)	4.3 0.6-19)	0.8 (0.3-17)
***p*-value**		0.0001			0.0331	

protein	**DUSP1** BPH (n =13)	untreated PCa (n = 67)	CRPC (n=61)	**SGK1** BPH (n = 13)	untreated PCa (n= 37)	CRPC (n= 61)
IHC 0/1+ (%)	0	88	97	0	43	53
IHC 2+/3+ (%)	100	12	3	100	57	47
***p*-value**		< 0.0001			0.0021	

2.3 Epigenetically regulated microRNAs in prostate cancer (III)

Array-based miRNA expression profiling after 5azadC and TSA treatments was performed on six prostate cancer cell lines (PC-3, DU145, LAPC-4, LNCaP, VCaP and 22Rv1) and on primary prostate epithelial cells, PrECs. For a normal prostate epithelial cell line, EP156T, miRNA expression was also profiled. Additionally, miRNA expression was studied in 13 clinical samples (BPH, $n = 4$; untreated PCa, $n = 5$; and CRPC, $n = 4$).

One hundred four miRNAs out of 470 had >1.5-fold increased expression in any of the prostate cancer cell lines after treatment, of which, 38 miRNAs had low expression at the initial untreated state and significant expression after treatment. To be a cancer-specific event, the expression increase after 5azadC+TSA should not take place in PrEC cells, and the basal expression should be higher in either of the normal prostate epithelial cell models than in cancer cell lines. These definitions further narrowed down the number of potential epigenetically silenced tumor suppressor miRNAs to 23 (Table 6). In clinical samples, 159 miRNAs had >2-fold higher expression in any BPH sample compared to any cancer sample, with 105 miRNAs having very low or absent expression in at least one cancer sample. Combining the 105 miRNAs from the clinical samples with the 23 miRNAs from the cell lines resulted in a list of six miRNAs (*miR-149, miR-193b, miR-203, miR-218, miR-370*, and *miR-512-3p*) fulfilling all our set criteria for a potentially epigenetically regulated tumor suppressor miRNA.

mir-149 had increased expression after the treatments in LNCaP cells, but methylation analysis revealed no significant methylation in these cells at the studied promoter CGI of the Glypican 1 gene hosting *miR-149*. *mir-203* was identified as a candidate for epigenetic silencing in the 22Rv1 cell line but was not significantly methylated at the studied CGI at the predicted 5’ end of *pri-miR-203*. Closer

inspection of *miR-370* expression in clinical samples showed its expression to be increased rather than decreased in CRPC samples compared to BPH samples. *miR-218* and *mir-512-3p* can both be produced from two different genomic loci, and the active loci cannot be deduced from the expression profile. For these reasons, none of these five miRNAs was studied further.

***Table 6**. miRNAs with upregulated expression after 5azadC and TSA treatment in prostate cancer cell lines but not in PrEC cells, and >2-fold higher expression in PrEC than in cancer cells. Bolded miRNAs represent the overlapping candidates from the analysis of clinical samples.*

Mature miRNA	pre-miR miRBase ID	pre-miR	Location (strand)	CGI	Upregulated by 5azadC+TSA in
miR-9	MI0000466	*miR-9-1*	1: intronic (*C1orf61*) (-)		LAPC-4
	MI0000467	*miR-9-2*	5: intergenic (-)		LAPC-4
	MI0000468	*miR-9-3*	15: intergenic (+)	X	LAPC-4
miR-27a	MI0000085	*miR-27a*	19: intergenic (-)		LNCaP, VCaP
miR-34a	MI0000268	*miR-34a*	1: intergenic (+)		PC-3
miR-149	**MI0000478**	***miR-149***	**2: intronic (*GPC1*) (+)**	**X**	**LNCaP**
miR-188	MI0000484	*miR-188*	X: intergenic (+)		22rv1, VCaP, DU145
miR-193b	**MI0003137**	***miR-193b***	**16: intergenic (+)**	**X**	**22rv1, VCaP**
miR-203	**MI0000283**	***miR-203***	**14: intergenic (+)**	**X**	**22rv1**
miR-218	**MI0000294**	***miR-218-1***	**4: intronic (*SLIT2*) (+)**	**X**	**LAPC-4**
	MI0000295	***miR-218-2***	**5: intronic (*SLIT3*) (-)**	**X**	**LAPC-4**
miR-370	**MI0000778**	***miR-370***	**14: intergenic (+)**	**X**	**22rv1, VCaP, DU145, PC-3**
miR-375	MI0000783	*miR-375*	2: intergenic (-)	X	LNCaP
miR-487b	MI0003530	*miR-487b*	14: intergenic (+)	X	VCaP
miR-512-3p	**MI0003140**	***miR-512-1***	**19: intergenic (+)**		**VCaP, DU145**
	MI0003141	***miR-512-2***	**19: intergenic (+)**		**VCaP, DU145**
miR-513a	MI0003191	*miR-513a-a*	X: intergenic (-)		22rv1
	MI0003192	*miR-513a-2*	X: intergenic (-)		22rv1, DU145, PC-3
miR-517a	MI0003161	*miR-517a*	19: intergenic (+)		DU145
miR-517b	MI0003165	*miR-517b*	19: intergenic (+)	X	LAPC-4, DU145
miR-518b	MI0003156	*miR-518b*	19: intergenic (+)		DU145
miR-526a	MI0003157	*miR-526a-1*	19: intergenic (+)		DU145
	MI0003168	*miR-526a-2*	19: intergenic (+)		DU145
miR-601	MI0003614	*miR-601*	9: intronic (*DENDD1A*) (-)	X	22rv1, PC-3
miR-629	MI0003643	*miR-629*	15: intronic (*TLE3*) (-)	X	22rv1, LNCaP, DU145
miR-630	MI0003644	*miR-630*	15: intergenic (+)		22rv1, VCaP, DU145, PC-3
miR-638	MI0003653	*miR-638*	19: intronic (*DNM2*) (+)	X	VCaP, DU145
miR-663	MI0003672	*miR-663*	20: intergenic (-)	X	22rv1, DU145
miR-765	MI0005116	*miR-765*	1: intronic (*ARHGEF11*) (-)	X	VCaP, PC-3

2.3.1 *miR-193b*, a putative epigenetically regulated tumor suppressor in prostate cancer (III)

miR-193b expression was increased >1.5-fold in the 22Rv1 and VCaP cell lines after the 5azadC and TSA treatments. Concordantly, when comparing all of the untreated cell lines, the basal *miR-193b* expression was lowest in the 22Rv1 and VCaP cell lines. In 22Rv1 cells, a CGI ~1 kb upstream of the mature *miR-193b* locus was densely methylated, and this methylation was partially removed upon the treatments. VCaP cells were methylated only at the edge of the studied CGI. In the

set of 13 clinical samples, the lowest *miR-193b* expression was observed among the CRPC samples (Figure 8). In a larger set of 108 clinical samples[*], the *mir-193b* expression decrease was clearly evident when comparing prostate cancer samples to adjacent normal prostate tissue ($p = 0.0002$) or TURP samples from cancer patients to those of BPH patients ($p = 0.0139$). Methylation analysis of the 13 samples in the initial sample set showed variable levels of methylation in almost all cancer samples, even though none of the samples was as densely methylated as in the 22Rv1 cell line. All four BPH samples were fully unmethylated.

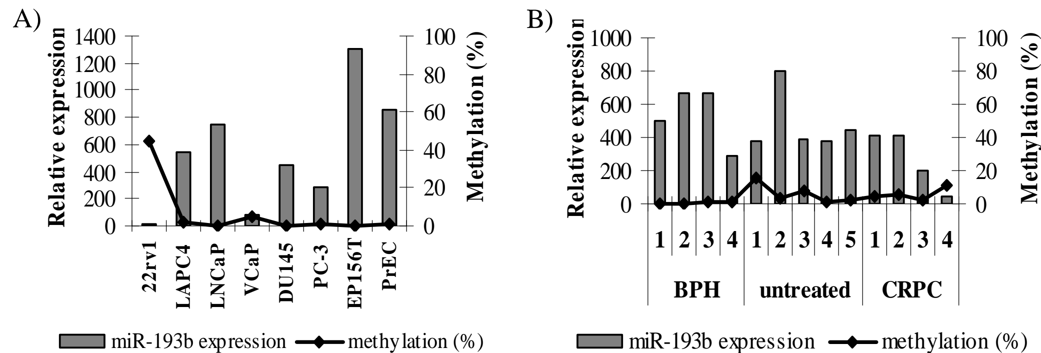

Figure 8. *Expression and methylation levels of miR-193b in cell lines (A) and in the set of 13 clinical samples (B).*

pre-miR-193b oligonucleotide transfections were performed on 22Rv1 cells to study the functional significance of this miRNA in prostate cancer cells (Table 7). More than a 200-fold increase in expression was observed after the transfections compared to scrambled-pre-miR-transfected cells. Increased *miR-193b* expression significantly reduced the proliferation of these cells ($p < 0.0001$) with a concurrent decrease in the amount of cells in S-phase ($p = 0.0018$). Additionally, *pre-mir-193b*-transfected cells had a significantly reduced ability to grow anchorage-independently compared to the control-transfected cells ($p = 0.0041$).

Table 7. *Functional outcome of pre-miR-193b expression in 22Rv1 cells.*

	pre-miR-scrambled	*pre-miR-193b*	*p*-value
Relative growth (relative to day 1)			
+1d	1	1	
+3d	1.8	1.3	0.001
+5d	4.7	2.9	0.0009
+7d	10.3	6.9	<0.0001
Cell cycle (% of cells)			
G1	55.1	67.1	
S	31.3	21.1	0.0018
G2/M	13.6	11.7	
Colony formation (% of cells)			
Colony forming cells	70.3	50.3	0.0041

[*]Martens-Uzunova ES, Jalava SE, Visakorpi T, Jenster G. Submitted for publication.

DISCUSSION

1. Molecular mechanisms of prostate cancer

Prostate cancer, like all cancers, is caused by the accumulation of genetic and epigenetic abnormalities that eventually lead to the transformation of the cells. The progression of prostate cancer is illustrated in Figure 9, with some of the most common genetic and epigenetic changes depicted. In prostate cancer, large chromosomal aberrations are commonly found, with recurrent losses at 5q, 6q, 8p, 10q, 13q, 16q, 17p, 18q and 22q, and gains at 1q, 3q, 7, 8q, 16p, 17q and Xq (Saramäki *et al.* 2006a). Regardless of their frequency (*e.g.*, the loss of 8p and the gain of 8q are found in >70% of advanced prostate cancers (Nupponen *et al.* 1998)), the actual target genes for many of these chromosomal changes are still unknown. In fact, for some of them, it is likely that there is no single target gene, but rather several genes are targeted. *MYC*, the well characterized oncogene residing at 8q24, can contribute to prostate cancer and is amplified in up to 90% of metastatic prostate cancers (Jenkins *et al.* 1997, Ellwood *et al.* 2003). However, even though it can be one of the targets of 8q24 amplification, results from breast and prostate cancer studies suggest that there must also be other genes targeted in that region, as *MYC* amplification rarely results in *MYC* overexpression (Nupponen *et al.* 1999, Bieche *et al.* 1999, Savinainen *et al.* 2004). In addition to *MYC*, other well-known cancer genes, like the TSGs *PTEN* (phosphatase and tensin homolog) and *TP53*, are involved in prostate cancer. Loss of *PTEN* at 10q23 and *PTEN* and *TP53* mutations are all found in advanced prostate cancers, while being relatively uncommon in early lesions (Navone *et al.* 1993, Cairns *et al.* 1997, Li *et al.* 1997, Steck *et al.* 1997).

Probably the best-established and most-studied molecular mechanism of prostate cancer is the role of androgens and AR. Approximately 30% of castration-resistant prostate cancers harbor *AR* amplifications (Visakorpi *et al.* 1995), and the amplification associates with elevated *AR* expression levels. However, only one-third of the *AR*-overexpressing cases can be explained by the amplification; for the rest the mechanism is unknown. *AR* overexpression sensitizes prostate cancer cells to low levels of androgens, as shown by an *in vitro* prostate cancer model (Chen *et al.* 2004), thus offering the cancer cells means to circumvent castration, the choice of treatment for locally advanced and metastatic prostate cancer. Additionally, mutations in the *AR* ligand-binding domain offer the receptor novel substrate specificities, thus enabling transactivation in an androgen-deprived setting (Newmark *et al.* 1992, Culig *et al.* 1993).

One of the most recurrent molecular alterations in prostate cancer (found in ~40% of prostate cancers) is the relatively recently described gene fusion on chromosome 21q, where the androgen-regulated *TMPRSS2* (transmembrane protease, serine 2) gene becomes fused together with *ETS* transcription factor family

members, such as *ERG* and *ETV1* (Tomlins *et al.* 2005, Saramäki *et al.* 2008, Kumar-Sinha *et al.* 2008). While *ERG* is known to fuse only with *TMPRSS2*, other *ETS* factors can have additional androgen-regulated, 5' fusion partners (Tomlins *et al.* 2007). These fusion genes offer one explanation for how androgens can drive cell proliferation in prostate cancer, instead of differentiation and cell survival as in normal prostate cells.

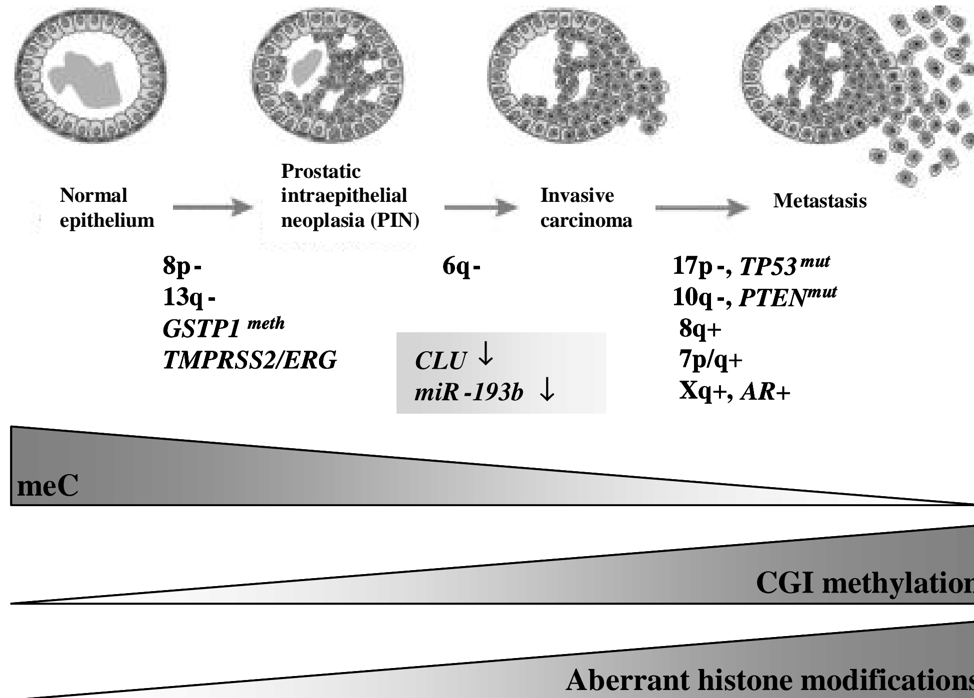

***Figure 9**. Model of prostate cancer progression with some of the most common genetic and epigenetic aberrations depicted. The potential novel tumor suppressors identified in the present study are shown in a shaded box. (Modified by permission from Cold Spring Harbor Laboratory Press: Abate-Shen and Shen, 2000).*

Regardless of the fact that some of these genetic abnormalities occur fairly commonly, the most common aberrations in prostate cancer are epigenetic, with methylation of *GSTP1* detectable in ~90% of prostate cancer cases. Several other epigenetic changes are also highly recurrent, and novel technological advances make them suitable candidate markers for the management of prostate cancer. The studies described here aimed to identify novel hypermethylated TSGs in prostate cancer that could help elucidate in more detail the molecular mechanisms of prostate cancer carcinogenesis and tumor progression, or have clinical value in diagnostic or prognostic settings.

2. Genomic characterization of the TRAMP-C2 cell line (II)

Despite the high incidence of prostate cancer, there are only a limited number of available *in vitro* and *in vivo* models for research, and many of these models (mainly cell lines) rather poorly resemble the clinical disease. For example, about half of the most commonly used prostate cancer cell lines do not express *AR*, while its amplification and overexpression are among the most recurrent changes in the clinical CRPCs (Visakorpi *et al.* 1995, Linja *et al.* 2001). Additionally, the chromosomal aberrations found in these cell lines only partially match the ones commonly found in clinical tumors.

TRAMP (transgenic adenocarcinoma of mouse prostate) is a transgenic mouse model of prostate cancer, with SV40 T large antigen under the prostate-specific probasin promoter, and these mice develop spontaneous prostate cancers (Greenberg *et al.* 1995). The prostate cancer progression in TRAMP mice proceeds through stages similar to human cancer, with premalignant prostatic intraepithelial neoplasias preceding adenocarcinomas, which are then followed by metastatic disease. Despite TRAMP being the most widely used mouse model for prostate cancer due to its clinical resemblance to human cancer, there have been no comprehensive studies on the genomic abnormalities of these mice. In addition to the TRAMP mice, three cell lines have been established from a 32-week old TRAMP mouse, namely TRAMP-C1, -C2 and –C3 (Foster *et al.* 1997). All of these cell lines express AR, and cytokeratin and CDH1, which are indicative of epithelial origin of the cells. Our study aimed to characterize the TRAMP-C2 cell line for its chromosomal alterations and to compare them to those commonly found in human prostate cancer, in order to gain further insight into the value of the TRAMP model in studying clinical prostate cancer.

Interestingly, we observed relatively few genomic aberrations in the TRAMP-C2 cell line, and these gains and losses were quite large, some of them comprising almost the entire chromosome arm. Even though no high level amplifications were found in this cell line, the observed low-level copy number changes significantly associated with the gene expression. Similar association between gene copy number and gene expression has been previously reported in human prostate cancer cell lines and xenografts (Saramäki *et al.* 2006a), as well as in other cancers (Pollack *et al.* 2002). This supports the idea that small changes in gene copy number can have functional effects on tumorigenesis.

Comparison to corresponding human chromosomal regions revealed that the genomic alterations found in TRAMP-C2 do not significantly overlap with the most commonly found genomic losses and gains in clinical prostate cancers (Sun *et al.* 2007). However, the few matches found (*e.g.*, the gains at 8q11.21 and 8q24.13-q24.3, and the losses at 6q14.1-q14.3 and 10q26.11-q26.3) further underline the role of these regions in prostate cancer. Of the suggested target genes for the gain of 8q24 (Sato *et al.* 1999, Porkka *et al.* 2004), mouse homologues of *MYC* and *KIAA0196* showed increased copy number and overexpression. For the proposed

targets of the other chromosomally altered regions in both clinical prostate cancers and the mouse model, no strong support was obtained from the TRAMP-C2 cell line. However, the fact that the few coinciding chromosomal changes are among the most commonly found ones in clinical cancers suggests that the TRAMP-C2 cell line can be useful in deciphering the genomic abnormalities behind prostate carcinogenesis (*i.e.*, this line will be useful in elucidating the targeted genes behind these common genomic aberrations).

3. Epigenetically regulated genes in various prostate cancer models (I, II, III)

Microarray-based screens were used to identify epigenetically regulated, specifically hypermethylated, genes coding for proteins and miRNAs in human and mouse prostate cancer cell lines. The indirect approach used here, where pharmacological inhibition of DNMTs and HDACs was employed to re-establish the active transcriptional status of epigenetically silenced TSGs, has been proven useful in identifying novel TSGs, just as well as in finding additional silencing mechanisms for already known TSGs (Cameron *et al.* 1999). Despite its wide use and the number of TSGs identified using this approach, it bears one major downside; the large number of false positive hits, *i.e.*, genes whose expression increases upon the treatments either as a secondary response or through cytotoxic effects.

In TRAMP mice, similar to human prostate cancers, overexpression of DNMTs is a common occurrence (Patra *et al.* 2002, Morey *et al.* 2006). It has been suggested that this would result from the inactivation of the RB tumor suppressor pathway, as E2F is a transcriptional activator of *DNMT1* (Kimura *et al.* 2003, McCabe *et al.* 2005). In TRAMP mice, 5azadC (a DNMT inhibitor) can effectively block tumorigenesis (McCabe *et al.* 2006), supporting the idea to screen for possibly epigenetically targeted genes not only in human prostate cancer cell lines but also in the TRAMP-derived TRAMP-C2 cell line. Indeed, several of the candidate genes for epigenetic silencing identified here in the TRAMP-C2 cell line were earlier reported to undergo similar expression induction in prostate cancer cell lines upon treatment with 5azadC or were shown to be methylated in prostate cancer cell lines or in clinical prostate tumors. This demonstrates the feasibility of the TRAMP-C2 cell line as an additional tool to human cancer cell lines and xenografts for studying the human disease at epigenetic level.

3.1 Clusterin (II)

Clusterin was identified as a potential epigenetically regulated gene in the TRAMP-C2 cell line. Earlier work has demonstrated that Clu expression dramatically decreases along with the prostate cancer progression in TRAMP mice (Caporali *et*

al. 2004). Here, we found strong methylation at the 5'UTR region of the *Clu* gene, while another CpG-rich region at the TSS was not methylated. Our results suggest that the cancer-related decrease in *Clu* expression could be due to epigenetic silencing of *Clu* in the TRAMP-C2 cell line, where the methylated 5'UTR region might harbor crucial methylation-sensitive elements needed for the transcriptional activity of *Clu* in mice. Methylation at the clusterin promoter and at a suggested enhancer region has also been observed in *HRAS*-transformed rat fibroblasts, where clusterin expression correlated with methylation levels (Lund *et al.* 2006).

In human prostate cancer cell lines, the lowest *CLU* expression was detected in LNCaP cells, where significant methylation at two adjacent CGIs residing on both sides of the *CLU* TSS was also observed. In clinical prostate samples, *CLU* expression was decreased in cancer samples compared to BPH, as has been previously shown (Bettuzzi *et al.* 2000, Scaltriti *et al.* 2004). However, DNA methylation at the *CLU* promoter was scarce at the few tumor samples studied, implying that DNA methylation is not the primary mode of silencing for clusterin in clinical prostate cancer. Nonetheless, in tumor endothelial cells, *CLU* expression has been shown to be similarly responsive to 5azadC and TSA treatments as in prostate cancer cells, and the silencing was shown to be mediated by histone deacetylation and loss of H3K4 methylation (Hellebrekers *et al.* 2007). These results suggest that in certain cell types, *CLU* expression can indeed be epigenetically regulated, and the mechanism by which it happens (*i.e.*, DNA methylation, histone modifications or both) may vary depending on the cellular context.

The clusterin puzzle is very complex overall. Over the past ten years, there have been dozens of reports on clusterin function, and the results have been all but uniform. For example in prostate cancer, in addition to reports of decreased *CLU* expression in cancer (as evidenced by several microarray expression studies, Oncomine database, www.oncomine.org), there are also opposing reports showing CLU as a mediator of prostate cancer progression to castration resistance through its antiapoptotic function (Miyake *et al.* 2000, July *et al.* 2002). These somewhat confusing results can be explained with the two protein isoforms of clusterin, nuclear (nCLU) and secreted (sCLU), which result from two distinct mRNA transcripts with differing N-termini (reviewed in Rizzi and Bettuzzi 2008, Trougakos *et al.* 2009). nCLU relocates to the nucleus through its N-terminal nuclear localization signal (NLS) and functions pro-apoptotically, possible through interacting with the Ku70 autoantigen (Yang *et al.* 2000, Caccamo *et al.* 2004, Rizzi *et al.* 2009). sCLU, in part, becomes heavily glycosylated and proteolytically cleaved in the endoplastic reticulum (ER), where it is targeted by an ER localization signal lacking from the transcript isoform producing nCLU. The two monomers are held together by several disulfide bonds, and the resultant sCLU heterodimer is secreted. sCLU functions mainly by promoting cell survival and proliferation. In prostate cancer, some consensus on clusterin functions has been reached, and it is thought that whereas the tumor suppressor nCLU becomes downregulated in prostate cancer, the possibly oncogenic sCLU expression is increased, and this might reflect the hormonal dependency of the tumor. Antisense oligonucleotide (ASO) therapy against clusterin is currently in clinical trials, and it aims to potentiate the effects of androgen withdrawal therapy, as well as to prevent

resistance to chemotherapy (reviewed in Gleave and Miyake 2005, Miyake *et al.* 2005, Sowery *et al.* 2008). Whereas the role of sCLU in prostate cancer still awaits clarification, a novel, recently described clusterin knock-out model, TRAMP/CluKO, clearly demonstrates the tumor suppressor properties of clusterin, with the TRAMP/CluKO(-/+) and TRAMP/CluKO(-/-) mice developing more poorly differentiated and metastatic tumors (Bettuzzi *et al.* 2008).

In addition to prostate cancer, other cancers like breast and colon cancer display high levels of sCLU (Krüger *et al.* 2007, Kevans *et al.* 2009). In both cases, high cytoplasmic clusterin staining significantly associates with poor prognosis, consistent with the pro-survival function of sCLU. However, a recently completed metastatic breast cancer phase II trial showed no significant improvement in chemotherapeutic treatment response with concurrent clusterin ASO therapy (Chia *et al.* 2009). In mice xenografted with neuroblastoma cells, ASO therapy has been found to significantly increase the therapeutic potential of the HDAC inhibitor, valproate (Liu *et al.* 2009). All in all, while there are still many open questions about the specific properties of the distinct clusterin isoforms and their usefulness in therapeutics, inhibition of sCLU and induction of nCLU might both prove useful in the future. Moreover, as shown by us and others, targeting their expression through epigenetic approaches seems a feasible and tempting option.

3.2 Dual specificity phosphatase 1 (I)

Dual specificity phosphatase 1 (*DUSP1, MKP-1*) was identified as a 5azadC and TSA treatment inducible gene in the PC-3 prostate cancer cell line. Analysis of *DUSP1* expression in clinical samples demonstrated significantly decreased expression in CRPC compared to BPH and untreated cancers. Previous reports have shown similarly decreases in *DUSP1* expression in poorly-differentiated and metastatic prostate cancers, but with a preceding increase in expression in prostate cancer precursor lesions, PIN (prostatic intraepithelial neoplasia), and in primary carcinomas as compared to normal prostate cells (Loda *et al.* 1996, Magi-Galluzzi *et al.* 1998). In our prostate cancer samples, no such increase was detected, and moreover, DUSP1 protein expression was already largely absent from the untreated tumor samples, with CRPC samples being totally negative. In other cancer types (*e.g.*, colon and ovarian cancers), *DUSP1* expression also decreases in advanced tumors (Loda *et al.* 1996, Manzano *et al.* 2002), and in hepatocellular carcinomas, loss of DUSP1 expression has been shown to be an independent predictor of poor outcome (Tsujita *et al.* 2005). On the contrary, in breast cancer, *DUSP1* levels stay elevated throughout the malignancy (Wang *et al.* 2003). This contradictory role of DUSP1 in cancer can be seen also from DUSP1 overexpression studies, where forced *DUSP1* expression in ovarian cancer cells decreases their tumorigenic properties, suggesting a tumor suppressor role for DUSP1 (Manzano *et al.* 2002). While in pancreatic cells, silencing *DUSP1* expression leads to suppression of cell growth and decreased tumorigenicity in nude mice, suggestive of an oncogenic function (Liao *et al.* 2003). In our studies, *DUSP1* overexpression in PC-3 cells did

not result to any significant changes in proliferation or anchorage-independent growth of these cells.

DUSP1 is a dual specificity phosphatase capable of inactivating mitogen-activated protein kinase (MAPK) pathway kinases by dephosphorylating both phosphotyrosine and phosphothreonine residues (reviewed in Boutros *et al.* 2008). Its preferential dephosphorylation targets are stress-activated protein kinase/c-jun N-terminal kinase (SAP/JNK) and p38 MAPK, and to a lesser extent extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Sun *et al.* 1993, Franklin and Kraft 1997). Apart from inactivation of the MAPK pathway, additional ways to present tumor suppressor actions of DUSP1 include inhibition of RAS-induced DNA synthesis and induction of cell cycle arrest at G1-phase (Sun *et al.* 1994, Brondello *et al.* 1995). These opposing outcomes from DUSP1 expression suggest it to behave in a cellular context-dependent manner with the endpoint depending on the targeted MAP kinase.

Our primary interest in *DUSP1* was to establish if it is an epigenetically silenced gene in prostate cancer. The methylation analysis at the dense CGIs located around the TSS of *DUSP1* showed no methylation in PC-3 at this region, suggesting that *DUSP1* is not a primary target for methylation-mediated silencing in prostate cells, or at least the methylated region resides further away from the core promoter. *DUSP1* promoter methylation has also been studied in papillary thyroid carcinomas (PTC), and similar to our results, no methylation was found to be involved in the decreased expression of *DUSP1* in PTCs (Huang *et al.* 2003). However, in hepatocellular carcinomas with poor prognosis, DUSP1 inactivation was shown to result either from ERK1/2-mediated ubiquitination and consequent proteosomal degradation of DUSP1 or from *DUSP1* promoter methylation and LOH at the *DUSP1* locus (Calvisi *et al.* 2008). Additionally, studies on mouse fibroblasts showed that *DUSP1* expression is responsive to TSA treatment, and the response was further augmented by arsenite (Li *et al.* 2001). The arsenite-mediated *DUSP1* induction also dramatically induced phosphorylation and acetylation at H3 of the *DUSP1* promoter. These studies demonstrate how epigenetics, either through DNA methylation, histone modifications or chromatin remodeling, can regulate the expression of *DUSP1* in normal and cancer cells and thus contribute also to the role of *DUSP1* in malignant transformation. Even though the data from other cancers, especially from hepatocellular cancer, supports a tumor suppressor role for *DUSP1*, our unpublished data on stable overexpression of *DUSP1* in the PC-3 prostate cancer cell line showed no effect on either cell proliferation or anchorage-independent growth, suggesting that *DUSP1* may not a TSG in prostate cancer.

3.3 Serum/glucocorticoid regulated kinase 1 (I)

Serum/glucocorticoid regulated kinase 1 (*SGK1*) was identified, similar to *DUSP1*, as an 5azadC and TSA-inducible gene in the PC-3 prostate cancer cell line, but methylation analysis did not support direct methylation of the *SGK1* promoter as a mechanism for *SGK1* silencing. In clinical samples, *SGK1* mRNA expression

decreased significantly in CRPCs, whereas SGK1 protein expression was already decreased in primary untreated carcinomas, with approximately half of all tumors being negative or only weakly positive for SGK1 protein expression. This is the only report of SGK1 expression in clinical prostate samples, while in some other cancers, increased *SGK1* levels in tumor samples compared to normal tissue have been reported (Adeyinka *et al.* 2002, Chung *et al.* 2002).

SGK1 functions downstream of phosphatidylinositol 3-kinase (PI3K) and has high similarity to AKT, another PI3K downstream kinase (Webster *et al.* 1993). SGK1 has been shown to play an important role in PI3K-mediated cell growth and survival (Park *et al.* 1999, Wu *et al.* 2004, Schoenebeck *et al.* 2005). The anti-apoptotic role of SGK1 is mediated through TP53-dependent SGK1 upregulation upon stress, leading to inactivation of the pro-apoptotic transcription factor forkhead box O3, FOXO3 (Brunet *et al.* 2001, You *et al.* 2004). On the other hand, SGK1 has been shown to be a negative regulator of B-Raf, thus functioning in silencing the proliferative signaling cascade (Zhang *et al.* 2001). SGK1 localization in cells is dependent on the cell cycle; serum-stimulated actively proliferating cells retaining SGK1 mainly in the nucleus, while in glucocorticoid-induced G1-arrested cells, SGK1 is predominantly cytoplasmic (Buse *et al.* 1999). The intracellular translocation potential of SGK1 offers it effective means to control its downstream targets in response to various stimuli.

Our results suggest that SGK1 becomes downregulated during prostate cancer progression. However, overexpressing *SGK1* in PC-3 cells did not result to measurable change in the phenotype of these cells. While there are no other reports on *SGK1* in clinical prostate samples, studies on prostate cancer cell lines show SGK1 as a mediator of androgen signaling (Shanmugam *et al.* 2007). SGK1 was demonstrated to be a downstream target of AR, and needed for the androgen-induced evasion of apoptosis in serum-starved prostate cancer cells (Shanmugam *et al.* 2007), as well as for androgen-induced cell growth (Sherk *et al.* 2008). Recently, a novel AR co-activator with high expression levels in advanced prostate cancers, ATAD2 (ATPase family, AAA domain containing 2) was shown to be required for AR-mediated *SGK1* upregulation (Zou *et al.* 2009). Moreover, small-molecule inhibitors against SGK1 have been developed and tested for their efficacy in inhibiting SGK1, and consequently AR downstream targets (Sherk *et al.* 2008). One such inhibitor was effective in blocking androgen-stimulated cell growth in the LNCaP prostate cancer cell line. It was suggested that, in addition to androgen ablation, targeting SGK1 might be a feasible way to target androgen-dependent prostate cancers, especially those that have mutations in *PTEN*, the inhibitor of PI3K, and thus have a constitutively active PI3K pathway. All of these data, together with our SGK1 expression data, suggest that while SGK1 may have a role in prostate cancer, additional studies are warranted in clinical tumors to establish the true nature of SGK1 function in androgen-dependent and androgen-independent tumors.

3.4 *miR-193b* (III)

miR-193b was identified as being downregulated by DNA methylation in the 22Rv1 prostate cancer cell line. *miR-193b* expression was also shown to be markedly decreased in prostate cancer samples compared to adjacent benign prostate tissue. While no high-level methylation (similar to 22Rv1 cells) was observed in any of the studied prostate cancer samples, heterogeneous methylation patterns were present in almost all cancer samples, and all BPH samples were totally unmethylated. A more homogeneous methylation could have possibly been reached if the tissue samples had been laser-capture microdissected, as the observed strongly methylated and almost fully unmethylated clones in bisulphite sequencing could very well result from tumor cells and adjacent normal cells, respectively. Previous work by Lujambio *et al.* (2007) suggested *miR-193b* to be epigenetically regulated in lymph node metastatic cell lines originating from colon, skin, and head and neck cancers. In these cell lines, similar to our prostate cancer cell lines, *miR-193b* expression increased in response to 5azadC treatment. Additionally, *miR-193b* was shown to be methylated at a 10 CpG dinucleotide-encompassing area around the pre-miR-193b~365-1 start site in their cell lines. However, this methylation was not cancer-specific being also found in normal tissues. In prostate cancer cell lines, we found these CpGs methylated in 22Rv1, VCaP, LNCaP, PC-3, EP156T, and PrEC cells, but not in DU145 and LAPC-4. Additional evidence on the epigenetic regulation of *miR-193b* in prostate cancer came from a recent report studying histone modifications in the PC-3 and EP156T cell lines (Ke *et al.* 2009). While in normal EP156T cells, *miR-193b* was enriched with active H3K4me3 marks, in the PC-3 cancer cell line, the most prominent mark was H3K27me3, indicating silenced chromatin. In our study, no DNA methylation was seen at the *miR-193b* promoter CGI in the PC-3 cell line nor was *miR-193b* expression induced with 5azadC+TSA treatment. *miR-193b* expression was also significantly higher in PC-3 cells than in 22Rv1 cells, suggesting that while *miR-193b* can be regulated through different epigenetic mechanisms, methylation might to be more potent for silencing expression.

miRNAs are fairly commonly found in clusters, from which they become transcribed as polycistronic transcripts (Zhang *et al.* 2009). According to bioinformatic analyses by Saini *et al.* (2008), *miR-193b* is transcribed together with *miR-365-1* as a *pre-miR-193b~365-1*. However, we saw no increase in *miR-365-1* expression upon 5azadC and TSA treatment. Rather, an expression reduction was observed in the 22Rv1 and VCaP cell lines. This observation suggests additional layers of regulation, apart from transcription itself, in the process to produce the functional mature miRNAs. Another possibility for the non-concordant expression of these assumingly co-transcribed miRNAs is that they are not truly transcribed together as predicted, but rather as separate transcription units with unique regulatory elements.

Even though the role of methylation in *miR-193b* silencing remains murky, its tumor suppressor properties were demonstrated *in vitro*. Re-introduction of *miR-193b* to 22Rv1 cells resulted in significant growth reduction, a decrease in cells in S-phase, and a decreased ability of the cells to grow anchorage-independently. All

of these results support a tumor suppressor role for *miR-193b* in prostate cancer. Currently, there are no verified targets for *miR-193b* in prostate cancer. Two recent reports showed *miR-193b* to target estrogen receptor alpha (*ESR1*) and urokinase-type plasminogen activator (*PLAU*) in breast cancer (Leivonen *et al.* 2009, Li *et al.* 2009). However, neither one of these genes is likely to be *mir-193b* target in prostate cancer. *ESR1* is expressed only at very low levels in prostate cancer cell lines and in prostate tumor samples (Linja *et al*. 2003). *PLAU* expression in prostate cancer cell lines does not support it to be targeted by *miR-193b*, as 22Rv1 cells do not express *PLAU* mRNA at all (Helenius *et al*. 2006). However, increased PLAU expression has been reported to be an independent predictor for biochemical recurrence after radical prostatectomy (Kumano *et al*. 2009). To establish whether *PLAU* is a *miR-193b* target also in prostate cancer and what other genes could be targeted, additional studies are needed. The miRNA target prediction programs, such as Miranda and TargetScan (microrna.sanger.ac.uk), predict several, but mainly different targets. For example, of the top 20 targets predicted by these programs, none are shared.

The other candidate miRNAs identified in this study, apart from *miR-512-3p*, have also been previously implicated in cancer. *miR-203* has indeed been shown to be epigenetically silenced in some ALL and AML (Bueno *et al.* 2008). Bueno *et al.* (2008) showed *miR-203* to target *ABL1* (c-abl oncogene 1) and *BCR* (breakpoint cluster region)-*ABL1* oncogenes, and the methylation-induced silencing of *miR-203* expression was specifically observed in ALL and CML patients harboring the Philadelphia chromosome (*i.e.* the *BCR-ABL1* translocation). Another study additionally demonstrated repressive chromatin marks (*i.e.* high H3K9me3 and low H3K4me3) at this CGI in the proximity of *miR-203* in ALL cell lines (Roman-Gomez *et al.* 2009). CpG methylation at *miR-203* has also been reported to occur at very high frequency in oral carcinomas (Kozaki *et al.* 2008). In our studies, *miR-203* was not methylated, at least at the same CGI as in ALL and CML. *miR-218* expression significantly decreases in HPV (human papillomavirus)-positive cervical cancers, and this reduction is caused by expression of the *E6* oncogene of high risk HPV16 (Martinez *et al.* 2008). This same study reported both *miR-193b* and *miR-203* to be overexpressed in HPV-positive cervical cancers compared to HPV-negative ones and normal cervical cells. *miR-149* downregulation has been observed in squamous cell carcinomas of the tongue (Wong *et al.* 2008), and a common single-nucleotide polymorphism (SNP) at the *miR-149* locus has been associated with an increased risk of breast cancer in Chinese women (Hu *et al.* 2009). *miR-370* has been reported by us and others to be significantly overexpressed in advanced prostate carcinomas as compared to normal or benign samples (Porkka *et al.* 2007, Ambs *et al.* 2008). Regardless, in cell lines, we saw an expression profile implicative of epigenetic silencing. Indeed, in human malignant cholangiocytes *miR-370* expression was shown to decrease in response to IL-6 (interleukin 6)-induced DNMT1 expression, while 5azadC treatment increased *miR-370* expression (Meng *et al.* 2008). Meng *et al.* (2008) also reported MAP3K8, mitogen-activated protein kinase kinase kinase 8, to be one of the *miR-370* targets, suggesting a tumor suppressor function for *miR-370* through targeting MAP3K8 in cholangiocytes. Thus, *miR-370* seems to be responsive to epigenetic modifications: in some cells,

(*e.g*. cholangiocytes), it is an epigenetically regulated tumor suppressor miRNA targeting oncogenes; while in others (*e.g*., prostate cancer cells) it is an onco-miR targeting tumor suppressors.

CONCLUSIONS

The work presented here aimed to elucidate the yet-undiscovered molecular mechanisms of prostate cancer, and more precisely, to identify epigenetically regulated genes contributing to prostate carcinogenesis. Such genes were screened in various prostate cancer models using an indirect microarray expression analysis-based approach. Additionally, the TRAMP-C2 cell line, derived from a widely-used prostate cancer *in vivo* model (transgenic adenocarcinoma of mouse prostate TRAMP), was characterized for genetic aberrations.

Gene copy number analysis of TRAMP-C2 showed relatively few genomic regions with losses or gains of genetic material. However, gene copy numbers significantly correlated with expression levels, similar to human cancers. When the TRAMP-C2 genomic aberrations were compared to the corresponding human homologue chromosomal areas, no significant overlap was observed with the most commonly altered genomic regions in clinical cancers. Among the few matching alterations were the gains at 8q11.21 and 8q24.13-q24.3 and the losses at 6q14.1-q14.3 and 10q26.11-q26.3. Their presence in this mouse model further underlines their meaning in prostate cancer, as well as provides an additional model to be used for establishing the targeted genes of these aberrations.

The epigenetic profiling of the TRAMP-C2 cell line identified *Clu* as a methylation-silenced gene in this model. In human prostate cancer cell lines, methylation at the *CLU* gene promoter was observed in LNCaP cells, which also exhibited the lowest expression levels of *CLU*. In clinical samples, *CLU* expression was significantly reduced in cancers compared to BPH samples. Our data, together with other recent studies, suggests that as the nuclear isoform of clusterin clearly functions as a tumor suppressor in prostate cancer, epigenetic silencing may be one of the contributing factors for its decreased expression in prostate cancer.

DUSP1 and *SGK1* were identified in a screen for epigenetically silenced genes in the PC-3 prostate cancer cell line. Neither gene proved to be methylated at their promoters in PC-3 cells, but their expression both at the mRNA and protein levels decreased markedly when comparing clinical cancers to BPH samples, suggesting that they might have a role in prostate tumorigenesis. However, our unpublished results on *DUSP1* and *SGK1* overexpression in PC-3 cells showed no major effects on either cell proliferation or anchorage-independent growth. Literature on *SGK1* in prostate cancer is relatively sparse, and there are clear contradictions between the suggested functions and the demonstrated expression profiles. Our current knowledge does not support a major tumor suppressor functions for either *DUSP1* or *SGK1* in prostate cancer.

The experiments to find miRNAs that were epigenetically regulated in prostate cancer identified *miR-193b* as a potential tumor suppressor. *miR-193b* was lowly expressed in the 22Rv1 cell line, with dense methylation observed at the predicted 5'UTR. The expression of *miR-193b* also decreased significantly in clinical cancer

samples compared to normal adjacent cells. While no strong methylation was seen in the clinical samples, some level of methylation was present in tumor samples, but not in any BPH samples. Epigenetic silencing of *miR-193b* has also been suggested to take place through aberrant histone modifications, as shown in the PC-3 cell line (Ke *et al.* 2009). Functional assays (with transiently *miR-193b*-overexpressing 22Rv1 cells) to establish the tumor suppressor properties of *miR*-193b demonstrated decreased cell proliferation, a decreased number of cells in S-phase, and a decreased ability to grow anchorage-independently, supporting the idea that *miR-193b* is an epigenetically silenced TSG in prostate cancer.

The present studies suggest *miR-193b* as a novel TSG in prostate cancer, as well as confirm the previously somewhat contradictory tumor suppressor role of *CLU* in prostate cancer. Both of these genes are also likely to be under some level of epigenetic transcriptional regulation. Figure 9 illustrates the current view of prostate cancer progression with some of the most common genetic and epigenetic aberrations depicted. The TSGs described in this study are also shown. Additional studies are needed to further clarify the tumor suppressor potential of *miR-193b* and *CLU* and to verify the mode and extent of their epigenetic regulation involved in clinical tumors. The latter will be studied in the future using more direct methods to inquire epigenetic modifications of the genome, such as enrichment of methylated DNA through methyl-CpG binding domain of MBD2 protein combined with deep sequencing.

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ORIGINAL COMMUNICATIONS

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Dual-specificity phosphatase 1 and serum/glucocorticoid-regulated kinase are downregulated in prostate cancer

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Inactivation of tumor suppressor genes through deletion, mutation and epigenetic silencing has been shown to occur in cancer. In our study, we combined DNA demethylation and histone deacetylation inhibition treatments with suppression subtraction hybridization (SSH) and cDNA microarrays to identify potentially epigenetically downregulated genes in PC-3 prostate cancer cell line. We found 11 genes whose expression was upregulated after relieving epigenetic regulation. Expression of 3 genes [dual-specificity phosphatase 1 (*DUSP1*), serum/glucocorticoid regulated kinase (*SGK*) and spermidine/spermine N1-acetyltransferase (*SAT*)] was subsequently studied in clinical sample material using real-time quantitative RT-PCR and immunohistochemistry. The *DUSP1* and *SGK* mRNA expression was lower in hormone-refractory prostate carcinomas compared to benign prostate hyperplasia (BPH) or untreated prostate carcinomas. BPH, normal prostate and high-grade prostate intraepithelial neoplasia (PIN) expressed high levels of DUSP1 and SGK proteins. Ninety-two percent and 48% of the prostate carcinomas showed almost complete lack of DUSP1 and SGK proteins, respectively, indicating common downregulation of these genes. The genomic bisulphite sequencing did not reveal dense hypermethylation in the promoter regions of either *DUSP1* or *SGK*. In conclusion, the data suggest that downregulation of *DUSP1* and *SGK* is an early event and could be important in the tumorigenesis of prostate cancer.
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Key words: prostate cancer; microarray; SSH; *DUSP1*; *SGK*

Prostate cancer is the most commonly diagnosed malignancy among men in many Western countries and the second-leading cause of cancer death.[1,2] Despite its prevalence and the extensive studies dedicated to understanding the disease, the molecular mechanisms of prostate cancer initiation and progression remain incompletely understood. Cancer arises through a series of genetic changes that provides the cell with growth advantage over the normal counterparts. Such genetic changes include amplifications or gain-of-function mutations of oncogenes and deletions together with loss-of-function mutations of the remaining allele of tumor suppressor genes. Recently, several studies have suggested that the loss of just 1 allele (*i.e.,* haploinsufficiency) may be enough for inactivation of some tumor suppressor genes, such as *PTEN*.[3] For prostate cancer, the hunt for oncogenes as well as classical tumor suppressor genes is ongoing, as several chromosomal aberrations have been found but in most cases the target genes for these amplifications and deletions are yet to be discovered.[4] Over the past years, epigenetic silencing of gene transcription has become a well-recognized alternative mean for inactivation of tumor suppressor genes.[5] Several genes, such as *GSTP1, RASSF1A,* E-cadherin and *CD44,* have been shown to become hypermethylated during prostate tumorigenesis.[6–9]

cDNA microarrays can be used to study aberrant DNA methylation in an approach where the reexpression of epigenetically silenced genes in cancer cell lines is induced by treatments with DNA demethylating agents and histone deacetylase inhibitors.[10] This method directly links hypermethylation to the transcriptional status of genes. In our present study, we used a combination of suppression subtraction hybridization (SSH), cDNA microarrays and DNA demethylation and histone deacetylase inhibitor treatments to identify novel potentially epigenetically downregulated genes in prostate cancer. We identified 11 genes whose expression was increased in PC-3 prostate cancer cell line after reversing the epigenetic modifications. We further demonstrated decreased expression of 2 of these genes at both mRNA and protein levels in clinical samples of prostate carcinomas even though no direct evidence of epigenetic regulation was seen.

Material and methods

Cell lines and tissue samples

The prostate cancer cell line PC-3 was obtained from American Type Culture Collection (Manassas, VA) and cultured under recommended conditions. For the RT-PCR analyses, freshly frozen prostate tumor specimens representing benign prostate hyperplasia (BPH, $n = 9$), untreated ($n = 30$) and hormone-refractory ($n = 12$) carcinomas were obtained from Tampere University Hospital (Tampere, Finland). The specimens were histologically examined for the presence of tumor cells using H&E staining. Only samples containing >60% cancerous or hyperplastic epithelial cells were selected for the analyses. The BPH samples were obtained from prostatectomy specimens from cancer patients and were histologically verified not to contain any cancerous cells. Samples from hormone-refractory carcinomas were obtained from transurethral resections of prostate from patients experiencing urethral obstruction despite ongoing hormonal therapy. The time from beginning of hormonal therapy to progression (transurethral resection of prostate) varied from 15–60 months. For the immunostainings, tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded tumor blocks obtained from the Tampere University Hospital according to published guidelines.[11] The TMA contained 68 samples of untreated prostate carcinomas obtained from prostatectomies. The pTNM stage distribution of the cases was 40 pT2N0M0, 5 pT2N1M0, 21 pT3N0M0 and 1 pT3N1M0. The Gleason score distribution was 21 Gleason <7, 33 Gleason 7 and 13 Gleason >7. In addition, the TMA contained 64 samples of hormone-refractory tumors obtained from transurethral resections of prostate (TURP) from patients experiencing urethral obstruction despite ongoing hormonal therapy. The time from

Abbreviations: BPH, benign prostate hyperplasia; *DUSP1,* dual-specificity phosphatase 1; IHC, immunohistochemistry; PIN, prostate intraepithelial neoplasia; *SAT,* spermidine/spermine N1-acetyltransferase; *SGK,* serum/glucocorticoid-regulated kinase; SSH, suppression subtractive hybridization; *TBP,* TATA-box binding protein; TMA, tissue microarray; TSA, trichostatin A, 5-azadC 5-aza-2′-deoxycytidine.

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TABLE I – PRIMER AND PROBE SEQUENCES FOR RT-PCR

Primer/probe	Sequence 5′ → 3′	T_{anneal}
DUSP1 for	AGTACCCCACTCTACGATCAGG	56°C
DUSP1 rev	TGATGGAGTCTATGAAGTCAATGG	
SGK for	GCCAAGGATGACTTCATGG	56°C
SGK rev	CAGGCTCTTCGGTAAACTCG	
SAT for	GAAGGACACAGCATTGTTGG	57°C
SAT rev	TCATTGCAACCTGGCTTAGA	
TBP for	GAATATAATCCCAAGCGGTTTG	57°C
TBP rev	ACTTCACATCACAGCTCCCC	
TBP-FL[1]	TTTCCCAGAACTGAAAATCAGTGCC-FL	
TBP-LC[1]	LC Red640-TGGTTCGTGGCTCTCTTATCCTCATG	
β-actin for	TGGGACGACATGGAGAAAAT	55°C
β-actin rev	AGAGGCGTACAGGGATAGCA	
β-actin-FL[1]	CCGCGAGAAGATGACCCACAGATCAT-FL	
β-actin-LC[1]	LC Red640-TTGAGACCTTCAACACCCCAGCCA	

[1]Designed and obtained from TibMolBiol, Berlin, Germany.

beginning of hormonal therapy to progression varied from 1–122 months with a mean of 31 months. Also, 13 TURP samples from patients with BPH were analyzed. The use of clinical tumor material has been approved by the Ethical Committee of Tampere University Hospital.

DNA demethylation and histone deacetylation inhibition treatments and RNA extraction

PC-3 prostate cancer cells were treated with DNA demethylating agent 5-aza-2′-deoxycytidine (5-azadC; Sigma-Aldrich, St. Louis, MO) and histone deacetylase inhibitor trichostatin A (TSA; Sigma-Aldrich) at different concentrations both alone and in combination. Briefly, PC-3 cells were seeded at low density and grown for 72 hr. Next, the cells were treated with final concentrations of 0.5 μM or 1 μM of 5-azadC for 48 hr. Subsequently, either the cells were collected (5-azadC treatment alone) or were further treated with 5-azadC and at final concentrations of 0.3 μM or 1 μM of TSA for an additional 24 hr. The cells treated only with TSA were first grown 72 hr and then treated with 0.3 μM TSA for 24 hr. Control cells were grown without these agents for 96 hr, with fresh medium changed every 48 hr. Total RNA was extracted from the cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA).

cDNA microarray expression analysis

For the cDNA microarray analyses, a slide containing 432 clones from the Suppression Subtraction Hybridization (SSH)-cDNA library was used. The construction of the SSH-cDNA library has been previously described.[12] Briefly, the library was constructed by subtracting cDNA from BPH with cDNA from PC-3 cell line using PCR-Select™ cDNA Subtraction Kit (BD Biosciences, Palo Alto, CA) according to manufacturer's instructions. The subtracted cDNA was cloned into pCR®II-TOPO TA vector (Invitrogen) and individual clones were picked, amplified and spotted in triplicates onto poly-L-lysine-coated glass slides at the Finnish DNA Microarray Center (Turku Center for Biotechnology, Turku, Finland). The labeling of the cDNAs and the hybridizations were done as described previously in more detail.[13] Briefly, for each hybridization, labeled first-strand cDNAs were generated from 40 μg of total control (Cy3) and 5-azadC- and/or TSA-treated (Cy5) RNAs using oligo $d(T)_{15}$ primer and SuperScript II Reverse Transcriptase (Invitrogen). The labeled probes were then combined, purified and concentrated using Microcon YM-30 columns (Millipore, Billerica, MA). During this purification step, the following blocking agents were included in the probe solution: 10 μg of Cot-1 DNA (Invitrogen), 20 μg of polyadenylic acid (Sigma-Aldrich) and 42 μg of Yeast tRNA (Invitrogen). The hybridization was carried out at +65°C overnight. After hybridization slides were washed and scanned using ScanArray 4000 laser confocal scanner (GSI Lumonics, Billerica, MA). QuantArray software program (Packard Bioscience, BioChip Technology LCC, Billerica, MA) was used to quantitate signal intensities. Cy5/Cy3 ratios were calculated from background subtracted and normalized Cy5 and Cy3 signals. For normalization, we used the mean signal intensities of 1 subarray. Median value of Cy5/Cy3 ratio from 3 replicates was calculated and median Cy5/Cy3 ratio >2 was regarded as increased expression.

Sequencing

Clones that had Cy5/Cy3 ratio >2, thus exhibiting increased expression after 5-azadC and/or TSA treatments, were amplified using NES1 and NES2R adaptor-specific primers (BD Biosciences). PCR reactions were purified using QIAquick PCR purification columns (Qiagen, Valencia, CA). Sequencing was done with the same NES-primers using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI 3100 sequencer (Applied Biosystems, Foster City, CA).

Real-time quantitative RT-PCR

Three micrograms of total RNA from control and treated cells were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and oligo $d(T)_{15}$ primer. Standard curve was prepared from cDNA reverse transcribed from 3 μg of total liver RNA (BD Biosciences). Serial dilution of the standard was done to obtain cDNA corresponding to 100, 20, 4, 0.8, 0.16 and 0.032 ng of original RNA. The first-strand cDNA synthesis of the clinical tumor material has previously been described.[14] Amplification primers were designed into different exons or to span exon-intron boundary to avoid amplification of any contaminating genomic DNA (Table I). PCR reactions of 50 cycles were performed with Light Cycler™ (Roche Diagnostics, Mannheim, Germany) using QuantiTech™ SYBR® Green RT-PCR Kit (Qiagen) according to manufacturer's instructions. The annealing temperatures are shown in Table I. Fluorescence signals were measured after the elongation step at 72°C for dual-specificity phosphatase 1 *(DUSP1)* and spermidine/spermine N1-acetyltransferase *(SAT)* and at 79°C for serum/glucocorticoid-regulated kinase *(SGK)*. After the amplification, melting curve analysis and agarose gel electrophoresis were performed to ensure that only 1 correct-sized PCR product was amplified. To normalize the expression levels of the studied genes, the expression of TATA-box binding protein *(TBP)* was measured with Light Cycler™ using primers and probes shown in Table I as described previously.[14] The fold induction of each sample was calculated from the *TBP*-normalized expression values by dividing each expression value with the expression value of the control sample. For comparison, the expression levels of the studied genes were also normalized to the expression levels of β-actin measured with LightCycler™ using primers and probes shown in Table I.

TABLE II – GENES UPREGULATED BY 5-azadC AND TSA TREATMENTS IN PC-3 CELLS

Gene name	Gene symbol	Chromosomal location	Upregulated with	Expression ratio[1]
Spermidine/spermine N1-acetyltransferase	*SAT*	Xp22.1	5-azadC or 5-azadC+TSA[2]	3.06
Dual-specificity phosphatase 1	*DUSP1*	5q34	5-azadC or 5-azadC+TSA[2]	2.91
Serum/glucocorticoid-regulated kinase	*SGK*	6q23	5-azadC or 5-azadC+TSA[2]	2.56
Haplotype A mitochondrion		mtDNA	5-azadC or 5-azadC+TSA[2]	2.30
Beta-2-microtubulin	*BM2*	15q21–q22.2	5-azadC or 5-azadC+TSA[2]	2.09
Ribosomal protein S27 (metallopanstimulin 1)	*RPS27*	1q21	5-azadC or 5-azadC+TSA[2]	2.07
Haplotype U5 mitochondrion		mtDNA	5-azadC or 5-azadC+TSA[2]	2.04
Thymosin, beta 4	*TMSB4X*	Xq21.3–q22	5-azadC or 5-azadC+TSA[2]	2.03
Mitochondrial cytochrome b		mtDNA	5-azadC or 5-azadC+TSA[2]	2.01
Heat shock protein 90, alpha	*HSPCA*	14q32.33	TSA alone or 5-azadC+TSA	3.04
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	*NFKBIA*	14q13	TSA alone or 5-azadC+TSA	2.49

[1]Median expression ratio from all the treatments where the gene was upregulated.–[2]5-azadC, 5-aza-2′-deoxycytidine, TSA, trichostatin A.

Immunohistochemical staining

The protein expressions were analyzed using a standard immunohistochemical technique with polyclonal rabbit antibodies against DUSP1 (M-3786, Sigma-Aldrich) and SGK (#3272, Cell Signaling Technology, Beverly, MA). To improve the staining, high-temperature antigen retrieval, with a 2 min incubation in 10 mM Na-citrate (pH 6.0) for DUSP1 or in Citra-solution (BioGenex, SanRamon, CA) for SGK in an autoclave, was used. The bound antibody was visualized with a conjugate of a secondary antibody and horseradish peroxidase with diaminobenzidine as a chromogen (PowerVision+ Detection System, ImmunoVision, Springdale, AR). Expression was classified into 2 groups from no staining to weak staining (IHC scores 0 and 1+) and from moderate to strong staining (IHC scores 2+ and 3+).

Genomic bisulphite sequencing

Methylation status of *DUSP1* and *SGK* genes in PC-3 cells was determined using bisulphite treatment and sequencing. Bisulphite treatments were done using both CpGenome™ DNA Modification Kit (Chemicon® International, Temecula, CA) according to the manufacturer's instructions and by a standard procedure as described.[15] In brief, 1 μg of shredded DNA from PC-3 was denatured with freshly prepared 3M NaOH and incubated for 15 min at 42°C. For deamination, freshly prepared 10 mM hydroquinone and 3M sodium bisulphite (pH 5.0) were added and incubated at 50°C overnight. Modified DNA was desalted using Wizard DNA Clean Up Kit (Promega, Madison, WI). DNA modification was completed by desulphonating the DNA with freshly prepared 3M NaOH and incubating 15 min at 37°C. Bisulphite-modified DNA was ethanol precipitated and resuspended into buffer. Two microliters of modified DNA was used as a template for the 1st round of PCR amplification with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), followed by a 2nd round of PCR using the same primers as in the 1st round with 4 μl of the 1st round PCR product as a template. Primer sequences can be obtained from the authors. Second round PCR products were purified using either Qiagen's QIAquick PCR Purification Kit or Qiagen's Gel Extraction Kit and subcloned into pCR®2.1-vector using TOPO TA Cloning® Kit (Invitrogen). Ten clones from bisulphite treatments for both *DUSP1* and *SGK* were then amplified using Dynazyme DNA Polymerase (Finnzymes, Espoo, Finland) and M13 primers. The PCR products were column purified and sequenced with M13 primers as described above.

Statistical analysis

The association of the mRNA expression levels with the tumor type was calculated with nonparametric Kruskal-Wallis test. The association of the protein expression with tumor type, clinical stage, Gleason score and prostate specific antigen (PSA) levels was calculated with Fisher's exact and χ^2 tests as well as with variance analysis.

Results

cDNA microarray expression analysis after epigenetic modifications

Of the 432 clones on the array, 17 clones (3.9%) were upregulated after the treatments with 5-azadC and/or TSA. Sequencing showed that these 17 clones represent 11 individual genes (listed in Table II). Nine of the 11 upregulated genes were upregulated when treated with 5-azadC or both 5-azadC and TSA at different concentrations but not when treated with TSA alone. Two genes were upregulated also when treated with TSA alone. From the 9 genes, *SAT, DUSP1* and *SGK* were selected for further studies based on not being upregulated by TSA treatment alone, their high Cy5/Cy3 ratios and known functions.

cDNA microarray result verification by real-time quantitative RT-PCR

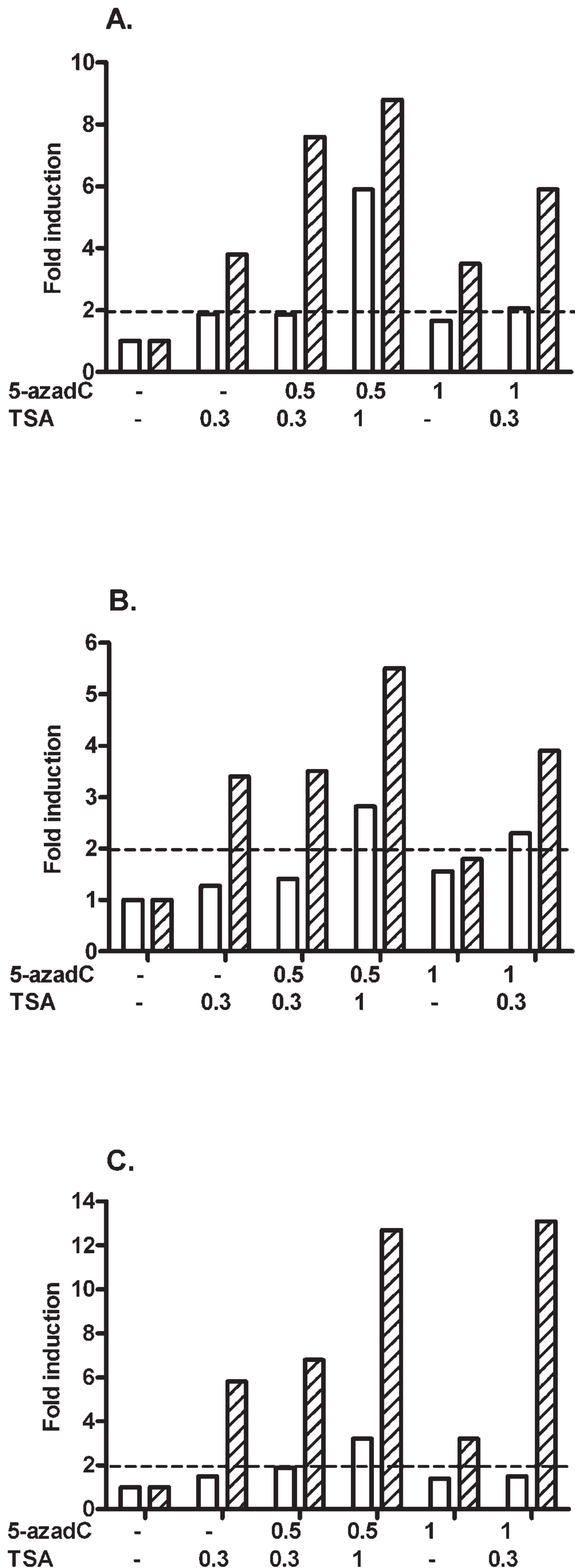
The microarray expression data of *DUSP1, SGK* and *SAT* were verified using real-time quantitative RT-PCR. The results from the microarray analyses and real-time quantitative RT-PCR are shown in Figure 1. RT-PCR confirmed the upregulation of these genes after the treatments with 5-azadC and TSA. With real-time RT-PCR, higher expression differences were detected than with microarrays. The real-time RT-PCR also exhibited increased expression of all 3 genes after TSA treatment alone. Normalization with β-actin gave similar results as the normalization with *TBP* (data not shown).

Expression of DUSP1, SGK and SAT mRNA in clinical samples

The results from real-time quantitative RT-PCR are represented in Figure 2. The expression of *DUSP1* was significantly lower in locally recurrent hormone-refractory prostate carcinomas compared to untreated prostate carcinomas or BPH ($p = 0.0001$). Also, the expression of *SGK* was significantly lower in hormone-refractory compared to untreated carcinomas or BPH ($p = 0.0331$). The expression of *SAT* was about equal in all sample groups ($p = 0.5636$).

Expression of DUSP1 and SGK proteins by immunohistochemistry

Protein levels of DUSP1 and SGK were assessed on TMAs using immunohistochemistry. The DUSP1 staining was detected uniformly in the cytoplasm of the normal epithelial cells with the strongest staining intensity in basal cells (Fig. 3*a*). In prostate intraepithelial neoplasia (PIN), DUSP1 was also strongly expressed in secretory luminal cells (Fig. 3*b*). High DUSP1 protein expression was also seen in atrophic glands (Fig. 3*c*). All BPH samples showed DUSP1 immunostaining; however, the staining intensity varied notably from one gland to another. In untreated prostate and hormone-refractory carcinomas, DUSP1 protein levels varied from undetectable to moderate, the majority (92%) being negative or weakly positive (Fig. 3*d–f* and Table III). Although completely DUSP1-negative (IHC 0) samples were found more often in hormone-refractory than in untreated carcino-



mas (70% compared to 46%), there was no statistically significant difference in the expression levels between the untreated and hormone-refractory tumors. DUSP1 protein expression was not associated with clinical stage, Gleason score or PSA levels in the untreated cases, either. However, the expression was significantly ($p < 0.0001$) lower in malignant than BPH samples (Table III).

SGK protein localized mainly into the nucleus of the secretory luminal cells. SGK was expressed uniformly at high levels in nonmalignant tissues and at varying levels from undetectable to high expression in untreated and hormone-refractory carcinomas (Fig. 3*g*–*i*). All BPH samples were positive for SGK, whereas 48% of all carcinomas exhibited no or weak staining ($p = 0.0021$) (Table III). SGK protein expression was not associated with clinical stage, Gleason score or PSA levels in the untreated cases.

Genomic bisulphite sequencing

Methylation status of *DUSP1* and *SGK* gene promoters in PC-3 cells was studied using genomic bisulphite sequencing. For *DUSP1,* a 755 bp region from −490 to +270 relative to transcription start site (TSS) and a 230 bp from −1210 to −980 at the suggested negative regulatory region[16] were studied. For *SGK,* a region of 460 bp from −335 to +125 relative to TSS was analyzed. No dense DNA methylation was seen at these GC-rich areas for either gene. Only a few nonrecurrent methylated CpG sites in some of the sequenced clones were found. Results were the same for CpGenome™-modified and standard bisulphite-treated DNAs.

Discussion

We combined suppression subtractive hybridization (SSH) and cDNA microarrays with DNA demethylation and histone deacetylase inhibition treatments to identify genes that could be epigenetically downregulated in prostate cancer. It has previously been shown that the combination of SSH and microarrays increases the sensitivity of the approach to detect epigenetically modified genes compared to using microarray analyses alone.[10] Suzuki *et al.*[10] used SSH to subtract the cDNA from treated and control cells to create a probe for cDNA microarray hybridization. Instead, we used the SSH as a preselective step for targets on the array. Our array was enriched with cDNAs present more abundantly in BPH compared to PC-3 prostate cancer cell line.[12] Therefore, the genes on the array potentially also included genes that become silenced during tumorigenesis by epigenetic modifications.

After cDNA microarray hybridization, we found 11 genes whose expression was upregulated in response to DNA demethylating agent 5-azadC and HDAC inhibitor TSA. According to the cDNA microarray analysis, 9 of the 11 genes were upregulated when treated with both 5-azadC and TSA but not with TSA alone. They account for genes whose possible epigenetic regulation could involve promoter hypermethylation. Two of the 11 genes were also upregulated when treated with TSA alone. According to the classification suggested by Suzuki *et al.,*[10] the possible epigenetic regulation of this type of gene does not necessarily involve DNA methylation but mainly histone deacetylation. For further analysis, we chose 3 of the 9 genes that exhibited the highest Cy5/Cy3 ratios and were upregulated by combined treatment but not with TSA alone, as well as were functionally interesting. The upregulated expression of *DUSP1,*

Figure 1 – Expression of (*a*) *DUSP1*, (*b*) *SGK* and (*c*) *SAT* after 5-azadC and TSA treatments in PC-3 cells analyzed by cDNA microarrays (open bars) and real-time quantitative RT-PCR (hatched bars). The Cy5/Cy3 ratios (treated/untreated) are represented for microarrays and *TBP*-normalized relative expression values for Q-RT-PCR. Relative expression is represented as fold induction where control experiment is designated as 1. The dashed line marks the cut-off value (Cy5/Cy3 > 2) used for defining upregulation in microarray experiments.

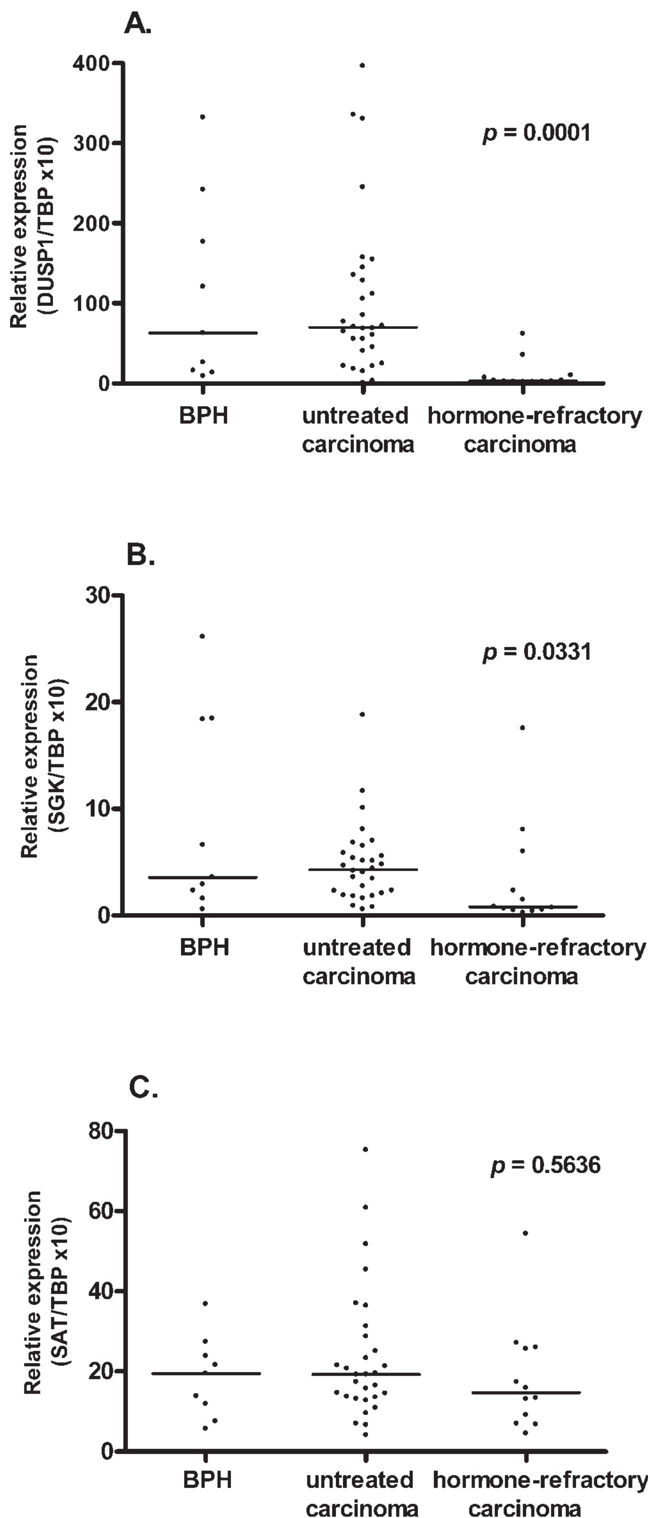


FIGURE 2 – Expression of (*a*) *DUSP1,* (*b*) SGK and (*c*) *SAT* in clinical samples (BPH, $n = 9$; untreated carcinoma, $n = 30$; hormone-refractory carcinoma, $n = 12$) analyzed by real-time quantitative RT-PCR. Median values are represented as horizontal bars.

SGK and *SAT* found in microarrays was first confirmed with real-time quantitative RT-PCR. Different concentrations of 5-azadC and TSA gave a dose-dependent response in gene expression, and a synergistic effect of 5-azadC and TSA on upregulation was found. Such a synergy has also been reported previously.[17,18] Real-time RT-PCR, as a more sensitive method, gave higher expression differences between treatments and showed that all 3 genes studied were upregulated by TSA alone as well (Fig. 1). This implies that the possible epigenetic regulation of *DUSP1, SGK* and *SAT* does not necessarily involve DNA hypermethylation.

Expression of *DUSP1, SGK* and *SAT* was then further studied in clinical samples of BPH, untreated and locally recurrent hormone-refractory prostate carcinomas. SAT mRNA expression did not vary between the sample groups studied. Both *DUSP1* and *SGK* mRNA expressions were statistically significantly decreased in hormone-refractory carcinomas compared to untreated carcinomas or BPH (Fig. 2). However, at the protein level, the decreased expression of the genes was also found in untreated carcinomas (Fig. 3, Table III). This discrepancy could be, at least in part, explained by normal cell contamination in primary tumor samples used for RT-PCR. Neither *DUSP1* nor *SGK* has an androgen-responsive element (ARE) in the promoter region of the gene. Thus, decreased androgen levels in hormone-refractory carcinoma cannot directly cause the downregulation of *DUSP1* and *SGK*.

DUSP1 protein was localized into the cytoplasm of the malignant cells as has also previously been demonstrated.[19–21] Also, nuclear localization of DUSP1 has been reported in ovarian cancer as well as in fibroblasts,[22,23] whereas we did not detect any strong nuclear staining. All prostate carcinomas exhibited decreased expression compared to BPH, the basal cells of normal prostate or PIN, and in most of the cases, there was complete or almost complete lack of staining. Our data are partly consistent with previous studies by Loda *et al.,*[21,24] who showed, by mRNA *in situ* hybridization, that *DUSP1* mRNA expression is increased in PIN and decreased in poorly differentiated prostate carcinomas and metastases. However, Loda *et al.* found that well-differentiated tumors express *DUSP1,* whereas our data suggest that all carcinomas irrespective of the grade contain reduced expression of the DUSP1 protein. In other carcinomas, such as colon, bladder and ovary, it has been shown that DUSP1 mRNA is expressed at relatively high levels in early stage tumors, whereas the expression decreases during tumor progression.[22,24] On the other hand, breast carcinomas seem to maintain relatively high expression of *DUSP1* in all stages.[24,25] The data on experimental reexpression of *DUSP1* in malignant cells have been controversial. In ovarian cancer cells, the reexpression decreased the malignant potential of the cells by inhibiting anchorage-dependent and -independent cell growth as well as cell motility.[22] Whereas, in pancreatic cancer cells, the downregulation decreased cell proliferation and tumorigenicity in nude mouse tumor model.[26]

DUSP1 (also referred to as MKP-1, CL100, HVH1, PTPN10) is a dual-specificity phosphatase that inactivates mitogen-activated protein kinases (MAPKs) by dephosphorylating both phosphotyrosine and phosphothreonine residues. DUSP1 has been shown to dephosphorylate and inactivate all members of the MAPK family, although stress-activated protein kinase/c-jun N-terminal protein kinase (SAPK/JNK) and p38 MAPK seem to be preferential targets.[20,27–29] Constitutive DUSP1 expression has been shown to lead to inhibition of cell cycle by inhibiting MAP kinase activity and blocking S-phase entry in fibroblasts.[23] In a senescence model of mammary fibroblasts, DUSP1 was upregulated and suggested to have a role in senescence induction through inhibition of AP-1 activity and the subsequent transcription of genes involved in DNA replication.[30] DUSP1 overexpression also inhibited MAPK-mediated Ras-induced DNA synthesis in fibroblasts.[31] In addition, it was recently shown that in breast cancer cell lines, the increased antiapoptotic activity of ERK1/2 by anthracyclines was caused by decreased DUSP1 expression.[32] However, various reports have shown that DUSP1 also has antiapoptotic effects.[20,25,33,34] This implies that both high and low DUSP1 expression levels can result in antiapoptotic activity depending on which MAP kinase DUSP1 is targeting.

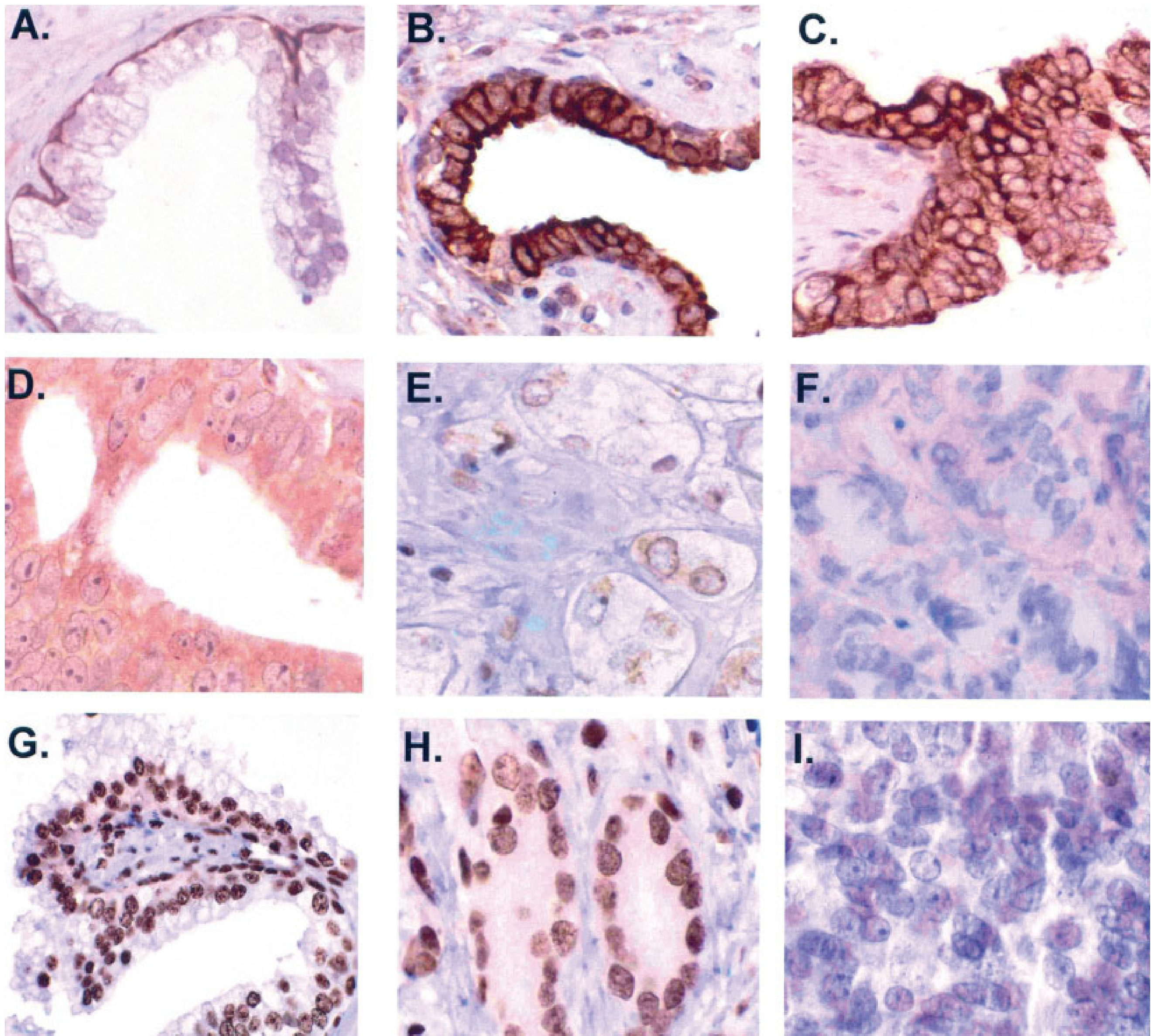


FIGURE 3 – Examples of the immunohistochemical staining. (*a–f*) DUSP1; (*g–i*) SGK. (*a*) Basal cells of the normal prostates express high levels of DUSP1. (*b*) Atrophic gland, as well as (*c*) high-grade prostate intraepithelial neoplasia (PIN) also stain strongly with anti-DUSP1 antibody. (*d*) A small fraction of prostate carcinomas exhibit diffuse and modest staining for DUSP1, whereas most of the (*e*) untreated and (*f*) hormone-refractory prostate carcinomas are negative for DUSP1. (*g*) BPH and (*h*) prostate carcinoma exhibit strong nuclear staining with anti-SGK antibody. (*i*) Hormone-refractory prostate cancer is negative for SGK.

TABLE III – EXPRESSION OF DUSP1 AND SGK BY IMMUNOHISTOCHEMISTRY IN BENIGN PROSTATE HYPERPLASIA (BPH) UNTREATED AND HORMONE-REFRACTORY PROSTATE CARCINOMAS

Immunostaining score[1]	DUSP1			SGK		
	BPH	Untreated	Hormone-refractory	BPH	Untreated	Hormone-refractory
0–1+	0 (0%)	60 (88%)	58 (97%)	0 (0%)	29 (43%)	34 (53%)
2+–3+	13 (100%)[2]	8 (12%)	2 (3%)	13 (100%)	38 (57%)	30 (47%)
Total	13 (100%)	68 (100%)	60 (100%)	13 (100%)	67 (100%)	64 (100%)
p-value		<0.0001			0.0021	

[1]Immunohistochemical score 0 indicates no staining, 1+ weak, 2+ moderate and 3+ strong stainings.–[2]Immunostaining of DUSP1 was heterogeneous in BPH samples.

We also analyzed protein expression of SGK by using immunohistochemistry. SGK was strongly expressed in all epithelial cells of normal prostate, BPH and high-grade PIN. In malignant prostate, SGK protein was detected at varying levels. About half of the carcinomas exhibited no or only weak immunostaining. SGK localized exclusively to the nucleus, where it has been shown to reside in actively proliferating cells.[35] Only a few studies on the expression of SGK in malignant tissues, but not in prostate, have

previously been reported, and increased expression of SGK in hepatocellular carcinoma and ductal carcinoma *in situ* of breast has been shown.[36,37]

SGK is a serum/glucocorticoid-regulated kinase whose transcription, enzymatic activity and subcellular localization are under simultaneous and stringent stimulus-dependent regulation. A wide variety of different stimuli can induce *SGK* transcription, including serum, glucocorticoids, p53, follicle stimulating hormone (FSH) and various stresses such as hyperosmotic stress, heat shock, oxidative stress and UV irradiation.[38–42] The phosphorylated, enzymatically active form of SGK has been found in serum-stimulated cells, whereas in the glucocorticoid-treated cells, only the unphosphorylated SGK was present. In the S and G_2/M phase of MCF-7 breast cancer cells, SGK mainly localized into the nucleus, whereas in cells arrested in G_1 phase, SGK resided in the cytoplasm or perinucleus.[35] This regulated translocation offers SGK means to produce different outcomes for different stimuli even though both stimuli would initially induce transcription.

SGK shares significant sequence similarity with another serine/threonine kinase, *Akt* and, as Akt, is a downstream target of phosphatidylinositol 3-kinase (PI3K).[38,43] SGK has been shown to act as a negative regulator of B-Raf kinase activity, thus offering it means to regulate the Raf-MEK-ERK pathway.[44] It has also been reported that SGK could possess antiapoptotic effects through negative regulation of forkhead transcription factor, FOXO3a and activation of IκB kinase.[42,45,46] The *SGK* gene is located in the chromosome 6q23 region, which has been shown to be commonly deleted in prostate cancer according to comparative genomic hybridization[47] and loss of heterozygosity analysis.[48] Thus, the gene should be considered as a putative target for the deletion.

The aim of our study was to identify genes with altered expression due to epigenetic regulation in prostate cancer. The screening experiment with DNA demethylating agent and histone deacetylase inhibitor indicated *DUSP1* and *SGK* as possible target genes for epigenetic modification. To directly demonstrate whether the promoter regions of the genes are hypermethylated in the PC-3 prostate cancer cell line, we used genomic bisulphite sequencing method. Despite the increased expression of the genes after 5-azadC and TSA treatments, we were not able to demonstrate excessive methylation in their core promoter regions. This may suggest that the hypermethylated regions downregulating the expression of the genes were located outside the promoter regions studied. It has also recently been suggested that even methylation of a few CpG sites could have a significant effect on the expression.[49] On the other hand, a recent study showed that *DUSP1* expression in PC-3 cells was induced after treatment with histone deacetylase inhibitor, FK228,[50] suggesting that maybe histone deacetylation, instead of promoter hypermethylation, is downregulating the *DUSP1* expression in these cells. Another possibility is that the genes themselves are not primary targets for DNA methylation; instead some epigenetically modified upstream factors could affect the transcription of *DUSP1* and *SGK*. 5-azadC also has cytotoxic effects on cells,[51] in addition to its function as an inhibitor of DNA methylation, which could, at least theoretically, cause the induction of expression of *DUSP1* and *SGK* in PC-3 cells. Thus, the mechanisms behind the downregulation of *DUSP1* and *SGK* expressions in prostate cancer remain unclear and need further studies.

In conclusion, we demonstrated here a common downregulation of mRNA and protein expressions of 2 genes, *DUSP1* and *SGK,* in prostate cancer. Downregulation of DUSP1 seemed to be an early event in the tumorigenesis of prostate cancer affecting almost all tumors. The expression of SGK was decreased in about half of the prostate carcinomas, whereas the expression was high in all nonmalignant prostate epithelial cells. Studies on the tumor suppressor potential as well as the mechanisms of the downregulation of both *DUSP1* and *SGK* are now warranted.

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Clusterin is epigenetically regulated in prostate cancer

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Lack of good models has complicated investigations on the mechanisms of prostate cancer. By far, the most commonly used transgenic mouse model of prostate cancer is TRAMP, which, however, has not been fully characterized for genetic and epigenetic aberrations. Here, we screened TRAMP-derived C2 cell line for the alterations using different microarray approaches, and compared it to human prostate cancer. TRAMP-C2 had relatively few genomic copy number alterations according to array comparative genomic hybridization (aCGH). However, the gene copy number and expression were significantly correlated ($p < 0.001$). Screening genes for promoter hypermethylation using demethylation treatment with 5-aza-2′-deoxycytidine and subsequent expression profiling indicated 43 putatively epigenetically silenced genes. Further studies revealed that clusterin is methylated in the TRAMP-C2 cell line, as well as in the human prostate cancer cell line LNCaP. Its expression was found to be significantly reduced ($p < 0.01$) in untreated and hormone-refractory human prostate carcinomas. Together with known function of clusterin, the data suggest an epigenetic component in the regulation of clusterin in prostate cancer.
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Key words: TRAMP; neoplasia; prostatic carcinoma; hypermethylation; array

Although the chromosomal aberrations commonly associated with different stages of prostate cancer are well known, only a few individual genes have been shown to be involved in a significant proportion of prostate cancers (reviewed in Ref. 1). Identification of genetically or epigenetically altered genes in prostate cancer has been hampered by the lack of good model systems. Prostate carcinoma cells do not grow well *in vitro*, and there are only a few commonly available human prostate cancer cell lines, such as, PC-3, DU-145, LNCaP, 22Rv1, LAPC-4, MDA-Pca-2b, NCI-H660 and VCaP. Generally, the cell lines do not resemble clinical prostate carcinomas very well, most of them do not contain the chromosomal aberrations that are typical for prostate cancer (*e.g.*, gains of 8q and Xq and losses of 6q, 8p, 10q and 13q; [2,3]), and only LAPC-4 and VCaP express wild-type androgen receptor.[3]

The transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model was established in 1995,[4] and subsequently it has become the most commonly utilized transgenic mouse model of prostate cancer. In this model, the SV40 large T antigen (tag) is located under the probasin promoter, driving the expression of tag to the epithelium of the mouse prostate. These mice develop prostate cancer spontaneously by the age of 8–12 weeks, and the development and progression of cancer closely mimics the human disease; they develop premalignant prostatic intraepithelial lesions that progress into invasive focal carcinomas, forming also distant metastases. Three cell lines have been derived from the TRAMP mouse model: TRAMP-C1, TRAMP-C2 and TRAMP-C3. All of them have been established from a prostate tumor of a single 32-week-old TRAMP mouse. The TRAMP cell lines express AR, cytokeratin and E-cadherin, and they do not express the transgenic T antigen.[5] TRAMP-C1 and TRAMP-C2 are also tumorigenic when grafted into syngeneic hosts.

Despite the extensive use of the TRAMP model, no genome-wide analysis of the genetic alterations of the model has previously been published. The goal of our study was to characterize the TRAMP-C2 cell line for gene copy number alterations and epigenetic changes in order to evaluate how closely the model mimics human prostate cancer. Another goal was to identify individual genes that may be altered in human prostate cancer.

Material and methods

Cell lines and tissue samples

The mouse prostate epithelial adenocarcinoma cell line TRAMP-C2, as well as the human prostate cancer cell lines PC-3, DU145 and LNCaP, were obtained from American Type Culture Collection (Manassas, VA) and cultured under recommended conditions. The human prostate cancer cell line LAPC-4 was kindly provided by Dr. Charles Sawyers (MSKCC, New York, NY). Freshly frozen prostate tumor specimens representing benign prostate hyperplasia (BPH, $n = 9$), androgen-dependent ($n = 29$) and hormone-refractory ($n = 11$) carcinomas were obtained from Tampere University Hospital (Tampere, Finland). The specimens were histologically examined for the presence of tumor cells using H&E staining. Only samples containing >60% cancerous or hyperplastic epithelial cells were selected for the analyses. The BPH samples were obtained from prostatectomy specimens from cancer patients and were histologically verified not to contain any cancerous cells. Samples from hormone-refractory carcinomas were obtained from transurethral resections of prostate (TURP) from patients experiencing urethral obstruction despite ongoing hormonal therapy. The time from the beginning of hormonal therapy to progression (TURP) varied from 15 to 60 months. The use of clinical tumor material has been approved by the Ethical Committee of Tampere University Hospital.

DNA demethylation and histone deacetylation inhibition treatments

TRAMP-C2 cells were treated with DNA demethylating agent 5-aza-2′-deoxycytidine (5azadC; Sigma-Aldrich, St. Louis, MO) and histone deacetylase inhibitor trichostatin A (TSA; Sigma-Aldrich), both separately and in combination. Briefly, TRAMP-C2 cells were seeded at low density and grown for 48 hr. For 5azadC treatment alone, the cells were then treated with a final concentration of 0.05 μM 5azadC and harvested after 48 hr. For combined treatment, the cells were first treated with 0.05 μM 5azadC for 24 hr, and then with TSA at a final concentration of 0.1 μM for another 24 hr, before RNA collection. The cells treated with TSA alone were first grown for 48 hr and then treated with 0.1 μM TSA for 24 hr. Control cells were grown without these agents for 72 hr before RNA collection, with fresh medium changed after 48 hr. Total RNA was extracted from the cells using the Trizol reagent (Invitrogen, Carlsbad, CA).

Additional Supporting Information may be found in the online version of this article.

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cDNA microarray expression analysis

Mou15K-1 cDNA microarray slides, containing about 15,000 mouse cDNA clones in duplicate, were obtained from the Finnish DNA Microarray Centre (Turku Centre for Biotechnology, University of Turku and Åbo Akademi, Turku, Finland). The slides were used for expression analyses of the TRAMP-C2 cell line, as well as for detecting epigenetically silenced genes. For the expression analysis, normal mouse total RNA (Clontech, Mountain View, CA) was used as reference. For the epigenetic analyses, RNA from TRAMP-C2 treated with 5azadC and/or TSA (described earlier) was used with RNA from the untreated TRAMP-C2 cells as reference. For all microarray hybridizations, 40 µg of total RNA from the test and reference samples were used for generating cDNAs labeled with Cy5 or Cy3, respectively. Labeling and hybridization were performed as described in Rauhala *et al*.[6] The fluorescence signals of Cy3 and Cy5 were measured using the Agilent DNA Microarray Scanner BA (Agilent Technologies, Santa Clara, CA). Feature Extraction Software version A.7.5.1 was used to quantitate the signal intensities (Agilent Technologies). Low-quality data points (*i.e*., low signal-to-noise ratio, saturated signals or nonuniform signals) were excluded from the analysis and treated as missing values. The nonbackground-subtracted signals were normalized using the LOWESS normalization method of the Feature Extraction software. Cy5/Cy3 signal ratios were calculated from the normalized data. The array data were submitted using MIAMExpress to the ArrayExpress database (acc.number E-MEXP-1610, and E-MEXP-1611).

Gene copy number analysis

The Mou15K-1 cDNA microarray slides (Finnish DNA Microarray Centre) described earlier were also used for detecting gene copy number changes in the TRAMP-C2 cell line by array comparative genomic hybridization (aCGH). The aCGH analysis was performed as described earlier[7] with slight modifications.[2] Briefly, genomic DNA from the TRAMP-C2 cells was extracted using the Blood and Cell Culture DNA Maxi kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Subsequently, the DNA preparations from TRAMP-C2, as well as from normal mouse whole blood cells (Clontech) that was used as a reference, were double-digested with AluI and RsaI restriction enzymes. Six microgram of the digested DNA preparations from TRAMP-C2 and from normal mouse blood cells were labeled with Cy5 and Cy3, respectively, using the BioPrime Labeling Kit (Invitrogen) and hybridized to the microarray slides. After washes, the signals were measured using the Agilent DNA Microarray Scanner BA (Agilent Technologies). Quantification, filtering, and normalization of the hybridization signals were carried out as described in the previous section.

Visualization of the copy number data and identification of the chromosomal regions of losses and gains were performed as described earlier.[2] First, chromosomal locations of the genes represented on the Mou15K-1 microarray were retrieved from the UCSC Genome Browser databases (http://hgdownload.cse.ucsc.edu/downloads.html). Subsequently, normalized and log2 transformed aCGH hybridization signal ratios were imported into GraphPadPrism4 software (GraphPad Software, San Diego, CA), in which Lowess curves of individual chromosomes were created and combined to visualize the genome-wide copy number alterations. To define regions of loss and gain, a cut-off value of mean log2 ratio $\pm$ 0.5 $\times$ SD was set. A chromosomal region was considered lost or gained when at least 4 adjacent clones showed log2 ratios below or above the cut-off value, respectively. Also, regions in which 5 of 6 adjacent clones showed log2 ratios lower or higher than the cut-off value, were considered lost or gained. The array data were submitted using MIAMExpress to the ArrayExpress database (acc.number E-MEXP-1609).

To find out the human chromosomal regions corresponding to regions of loss and gain in the mouse TRAMP-C2 cell line, homologous chromosomal regions were retrieved from the web pages of the Ensembl Mouse Synteny View (http://www.ensembl.org/Mus_musculus/syntenyview).

Fluorescent in situ *hybridization*

TRAMP-C2 aCGH data was validated for 1 area of gain (16qC4) and 1 area of loss (7qD1) using fluorescent *in situ* hybridization (FISH). As a reference was used a probe for an unaltered chromosomal region (9qA2). Mouse genomic BAC clones (RP23-247E2 for 16qC4, RP23-70H15 for 7qD1 and RP23-149D5 for 9qA2) were ordered from ResGen™; Invitrogen. Probes for gain and loss were labeled with digoxigenin-dUTP (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland) by nick translation. The reference probe was labeled with Alexa Fluor 594 (Molecular Probes, Eugene, OR). Dual color FISH analyses of the TRAMP-C2 cell line were done as previously described.[8] After stringent washes, the slides were stained with antidigoxigenin-FITC and counterstained with antifade solution (Vectashield, Vector Laboratories, Burlingame, CA) containing 4′-6-diamino-2-phenylindole. Signals were scored from non-overlapping nuclei using Olympus BX50 epifluorescence microscope (Tokyo, Japan).

Real-time quantitative RT-PCR

Gene expression data obtained from the cDNA microarray analyses after DNA demethylation and histone deacetylase inhibition were verified using real-time quantitative RT-PCR (Q-RT-PCR) for selected genes. Five microgram of total RNA from control and treated cells was reverse transcribed using AMV Reverse Transcriptase (Finnzymes Oy, Espoo, Finland) and random hexamer primers (Invitrogen) according to the enzyme manufacturer's instructions. Standard curve was prepared from cDNA reverse transcribed from 5 µg of mouse kidney total RNA (Clontech). To avoid amplification of any contaminating genomic DNA, amplification primers were designed into different exons or, when possible, to span an exon–intron boundary. PCR reactions were performed with LightCycler™; machine using LightCycler® FastStart DNA Master SYBR Green I kit according to the manufacturer's instructions (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland). The amplification protocol included 10 min of initial denaturation/activation at 95°C, followed by 45–50 cycles of denaturation 95°C 10 sec, annealing $\times$°C 5 sec (Supplementary Table S1), and elongation 72°C 10 sec. After the amplification, melting curve analysis and agarose gel electrophoresis were performed to ensure that only one, correct-sized PCR product was amplified. To normalize the expression levels of the studied genes, the expression of TATA-box binding protein (TBP) was measured. The relative expression levels of each sample were calculated from the TBP-normalized expression values by dividing each expression value with the expression value of the control sample.

Clusterin expression was also studied in human prostate cancer cell lines and clinical samples using real-time Q-RT-PCR. Preparation of cDNA from tissue RNA has been described in Linja *et al*.[9] Human Universal Total RNA (Clontech) was used for creating a standard curve. The primer sequences and annealing temperatures used are shown in Supplementary Table S1.

Genomic bisulphite sequencing

Methylation status of selected genes was determined using genomic bisulphite sequencing. Genomic DNA from cell lines was extracted using standard procedures including proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation. Bisulphite treatments were done for 2 µg of genomic DNA by a standard procedure described in Aapola *et al*.[10]

Two microliter of modified DNA was used as a template for the 1st round of PCR amplification with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. A 2nd round of PCR was done when needed, using the same primers as in the 1st round, with 2–4 µl of the 1° PCR product as a template. Primers to amplify bisulphite

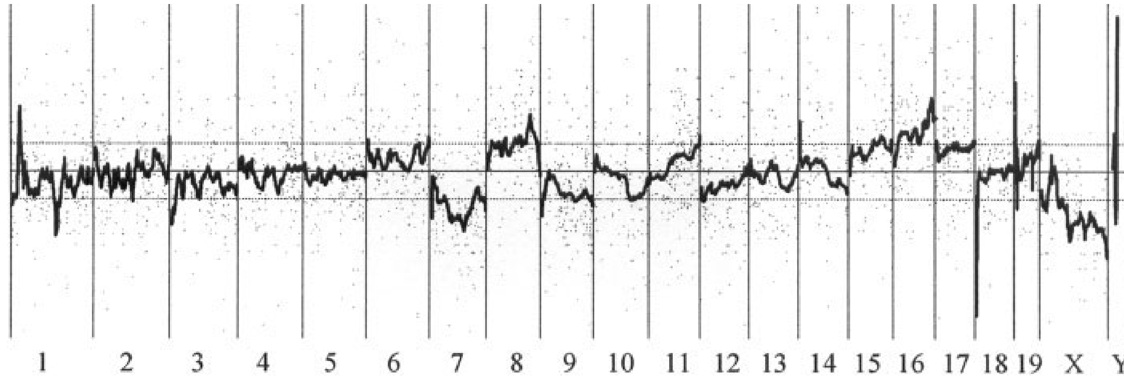

Figure 1 – Gene copy number analysis of TRAMP-C2. The log2 ratio values of all data points obtained by the aCGH are plotted against their genomic locations from chromosome 1p-telomere to chromosome Y q-telomere. Lowess curve is created from the same data. Dotted lines indicate the cut-off values for losses and gains, defined as the mean ± 0.5 × SD of the log2 ratio values.

Table I – Regions of losses and gains identified in TRAMP-C2 by aCGH

Chromosome	Cytogenetic region in mouse	Distance from p-telomere (Mbp)	Size (Mbp)	Corresponding cytogenetic regions in human
Areas of loss				
7	A3-F3	19.5–109.4	89.9	1q44; 11p14.3-p15.4; 11q13.4-q14.3; 15q11.2-q13.3; 15q25.1-q26.3
7	F4-F5	122.0–132.6	10.6	10q26.11-q26.3; 16p11.2
9	E3.1-E3.4	83.3–95.2	11.9	3q24; 6q14.1-q14.3; 15q24.3-q25.1
9	F3-F4	115.2–120.6	5.4	3p22.1-p22.3; 3p24.1
10	C2-C3	87.6–101.7	14.1	12q21.32-q23.2
Areas of gain				
8	A2-A3	13.9–20.1	6.2	8p23.1-p23.3; 16q22.1
8	B3.3-C4	64–84.4	20.4	4q31.21-q32.3; 8p21.3-p22; 19p12-p13.13
8	C5-E2	92.8–123.3	30.5	1q42.13; 16q12.2-q24.3
15	D2-F1	59.1–89.4	30.3	8q24.13-q24.3; 22q12q.3-q13.33
15	F3	99.7–103.8	4.1	12q13.12-q13.2
16	B1-C4	12.8–97.0	84.2	3p11.2-p12.3; 3q11.2-q21.2; 3q27.1-q29; 8q11.21; 21q21.2-q22.3; 22q11.21-q11.22

modified DNA were designed using MethPrimer program ([11]; www.urogene.org/methprimer/). For primer designing, we used the following criteria: CpG island size >100 bp, CG content >50% and observed/expected CpG ratio >0.6. Primer sequences are shown in Supplementary Table S1. 2° PCR products were purified using either Qiagen's QIAquick PCR Purification Kit or Qiagen's Gel Extraction Kit. The purified PCR products were subcloned into pCR®2.1-vector using TOPO TA Cloning® Kit (Invitrogen). Several clones were picked and grown, followed by PCR using M13 primers. The PCR products were purified using Multisqreen $PCR_{\mu}96$ Filter Plates (Millipore, Billerica, MA) and sequenced with ABI PRISM® BigDye™; Terminator Cycle Sequencing Ready Reaction Kit and ABI 3130*xl* sequencer (Applied Biosystems).

Results

Gene copy number analysis

To characterize the TRAMP-C2 cell line at the genomic level, gene copy number analysis was performed using the aCGH method. The aCGH analysis revealed both losses and gains of genetic material, as illustrated in Figure 1. All together, 11 altered regions were identified in 6 different chromosomes: five regions of loss in chromosomes 7, 9 and 10, and 6 regions of gain in chromosomes 8, 15 and 16. Generally, the altered regions were quite large, ranging from 4.1 to 89.0 Mb. No high-level amplifications were detected by the aCGH. These genomic alterations with the corresponding human homolog cytogenetic areas are listed in Table I. The aberrations are also depicted graphically on human chromosomes in Supplementary Figure S1.

Two chromosomal alterations found by aCGH, gain of 16q and loss of 7q, were verified using FISH. Analysis showed 4–6 locus-specific signals of 16qC4 and 3–4 reference signals (9qA2) confirming the gain, whereas 2–3 locus-specific signals of 7qD1 and 3–4 reference signals (9qA2) were seen verifying the loss. Representative images of FISH analyses are shown in Supplementary Figure S2.

Association of gene expression and copy number

The same cDNA microarray platform used for the copy number analysis described earlier was also utilized in studying gene expression in TRAMP-C2 compared to normal mouse prostate. To study whether gene expression was associated with gene copy number globally, the genes were divided into 3 categories: those showing loss, gain and no change in gene copy number compared to the normal mouse genome. There was a statistically significant association between global expression and gene copy number ($p < 0.001$, Kruskal–Wallis test): genes in the category of no copy number change showed higher expression, on average, than genes in the category of copy number loss. Similarly, genes in the category of copy number gain were expressed at higher levels, on average, compared to genes with no copy number change (Fig. 2).

Detection of epigenetically silenced genes

Epigenetic modifications of TRAMP-C2 were assessed by treating the cells with a combination of the demethylating agent 5azadC and the histone deacetylase inhibitor TSA, followed by gene expression analysis using the microarrays to detect upregulated genes. This data was combined with the gene expression data comparing TRAMP-2C with the normal mouse. Only genes that showed both upregulation after 5azadC+TSA treatment

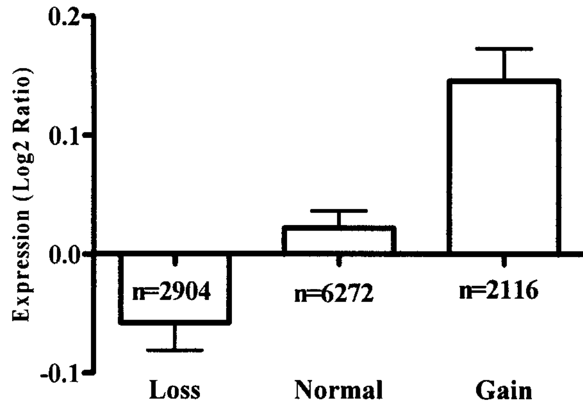

FIGURE 2 – Association of gene copy number and expression in TRAMP-C2 was found highly significant ($p < 0.001$, Kruskal–Wallis test). *n* refers to the number of data points in each category.

(expression ratio >2) and low expression in the TRAMP-C2 cells compared to the normal mouse prostate (expression ratio <0.5) were picked. There were 43 genes that fulfilled these criteria, and thus, were considered potentially epigenetically silenced in TRAMP-C2. The names and expression ratios of the genes are listed in Table II.

The gene copy number data obtained by the aCGH were also combined with the 2 other data sets in order to detect genes that would show both epigenetic silencing and loss of a gene copy. Of the 43 potentially epigenetically silenced genes (Table II), 4 (decorin, nucleobindin 2, BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3; and eukaryotic translation initiation factor 3, subunit 5) were located in regions that showed losses in TRAMP-C2. In addition, human homologs of 7 of the genes (clusterin, integral membrane protein 2B, forkhead box O1, matrix gamma-carboxyglutamate protein, microsomal glutathione *S*-transferase 1, glutathione peroxidase 3 and lactate dehydrogenase 2, B chain) are located in the chromosomal regions commonly deleted in human prostate cancer.

TABLE II – CANDIDATE GENES FOR EPIGENETIC SILENCING

Gene name	Gene symbol	Expression ratio (5′aza2dC+TSA vs. untreated TRAMP-C2)	Expression ratio (TRAMP-C2 *vs.* normal mouse)	Mouse chrom	Human chrom
Decorin	*Dcn*	15.2	0.04	**10**	12q21.33
Clusterin[1]	*Clu*	5.09	0.08	14	**8p21-p12**
Selenoprotein P, plasma, 1	*Sepp1*	4.54	0.147	15	5q31
Lipopolysaccharide binding protein	*Lbp*	3.89	0.146	2	20q11.23-q12
Hexosaminidase A	*Hexa*	3.77	0.449	9	15q23-q24
Regulator of G-protein signaling 2	*Rgs2*	3.62	0.171	1	1q31
Integral membrane protein 2B	*Itm2b*	3.42	0.087	14	**13q14.3**
Forkhead box O1	*Foxo1*	2.93	0.458	3	**13q14.1**
Epoxide hydrolase 1, microsomal	*Ephx1*	2.91	0.209	1	1q42.1
Alpha-*N*-acetylglucosaminidase (Sanfilippo disease IIIB)	*Naglu*	2.79	0.391	11	17q21
Matrix gamma-carboxyglutamate (gla) protein[1]	*Mgp*	2.76	0.165	6	**12p13.1-p12.3**
DnaJ (Hsp40) homolog, subfamily B, member 9	*Dnajb9*	2.72	0.156	12	14q24.2-q24.3
Nucleobindin 2	*Nucb2*	2.66	0.023	**7**	11p15.1-p14
Myosin, light polypeptide 9, regulatory	*Myl9*	2.64	0.33	2	20q11.23
Estrogen receptor 1 (alpha)[2]	*Esr1*	2.58	0.346	10	6q25.1
Microsomal glutathione *S*-transferase 1	*Mgst1*	2.55	0.203	6	**12p12.3-p12.1**
Histocompatibility 13	*H13*	2.5	0.309	2	6p21.3
Leukotriene B4 12-hydroxydehydrogenase	*Ltb4dh*	2.44	0.42	4	9q31.3
Glutathione *S*-transferase, mu 2[1]	*Gstm2*	2.41	0.193	3	1p13.3
Inhibitor of DNA binding 2	*Id2*	2.37	0.44	12	2p25
Beta-2 microglobulin	*B2m*	2.36	0.069	2	15q21-q22.2
Neighbor of Brca1 gene 1	*Nbr1*	2.33	0.423	11	17q21.31
Glutathione peroxidase 3[3,4]	*Gpx3*	2.3	0.054	11	**5q23**
Lysosomal-associated protein transmembrane 5	*Laptm5*	2.3	0.053	4	1p34
Elongation factor RNA polymerase II-like 3	*Ell3*	2.25	0.311	2	15q15.3
N-myc downstream regulated 2	*Ndrg2*	2.25	0.404	14	14q11.2
SH3 domain binding glutamic acid-rich protein-like 3	*Sh3bgrl3*	2.2	0.489	4	1p35-p34.3
RAB11a, member RAS oncogene family	*Rab11a*	2.19	0.475	9	15q21.3-q22.31
PTEN induced putative kinase 1	*Pink1*	2.17	0.17	1	1p36
BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3	*Bnip3*	2.15	0.365	**7**	10q26.3
ATP synthase, H+ transporting, V1 subunit D	*Atp6v1d*	2.14	0.437	12	14q23-q24.2
Glutathione *S*-transferase, mu 5[1]	*Gstm5*	2.13	0.274	3	1p13.3
Nedd4 family interacting protein 1	*Ndfip1*	2.13	0.374	18	5q31.3
Glutathione *S*-transferase, mu 6	*Gstm6*	2.1	0.118	3	
Lactate dehydrogenase 2, B chain[5]	*Ldhb*	2.09	0.378	6	**12p12.2-p12.1**
X-box binding protein 1	*Xbp1*	2.08	0.141	11	22q12
Bone morphogenetic protein 15	*Bmp15*	2.07	0.268	X	Xp11.2
Glutathione *S*-transferase, mu 1[3,4]	*Gstm1*	2.07	0.108	3	1p13.3
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	*Atp5l*	2.05	0.451	9	11q23.3
Argininosuccinate lyase	*Asl*	2.04	0.461	5	7cen-q11.2
Cystatin C	*Cst3*	2.04	0.479	2	20p11.21
Eukaryotic translation initiation factor 3, subunit 5 (epsilon)	*Eif3s5*	2.00	0.405	**7**	11p15.4
Interferon gamma inducible protein 30[1]	*Ifi30*	2.00	0.39	8	19p13.1

Genes that had expression ratio ≥2 after relieving epigenetic regulations in TRAMP-C2 and had a decreased expression (ratio ≤0.5) in TRAMP-C2 compared to normal mouse are listed. The mouse genomic location as well as the genomic location of the human homolog of the gene is shown. Bolded genomic locations indicate loss in the TRAMP-C2 genome or commonly deleted regions in human prostate cancers.[12]

[1]Found to be upregulated by 5azadC treatment in prostate cancer cell lines.[12]–[2]Found to be methylated in clinical prostate carcinomas and prostate cancer cell lines.[14]–[3]Found to be upregulated by 5azadC treatment in prostate cancer cell lines.[13]–[4]Found to be methylated in clinical prostate carcinomas and prostate cancer cell lines.[13]–[5]Found to be methylated in clinical prostate carcinomas.[15]

To assess whether these genes might be epigenetically modified also in human prostate cancer, the data were compared to previously published epigenetic studies on human prostate cancer. Seven human homologs (clusterin, matrix gamma-carboxyglutamate protein, glutathione S-transferase, mu 2; glutathione peroxidase 3, glutathione *S*-transferase, mu 5; glutathione *S*-transferase, mu 1; and interferon gamma inducible protein 30) of the listed 43 mouse genes have been shown to be upregulated in human prostate cancer cell lines after treating the cells with 5azadC.[12,13] In addition, 4 of the genes (estrogen receptor 1, lactate dehydrogenase 2, B chain; glutathione peroxidase 3 and glutathione *S*-transferase, mu 1) have been shown to be methylated in human prostate cancer cell lines and/or clinical carcinomas, either by bisulphite sequencing or by methylation-specific PCR.[13–15]

Methylation analysis of clusterin and decorin in mouse

Of the 43 genes, clusterin and decorin were selected for further studies of epigenetic modifications for the following reasons: (*i*) both of these genes were significantly upregulated by treating the TRAMP-C2 cells with 5azadC and TSA, (*ii*) their expression was significantly lower in TRAMP-C2 compared to normal mouse prostate, and (*iii*) they were located on chromosomal regions of loss in either TRAMP-C2 (decorin) or human prostate cancer (clusterin). Clusterin is also one of the genes that have already been shown to be upregulated by treating human prostate cancer cells with 5azadC.[12]

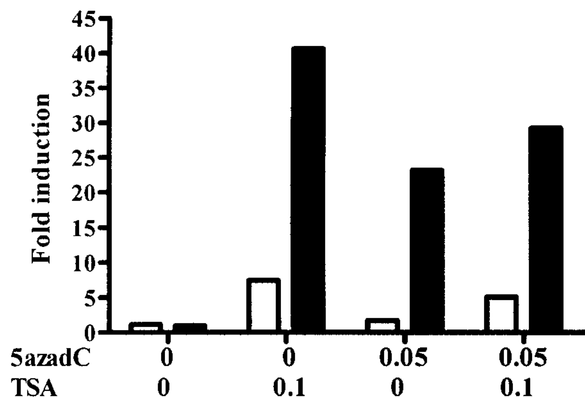

Figure 3 – Clusterin expression in the TRAMP-C2 cell line after treating the cells with 5azadC and TSA as indicated. Relative expression is represented as fold induction, where the control experiment (untreated) is designated as 1. For microarrays (white bars), the fold induction is calculated from the Cy5/Cy3 ratios (treated/untreated) and for real-time Q-RT-PCR (black bars), from the *TBP*-normalized expression values.

First, the microarray expression results after 5azadC and/or TSA treatments were verified using real-time quantitative RT-PCR. Both clusterin and decorin expression were shown to be upregulated in the TRAMP-C2 cells as a result of the treatments (Fig. 3 and Supplementary Fig. S3*a*.). For both genes, the detected induction of gene expression was higher when measured using Q-RT-PCR as compared to microarray analysis, reflecting the better dynamic range of Q-RT-PCR.

To assess whether the increased expression of clusterin and decorin after 5azadC treatment was caused by methylation of these genes, we analyzed the promoter/5′UTR (untranslated region) regions of the genes by bisulphite sequencing the genomic DNA of the TRAMP-C2 cell line. For clusterin, we sequenced 2 CpG islands in the promoter/5′UTR of the gene (CpG island no. 1 from −54 to +73 bp and CpG island no. 2 from +306 to +442 bp relative to transcription start site, TSS, NM_013492, Fig. 4*a*). The first CpG island lacked methylation completely, while the second CpG island was 64% methylated (Fig. 4*b*). Even though the decorin gene does not have true CpG islands in the promoter/5′UTR regions of the gene, 2 sets of primers were designed to cover a total number of 8 CpG dinucleotide sequences located in the promoter region ~2.5 kb upstream of the TSS and within the 5′UTR region. Sequencing showed the CpGs located in the promoter region to be fully unmethylated and the CpGs surrounding the TSS to be almost fully methylated (on average 88%) (Supplementary Fig. S3*b*).

Expression and methylation analyses of clusterin in human prostate cancer cell lines and clinical tumor samples

Since it has previously been demonstrated that decorin is expressed solely in the stromal cells in the human prostate,[16] it is an unlikely target of epigenetic silencing in prostate cancer. Therefore, only clusterin was further analyzed. Its expression was studied in clinical samples of BPH, untreated prostate carcinomas and hormone-refractory carcinomas using Q-RT-PCR. As shown in Figure 5, expression of clusterin was significantly higher in

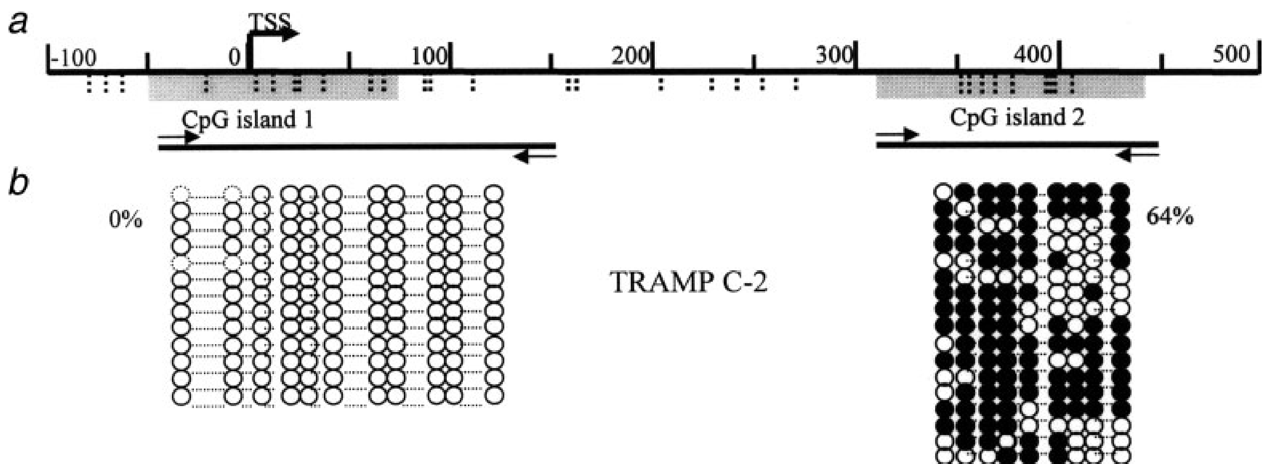

Figure 4 – Methylation analysis of the clusterin gene in TRAMP-C2. (*a*) The mouse clusterin gene promoter/5′UTR CpG islands and bisulphite sequencing primer positions. Dashed vertical lines represent single CpG dinucleotides and shadowed areas represent CpG islands predicted by the MethPrimer program. Horizontal solid lines represent the amplified and sequenced genomic regions and the arrows the used primers. TSS indicates transcription start site. (*b*) Bisulphite sequencing results for the TRAMP-C2 cell line. CpGs are represented by open dots if unmethylated and by black dots if methylated. Dashed line dots represent CpGs where sequencing results were not obtained. Each row represents a single PCR clone sequenced. The percentage of CpG methylation is indicated.

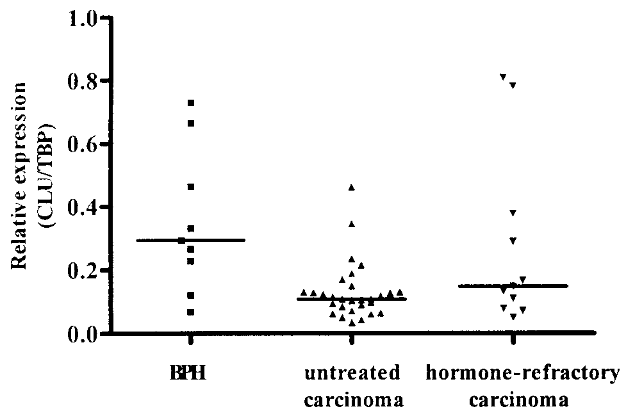

FIGURE 5 – Clusterin expression in clinical samples of benign prostate hyperplasia (n = 9), untreated prostate carcinomas (n = 28), and hormone-refractory prostate carcinomas (n = 12) measured using real-time Q-RT-PCR. Median values are represented as horizontal bars. Clusterin expression was significantly higher in BPH than in carcinomas (p = 0.0095, Kruskal–Wallis test).

BPH than in untreated and hormone-refractory carcinomas (p = 0.0095, Kruskal–Wallis test).

Expression and methylation of clusterin was next studied in human prostate cancer cell lines to find out if clusterin is epigenetically silenced by hypermethylation also in human prostate cancer cells. Q-RT-PCR showed that of the 4 cell lines studied (PC-3, DU145, LNCaP and LAPC-4), LNCaP had the lowest level of clusterin expression (Fig. 6*a*). Bisulphite sequencing was performed for 2 CpG islands situated on both sides of the TSS of the gene (CpG island no. 1 from −210 to −2 bp and CpG island no. 2 from +14 to +177 relative to the TSS of the transcript variant 1, NM_001831, Fig. 6*b*). LNCaP showed the highest level of methylation of the clusterin gene: it was on average 50% methylated on the CpG island no. 1 and 23% methylated on the CpG island no. 2 (Fig. 6*c*). In LAPC-4, clusterin was on average 9.5% methylated on CpG island no. 1, but unmethylated in CpG island no. 2. PC-3 and DU145 did not show significant methylation on either of the CpG islands of the clusterin gene.

Discussion

One of the aims of our study was to characterize gene copy number alterations in the TRAMP-C2 cell line in order to evaluate how well it mimics human prostate cancer. According to aCGH, TRAMP-C2 contains relatively few chromosomal aberrations: only 6 of the 21 chromosomes were identified to contain either losses or gains of genetic material. No high-level amplifications were found. On the other hand, most of lost and gained regions were quite large, some of them covering almost the entire chromosome, as did one of the losses on chromosome 7 and the gain on chromosome 16. Therefore, the number of genes potentially affected by the chromosomal alterations is fairly high. There was a positive association between global gene expression and gene copy number, indicating that also low-level changes in gene copy number can significantly affect gene expression. We have previously demonstrated similar strong association of gene copy number and expression in human prostate cancer cell lines and xenografts.[2] These findings are in good agreement with experiments showing association between aneuploidy and gene expression, as well as phenotype.[17,18] Altogether, there is a growing amount of evidence that low copy number changes (*i.e.*, gene dosage) may be functionally significant in the development of cancer.

The human cytogenetic regions corresponding to the regions of losses and gains identified in the TRAMP-C2 genome are not the ones that are typically altered in human prostate cancer. However, 4 interesting matches were identified: 6q14.1-q14.3 and 10q26.12-q26.3, which are commonly lost, as well as 8q11.21 and 8q24.13-q24.3, which are frequently gained in prostate carcinomas.[1] Target genes of these alterations in human prostate cancer have been searched for more than a decade, but they are still not known. For example, for the 8q24.13-q24.3 gain, suggested targets include the MYC and KIAA0196 genes,[19,20] the mouse homologs of both of which showed copy number gains and overexpression in TRAMP-C2 by the microarray expression analyses (data not shown). Other candidate target genes of this region of gain, such as EIF3S3[21] and RAD21[20] did not have their mouse homologs on the array. For the other 3 common regions of alterations, no promising target genes have been suggested. The fact that some of the common genomic alterations of human prostate cancer were detected also in the homologous segments of the mouse chromosomes in TRAMP-C2 indicates that the molecular mechanisms of the development of prostate cancer in the transgenic TRAMP mouse may well be similar to those of human prostate cancer. Thus, the TRAMP-C2 cell line could be useful in identification of genes that are involved in the development of human prostate cancer.

Next, genome-wide characterization of epigenetic silencing was performed. Approximately 12% of the genes on the array responded to 5azadC and TSA treatments. Most of them are likely to be induced through secondary effects such as cytotoxicity. Thus, further criteria were needed to recognize truly epigenetically silenced genes. Based on the increased expression after 5azadC+TSA treatment and the low basic level of expression in the untreated TRAMP-C2 cells compared to normal mouse prostate, a total number of 43 genes were considered potentially epigenetically silenced in TRAMP-C2. Seven of these 43 genes have been previously shown to be induced by 5azadC treatment also in human prostate cancer cells,[12,22] and methylation of 4 of them have been shown at the DNA level in human prostate cancer cell lines and clinical prostate carcinomas.[12–14] These similarities suggest that some of the genes that were detected to be potentially epigenetically silenced in TRAMP-C2 could contain epigenetic modifications also in human prostate cancer cells. In addition, it has been shown that as in clinical prostate carcinomas, also in TRAMP mouse the expression levels of the DNA methyl transferases are increased and aberrant DNA methylation correlates with altered gene expression levels.[23] Therefore, TRAMP-C2 can be considered a useful model for studying epigenetic changes in prostate cancer.

The other aim of our study was to identify individual genes that are genetically and epigenetically altered in human prostate cancer by utilizing the TRAMP-C2 cell line as the model system. Decorin and clusterin were the 2 highest-ranking genes (Table II). However, since decorin is known to be expressed only in human prostate stromal cells, and not in cancer cells,[16] we decided not to study it further. Clusterin was the second most highly induced gene in the 5azadC+TSA treated TRAMP-C2 cells, and its human homolog is located in the chromosomal region 8p21-p12, which is frequently deleted in human prostate cancer, making clusterin a putative tumor suppressor gene. Bisulphite sequencing showed clusterin to be, on average, 64% methylated in the 5′UTR CpG island. It has previously been shown that the expression of clusterin is downregulated during the development of cancer in the TRAMP model.[24] The mechanism of the downregulation has been unknown. Our finding suggests that it could be due to hypermethylation. Further support for the epigenetic silencing of clusterin comes from a recent study showing that the expression of clusterin is suppressed in *HRAS*-transformed rat fibroblasts through methylation.[25]

Of the 4 human prostate cancer cell lines studied (PC-3, DU145, LNCaP and LAPC-4), LNCaP showed the lowest level of clusterin mRNA expression, and the highest level of clusterin gene methylation, indicating epigenetic silencing of clusterin also in the LNCaP cell line. It has previously been shown that 5azadC treatment induces the clusterin expression in human prostate cancer cell line MDA-Pca-2a, suggesting epigenetic silencing of clusterin also in that cell line.[12] Despite the difference in mRNA levels

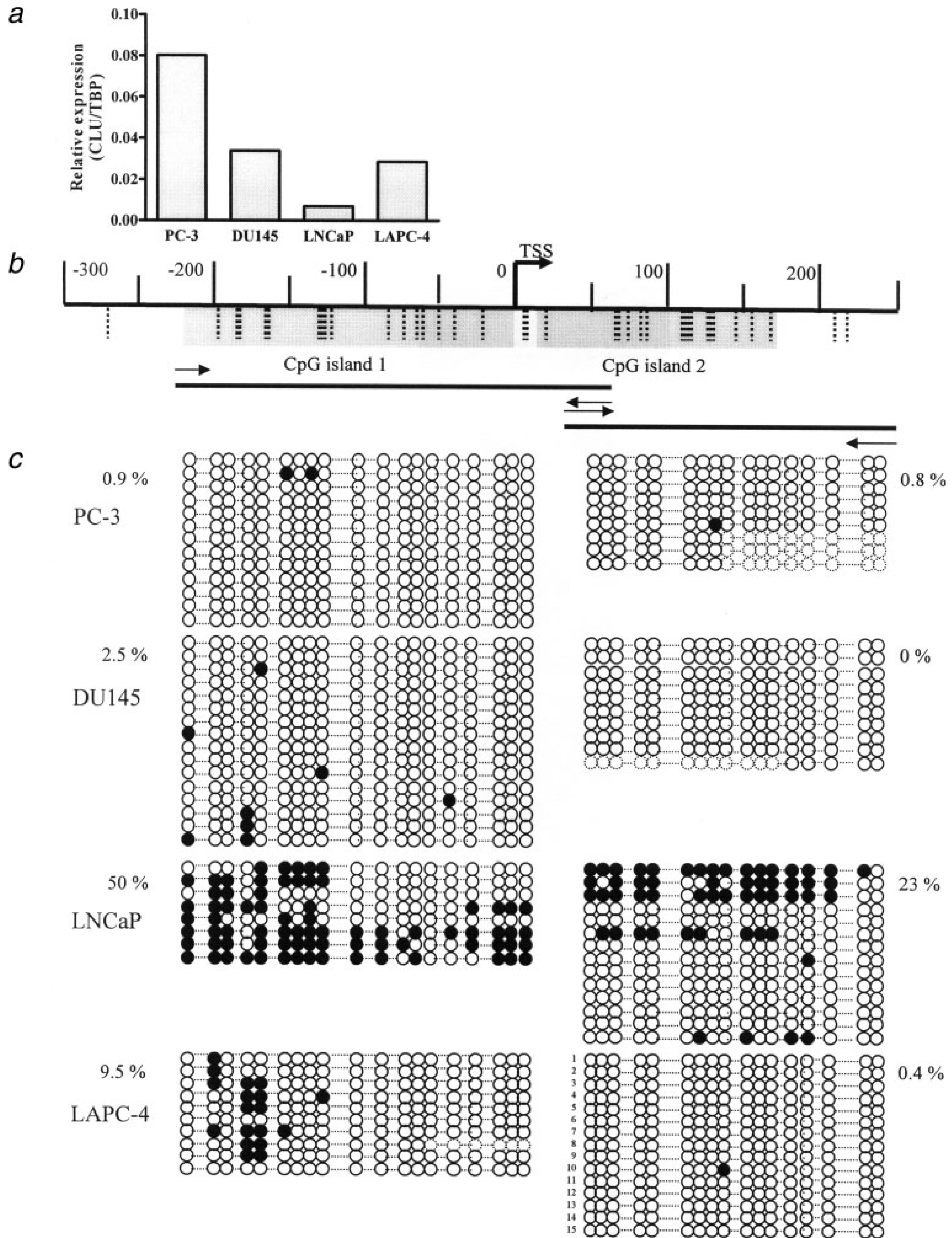

Figure 6 – Expression and methylation analyses of the clusterin gene in human prostate cancer cell lines. (*a*) Expression of clusterin in PC-3, DU145, LNCaP and LAPC-4 prostate cancer cell lines by Q-RT-PCR. (*b*) The human clusterin gene promoter/5′UTR CpG islands and bisulphite sequencing primer positions. Dashed vertical lines represent single CpG dinucleotides and shadowed areas represent CpG islands predicted by the MethPrimer program. Horizontal solid lines represent the amplified and sequenced genomic regions and the arrows the used primers. TSS indicates transcription start site. (*c*) Bisulphite sequencing results for PC-3, DU145, LNCaP and LAPC-4 prostate cancer cell lines. CpGs are represented by open dots if unmethylated and by black dots if methylated. Dashed line dots represent CpGs where sequencing results were not obtained. Each row represents a single PCR clone sequenced. The percentage of CpG methylation is indicated.

of clusterin between the cell lines, no significant differences were observed in clusterin protein levels according to Western blotting (Fig. S4). This apparent discrepancy reflects probably the poorer quantitativeness of the Western blotting compared to Q-RT-PCR.

Clusterin is generally expressed ubiquitously in various cell and tissue types and participates in numerous cellular functions including cell adhesion, tissue remodeling and apoptosis (reviewed in Ref. 26). It has been proposed to be expressed as 2 different mRNA isoforms (isoform 1, NM_001831 and isoform 2, NM_203339) giving rise to N-terminally differing proteins (NP_001822 and NP_976084, respectively) targeted ultimately for secretion. Secreted clusterin (sCLU) becomes heavily glycosylated and cleaved into its α and β subunits in the endoplastic reticulum (ER) before its secretion. A recent report showed that under certain stress conditions sCLU can be retrotranslocated into the cytoplasm, thus evading the secretory pathway.[27] In addition to sCLU, a nuclear form of clusterin (nCLU) has been reported.[28] This results from an alternative splicing event of transcript isoform 1, leading to the exclusion of exon 2 including the ER-targeting signal, and resulting in a nuclear, unglycosylated, uncleaved form of the protein. Another mechanism for producing this nuclear isoform was proposed recently by.[29] They showed that nCLU can be produced from a full length clusterin cDNA construct, suggesting nCLU to be a product of alternative initiation of translation rather than alternative splicing. Several reports have shown that sCLU and nCLU have opposing roles in various cell functions. While sCLU is antiapoptotic, helping cells evade apoptosis by interfering with Bax-activation, nCLU is proapoptotic, promoting apoptosis through complexing with the Ku70 autoantigen.[28,30–32] This has led to apparently contradictory reports that can be explained by the differing expression patterns and properties of sCLU and nCLU.

The data on clusterin expression in prostate cancer have been controversial. In clinical tumors both up- and downregulation has been reported, and the gene has been suggested to be either a tumor suppressor or promoter of prostate cancer.[33–36] It has been shown that castration induces the expression of clusterin.[35] This has led to clinical trials studying antisense oligo (ASO) therapy against clusterin to enhance the effects of androgen ablation and chemotherapy in prostate cancer (reviewed in Ref. 37). On the other hand, the chemopreventive action of green tea catechins has been shown to correlate with increased expression of clusterin in the TRAMP mouse model.[24] Our data here clearly indicate that, at least, the mRNA expression of clusterin is downregulated in clinical prostate cancer. Both untreated and hormone-refractory tumors showed downregulation, suggesting that it is a relatively early event in the development of prostate cancer and not related to the emergence of hormone-refractory disease. To further assess the role of epigenetic silencing in controlling clusterin expression, bisulphite sequencing was performed on a few tumor samples. Only marginal methylation of clusterin promoter/5′-UTR CpGs was found in any of these tumors (data not shown). This suggests that methylation may not be the only contributor to regulate clusterin expression in clinical prostate tumors.

There are discrepancy in the published data on the location of clustering expression in prostate, some suggesting stromal,[36] others epithelial expression.[38] We used immunohistochemistry to study the location of clusterin expression in normal prostate (Fig. S5). It was found to be mainly in epithelial cells. Since the staining pattern varied a great deal from sample to sample, it was not possible to reliably quantify the protein expression. However, the protein expression appeared to be lower in tumors than in normal prostate (data not shown).

In conclusion, we show here that the TRAMP-C2 mouse prostate cancer cell line shows some similarities with human prostate cancer in terms of genetic and epigenetic alterations, suggesting that the model could be used to identify the target genes for those alterations. We also demonstrate the downregulation of clusterin due to promoter hypermethylation in the TRAMP-C2 and LNCaP cell lines. In addition, clusterin was found to be downregulated in both untreated and hormone-refractory human prostate cancer. Thus, further studies are warranted to investigate the role of clustering as a putative prostate cancer tumor suppressor gene.

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