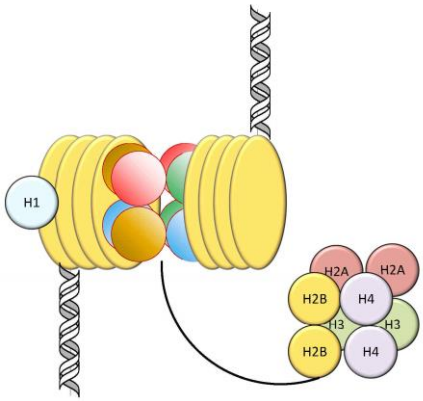


PROSTATE CANCER AND CHROMATIN CONFORMATION

"Nothing is necessarily what it appears to be"

ABSTRACT

Chromatin Confirmation describes the complexity of DNA in the nucleus as it is intertwined amongst histones and other related strands. The result can be changes in gene expressions. Recent work argues for using this result as an adjunct to PSA testing in determining diagnosis and prognosis of prostate cancer. We examine some of these issues in detail and the putative gene targets as well.

Terrence McGarty
TGL 197, March 2023

Notice

This document represents the personal opinion of the author and is not meant to be in any way the offering of medical advice or otherwise. It represents solely an analysis by the author of certain data which is generally available. The author furthermore makes no representations that the data available in the referenced papers is free from error. The Author also does not represent in any manner or fashion that the documents and information contained herein can be used other than for expressing the opinions of the Author. Any use made and actions resulting directly or otherwise from any of the documents, information, analyses, or data or otherwise is the sole responsibility of the user and The Author expressly takes no liability for any direct or indirect losses, harm, damage or otherwise resulting from the use or reliance upon any of the Author's opinions as herein expressed. There is no representation by The Author, express or otherwise, that the materials contained herein are investment advice, business advice, legal advice, medical advice or in any way should be relied upon by anyone for any purpose. The Author does not provide any financial, investment, medical, legal or similar advice in this document or in its publications on any related Internet sites.

Furthermore, this document contains references to and quotes and modified charts and figures from papers and documents under the premise of “Fair Use” in order to present ideas and understandings in context. The Author has attempted to make any and all references to such material separate from those of the author per se and has referenced the source expressly in all cases. These documents are for the dissemination of ideas and have no commercial intent. Our approach herein is to take elements of what is recent in the literature focused on a specific topic and attempt to develop a tapestry image of these connectable elements. We do not provide any new or fundamental results but merely attempt to assemble elements in a systematic and holistic manner.

The Author would appreciate any communications relating to these documents and these should be sent to:

mcgarty@alum.mit.edu.

Contents

1 INTRODUCTION

Prostate cancer is common, complex, and at times, confusing[1]. It can range from indolent to highly aggressive. Finding markers to identify its presence, without invasive biopsies, which themselves are at best a reasonable tool, has been an ongoing process.

PSA has been a tool used to assess the presence of prostate cancer, PCa, since the early 1990s. We have examined this in detail elsewhere and added such measures as %Free, velocity, density, and temporal analysis. The results are still problematic and have not presented a basis for avoiding biopsies. Even the use of mpMRI is problematic with patients who have already had multiple previous biopsies, since the resulting scar tissue may result in a positive results. All too often MRIs on patients with previous biopsies have PIRADs of 4.

A recent work using ctDNA, circulating tumor DNA, as well as cfDNA, circulating free DNA, and examining it for binding of the DNA due to chromatin protein interactions on a specific set of genes has putatively given metrics for both diagnostic and prognostic values. Unlike PSA along, which is poor in both sensitivity and specificity, the proposed gene analysis is highly sensitive and specific for both diagnostic and prognostic purposes.

1.1 CHROMATINS CONFORMATIONS

In this report we focus on a different method of assessing the presence of prostate cancer, PCa, and further in assessing a prognosis. The approach here is to examine genes that have been modified, via their DNA as a result of interactions in their chromatin state. Now simply, chromatin is the amalgam of histones and DNA, which wraps around the histones. The wrapping is not always a neat process and frequently there is an interaction between parts of the DNA and the histones. The result can be a loop created from the interactive process, a loop of the same chromosome or another chromosome. Depending on the loops the gene expressions may be dramatically altered and thus drive a cell into a malignant state.

Now it is argued by authors that this morphed DNA may be detectable in the blood by the use of recent complex analytical tools. By choosing a robust set of genes it can be argued that along with using PSA and detecting these morphed genes from the chromatin conformations, we now have excellent metrics for diagnosis and prognosis.

We examine these in some detail herein. Our specific interest is in recent literature espousing the use of chromatin conformation analyses for segments of genes, DNA, which have been impacted upon by the interactions of genes in the nucleus. Unlike the classic simple view of DNA being a linear sequence of base pairs in reality it is a highly tangles mass that may see interactions between the chromosome itself as well as between chromosomes. This set of interactions become markers for certain cancers via circulating free DNA, cfDNA.

[1] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

Now determining these chromatin conformation issues Han et al note:

During the higher eukaryotic cell cycle, the spatial volumes of each chromosome are not random but are organized into specific patterns, in which individual chromosomes occupy defined, mutually exclusive regions of the nuclear volume that represent a structural unit referred to as a chromosome territory (CT).

With extensive effort, the spatial organizations of individual chromosomes and the entire genome, with resolutions down to 1kbp, have been described.

It has now been widely accepted that genome architecture is a crucial aspect of gene regulation and genome stability because the highly ordered chromatin arrangement facilitates communication between genes and their regulatory elements.

Early studies of genomic conformation were largely based on cytological techniques, such as fluorescence in situ hybridization (FISH), which allows direct evaluation of the proximity between genetic loci using probes. Observations of genome architecture by FISH have revealed the existence of CTs, looping out from CTs, and the tendency for clustering of active chromatin domains. While this method has been a widely used tool to study topography of chromosomes or DNA fragments of interest in individual cells, and allow us to determine how the chromosomes are organized by directly viewing their position with microscopy.

However, technical limitations such as low throughput, low resolution and probe sequence specificity make it unsuitable for elaborate genome-wide studies of chromosomal topology.

Recently, chromosome conformation capture (3C) and 3C-based techniques using high-throughput sequencing data have emerged as powerful tools to reconstruct the spatial topology at regional, whole chromosome and genome levels.

These techniques have become the most effective way to elucidate the functional impact and the potential mechanisms establishing and maintaining spatial genome organization.

Protocols such as 3C as modified are at the center of the discussions herein.

1.2 Recent Results

There is a continuing effort to diagnose PCa without the use of a biopsy. Prostate biopsy may have some morbidity associated with it as well as the resulting pathological analysis having some limits. Namely the Gleason grade is often increased after prostatectomy from what was present on the biopsy. Thus is a more cost effective method has been sought.

As Pchejetski et al (2023) have recently noted:

Prostate cancer (PCa) has a high lifetime prevalence (one out of six men), but currently there is no widely accepted screening programme. Widely used prostate specific antigen (PSA) test at cut-off of 3.0 ng/mL does not have sufficient accuracy for detection of any prostate cancer,

resulting in numerous unnecessary prostate biopsies in men with benign disease and false reassurance in some men with PCa.

The assertion of a specific value frankly has rarely been accepted. PSA depends on the size of the prostate since the cells produce the protein and the more cells the more protein. %Free is a measure of non-malignant cells, and velocity is a measure of change. The latter is a universally used metric, namely has anything changed. We have seen high PSA in older patients (80+) with BPH but no discernable PCa whereas we have also seen PCa in patients with PSA just above 2. PSA is just one of many measures but we believe that having a history of the measures carries substantial weight.

We have recently identified circulating chromosome conformation signatures (CCSs, Episwitch® PCa test) allowing PCa detection and risk stratification in line with standards of clinical PCa staging[2].

The purpose of this study was to determine whether combining the Episwitch PCa test with the PSA test will increase its diagnostic accuracy. …

PSA > 3 ng/mL alone showed a low positive predicted value (PPV) of 0.14 and a high negative predicted value (NPV) of 0.93. EpiSwitch alone showed a PPV of 0.91 and a NPV of 0.32. Combining PSA and Episwitch tests has significantly increased the PPV to 0.81 although reducing the NPV to 0.78[3].

Furthermore, integrating PSA, as a continuous variable (rather than a dichotomised 3 ng/mL cut-off), with EpiSwitch in a new multivariant stratification model, Prostate Screening EpiSwitch (PSE) test, has yielded a remarkable combined PPV of 0.92 and NPV of 0.94 when tested on the independent prospective cohort*. …*

Our results demonstrate that combining the standard PSA readout with circulating chromosome conformations (PSE test) allows for significantly enhanced PSA PPV and overall accuracy for PCa detection. The PSE test is accurate, rapid, minimally invasive, and inexpensive, suggesting significant screening diagnostic potential to minimise unnecessary referrals for expensive and invasive MRI and/or biopsy testing. Further extended prospective blinded validation of the new combined signature in a screening cohort with low cancer prevalence would be the recommended step for PSE adoption in PCa screening

---

[2] https://www.oxfordbiodynamics.com/episwitch-platform *Due to the prodigious response to our press release regarding the Prostate Screening EpiSwitch® (PSE), we wanted to give everyone a quick update. Currently, the* ***PSE is not commercially available, and no clinical trials are underway****. We are working hard to bring this important test into the clinical lab for routine diagnostic testing. For more information regarding the PSE, please check out our press release and published article in Cancers. Thank you for being so supportive, and please check our website for future updates on this subject.*

[3] Just to note, the PPV is P[have PCa|PSA>3] and NPV is P[no PCa|PSA<3]. Thus having a high PSA is no basis for saying PCa whereas having a low PSA is no assurance that one does not have PCa. Thus by changing this from a binary to a continuous significantly improves the outcome.

Thus, as of the present, this technique has seen quite limited testing. Moreover there appears to be a material variance in the target genes and their products. However we fells that this approach opens another avenue for exploring genetic variances arising in and reflective of PCa. In a prior result by Alshaker et al (2021), of the same team as above they note:

We have detected specific chromosome conformation changes in the loci of ETS1, MAP3K14, SLC22A3 and CASP2 genes in peripheral blood from PCa patients yielding PCa detection with 80% sensitivity and 80% specificity.

Further analysis between PCa risk groups yielded prognostic validation sets consisting of HSD3B2, VEGFC, APAF1, BMP6, ERG, MSR1, MUC1, ACAT1 and DAPK1

genes that achieved 80% sensitivity and 93% specificity stratifying high-risk category 3 vs low risk category 1 and 84% sensitivity and 89% specificity stratifying high risk category 3 vs intermediate risk category 2 disease.

In contrast, the more recent paper targets a different set as noted:

Our data demonstrate the presence of stable chromatin loops in the loci encoding for ***DAPK1, HSD3B2, SRD5A3, MMP1, and miRNA98*** *in the circulation of PCa patients. We have previously described their implication in PCa pathology [22]. String analysis has shown that at the protein level of four out of five markers belong to the same network with a high confidence of interaction. Despite the identification of these epigenetic loci, until recently, the mechanism of cancer-related epigenetic changes in PBMCs remained unidentified. We have previously identified that similar signatures existed in primary tumours [20,22]. Our recently published data show for the first time a proof of concept for horizontal transfer of chromosome conformations in cancer cell-monocyte co-culture without direct cell-cell contact [23]…*

The limitations of this study include small number of patients, unavailability of other clinical indices like PHI and 4K *(which are not part of the standard of care in the UK) established PCa EpiSwitch biomarkers from the regulatory genome architecture of chromosome conformations (Episwitch).*

When tested in the context of screening population at risk, PSE yields a rapid and minimally invasive PCa diagnosis with a PPV of 0.92 and a NPV of 0.94.

Due to its high PPV that significantly exceeds current screening modalities (due to its non-invasive nature and low costs), the PSE test can be utilized for both diagnostic and screening purposes, minimizing unnecessary referrals for expensive and invasive MRI and/or biopsy testing. Further prospective larger scale studies of the new PSE test in a population screening cohort with low cancer prevalence would be an immediate next step in confirming and expanding PSE test utility.

1.3 Target Genes and Related Chromatin Conformations

The basic principle in these tests is as follows.

1. There are certain genes and their products that have a relationship to PCa

2. These genes may get entangled in a histone and the result may be the creation of a “loop which results in that gene not being able to be read properly.

3. The resulting DNA appears in the blood of the patient.

4. Using sophisticated techniques the presence of these looped DNA elements may be detected.

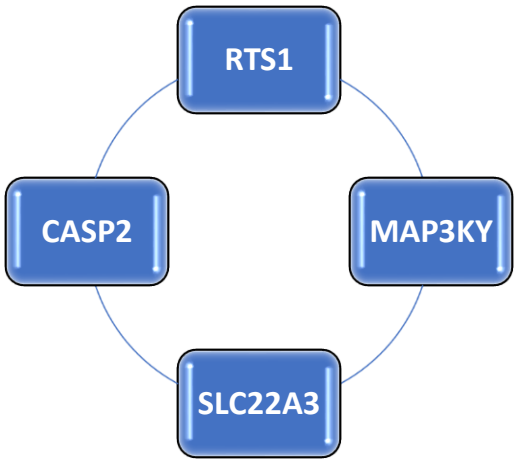
5. Using PSA along with a “profile” of the looped gene DNAs, a metric is calculated.

6. This metric then can be used to determine either the presence of PCa or the prognosis of an existing PCa.

We delineate the targets genes/products for three cases considered below.

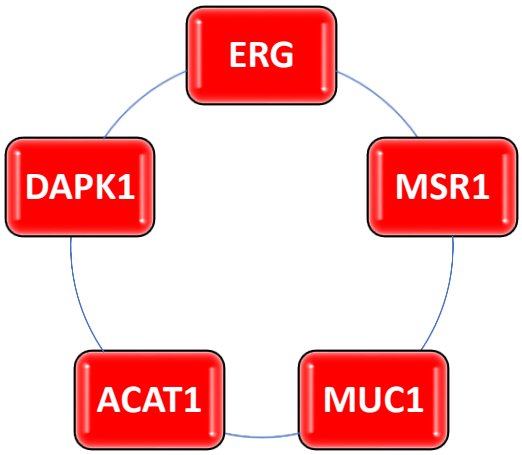
Case 1: Diagnostic (2021)

The first case is a diagnostic set of genes being targeted as shown below:



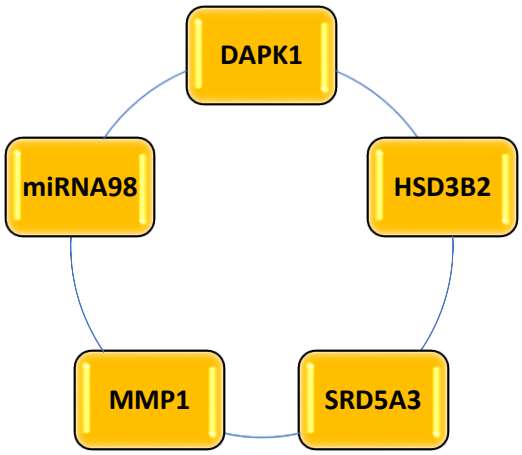
Case 2: Prognostic (2021)

The second case is a set of prognostic genets targeted as shown below:



Case 3: Diagnostic (2023)

The third case is from the most recent paper and is for diagnostic purposes:



We shall examine each of these in some detail. Specifically we shall discuss what these may be of use as metrics to examine.

2 CONFORMATION

The nucleus of a cell a highly compacted region containing the chromatin. Generally speaking chromatin is the amalgam of the DNA wrapped about sets of histones.

As Almeida et al note:

Chromatin is the template for the basic processes of replication and transcription, making the maintenance of chromosomal integrity critical for cell viability.

To elucidate how dividing cells respond to alterations in chromatin structure, here we analyse the replication programme of primary cells with altered chromatin configuration caused by the genetic ablation of the HMGB1 gene, or three histone H1 genes.

We find that loss of chromatin compaction in H1- depleted cells triggers the accumulation of stalled forks and DNA damage as a consequence of transcription–replication conflicts.

In contrast, reductions in nucleosome occupancy due to the lack of HMGB1 cause faster fork progression without impacting the initiation landscape or fork stability. Thus, perturbations in chromatin integrity elicit a range of responses in the dynamics of DNA replication and transcription, with different consequences on replicative stress. These findings have broad implications for our understanding of how defects in chromatin structure contribute to genomic instability

2.1 CHROMATIN AND CONFORMATION

We can now examine Chromatin Confirmation, of how DNA is effected by the chromatin it finds itself in. As Crutchly et al note:

Chromatin conformation signatures: Chromatin conformation signatures (CCSs) are collections of DNA contacts associated with specific gene expression states. There are four types of CCSs:

- *Local chromatin organization*
- *Intrachromosomal contacts*
- *Interchromosomal contacts*
- *Genomic environment*

We shall examine the above in some detail below. But simply as the DNA finds itself wrapped about the histones and then unwinds one sees interactions at various levels. These may be close interactions and distant interactions on the same DNA strand, or inter-strand interactions, and finally global interactions. These interactions may be in a variety of differing ways and thus produce gene products that may be different or produced in a more or less manner, all effecting the cells.

CCSs can be complex and include several types of DNA contacts. CCSs as ideal biomarkers

- *CCSs may integrate multiple variations into single signatures.*
- *CCSs may identify gene expression states regardless of mechanisms.*
- *CCSs uniquely identify 3D mechanisms of gene regulation.*

As Steensel notes:

Principles of chromatin organization:

1. Three-dimensional architecture

- *Architecture is driven by a combination of polymer biophysics and biochemical interactions.*
- *Most DNA is in a beads-on-a-string configuration with varying degrees of poorly understood local compaction.*
- *There are many long-range contacts between genomic loci, but most of these contacts are transient.*
- *Tethering to landmark structures contributes to the overall folding of chromosomes.*

2. Chromatin composition

- *Chromatin harbours thousands of proteins and dozens of histone marks.*
- *Distinct combinations of proteins and histone marks form a limited number of chromatin types.*
- *Some chromatin types mediate gene repression, others are conducive to transcription.*
- *Each chromatin type assists the binding of specific DBFs to their cognate DNA motifs.*

As Almeida et al note:

Every time a cell divides, its entire genetic and epigenetic information must be accurately replicated. In eukaryotic cells this occurs during the S-phase of the cell cycle through the activity of hundreds to thousands of replication origins (ORIs) distributed along their large genomes, in a context of a tightly packaged chromatin structure. Chromatin encodes epigenetic information and governs genome stability by protecting DNA to mutagenic agents and by regulating the accessibility of protein complexes to DNA.

Two outstanding recent reports have successfully reconstituted efficient and regulated budding yeast chromatin replication in vitro, providing important clues on the regulatory role of chromatin both in ORI specification and replisome progression. The basic unit of

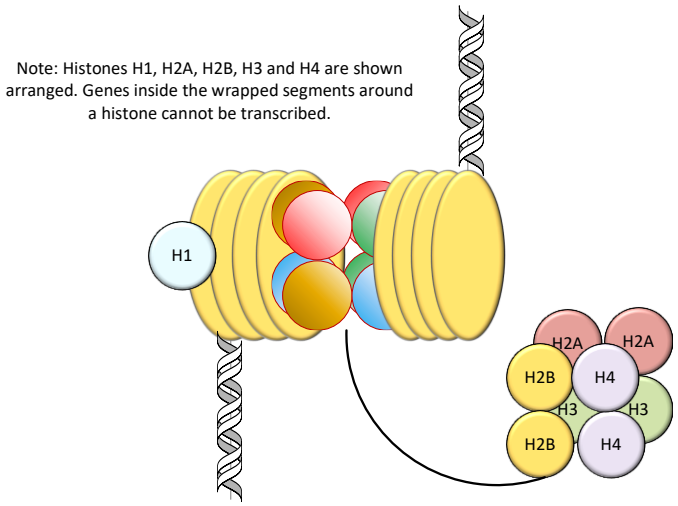
chromatin is the nucleosome, in which 147 bp of duplex DNA are wrapped around a histone octamer containing two copies of each of the four core histones: H2A, H2B, H3 and H43.

The higher-order organization of nucleosome core particles is controlled by the association of the intervening DNA with either the linker histone H1 or with non-histone proteins such as the high mobility group box (HMGB) family, that seem to exert different. While histone H1 is believed to stabilize the nucleosome by preventing DNA unwrapping, HMGB proteins impose a bending of the DNA that might destabilize the nucleosome structure, facilitating its remodelling. In agreement with this, histone H1 depletion alters global chromatin compaction in mammalian cells and causes derepression of heterochromatin transposable elements in Drosophila. On the other hand, HMGB1 depletion associates with reduced nucleosome occupancies and increased amounts of RNA transcripts both in Saccharomyces cerevisiae and in mammalian cells.

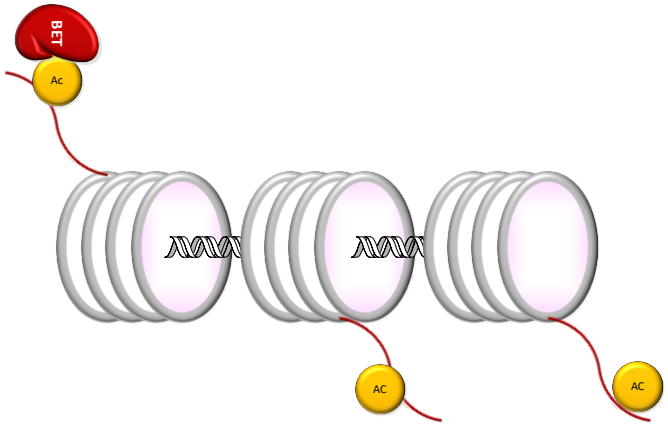
Interestingly, replisome progression studies with purified proteins on a chromatin template have found that Nhp6a, the yeast ortholog of mammalian HMGB1, additively stimulate the rate of replication in the presence of the histone chaperone FACT2. Here, by employing genetic ablation of three of the somatic isoforms of histone H1 or for HMGB1, we address how alterations in chromatin structure affect the definition of the sites of replication initiation and the kinetics of replication elongation in vivo.

We find that histone H1 depletion generates massive replication fork stalling and DNA damage signalling as a consequence of transcription–replication conflicts, while the increased chromatin dynamics associated with HMGB1 depletion allows faster fork progression without altering the replication initiation landscape or generating fork instability.

We show below the histone structure and the DNA wrapped around it. The histones are proteins which allow for the warped condensation of the DNA.

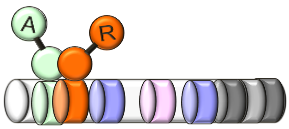


As a side note we also have as shown below methylation or acetylation of these histones. They are not to be discussed herein but they have dramatic effects on gene expressions.

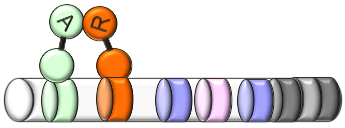


2.2 DNA

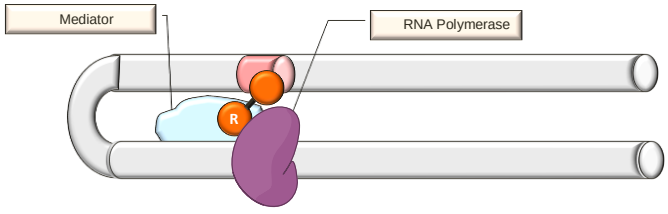
DNA functions are briefly shown below. This is a highly simplistic view as we shall see and as we better understand multiple epigenetic factors such as those in chromatin conformation we see dramatic changes in gene expression.



Competition: Activator is blocked by an overlapping Repressor



Inhibition: Activator is blocked by a binding Repressor.



Direct Repression: Repressor blocks transcription

From Crutchley et al:

The ability to store, retrieve and translate instructions from the genetic code is essential to maintain life in all cells. This process is not trivial by any means in human cells given the size of our genome. In fact, understanding this process is not trivial even for much smaller genomes. The human genetic code is composed of over 3 billion nucleotides, which when pieced together,

would measure almost a meter in length. Therefore, our genome must be tightly packaged and organized in order to fit within each micron-sized nuclei. Packaging of the human genome is functional rather than random, and there are three defined hierarchical levels of organization.

The first level of genome organization is characterized by the linear arrangement of genes and regulatory sequences (or 'DNA elements') along chromosomes.

This first dimension includes clusters of genes and their regulatory DNA elements. Gene clusters composed of evolutionarily duplicated genes tend to encode proteins with similar functions and with tissue-specific expression patterns defined by their regulatory elements. Examples of this level of organization include the Hox gene clusters and a/b-globin loci, both of which will be further described in sections later.

The second level of genome organization is defined by the interaction between DNA and proteins.

This second dimension is dominated by the relationship between genomic DNA and histones, where DNA is wrapped around nucleosomes to form the 10 nm chromatin fiber. At this level, chromatin appears as beads on a string, with beads corresponding to nucleosomes composed of two copies each of histone H2A, H2B, H3 and H4.

Histones can be extensively modified post-translationally by acetylation, methylation, phosphorylation, sumoylation, ADP-ribosylation and ubiquitinylation.

These epigenetic marks are mostly added to histone amino-terminal tails, and regulate their affinity to DNA and the recruitment of regulatory chromatin binding proteins. Histone modifications can also affect formation of the 30 nm chromatin fiber, which consists of a folded basic 10 nm fiber with nucleosomes stacked on top of each other. Very little is known about genome organization beyond the 30 nm fiber, of which the in vivo structure remains to be established.

Even at this level of packaging, a stretched out 30 nm chromatin fiber with the DNA content of an average chromosome would not fit into a nucleus. Therefore, additional folding and organization is essential for genome function.

The third level of genome organization is defined by the packaging and spatial arrangement of chromatin in the nuclear space.

This 3D organization is controlled by specialized proteins that bind and fold the 30-nm fiber into higher levels of organization such as loops. In addition to facilitating the accurate retrieval and translation of instructions from our genetic code, the spatial chromatin architecture of our genome is also used as a mechanism to regulate gene expression. Indeed, it was shown that DNA elements can regulate the expression of distal target genes by physically interacting with them.

This relatively recent discovery explains why the functional organization of the genome is not strictly linear along chromosomes and how DNA elements can regulate genes located very far away on the same or even on different chromosomes.

Thus, long-range chromatin contacts in cis (intrachromosomal) or in trans (interchromosomal) can regulate gene expression by bringing regulatory elements in close physical proximity to target genes.

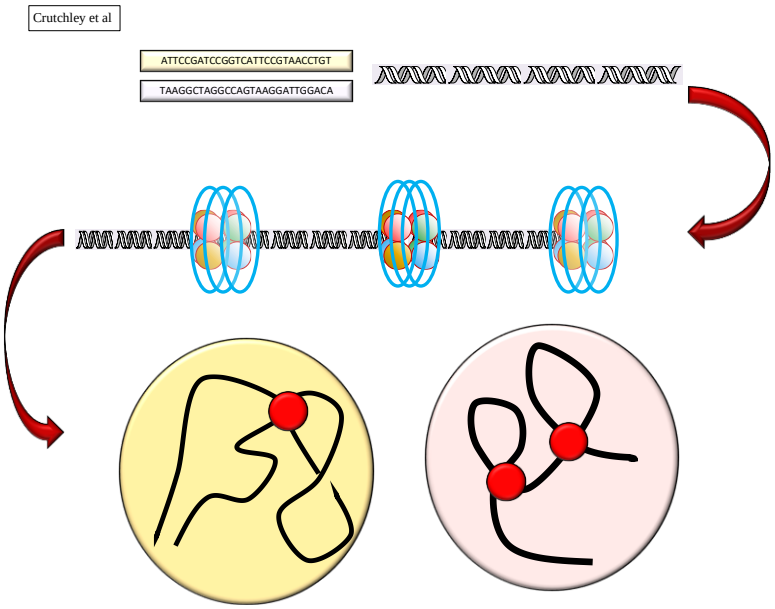
Here, we refer to 'long-range' chromatin contacts from an empirical standpoint as interactions stronger than those originating from random collisions surrounding regions of interest.

Long range chromatin contacts were found to regulate genes from diverse cellular pathways, indicating that this form of control is a general regulation mechanism.

However, at least for some genomic regions, regulation through long-range DNA contacts has remained unclear. Nonetheless, coregulated genes located far from each other or on different chromosomes also can co-localize and form foci in the nuclear space.

This type of organization likely participates in coordinating the proper timing and/or relative expression levels of various genes. 3D genome organization also includes positioning chromosomes into distinct territories within the nucleus, with gene-rich chromosomes at the center and gene-poor chromosomes near the periphery

The above authors reflect on these structural complexities as shown below. Our classic view if linear DNA which then gets spun about histones and then can cross-connect between themselves or with other DNA strands. One of the issue discussed herein is the impact of such effects.



As Hall et al note:

Genomic DNA is packaged by tightly wrapping around histone proteins in a complex known as chromatin to allow the almost 2 m of linear DNA in each human cell to fit into the nucleus.

Chromatin has a repeating and functional subunit known as a nucleosome, consisting of eight histone proteins and approximately 146 DNA bp, and regulates the access of transcriptional machinery to DNA that must unravel before transcription.

This unravelling, known as chromatin remodeling, is an epigenetic process regulated by cell-specific histone modifications, such as methylation, acetylation and phosphorylation, that mark genes, transcription start sites and stretches of regulatory DNA to control gene expression.

Near the start of a gene is the core promoter, serving as a docking site for RNA polymerase II and transcription factors to form the transcription pre-initiation complex. Approximately 250 bp upstream lies the proximal promoter, which is a sequence containing primary regulatory elements where general transcription factors bind.

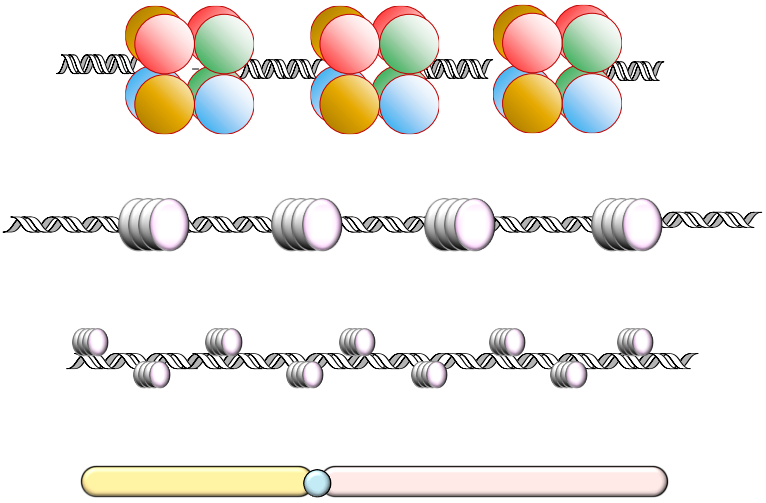
Enhancer regions are often located hundreds or thousands of bp away from transcription start sites, and are short stretches of DNA that can increase the transcription of genes. As these regulatory elements of the DNA sequence are not adjacent to one another, loops in the DNA bring distal enhancer regions closer to the proximal promoter.

Functional organisation of the genome is, therefore, not simply linear along chromosomes, as DNA elements can regulate genes located far away on the same (intrachromosomal) or different (interchromosomal) chromosomes, due to the organisation of the genome in three-dimensional nuclear.

Importantly, the capacity for distal chromosomal regions to enter close physical proximity and facilitate interaction of DNA elements makes spatial chromatin organisation a key mechanism in regulating gene expression.

Critically, genome packaging and rearrangement s non-random, with spatial chromatin organisation assuming a key functional role in the retrieval and translation of genetic instructions. While the analysis of spatial chromatin organisation has the potential to provide valuable insight into the role of genome packaging on gene expression in various settings, how this affects the expression of genes involved in the response to exercise and related stimuli is unknown.

We show this progression below:



Hall et al further note:

Chromosome conformation signatures represent a novel epigenetic biomarker of structural epigenetic changes in genomic architecture, documenting collections of DNA contacts associated with specific physiological outcomes.

With multiple genomic loci contributing to phenotypic differences, a signature of multiple DNA contacts is likely to provide greater biological insight than a single contact alone. An analogy would be how singular factors underpinning running performance (VO2max, lactate threshold, running economy, VO2 kinetics) provide better informative value when analysed in combination. Similarly, the polygenic influence on physical performance lead scientists in the field of sports genomics to consider the combined influence of multiple genetic variants as opposed to single genomic loci. That the expression of numerous genes is increased/decreased according to function and drives physiological adaptation leads us to hypothesise that there are detectable conditional CCSs associated with acute and chronic responses to exercise and related stimuli.

Specifically, the dynamic nature of chromatin organisation suggests there may be signatures associated with both transient responses and more persistent phenotypic changes, reflecting the underlying epigenetic regulatory landscape. Recent evidence that transcription factor activity, in particular, is affected by promoter region interactions demonstrates the permissiveness of CCSs to facilitate acute and persistent physiological alterations.

Recent application of CCSs has shown that this biomarker modality can be applied to whole blood samples to provide stable, binary readouts between two states (pre-intervention vs. post-intervention, disease vs. non-diseased) based on the presence or absence of a signature. It is important to note that study of CCSs requires consideration of participants individually, as opposed to their contribution to a collective group mean.

Mean values are routinely calculated and reported in sport and exercise research to summarise group data, providing a measure of central tendency. However, extreme values influence group

means, particularly in small samples, and are less suited to investigating variability between individuals.

The binary nature of CCS readouts offers a different approach, with samples (participants) grouped according to the presence or absence of a specific signature, as opposed to each sample contributing a numerical value on a scale, such as when measuring VO2max or jump height.

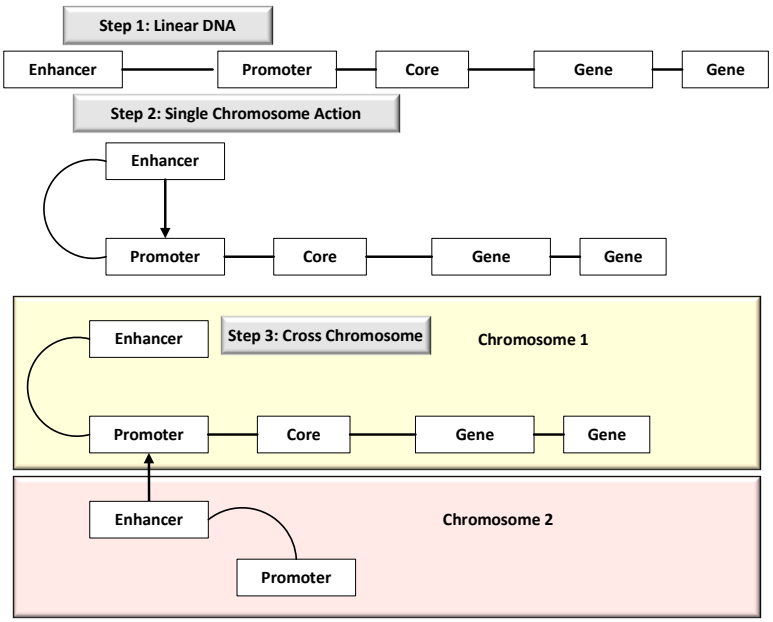
Hence, CCS technologies identify the flexibility or inflexibility of epigenomic states, rather than reporting the magnitude of gene expression, across multiple genomic loci.

Importantly, the way in which CCSs reorganise in response to stimuli to regulate gene expression appears to be one of the earliest detectable events, preceding other epigenetic modifications, transcription factor binding and transcription.

Due to the fact that DNA is spatially organized into 3D structures, and distal genomic regions can be brought into proximity through chromatin folding, it would be expected that such DNA sequences may also exhibit coordinated epigenetic marks, such as histone modifications and DNA methylation.

Indeed, a recent study using a variation of CCS, termed Methyl-HiC, revealed coordinated DNA methylation status between distal genomic segments that are in spatial proximity in the nucleus. Such combined approaches would be important to understand how epigenetic marks are dynamically regulated with characteristic patterns in different tissues. The ability to detect these early molecular changes may provide considerable benefit to sport and exercise scientists who seek to understand the initial drivers of adaptation.

Finally we show below this three step understanding from linear DNA to self-Enhancer/Promoter action and finally to inter chromosome Enhancer/Promoter actions.



2.3 HISTONES

Histones are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fiber in eukaryotes. Two molecules of each of the four core histones (H2A, H2B, H3, and H4) form an octamer, around which approximately 146 bp of DNA is wrapped in repeating units, called nucleosomes. The linker histone, H1, interacts with linker DNA between nucleosomes and functions in the compaction of chromatin into higher order structures. This gene encodes a member of the histone H2A family, and generates two transcripts through the use of the conserved stem-loop termination motif, and the polyA addition motif.[4]

We now want to discuss methylation and gene expression. Reference will be made to the work of Herman and Baylin, Jones and Takai, McCabe et al, Allis et al, and Issa and Kantarjian.

We begin with Herman and Baylin and their description of the diagram below:

In most of the mammalian genome, which is depicted here as exons 1, 2, and 3 of a sample gene (boxes 1, 2, and 3), introns of the gene (line between the exons), and regions outside the gene, the CpG dinucleotide has been depleted during evolution, as shown by the small number of such sites (circles).

Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome (numerous circles surrounding and within exon 1 of

[4] http://www.ncbi.nlm.nih.gov/gene/3014

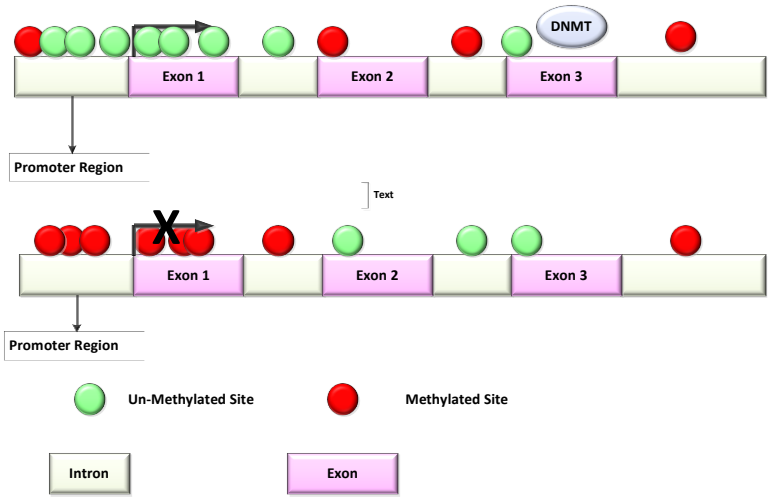
the sample gene). In normal cells, most CpG sites outside of CpG islands are methylated (black circles), whereas most CpG-island sites in gene promoters are unmethylated (white circles).

This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription (arrow in upper panel). In cancer cells, the DNA-methylation and chromatin patterns are shifted.

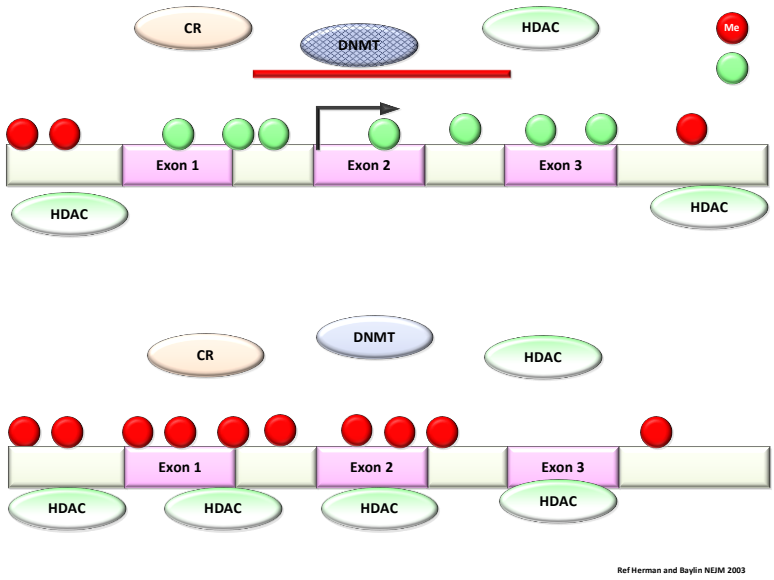
Many CpG sites in the bulk of the genome and in coding regions of genes, which should be methylated, become unmethylated, and a growing list of genes have been identified as having abnormal methylation of promoters containing CpG islands, with associated transcriptional silencing (red X at the transcription start site).

Although there are possible explanations and findings from ongoing investigations, it is not known why the DNA-methylating enzymes fail to methylate where they normally would and which of these enzymes are mediating the abnormal methylation of CpG islands in promoters.

We depict a modified version of their Figure below:



Thus methylation in this case blocks the expression of the targeted gene. Herman and Baylin also use the following Figure to describe more regarding methylation:



As to the above they state:

The chromatin around the transcriptionally active (green arrow), unmethylated promoter is occupied by widely spaced nucleosomes composed of histone complexes in which key residues in the tails of histone H3 are in the acetylated state (green ovals), and those in the tails of histone H3 are methylated at lysine 4 (yellow asterisks).

The region is accessible to key components of the gene-transcription apparatus, including primary transcription factors (TF); proteins with histone acetyltransferase activity (HAT), which maintain the histones in an acetylated state; and transcriptional coactivators (CA), which may also have histone acetyltransferase activities.

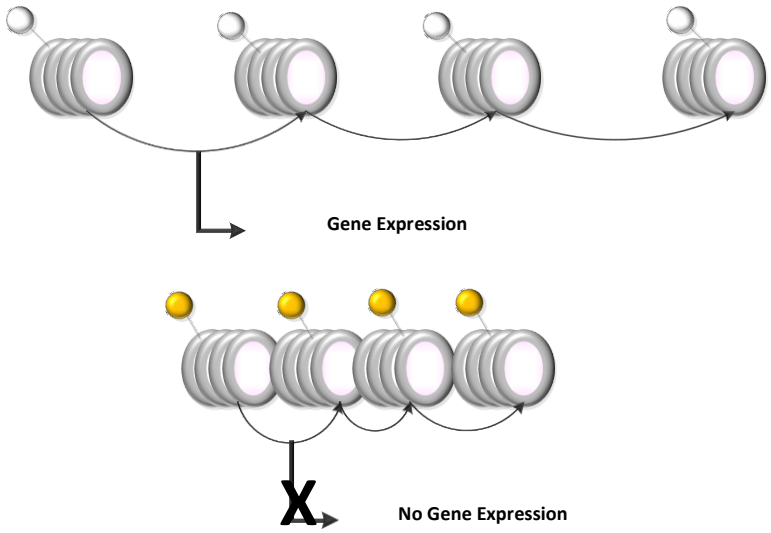
The flanking regions to either side of the unmethylated CpG island contain methylated cytosines. These regions are embedded in chromatin characteristic of transcriptionally silenced regions that is characterized by the binding of methylcytosine–binding proteins (MBPs) to the DNA methylated sites, and by nucleosomes that are more tightly compacted, with deacetylated histones (purple ovals) and methylated lysine 9 residues on the tails of histone H3 (black asterisks). The MBPs are part of complexes containing histone deacetylases (HDAC) that facilitate the deactivated state of the histones.

The blue vertical bars on either side of the unmethylated CpG island depict the molecular events, still to be determined, that prevent the spread of DNA methylation and of transcriptionally repressive chromatin across the CpG island in the promoter region of normal cells. The apparatus for DNA methylation, consisting of the DNA methyltransferases (DNMTs) and their complexes with transcriptional corepressors (CR) and histone deacetylases (HDAC), have access to the flanking areas but not to the CpG island in the promoter region within the barriers.

The lower panel depicts the breakdown of the barriers in a cancer cell, in which the transcriptionally repressive chromatin and DNA methylation have spread into the CpG island in the promoter region and correlate with transcriptional repression (red arrow with X) of the

gene. The DNA-methylating complex now has access to the region, and the transcriptional machinery (transcriptional coactivators, histone acetyltransferase, and transcription factors) is excluded.

Now the histones may also be acetylated and drawn together. When histones are drawn closer the genes in between cannot be read and thus they are not expressed. We show that below:



Now we can summarize this as follows:

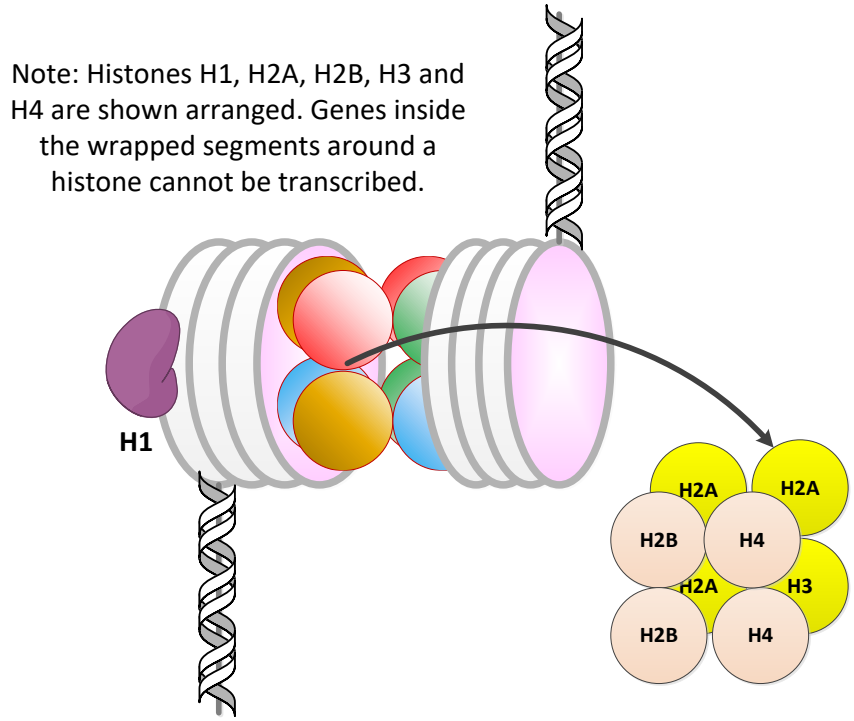
	Hypermethylated	Hypomethylated
Benign	Suppresses Proliferation Gene	Activates Suppressor Gene
Malignant	Suppresses Control Gene	Activates Proliferation Gene

What this shows is that methylation is good and bad. It is good if it suppresses the bad gene and bad if it suppresses a good gene, and vice versa.

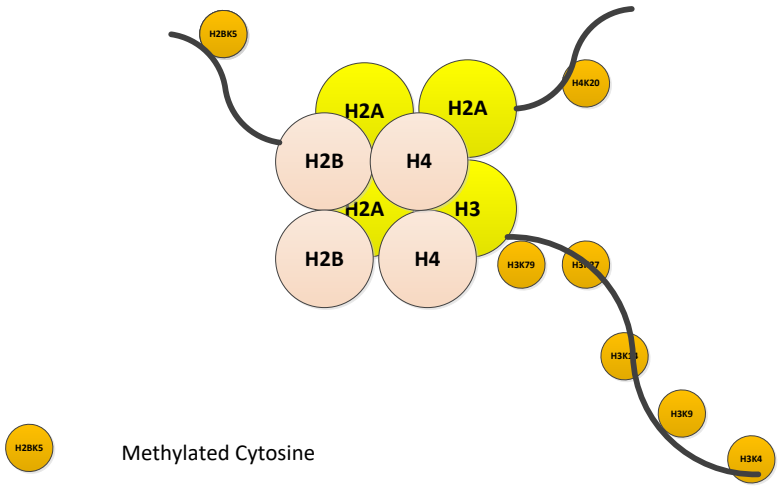
Methylation consists of the attachment of methyl groups on various elements of the genome. For our purposes we consider methylating the DNA on the CpG islands and methylation of the histones around which the DNA is wrapped. These effects have shown significant impact as well on PCa as well as many other cancers.

We have now described methylation, a rather simple process, and now we seek to discuss its influence on DNA. We start first at the top level of DNA, namely the chromosome. The DNA is often wrapped around histones, which are large protein masses that arrange themselves in a specific group. There are five main histones, H1, H2A, H2B, H3, and H4. They arrange themselves as shown below.

It appears as if one has eight large globes, each a histone, and they then allow the DNA to coil about them and in effect make certain that that specific segment of DNA is not read. Histones are another mechanism for DNA expression. They must be released so the DNA can be opened and then read in order for it to be expressed.



The specific arrangement of the histones is as shown below. It is not arbitrary but is a result of the specific surface charge arrangements on the histone proteins. We also depict the presence of methylated cytosines on this graphic, thus depicting the two major influences of methylation as well as acetylation, which we shall discuss.



Now what can happen is that the histone tails may become methylated, or acetylated, and when this occurs the histones may bind together or open up, depending on which lysine on the tail is affected. The open and close as a result of a methylation or acetylation is also called the histone code. Methylate or acetylate the right ones and the DNA is curled and not expressible and do another set and the DNA can be expressed.

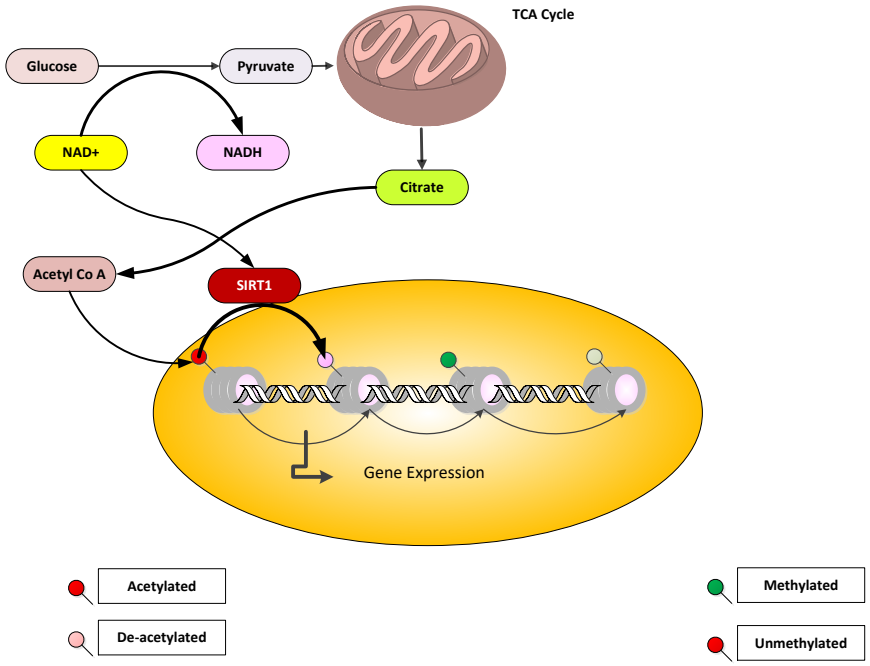
This Histone Code is shown below in the following Table.

	H3K4	H3K9	H3K14	H3K27	H3K79	H4K20	H2BK5
Mono-meth	Active	Active		Active	Active	Active	Active
Di-meth		Repress		Repress	Active		
Tri-meth	Active	Repress		Repress	Active		Repress
Acetyl		Active	Active		Repress		

Now we can use the above to understand the impact of these epigenetic factors via the interactions between Sirt1 and diet. In a recent paper by Labbe et al the authors examine dies and PCa. In particular they discuss the effect of Sirt1[5]. We show a modification of the Figure in the paper below. Glucose is converted to pyruvate via the action of NAD+ to NAH. Likewise this activates citrate to Acetyl-Co A and acetylates the histone changing its code but Sirt1 then deacetylates it to the ground state again. Thus loss of Sirt1 can potentially allow excess acetylated states which in turn does not allow the related genes to be expressed. Now from our discussions of miRNA exosomes we also understand that perhaps this down regulation of Sirt1

[5] http://www.nature.com/onc/journal/vaop/ncurrent/pdf/onc2014422a.pdf

could be a result of metastatic spread of deregulating miRNAs. Although conjecture, the spread of miR34 via exosomes would result in suppression of Sirt1 as well as many other critical genes.



The authors state as flows in their paper:

SIRT1 activity depends on the NAD+/NADH ratio modulated by glycolysis, while O-linked N-acetylglucosamine transferase uses GlcNAc produced by the hexosamine pathway. Pyruvate entering the tricarboxylic acid (TCA) cycle produces alpha-ketoglutarate, a critical cofactor for Jumonji domain-containing histone demethylase and TET. Acetyl-CoA is converted from the citrate generated by the TCA cycle and used as a donor by histone acetyltransferases.

Finally, the increase in ATP/ADP ratio from the TCA cycle also inactivates AMPK…. Under low-nutrient conditions, the NAD+/NADH ratio increases, activates SIRT1, which in turn de-acetylates and triggers ACECSs activity. Therefore, the pool of acetyl-CoA, which is governed by nutrient availability, controls the acetylation of metabolic enzymes as well as of histones at any given time.

As Melo et al state:

Exosomes are secreted by all cell types and contain proteins and nucleic acids. Here, we report that breast cancer associated exosomes contain microRNAs (miRNAs) associated with the RISC-Loading Complex (RLC) and display cell-independent capacity to process precursor microRNAs (pre-miRNAs) into mature miRNAs. Pre-miRNAs, along with Dicer, AGO2, and TRBP, are present in exosomes of cancer cells. CD43 mediates the accumulation of Dicer specifically in cancer exosomes.

Cancer exosomes mediate an efficient and rapid silencing of mRNAs to reprogram the target cell transcriptome. Exosomes derived from cells and sera of patients with breast cancer instigate nontumorigenic epithelial cells to form tumors in a Dicer-dependent manner. These findings offer opportunities for the development of exosomes based biomarkers and therapies.

It would be expected that this may be found elsewhere, especially in PCa, since both PCa and Breast Cancer have great similarity[6].

Moreover, Braicu et al have presented a more comprehensive understanding of exosomes. Their observations are as follows:

Exosomes are key elements that facilitate intercellular communication; depending on their vesicular content ('cargo'), they can modulate tumor cells by influencing major cellular pathways such as apoptosis, cell differentiation, angiogenesis and metastasis. This communication can involve the exchange of molecules such as small noncoding RNAs (e.g. miRNAs) between malignant, non-transformed and stromal cells (in all directions). Exosomal miRNAs represent ideal candidates for biomarkers, with multiple applications in the management of an array of pathologies such as cancer. Manipulating exosomal miRNAs suggests new alternatives for patient-tailored individualized therapies.

They continue:

MiRNAs are short single-stranded (19–25 nucleotides in length) nonprotein-coding RNA transcripts (ncRNA) that are initially produced in the nucleus and then transported into the cytoplasm, where they undergo a series of steps to acquire maturation. Mature miRNAs regulate gene expression by binding (through watsonian complementarity) to the sequence of a target mRNA. This interaction results in translational repression and/or mRNA cleavage, which consequently decreases the levels of the mRNA coding protein.

miRNAs have been found to be aberrantly expressed in many diseases. For example, in cancer, the tumor microenvironment contains deregulated miRNA levels, and a reason for their altered levels is because they are being actively secreted as membrane-bound vesicular content.

Finally they state:

Immediately after their synthesis, exosomes are released and can remain in the extracellular space near the cell they originated from. Alternatively, they can also travel through body fluids such as blood, urine, amniotic fluid, saliva, lung surfactant, malignant effusions or breast milk. The end result of this dynamic process is a variety of regulative molecules being transported to different tissues in different places, and influencing cellular processes. Exosomes have been shown to carry proteins, many of which have the potential to influence multiple regulatory mechanisms. For example, exosomes can transport annexins that have the ability of altering the dynamics of the cytoskeleton.

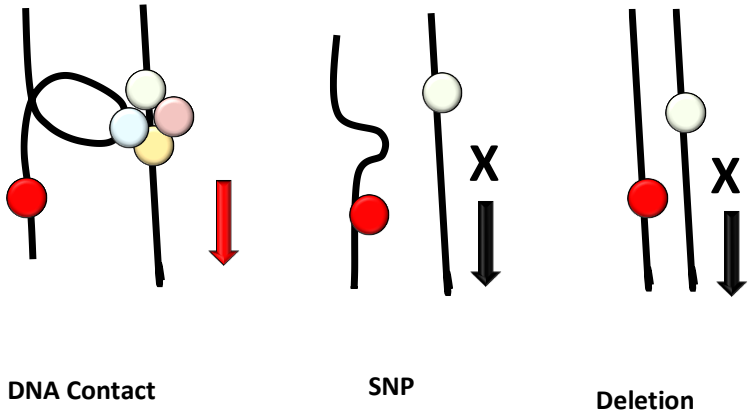
[6] See Telmarc White Paper 112 Prostate Cancer: miR-34, p53, MET and Methylation for detailed analysis.

Thus it is well understood that exosomes have not only the potential to allow one to see inside the cell, not only to transport to other cells but more importantly to act and a distributed means of control.

2.4 LOOPS

Chromatin conformations come in various forms. Some share promoters and some result in inhibitions. Below we provide three simple examples. The first is a shared environment where DNA contact results in the production of RNA. The latter two, SNP and Deletions, inhibit any RNA production.

Thus the linear model of DNA is in reality a much more complex issue than initially thought.



There are several modes of contact.

2.4.1 Local Contact

This occurs when the mutual contact is in a single strand of DNA chromatin generally is a localized area of a strand.

2.4.2 Intrachromosomal Contact

This occurs when the contacts are in the same chromosome but at a significant distance.

2.4.3 Interchromosomal Contact

This is contact on separate chromosomes.

2.4.4 Multi-chromosomal Contact

This set of configurations is across multiple chromosomes.

As Boltsis et al note:

Gene transcription is tightly regulated by regulatory elements (enhancers, insulators, silencers), which can be located at various distances from their cognate gene(s) on the linear DNA strand. In order to carry out their function, regulatory elements have to be in close proximity to their target gene(s).

"Loops" between enhancers and promoters usually result in local interactions, as opposed to CTCF-mediated long-range chromatin loops (TADs), which could facilitate enhancer-promoter interactions either by bringing them closer or by segregating the genome according to its chromatin state.

Recently it was shown that TFs (e.g., YY1 and LDB1), ncRNAs, the Mediator complex, p300 acetyltransferase and the cohesin complex proteins play key roles in the stabilization of chromatin looping or transcription factories The function of cohesin varies between various promoter-enhancer interactions. Some promoter–enhancer interactions could also be established only by transcription factors without the involvement of cohesin.

Four models have been proposed to explain how promoters and enhancers may regulate gene expression with the looping and the transcription factory model being the most prominent. Notably, the general notion of the looping model is that an enhancer is in close proximity to its target promoter(s) leading to gene activation, while the gene is silenced when the enhancer and promoter are not in close proximity.

Gene regulation from distal regulatory elements through local looping is now a commonly accepted concept.

Before the development of chromosome conformation capture technologies, which are essentially biochemical techniques, there was already strong evidence from biochemical and genetic type experiments that loop formation mediates transcription in both prokaryotic and eukaryotic systems. That was depicted in vitro with the lac repressor system.

In eukaryotic systems, in vitro assays using a plasmid suggested that an enhancer and a gene could be separated by a protein bridge invoking looping. Strong evidence in eukaryotes, with genes in the normal genome environment, was obtained at the b-globin locus after discovery of the Locus Control Region (LCR, (now called super-enhancers), which is located 70 kb upstream of the b-globin gene(s). Changing the distance or order of the b-globin genes and the LCR could only be explained by looping.

A few years later, the effect of natural mutations by defective enhancers located at very long distance, like in the case of polydactyly, was very difficult if not impossible to explain by mechanisms other than looping.

3 GENE TARGETS

We now examine in some details the gene targets that have been proposed. We discuss in the next section the methodology employed. The intent in this section is to attempt to provide a post hoc justification for why these targets are of merit and especially in the contest of aberrant chromatin conformation issues.

We return to Alshaker et al (2021) who further note:

In this study, we identified and validated ***chromosome conformations*** *as distinctive biomarkers for a non-invasive blood-based epigenetic signature for PCa.*

Our data demonstrate the presence of stable chromatin loops in the loci of ETS1, MAP3K14, SLC22A3 and CASP2 genes present only in PCa patients.

Validation of these markers in an independent set of 20 blinded samples showed 80% sensitivity and 80% specificity, which is remarkable for a PCa blood test.

Interestingly, the expression of some of these genes has already been linked to cancer pathophysiology.

ETS1 is a member of ETS transcription factor family. ETS1-overexpressing prostate tumours are associated with increased cell migration, invasion and induction of epithelial-to-mesenchymal transition.

MAP3K14 (also known as nuclear factor-kappa-beta (NF-kβ)-inducing kinase (NIK)) is a member of MAP3K group (or MEKK).

Physiologically, MAP3K14/NIK can activate noncanonical NF-kβ signalling and induce canonical NF-kβ signalling, particularly when MAP3K14/NIK is overexpressed. ***A novel role for MAP3K14/NIK in regulating mitochondrial dynamics to promote tumour cell invasion has been described .***

SLC22A3 (also known as organic cation transporter 3) is a member of SLC group of membrane transport proteins. ***SLC22A3 expression is associated with PCa progression.***

CASP2 is a member of caspase activation and recruitment domains group. Physiologically, CASP2 can act as an endogenous repressor of autophagy.

Two of the identified genes (SLC22A3 and CASP2) were previously shown to be inversely correlated with cancer progression*. Importantly, the presence of the chromatin loop can have indeterminate effect on gene expression.*

To screen for PCa prognostic markers we performed the EpiSwitch™ custom array to analyse competitive hybridization of DNA from peripheral blood from patients with low-risk PCa (category 1) and high risk PCa (category 3).

Six-marker set was identified for ***high-risk category 3 vs low-risk category 1,*** *including*

BMP6, ERG, MSR1, MUC1, ACAT1 and DAPK1.

Six-biomarkers were identified for high-risk category 3 vs intermediate-risk category 2, *including*

HSD3B2, VEGFC, APAF1, MUC1, ACAT1 and DAPK1.

*Three of these biomarkers (****MUC1, ACAT1 and DAPK1****) were shared between these sets.*

Our data show high concordance between chromosomal conformations in the primary tumour and in the blood of matched PCa patients at stages 1 and 3. The prognostic significance and diagnostic value of some of these genes have previously been suggested.

BMP6 plays an important role in PCa bone metastasis.

In addition to ETS1,

ERG is another member of the ETS family of transcription factors. Overwhelming evidence, reviewed in , suggested that ERG is implicated in several processes relevant to PCa progression including metastasis, epithelial–mesenchymal transition, epigenetic reprogramming, and inflammation .

MSR1 may confer a moderate risk for PCa .

MUC1 is a membrane-bound glycoprotein that belongs to the mucin family. MUC1 high expression in advanced PCa is associated with adverse clinicopathological tumour features and poor outcomes.

ACAT1 expression is elevated in high-grade and advanced PCa and acts as an indicator of reduced biochemical recurrence-free survival.

DAPK1 could function either as a tumour suppressor or as an oncogenic molecule in different cellular context.

HSD3B2 plays a crucial role in steroid hormone biosynthesis and it is up-regulated in a relevant fraction of PCa that are characterized by an adverse tumour phenotype, increased androgen receptor signalling and early biochemical recurrence..

VEGFC is a member of VEGF family and its increased expression is associated with lymph node metastasis in PCa specimens.

In a comprehensive biochemical approach,

APAF1 has been described as the core of the apoptosome.

Despite the identification of these loci, ***the mechanism of cancer-related epigenetic changes in PBMCs remains unidentified****. The interaction, however, could be detected systemically and under same conditions in the primary site of tumourigenesis. It is therefore assumed that the acquired changes must be directed by an external factor; presumably generated by the tumour cells. It is known that a significant proportion of chromosomal conformations are controlled by non-coding RNAs, which regulate the tumour-specific conformations.*

Tumour cells have been shown to secrete non-coding RNAs that are endocytosed by neighbouring or circulating cells and may change their chromosomal conformations, and are possible regulators in this case. RNA detection as a biomarker remains highly challenging (low stability, background drift, continuous variable for statistical stratification analysis).

Chromosome conformation signatures offer well recognized stable binary advantages for the biomarker targeting use, specifically when tested in the nuclei, since the ***circulating DNA*** *present in plasma does not retain 3D conformational topological structures present in the intact cellular nuclei.*

It is important to mention, that looking at one genetic locus does not equate to looking at one marker, as there may be multiple chromosome conformations present, representing parallel pathways of epigenetic regulation over the locus of interest. Other technologies for plasma-based cancer detection such as using plasma cell-free DNA (cfDNA) methylomes were recently introduced.

The validity of this assay was tested to identify patients with renal cell carcinoma using urine cfDNA[7] *with* ***area under the ROC curve (AUROC) of 0.86****.*

It is worth noting that ***cfDNA is capturing post-apoptotic and necrotic passive distribution of free DNA, with significant variations, while EpiSwitch is measuring 3D genomic profiling in intact cells, capturing systemic surrogate readouts,*** *which, for epigenetic modalities, have been shown to contain synchronized modulation at specific genetic loci concordant with primary sites of deregulation. Systemic surrogate signatures at selective loci at the level of 3D genomics are sustained through exosome signalling and are not restricted to oncology.*

3.1 DIAGNOSTIC I

From the 2021 Alshaker et al paper we examine the set of diagnostic markers as follows.

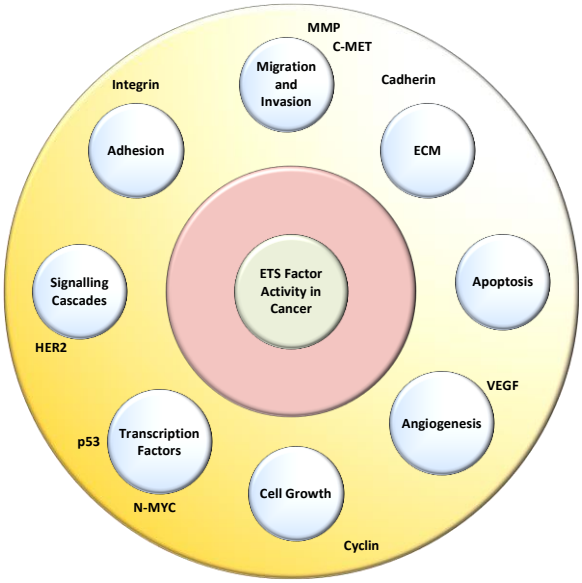
3.1.1 ETS1

[7] Cell free DNA, cfDNA

As noted in NCBI[8]:

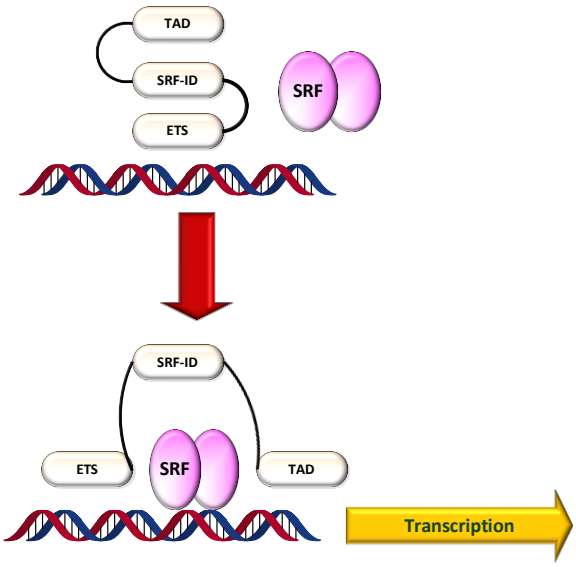
*This gene **encodes a member of the ETS family of transcription factors**, which are defined by the presence of a conserved ETS DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T in target genes. These proteins function either as transcriptional activators or repressors of numerous genes, and are involved in stem cell development, cell senescence and death, and tumorigenesis.*

The chart below demonstrates the complexity of genes in the ETS family

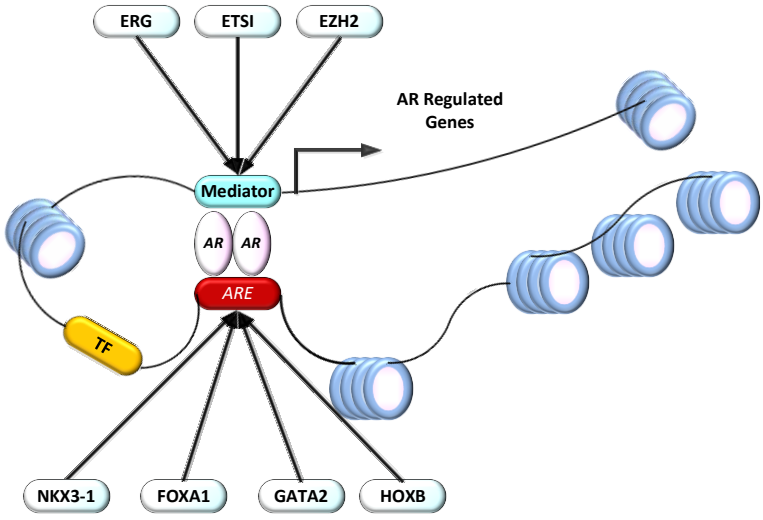


A simplified view in the nucleus regarding the use as a transcription factor is demonstrated below. ETS enters the nucleus and joining with three other proteins creates an effective transcription factor.

[8] https://www.ncbi.nlm.nih.gov/gene/2113



The following depicts the ETS1 control over an androgen receptors, AR. The dynamics of the AR in PCa are critical. Thus understanding its controllers provides additional control points for consideration.



Now Dittmer notes:

The Ets1 proto-oncoprotein is a member of the Ets family of transcription factors that share a unique DNA binding domain, the Ets domain.

The DNA binding activity of Ets1 is controlled by kinases and transcription factors. Some transcription factors, such as AML-1, regulate Ets1 by targeting its autoinhibitory module. Others, such as Pax-5, alter Ets1 DNA binding properties. Ets1 harbors two phosphorylation sites, threonine-38 and an array of serines within the exon VII domain.

Phosphorylation of threonine-38 by ERK1/2 activates Ets1, whereas phosphorylation of the exon VII domain by CaMKII or MLCK inhibits Ets1 DNA binding activity. Ets1 is expressed by numerous cell types. In haemotopoietic cells, it contributes to the regulation of cellular differentiation. In a variety of other cells, including endothelial cells, vascular smooth muscle cells and epithelial cancer cells, Ets1 promotes invasive behavior. Regulation of MMP1, MMP3, MMP9 and uPA as well as of VEGF and VEGF receptor gene expression has been ascribed to Ets1. In tumors, Ets1 expression is indicative of poorer prognosis

As Jiang et al note:

Ets1, a member of the ETS family of transcription factors, has been reported to participate in hyperglycemia-induced endothelial-to-mesenchymal transition (EMT), thus mediating endothelial injury.

Moreover, Ets1 has been demonstrated to play an important role in modulation of endothelial adhesion molecule expressions in a model of carotid artery balloon injury.

However, the exact mechanism by which Ets1 regulates endothelial adhesion molecule expression in hyperglycemia condition is still not well known.

As Xiao et al note:

ETS1 functions in RPG transcription regulation; to functionally test whether down-regulated ETS1 expression decreases RPG expression, we first constructed ETS1 knockdown 293T cells using short hairpin RNA (shRNA) expression plasmid. Transcriptome analysis showed that the genes in the ribosome pathway were significantly down-regulated, among which several randomly selected RPGs (e.g., RPL3 and RPS13) were validated by RT-qPCR. We also analyzed publicly available Ets1 (mouse homolog of ETS1) chromatin immunoprecipitation sequencing (ChIP-seq) data from primary mouse B cells to screen Ets1-binding sites across the genome and found that the Ets1 target genes were significantly enriched in the ribosome pathway. A similar result was also observed in human lymphoblastoid cells (GM12878).

Thus, these findings indicate that the ETS1 likely plays a role in regulating RPG transcription.

3.1.2 MAP3KY

From NCBI[9]:

This gene product is a 626-amino acid polypeptide that is 96.5% identical to mouse Mekk3. Its catalytic domain is closely related to those of several other kinases, including mouse Mekk2, tobacco NPK, and yeast Ste11. Northern blot analysis revealed a 4.6-kb transcript that appears to be ubiquitously expressed.

[9] https://www.ncbi.nlm.nih.gov/gene/4215

This protein directly regulates the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by activating SEK and MEK1/2 respectively; it does not regulate the p38 pathway.

In cotransfection assays, it enhanced transcription from a nuclear factor kappa-B (NFKB)-dependent reporter gene, consistent with a role in the SAPK pathway. Alternatively spliced transcript variants encoding distinct isoforms have been observed.

From Burotto et al,

The MAPK/ERK pathway is activated by upstream genomic events and/or activation of multiple signaling events where information coalesces at this important nodal pathway point. This pathway is tightly regulated under normal conditions by phosphatases and bidirectional communication with other pathways, such as the AKT/m-TOR pathway. Recent evidence indicates that the MAPK/ERK signaling node can function as a tumor suppressor as well as the more common prooncogenic signal.

The effect that predominates depends on the intensity of the signal and the context or tissue in which the signal is aberrantly activated. Genomic profiling of tumors has revealed common mutations in MAPK/ERK pathway components, such as BRAF. Currently approved for the treatment of melanoma, inhibitors of B-RAF kinase (BRAFi) are being studied alone and in combination with inhibitors of the MAPK and other pathways to optimize treatment of many tumor types.

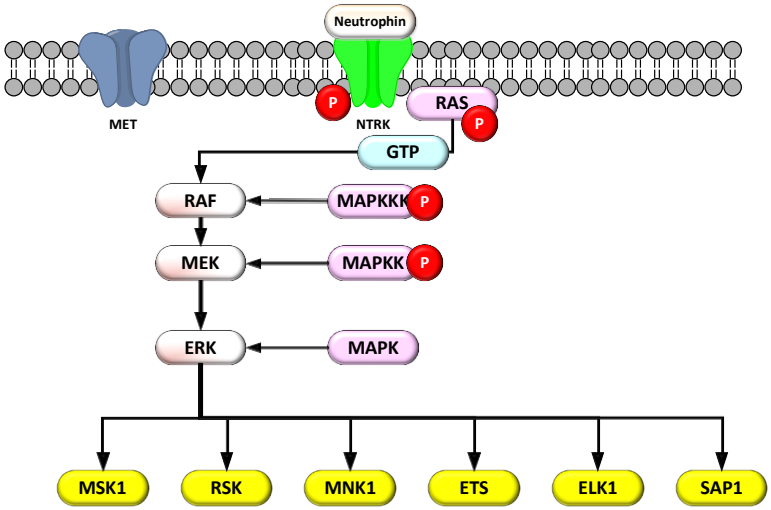
Therapies targeted toward MAPK/ERK components have variable response rates when used in different solid tumors, such as colorectal cancer and ovarian cancer. Understanding the differential nature of activation of the MAPK/ERK pathway in each tumor type is critical in developing single and combination regimens, as different tumors have unique mechanisms of primary and secondary signaling and subsequent sensitivity to drugs. …

There are four independent MAPK pathways composed of four signaling families: the MAPK/ERK family or classical pathway, and Big MAP kinase-1 (BMK-1), c-Jun Nterminal kinase (JNK), and p38 signaling families.

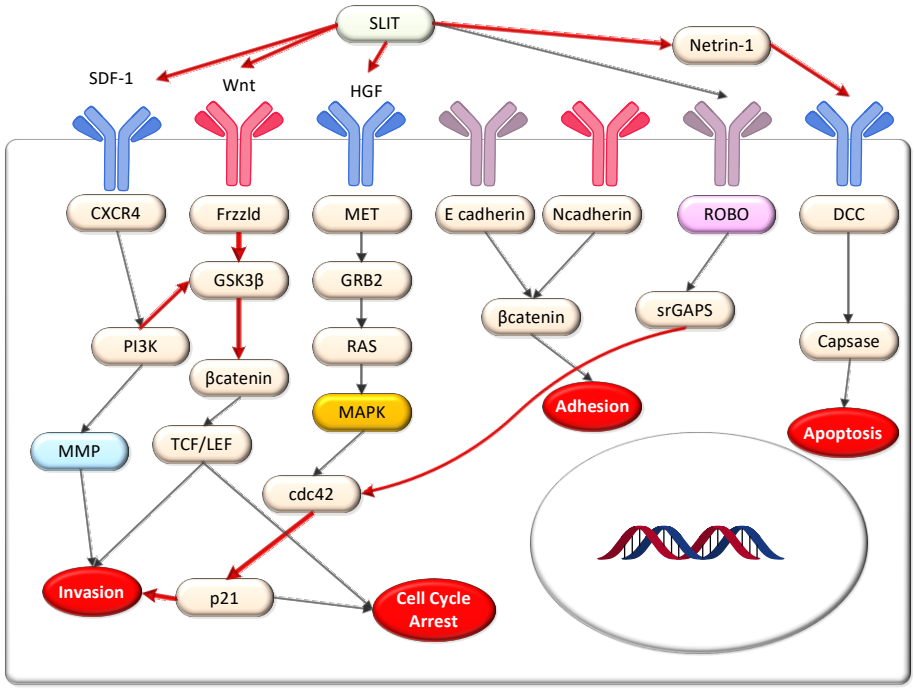
These families share a basic organization composed of two serine/threonine kinases and one double specificity threonine/ tyrosine kinase. Generically, these kinases are designated from upstream to downstream, closer to the nucleus, as MAPK kinase-kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. The canonical MAPK/ERK pathway is composed of three types of MAPKKK: A-RAF, B-RAF and RAF-1 or C-RAF kinases.

BRAF is the gene most commonly mutated at this level in human cancer. One level below are the MAPKKs, which are composed of MEK1 and MEK2. Finally, further downstream are ERK1 and ERK2, which are the final effectors of the MAPK pathway….

The figure below shows the MAPKKK element in its pathway.

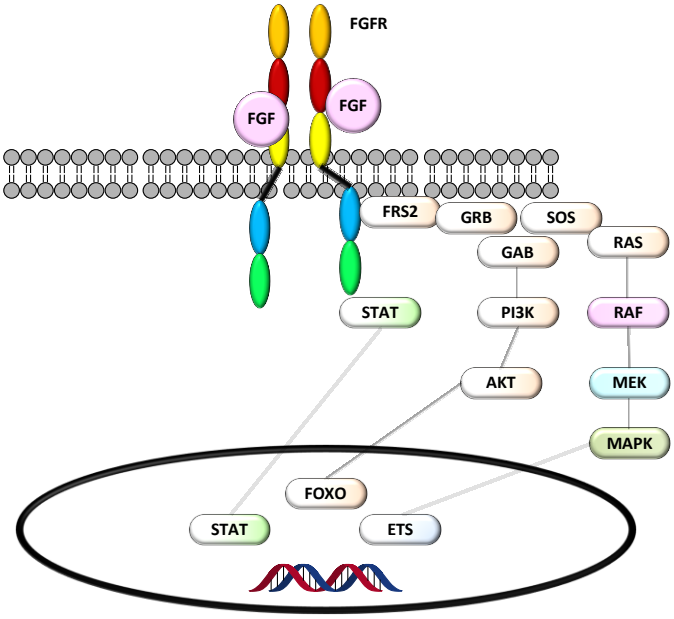


The effects of the various pathways are shown below. The insertion of MAPK and its derivatives play a significant role in invasion and cell cycle control.



From: Dickinson and Duncan

The figure below incorporates details regarding receptors, here FGFR and ligand FGF. They un turn activate MAK and derivatives.



3.1.3 SLC22A3

SLC22A3 is also known as OCT3. Now from NCBI[10] we have the following description:

Polyspecific organic cation transporters in the liver, kidney, intestine, and other organs are critical for elimination of many endogenous small organic cations as well as a wide array of drugs and environmental toxins. This gene is one of three similar cation transporter genes located in a cluster on chromosome 6. The encoded protein contains twelve putative transmembrane domains and is a plasma integral membrane protein.

Adding to this we have from Chen et al:

Human organic cation transporter 3 (OCT3 and SLC22A3) mediates the uptake of many important endogenous amines and basic drugs in a variety of tissues.

OCT3 is identified as one of the important risk loci for prostate cancer, and is markedly underexpressed in aggressive prostate cancers. The goal of this study was to identify genetic and epigenetic factors in the promoter region that influence the expression level of OCT3. …

Our studies demonstrate that genetic polymorphisms in the proximal promoter region of OCT3 alter the transcription rate of the gene and may be associated with altered expression levels of OCT3 in human liver. Aberrant methylation contributes to the reduced expression of OCT3 in prostate cancer.

As Khanppnavar et al note:

[10] https://www.ncbi.nlm.nih.gov/gene/6581

Organic cation transporters (OCTs, also SLC22A3) facilitate the translocation of catecholamines, drugs and xenobiotics across the plasma membrane in various tissues throughout the human body.

OCT3 plays a key role in low-affinity, highcapacity uptake of monoamines in most tissues including heart, brain and liver. Its deregulation plays a role in diseases. Despite its importance, the structural basis of OCT3 function and its inhibition has remained enigmatic. Here we describe the cryo-EM structure of human OCT3 at 3.2 Å resolution. Structures of OCT3 bound to two inhibitors, corticosterone and decynium-22, define the ligand binding pocket and reveal common features of major facilitator transporter inhibitors. In addition, we relate the functional characteristics of an extensive collection of previously uncharacterized human genetic variants to structural features, thereby providing a basis for understanding the impact of OCT3 polymorphisms. …

The SLC22 family comprises >30 transporters, which facilitate the transport of organic cations (OCTs), anions (OATs) and zwitterions (OCTNs). Collectively, these transporters define the pharmacokinetics of a vast array of drugs and xenobiotics. Herein, we describe the cryo-EM structure of OCT3 and provide the first direct insights into the organization of a SLC22 member, its substrate permeation pathway and ligand binding pocket. Both ligands of which we herein report cryo-EM structures, are handled by OCT3 in different ways which only partially overlap. It is not surprising, however, that the binding site of OCT3 allows accommodation of many diverse binding partners; the behavior rather substantiates the poly-specificity of a class of transporters which interact with a wide and complex array of compounds: from the antiviral drug abacavir and the antidiabetic drug metformin to the antineoplastic drug sunitinib

3.1.4 CASP2

As NCBI notes[11]:

This gene encodes a member of the cysteine-aspartic acid protease (caspase) family. Caspases mediate cellular apoptosis through the proteolytic cleavage of specific protein substrates.

The encoded protein may function in stress-induced cell death pathways, cell cycle maintenance, and the suppression of tumorigenesis. *Increased expression of this gene may play a role in neurodegenerative disorders including Alzheimer's disease, Huntington's disease and temporal lobe epilepsy. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene.*

As Tiwari et al note:

cAsP2/caspase 2 plays a role in aging, neurodegeneration, and cancer. The contributions of cAsP2 have been attributed to its regulatory role in apoptotic and nonapoptotic processes including the cell cycle, DNA repair, lipid biosynthesis, and regulation of oxidant levels in the

[11] https://www.ncbi.nlm.nih.gov/gene/835

cells. Previously, our lab demonstrated cAsP2-mediated modulation of autophagy during oxidative stress. here we report the novel finding that cAsP2 is an endogenous repressor of autophagy. Knockout or knockdown of cAsP2 resulted in upregulation of autophagy in a variety of cell types and tissues. Reinsertion of caspase-2 gene (Casp2) in mouse embryonic fibroblast (MeFs) lacking Casp2 (casp2−/−) suppresses autophagy, suggesting its role as a negative regulator of autophagy.

Loss of cAsP2-mediated autophagy involved AMP-activated protein kinase, mechanistic target of rapamycin, mitogen-activated protein kinase, and autophagy-related proteins, indicating the involvement of the canonical pathway of autophagy. The present study also demonstrates an important role for loss of cAsP2-induced enhanced reactive oxygen species production as an upstream event in autophagy induction.

Additionally, in response to a variety of stressors that induce cAsP2-mediated apoptosis, casp2−/− cells demonstrate a further upregulation of autophagy compared with wild-type MeFs, and upregulated autophagy provides a survival advantage. in conclusion, we document a novel role for cAsP2 as a negative regulator of autophagy, which may provide important insight into the role of cAsP2 in various processes including aging, neurodegeneration, and cancer …

Loss of CASP2 upregulates endogenous levels of autophagy under normal conditions Our previous study demonstrated that CASP2 modulates the autophagic response against mitochondrial oxidative stress in primary neurons. Of note, we also observed that even the basal level of autophagy was higher in the primary neurons cultured from Casp2 knockout (casp2−/−) mice compared with wild type (WT)….

AMPK and MTOR are involved in CASP2-modulated autophagy: Autophagy is controlled by several kinases including MTOR, which suppresses autophagy40 and AMPK, which induces autophagy. …

CASP2 levels are not regulated by autophagy Since we observed that CASP2 functions as a negative regulator of autophagy, we also examined whether CASP2 levels were modulated by autophagy upregulation. WT MEFs were treated with an autophagy inducer (rapamycin) as well as an early and a late stage inhibitor of autophagy. …

Role of MAPK in CASP2-mediated regulation of autophagy To identify other mediators involved in autophagy induced by loss of CASP2 further studies were conducted.

Regulation of autophagy by the members of the MAPK family, including MAPK1/ERK2 (mitogen-activated protein kinase 1) and MAPK3/ERK1, MAPK11/12/13/14 (p38 β, γ, δ and α, respectively) and MAPK8/9/10 (JNK1/2/3, respectively) has been documented.

Furthermore, previous reports have identified a regulatory association between CASP2 and MAPKs. …

Increased ROS production following loss of CASP2 occurs upstream of AMPK, MTOR and MAPK1/3 activation Loss of CASP2 results in an upregulation of ROS levels. A role for ROS is well established as an upstream mediator of autophagy by modulating activation of AMPK, MTOR, and MAPKs. Thus, we investigated a possible involvement of ROS as an upstream event in loss of CASP2-induced autophagy. …

Loss of CASP2 leads to protection from oxidative stress and enhances autophagy compared with the WT CASP2 is involved in induction of cell death via apoptosis under various conditions including oxidative stress,26,61-63 heat shock,30 and microtubule disruption …

3.2 DIAGNOSTIC II

As we noted previously from Pchejetski et al (2023) paper we examine the following set of markers:

Samples were tested for PSA, and the presence of CCSs in the loci encoding for of DAPK1, HSD3B2, SRD5A3, MMP1, and miRNA98 associated with high-risk PCa identified in our previous work.

Thus a set of 5 genes and miRNA are used with improved sensitivity and specificity. The authors continue:

Our data demonstrate the presence of stable chromatin loops in the loci encoding for DAPK1, HSD3B2, SRD5A3, MMP1, and miRNA98 in the circulation of PCa patients.

We have previously described their implication in PCa pathology. String analysis has shown that at the protein level of four out of five markers belong to the same network with a high confidence of interaction. Despite the identification of these epigenetic loci, until recently, the mechanism of cancer-related epigenetic changes in PBMCs remained unidentified. We have previously identified that similar signatures existed in primary tumours. Our recently published data show for the first time a proof of concept for horizontal transfer of chromosome conformations in cancer cell-monocyte co-culture without direct cell-cell contact

We shall examine each in some detail.

3.2.1 DAPK1

We discuss this in the next section in detail

3.2.2 HSD3B2

From NCBI[12]:

[12] https://www.ncbi.nlm.nih.gov/gene/3284

The protein encoded by this gene is a bifunctional enzyme that catalyzes the oxidative conversion of delta(5)-ene-3-beta-hydroxy steroid, and the oxidative conversion of ketosteroids. It plays a crucial role in the biosynthesis of all classes of hormonal steroids. This gene is predominantly expressed in the adrenals and the gonads. Mutations in this gene are associated with 3-beta-hydroxysteroid dehydrogenase, type II, deficiency. Alternatively spliced transcript variants have been found for this gene.

As Chang et al have noted:

3β-hydroxysteroid dehydrogenases (HSD3Bs), encoded by the HSD3B gene family at 1p13, have long been hypothesized to have a major role in prostate cancer susceptibility.

The recent reports of a prostate cancer linkage at 1p13 provided additional evidence that HSD3B genes may be prostate cancer susceptibility genes. To evaluate the possible role of HSD3B genes in prostate cancer, we screened a panel of DNA samples collected from 96 men with or without prostate cancer for sequence variants in the putative promoter region, exons, exon-intron junctions, and 3β-untranslated region of HSD3B1 and HSD3B2 genes by direct sequencing. Eleven single nucleotide polymorphisms (SNPs) were identified, four of which, including a missense change (B1-N367T), were informative. These four SNPs were further genotyped in a total of 159 hereditary prostate cancer probands, 245 sporadic prostate cancer cases, and 222 unaffected controls.

Although a weak association between prostate cancer risk and a missense SNP (B1-N367T) was found, stronger evidence for association was found when the joint effect of the two genes was considered. Men with the variant genotypes at either B1-N367T or B2-c7519g had a significantly higher risk to develop prostate cancer, especially the hereditary type of prostate cancer. Most importantly, the subset of hereditary prostate cancer probands, whose families provided evidence for linkage at 1p13, predominantly contributed to the observed association. Additional studies are warranted to confirm these findings.

3.2.3 SRD5A3

From NCBI[13]:

*The protein encoded by this gene belongs to the steroid 5-alpha reductase family, and polyprenol reductase subfamily**. It is involved in the production of androgen 5-alpha-dihydrotestosterone (DHT) from testosterone, and maintenance of the androgen-androgen receptor activation pathway.** This protein is also necessary for the conversion of polyprenol into dolichol, which is required for the synthesis of dolichol-linked monosaccharides and the oligosaccharide precursor used for N-linked glycosylation of proteins. Mutations in this gene are associated with congenital disorder of glycosylation type Iq.*

Zhang et al note:

[13] https://www.ncbi.nlm.nih.gov/gene/79644

…the mRNA expression levels of SRD5A3 in different cancers. Currently, Oncomine database is the largest oncology gene chip database and integrated data platform. SRD5A3 mRNA expression levels in tumor and normal tissues from different datasets were shown by setting P value <0.05, fold change > 2, and gene rank top 10% as the parameters…

As Uemura et al note:

Prostate cancer often relapses during androgen-depletion therapy, even under conditions in which a drastic reduction of circulating androgens is observed. There is some evidence that androgens remain present in the tissues of hormone-refractory prostate cancers (HRPC), and enzymes involved in the androgen and steroid metabolic pathway are likely to be active in HRPC cells. We previously carried out a genome-wide gene expression profile analysis of clinical HRPC cells by means of cDNA microarrays in combination with microdissection of cancer cells and found dozens of transactivated genes.

Among them, we here report the identification of a novel gene, SRD5A2L, encoding a putative 5α-steroid reductase that produces the most potent androgen, 5α-dihydrotestosterone (DHT), from testosterone.

Liquid chromatography-tandem mass spectrometry analysis following an in vitro 5α-steroid reductase reaction validated its ability to produce DHT from testosterone, similar to type 1 5α-steroid reductase. Because two types of 5α-steroid reductase were previously reported, we termed this novel 5α- steroid reductase 'type 3 5α-steroid reductase' (SRD5A3).

Reverse transcription–polymerase chain reaction and northern blot analyses confirmed its overexpression in HRPC cells, and indicated no or little expression in normal adult organs. Knockdown of SRD5A3 expression by small interfering RNA in prostate cancer cells resulted in a significant decrease in DHT production and a drastic reduction in cell viability.

These findings indicate that a novel type 3 5α-steroid reductase, SRD5A3, is associated with DHT production and maintenance of androgen–androgen receptor-pathway activation in HRPC cells, and that this enzymatic activity should be a promising molecular target for prostate cancer therapy.

3.2.4 MMP1

From NCBI[14]:

This gene encodes a member of the peptidase M10 family of matrix metalloproteinases (MMPs). ***Proteins in this family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis***.

[14] https://www.ncbi.nlm.nih.gov/gene/4312

The encoded preproprotein is proteolytically processed to generate the mature protease. This secreted protease breaks down the interstitial collagens, including types I, II, and III. The gene is part of a cluster of MMP genes on chromosome 11. Mutations in this gene are associated with chronic obstructive pulmonary disease (COPD). Alternative splicing results in multiple transcript variants, at least one of which encodes an isoform that is proteolytically processed.

As Alpha et al note:

Cells undergoing mesenchymal migration degrade the ECM by several methods. Expression of membrane-bound membrane-type 1 MMP (MT1-MMP/MMP14) and of secreted MMP1, MMP9, MMP10, MMP11, and MMP13 is often upregulated in almost every cancer type and plays a key role in degrading the ECM, basement membranes, and vascular basal lamina to promote invasion and metastasis. Hic-5 has been implicated in regulation of several of these proteins, which will be discussed in later sections. Briefly, Hic-5 is known to promote MT1-MMP localization to the membrane in both endothelial cells and fibroblasts and to promote MMP-9 expression in several cancer lines.

Cancer cells also utilize specialized adhesion structures known as invadopodia, which are actin-rich membrane protrusions that exhibit abundant MMP localization and activity, including MT1-MMP. Although invadopodia have primarily been studied in vitro, intravital imaging and careful immunohistochemical staining provide evidence of their existence and importance for directed ECM degradation in vivo, where they have been observed in close proximity to areas of basement membrane degradation and at sites of tumor cell extravasation through the endothelium.

Broadly speaking, individual invadopodia consist of a core of F-actin and actin regulatory and binding proteins, which are often surrounded by a ring of adhesion-associated proteins, including integrins, and various Rho GTPase family members. The tyrosine kinase Src is particularly important in invadopodia formation, while cortactin, TKS4, and TKS5 play key roles in modulating their maturation and activity.

In 2D, invadopodia (and related structures such as podosomes) are visualized as small, actin-rich puncta that colocalize with areas of matrix degradation. However, invadopodia can also self-assemble in 2D culture to form large superstructures known as rosettes which can degrade much larger areas of matrix. Both paxillin and Hic-5 localize to invadopodia and regulate their dynamics

3.2.5 miRNA98

From NCBI[15]:

microRNAs (miRNAs) are short (20-24 nt) non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs.

[15] https://www.ncbi.nlm.nih.gov/gene/723947 and https://www.ncbi.nlm.nih.gov/gene/407054

miRNAs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding.

The primary transcript is cleaved by the Drosha ribonuclease III enzyme to produce an approximately 70-nt stem-loop precursor miRNA (pre-miRNA), which is further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature miRNA and antisense miRNA star (miRNA*) products.

The mature miRNA is incorporated into an RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA …

Ting et al note:

The anti-tumor effect of vitamin D has been well recognized but its translational application is hindered by side effects induced by supra-physiological concentration of vitamin D required for cancer treatment.

Thus, exploring the vitamin D tumor suppressive functional mechanism can facilitate improvement of its clinical application.

We screened miRNA profiles in response to vitamin D and found that a tumor suppressive miRNA, miR-98, is transcriptionally induced by 1,25- dihydroxyvitamin D3 (1,25-VD) in LNCaP. Mechanistic dissection revealed that 1,25-VD-induced miR-98 is mediated through both a direct mechanism, enhancing the VDR binding response element in the promoter region of miR-98, and an indirect mechanism, down-regulating LIN-28 expression.

Knockdown of miR-98 led to a reduction of 1,25-VD anti-growth effect and overexpression of miR-98 suppressed the LNCaP cells growth via inducing G2/M arrest.

And CCNJ, a protein controlling cell mitosis, is down-regulated by miR-98 via targeting 3-untranslated region of CCNJ. Interestingly, miR-98 levels in blood are increased upon 1,25-VD treatment in mice suggesting the biomarker potential of miR-98 in predicting 1,25-VD response. Together, the finding that growth inhibitive miR-98 is induced by 1,25-VD provides a potential therapeutic target for prostate cancer and a potential biomarker for 1,25-VD anti-tumor action. …

***Vitamin D Induces miR-98 Expression in LNCaP Cells**—In search of miRNAs mediating the anti-proliferative effect of 1,25-VD, we performed systemic miRNA array profiling comparing LNCaP cells treated with ethanol (EtOH) vehicle control and 1,25-VD (LC Sciences). We performed two independent experiments in microarray study. There are 8 miRNAs consistently up- or down-regulated by 1,25-VD with statistical significance in individual experiments (Fig. 1A). … miR-98 is the miRNA showing strong signals (500) in array; therefore it was chosen for further validation and exploration for its roles in mediating 1,25-VD anti-tumor effects.*

First, we confirmed 1,25-VD effects on the miR-98 expression by Q-PCR in LNCaP sublines with different vitamin D responsiveness: vitamin D-resistant line (LNCaP-r) versus parental cells (LNCaP-p) . …

miR-98 expression was induced by 1,25-VD in LNCaP-p cell, but this miR-98 induction by 1,25-VD was diminished in the LNCaP-r cells. This suggests the regulation of miR-98 is through 1,25-VD/VDR transcriptional activity.

To further verify VDR's involvement in this regulation, we knocked down VDR by shRNA against VDR and examined the miR-98 expression upon 1,25-VD treatment. We found that VDR expression was reduced in VDR shRNA clones as compared with scramble shRNA controls (Fig. 1C), and 1,25-VD induced miR-98 in scramble shRNA expressed control lines, sc8 and sc10, but not in VDR knockdown clones, shVDR3 and shVDR7. In summary, 1,25-VD-induced miR-98 is VDR-dependent.

The Induction of miR-98 Contributes to the Anti-proliferative Effect of 1,25-VD—MiR-98 belongs to the Let-7 family which is well categorized as a tumor suppressive miRNA. It has been shown to regulate HMGA2, a gene controlling growth and tumorigenesis (25). Therefore, we suspect that miR-98 is involved in the anti-proliferative mechanism of 1,25-VD. Cells were transfected with antagomirs of miR-98 (anti-miR-98) to knock down miR-98 expression; the 1,25-VD responsiveness was examined.

As expected, the 1,25-VD induction of miR-98 was diminished in the anti-miR-98 transfected cells; consequently, 1,25-VD-mediated growth inhibition effect was significantly reduced in the anti-miR-98 cells as compared with cells that were transfected with control antagomirs. This indicates the importance of miR-98 in mediating the antiproliferative effect of 1,25-VD.

3.3 PROGNOSTIC

We focus on Stage 1 and 3 discrimination targets.

3.3.1 BMP6

From NCBI we have[16]:

*This gene encodes a **secreted ligand of the TGF-bet**a (transforming growth factor-beta) superfamily of proteins. Ligands of this family bind various TGF-beta receptors leading to **recruitment and activation of SMAD family** transcription factors that regulate gene expression[17]. The encoded preproprotein is proteolytically processed to generate each subunit of the disulfide-linked homodimer. This protein regulates a wide range of biological processes including iron homeostasis, fat and bone development, and ovulation. Differential expression of*

[16] https://www.ncbi.nlm.nih.gov/gene/654

[17] https://www.researchgate.net/publication/333704252_EMT_lncRNA_TGF_SMAD_and_Cancers

this gene may be associated with progression of breast and prostate cancer. Mutations in this gene may be associated with iron overload in human patients

As Garcia Muro et al note[18]:

The BMP6 protein (Bone Morphogenetic Protein 6) is part of the superfamily of transforming growth factor-beta (TGF-β) ligands, participates in iron homeostasis, inhibits invasion by increasing adhesions and cell-cell type interactions and induces angiogenesis directly on vascular endothelial cells.

BMP6 is coded by a tumor suppressor gene whose subexpression is related to the development and cancer progression; during neoplastic processes, methylation is the main mechanism by which gene silencing occurs.

This work presents a review on the role of BMP6 protein in breast cancer (BC) and other types of cancer. The studies carried out to date suggest the participation of the ***BMP6 protein in the epithelial-mesenchymal transition (EMT) phenotype, cell growth and proliferation****; however, these processes are affected in a variable way in the different types of cancer, the methylated CpG sites in BMP6 gene promoter, as well as the interaction with other proteins could be the cause of such variation.*

3.3.2 ERG

From NCBI[19]:

This gene encodes a member of the ***erythroblast transformation-specific (ETS) family of transcriptions factors****.*

All members of this family are key regulators of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis. The protein encoded by this gene is mainly expressed in the nucleus. It contains an ETS DNA-binding domain and a PNT (pointed) domain which is implicated in the self-association of chimeric oncoproteins.

This protein is required for platelet adhesion to the subendothelium, inducing vascular cell remodeling. It also regulates hematopoesis, and the differentiation and maturation of megakaryocytic cells. This gene is involved in chromosomal translocations, resulting in different fusion gene products, such as TMPSSR2-ERG and NDRG1-ERG in prostate cancer, EWS-ERG in Ewing's sarcoma and FUS-ERG in acute myeloid leukemia.

More than two dozens of transcript variants generated from combinatorial usage of three alternative promoters and multiple alternative splicing events have been reported, but the full-length nature of many of these variants has not been determined.

[18] https://pubmed.ncbi.nlm.nih.gov/34706618/

[19] https://www.ncbi.nlm.nih.gov/gene/2078

As Adamo and Ladomery have noted:

ETS-related gene (ERG) is a member of the E-26 transformation-specific (ETS) family of transcription factors with roles in development that include vasculogenesis, angiogenesis, haematopoiesis and bone development.

ERG's oncogenic potential is well known because of its involvement in Ewing's sarcoma and leukaemia. However, in the past decade ERG has become highly associated with prostate cancer development, particularly as a result of a gene fusion with the promoter region of the androgeninduced TMPRRSS2 gene.

We review ERG's structure and function, and its role in prostate cancer. We discuss potential new therapies that are based on targeting ERG. … ERG (ETS-related gene) is a member of the E-26 transformationspecific (ETS) family of transcription factors.

There are 30 identified ETS family genes, of which in the human genome. ETS genes are evolutionarily conserved across metazoa and are thought to have arisen 600–700 million years ago. Research in several vertebrate model organisms shows that ETS proteins are nuclear DNA-binding phosphoproteins that act as activators or repressors of transcription.

The ETS transcription factors are required for development and differentiation impacting across a wide range of tissue and cell types with roles in embryogenesis, vasculogenesis, angiogenesis, haematopoiesis and neuronal development. Their target genes are involved in the regulation of cellular architecture, cell migration, invasion and cell permeability. The ERG gene was first described in 1987 … in human colorectal carcinoma cells and gene resides on chromosome 21. Phylogenetic research suggests that ERG evolved from a series of ETS gene duplications during the Cambrian explosion around 542 million years ago. …

Over the last decade, ERG has been increasingly implicated in the aetiology of prostate cancer. In 2005, a paper published by Tomlins et al. showed that ERG is overexpressed in a high proportion of prostate carcinomas as a result of a gene fusion with the androgen-driven promoter of the TMPRSS2 gene.

Prostate epithelia do not normally express ERG.

ERG is one of the most consistently overexpressed oncogenes in malignant prostate cancer and is a driver event in the transition from prostatic intraepithelial neoplasia (PIN) to carcinoma. In prostate cancer, high expression of ERG is also associated with advanced tumour stage, high Gleason score, metastasis and shorter survival times.

ERG is also implicated in other cancers, including Ewing's sarcoma and leukaemia. For example, ERG-positive acute T-lymphoblastic leukaemias are four times more likely to relapse.

The overexpression of ERG is one of the key factors in transforming localised, aggressive cancer into metastatic cancer.

High levels of ERG are implicated in loss of cell polarity, changes in cell adhesion, nuclear pleomorphism promoting hyperplasia and PIN in mouse prostate epithelia.

Aberrant ERG expression has a major impact on cell invasion and epithelial–mesenchymal transition (EMT) through the upregulation of the FZD4 gene, a member of the frizzled family of receptors. Higher levels of FZD4 increase the expression of mesenchymal markers and reduce the expression of epithelial markers. ERG overexpression also leads to the loss of E-cadherin expression (a marker of EMT), as well as increased cell mobility and invasion.

Enhanced cell mobility and migration also results from ERG's transactivation of the EMT-related gene vimentin. Vimentin is highly expressed in actively migrating cells but not stationary in cells. It is a key component of the cytoskeleton in which it has a role in the re-organisation of actin filaments in migrating cells. High levels of ERG increase cell invasion via the activation of matrix metalloproteases (MMPs), the plasminogen activator pathway and the WNT-signalling pathway. ERG upregulates MMP1 and indirectly modulates the activation of MMP3 and of secreted protein acidic and rich in cysteine.

These genes regulate EC proliferation and induce loss of focal adhesion, alteration of cell morphology and barrier function. Other ERG-regulated genes involved in EMT and cell invasion include RhoA, VEGF-R2/FLK1 and Zeb1/Zeb2. ERG is clearly implicated in metastasis. CXCR4 is a type 4 C-X-C chemokine receptor that is upregulated by ERG in ~ 80% of primary prostate cancers and promotes metastasis to bone tissue. Its ligand, the chemokine stromal-derived factor-1 is produced by the bone marrow.

Cells that express the membrane-bound CXCR4 receptor metastasise to sites of stromal derived factor-1 release. Furthermore, the ADAMTS1 gene (encoding a disintegrin and metalloproteinase with a thrombospondin motif) is upregulated by ERG in prostate cancer cells. Cells that overexpress ADAMTS1 display excessive matrix deposition and chemotactic attraction towards fibroblasts. The downregulation or inactivation of the tumour-suppressor SMAD4 and the upregulation of osteopontin are associated with biochemical recurrence and lethal metastasis.

ERG activates osteopontin transcription; and there is evidence of a reciprocal relationship between the expression of ***SMAD4 and ETS-regulated genes*** *such as VEGF-A and MMP-9. ERG represses a number of prostate epithelium-specific genes (****KLK3—best known as PSA, SLC45A3/prostein, C15ORF, MSMB/ PSP94 and SCGB1D2****).*

This suggests that ERG promotes the de-differentiaton of prostate epithelium. ERG may also have a role in cell lineage selection as its overexpression causes stem cell surface markers (such as CD49F) normally expressed by the basolateral layer of the prostate to be expressed in luminal cells. It is the basal cell layer and stem cells of the prostate that show the biggest response to ERG overexpression resulting in ductal dysplasia and PIN lesions.

From Kish et al:

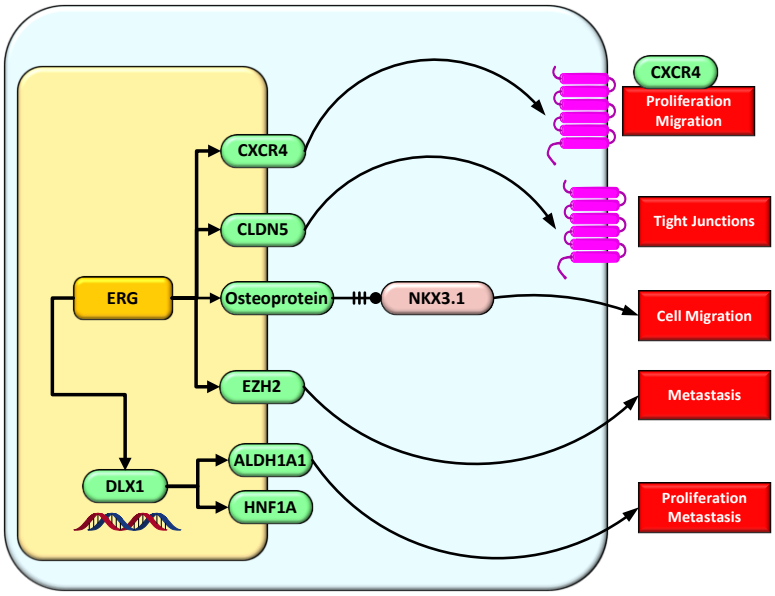
The ETS-related gene (ERG) is proto-oncogene that is classified as a member of the ETS transcription factor family, which has been found to be consistently overexpressed in about half of the patients with clinically significant prostate cancer (PCa).

The overexpression of ERG can mostly be attributed to the fusion of the ERG and transmembrane serine protease 2 (TMPRSS2) genes, and this fusion is estimated to represent about 85% of all gene fusions observed in prostate cancer.

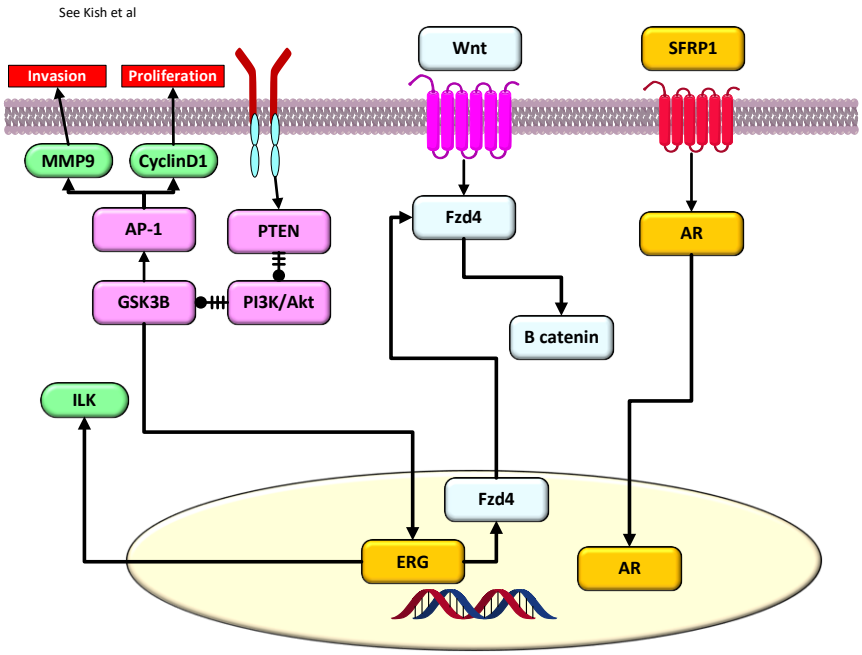
Clinically, individuals with ERG gene fusion are mostly documented to have advanced tumor stages, increased mortality, and higher rates of metastasis in non-surgical cohorts. In the current review, we elucidate ERG's molecular interaction with downstream genes and the pathways associated with PCa.

Studies have documented that ERG plays a central role in PCa progression due to its ability to enhance tumor growth by promoting inflammatory and angiogenic responses. ERG has also been implicated in the epithelial–mesenchymal transition (EMT) in PCa cells, which increases the ability of cancer cells to metastasize.

In vivo, research has demonstrated that higher levels of ERG expression are involved with nuclear pleomorphism that prompts hyperplasia and the loss of cell polarity



Further details on pathway elements are shown below from Kish et al.



3.3.3 MSR1

From NCBI[20]:

This gene encodes the class A macrophage scavenger receptors, which include three different types (1, 2, 3) generated by alternative splicing of this gene.

These receptors or isoforms are macrophage-specific trimeric integral membrane glycoproteins and have been implicated in many macrophage-associated physiological and pathological processes including atherosclerosis, Alzheimer's disease, and host defense.

The isoforms type 1 and type 2 are functional receptors and are able to mediate the endocytosis of modified low density lipoproteins (LDLs). The isoform type 3 does not internalize modified LDL (acetyl-LDL) despite having the domain shown to mediate this function in the types 1 and 2 isoforms. It has an altered intracellular processing and is trapped within the endoplasmic reticulum, making it unable to perform endocytosis. The isoform type 3 can inhibit the function of isoforms type 1 and type 2 when co-expressed, indicating a dominant negative effect and suggesting a mechanism for regulation of scavenger receptor activity in macrophages.

As Gudgeon et al note:

Macrophage scavenger receptor 1 (MSR1), also named CD204, holds key inflammatory roles in multiple pathophysiologic processes. Present primarily on the surface of various types of

[20] https://www.ncbi.nlm.nih.gov/gene/4481

macrophage, this receptor variably affects processes such as atherosclerosis, innate and adaptive immunity, lung and liver disease, and more recently, cancer.

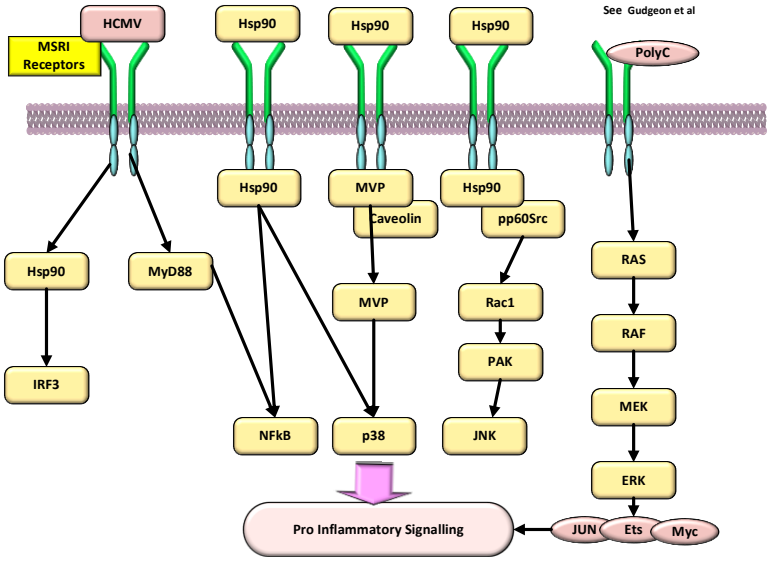
As highlighted throughout this review, the role of MSR1 is often dichotomous, being either host protective or detrimental to the pathogenesis of disease.

We will discuss the role of MSR1 in health and disease with a focus on the molecular mechanisms influencing MSR1 expression, how altered expression affects disease process and macrophage function, the limited cell signalling pathways discovered thus far, the emerging role of MSR1 in tumour associated macrophages as well as the therapeutic potential of targeting MSR1. …

The immunomodulatory effects of MSR1 were also confirmed during radiation therapy for prostate cancer.

Combination of radio and immunotherapy is beneficial in local tumour control as irradiation results in tumour-specific antigen shedding. These antigens can then be processed by antigen presenting cells such as DCs, ultimately resulting in an anti-tumour immune response. In situ vaccination with DCs in which MSR1 had been downregulated, alongside ionizing radiation, significantly suppressed the growth of murine prostate cancer and a reduction in distant metastases was also seen. Recapitulating earlier findings, a significant increase in tumour infiltrating CD8+ T cells was identified …

MSR1 as a ligand shows its control over multiple pathways as shown below.



3.3.4 MUC1

As NCBI notes[21]:

This gene encodes a ***membrane-bound protein that is a member of the mucin family****.*

Mucins are O-glycosylated proteins that play an essential role in forming protective mucous barriers on epithelial surfaces. These proteins also play a role in intracellular signaling.

This protein is expressed on the apical surface of epithelial cells that line the mucosal surfaces of many different tissues including lung, breast stomach and pancreas. This protein is proteolytically cleaved into alpha and beta subunits that form a heterodimeric complex. The N-terminal alpha subunit functions in cell-adhesion and the C-terminal beta subunit is involved in cell signaling.

Overexpression, aberrant intracellular localization, and changes in glycosylation of this protein have been associated with carcinomas. This gene is known to contain a highly polymorphic variable number tandem repeats (VNTR) domain. Alternate splicing results in multiple transcript variants

As Brayman et al note:

Mucins have numerous functions in the glycocalyx. Their high degree of glycosylation provides lubrication, prevents dehydration, and offers protection from proteolysis. Microbial challenge is frequent in most mucous membranes, and mucins protect against attack by sterically inhibiting microbial access to the cell surface. Bacterial adhesins bind mucin carbohydrates at the cell surface, a process that normally protects against infection. In addition, extended transmembrane mucins, such as MUC1 and MUC4, modulate cell-cell and cell-extracellular matrix (ECM) interactions by steric hindrance.

In fact, overexpression of MUC1 in tumor cells is suggested to promote metastasis through disruption of these interactions. This activity is directly related to the number of tandem repeats in the ectodomains of MUC1 and MUC4 since reduction of these motifs alone makes these molecules ineffective inhibitors of cell-cell and cell-ECM interactions.

In addition to mechanical functions, the MUC1 cytoplasmic tail has been shown to associate with β-catenin, as well as with other signaling molecules, e.g., Grb2/Sos, suggesting a potential role for MUC1 in cell signaling. In the mammary gland, MUC1 expression increases markedly during lactation along with increased MUC1:erbB1 interactions. Tyrosine phosphorylation of the MUC1 cytoplasmic tail occurs in both intact MUC1 and chimeric molecules consisting of CD8 ectodomains and the MUC1 cytoplasmic tail. It is not clear if MUC1 phosphorylation or interactions with signal transducing proteins change in response to physiological stimuli. Activation of erbB1 with EGF induces tyrosine phosphorylation of the MUC1 cytoplasmic tail and activation of ERK 1/2.

[21] https://www.ncbi.nlm.nih.gov/gene/4582

Moreover, EGF mediated activation of ERK 1/2 is drastically enhanced in the presence of high levels of MUC1 in the mouse mammary gland. Thus, potential stimuli, including growth factors or cytokines directly or through activation of their receptors may affect MUC1 stability, localization at the cell surface, or phosphorylation state. Direct interactions with the MUC1 ectodomain, e.g., by microbes or selectins, also could conceivably trigger signaling events. In this regard, increased tyrosine phosphorylation of the MUC1 cytoplasmic tail is associated with cell-substratum adhesion.

Thus, MUC1, and perhaps other mucins, have the potential to function as receptors either alone or in cooperation with known signal transducing proteins.

3.3.5 ACAT1

As NCBI notes[22]:

This gene encodes a mitochondrially localized enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA. *Defects in this gene are associated with 3-ketothiolase deficiency, an inborn error of isoleucine catabolism characterized by urinary excretion of 2-methyl-3-hydroxybutyric acid, 2-methylacetoacetic acid, tiglylglycine, and butanone*

As Goudarzi notes:

Acetoacetyl-CoA thiolase also known as acetyl-CoA acetyltransferase (ACAT) corresponds to two enzymes, one cytosolic (ACAT2) and one mitochondrial (ACAT1), which is thought to catalyse reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA during ketogenesis and ketolysis respectively. In addition to this activity, ACAT1 is also involved in isoleucine degradation pathway.

Deficiency of ACAT1 is an inherited metabolic disorder, which results from a defect in mitochondrial acetoacetyl-CoA thiolase activity and is clinically characterized with patients presenting ketoacidosis.

In this review I discuss the recent findings, which unexpectedly expand the known functions of ACAT1, indicating a role for ACAT1 well beyond its classical activity. Indeed ACAT1 has recently been shown to possess an acetyltransferase activity capable of specifically acetylating Pyruvate DeHydrogenase (PDH), an enzyme involved in producing acetyl-CoA. ACAT1-dependent acetylation of PDH was shown to negatively regulate this enzyme with a consequence in Warburg effect and tumor growth. Finally, the elevated ACAT1 enzyme activity in diverse human cancer cell lines was recently reported. These important novel findings on ACAT1's function and expression in cancer cell proliferation point to ACAT1 as a potential new anti-cancer target.

[22] https://www.ncbi.nlm.nih.gov/gene/38

3.3.6 DAPK1

As NCBI notes[23]:

Death-associated protein kinase 1 is a positive mediator of gamma-interferon induced programmed cell death.

DAPK1 encodes a structurally unique 160-kD calmodulin dependent serine-threonine kinase that carries 8 ankyrin repeats and 2 putative P-loop consensus sites. It is a tumor suppressor candidate. Alternative splicing results in multiple transcript variants.

As Singh et al note:

Death-Associated Protein Kinase 1 (DAPK1) belongs to a family of five serine/threonine (Ser/Thr) kinases that possess tumor suppressive function and also mediate a wide range of cellular processes, including apoptosis and autophagy.

The loss and gainof–function of DAPK1 is associated with various cancer and neurodegenerative diseases respectively.

In recent years, mechanistic studies have broadened our knowledge of the molecular mechanisms involved in DAPK1-mediated autophagy/apoptosis. In the present review, we have discussed the structural information and various cellular functions of DAPK1 in a comprehensive manner. …

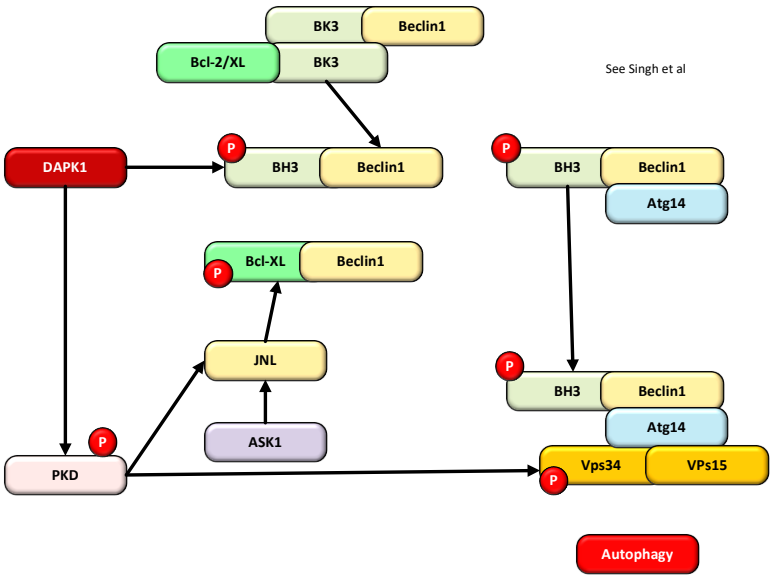
Death-associated protein kinase 1 (DAPK1), a part of a family of Ser/Thr kinase, was originally isolated in an unbiased antisense based genetic screen for genes whose protein products were imperative for interferon Gamma (IFN-g) induced death in HeLa cells, and identified by a functional cloning based on its involvement in interferon-g-induced apoptosis. DAPK1 is an important regulator of cell death and autophagy which act as a critical component in the ER stress-induced cell death pathway. It is a stress-responsive serine/threonine (Ser/Thr) kinase, which constitutes a critical integration point in ER stress signaling, transmitting these signals into two distinct directions, caspase activation and autophagy, leading to cell death.

DAPK1 is a mediator of pro-apoptotic pathway, involved in multiple cell death processes induced by various internal and external apoptotic stimulants.

This pro-apoptotic Ser/Thr kinase regulates both type I apoptotic (caspase-dependent) and type II autophagic (caspaseindependent) cell death signal. On the other hand, DAPK1 is a tumor suppressor gene, known to suppress tumor growth and metastasis by promoting autophagy and apoptosis

The authors note the pathway as shown below:

[23] https://www.ncbi.nlm.nih.gov/gene/1612



3.4 SMAD: A COMMON ELEMENT

We now consider SMADs. Although not one of the targeted genes it does have an integrating property. SMADs are a significant family of gene products that facilitate a variety of transcriptions. They are driven by the TGF family of ligands and have been known to have a place in cancer proliferation.

3.4.1 SMAD Family

SMADs are a set of signal transducers that assist the TGF bindings to effect cellular action via expression of a variety of genes. As Hill notes:

The transforming growth factor-β (TGF-β) family of ligands elicit their biological effects by initiating new programs of gene expression.

The best understood signal transducers for these ligands are the SMADs, which essentially act as transcription factors that are activated in the cytoplasm and then accumulate in the nucleus in response to ligand induction where they bind to enhancer/promoter sequences in the regulatory regions of target genes to either activate or repress transcription. …

The SMAD complexes have weak affinity for DNA and limited specificity and, thus, they cooperate with other site-specific transcription factors that act either to actively recruit the SMAD complexes or to stabilize their DNA binding. In some situations, these cooperating transcription factors function to integrate the signals from TGF-β family ligands with environmental cues or with information about cell lineage. Activated SMAD complexes regulate transcription via remodeling of the chromatin template.

Consistent with this, they recruit a variety of coactivators and corepressors to the chromatin, which either directly or indirectly modify histones and/or modulate chromatin structure.

SMADs thus act in conjunction with other transcription factors. Moustakas et al have noted:

Smad proteins transduce signals from transforming growth factor-β (TGF-β) superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases. Phosphorylation of ***receptor-activated*** *Smads (R-Smads) leads to formation of complexes with the common mediator Smad (Co-Smad), which are imported to the nucleus. Nuclear Smad oligomers bind to DNA and associate with transcription factors to regulate expression of target genes. Alternatively, nuclear R-Smads associate with ubiquitin ligases and promote degradation of transcriptional repressors, thus facilitating target gene regulation by TGF-β. Smads themselves can also become ubiquitinated and are degraded by proteasomes.*

Finally, the ***inhibitory Smads (I-Smads)*** *block phosphorylation of R-Smads by the receptors and promote ubiquitination and degradation of receptor complexes, thus inhibiting signalling.*

Namely there are multiple functions with various SMADs, some activate, some repress, some assist. Yet in all cases they have a close relationship with TGF.

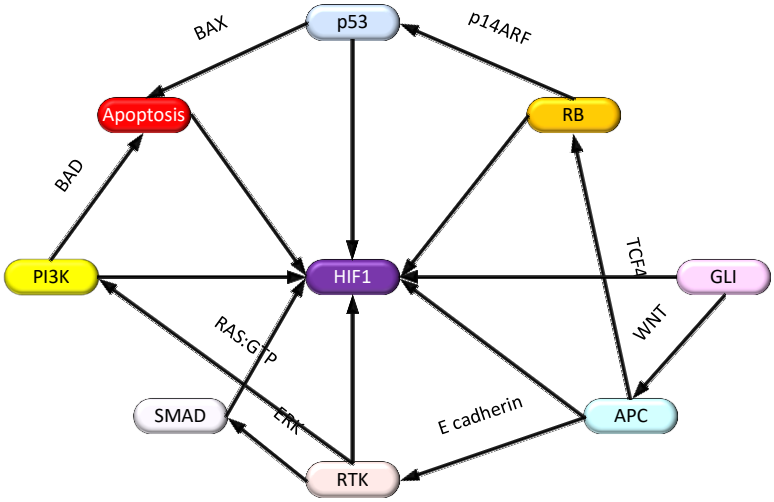
As Lamouille and Derynck have noted:

TGF-β family proteins signal through Smads, which combine with DNA sequence-specific transcription factors to activate or repress transcription. The Smad pathway, which, in the case of TGF-β, is mediated by Smad2 and Smad3 in combination with Smad4, is considered to be the major TGF-β family signaling pathway and accounts for the many changes in gene expression observed in response to TGF-β family proteins. TGF-β–induced non- Smad pathways have been identified and lead to the activation of Erk and JNK MAPK or RhoA, but how these pathways are activated in response to TGF-β is not well understood. In TGF-β– induced EMT, Smad signaling represents an essential pathway that confers changes in gene expression through cooperation with transcription factors such as Snail, Slug, and/or Id. Non-Smad signaling in response to TGF-β (e.g., activation of RhoA) also contributes to EMT and is important for the associated cytoskeletal and phenotypic changes.

Our results now show that TGF-β can increase protein synthesis in EMT through the mTOR pathway, leading to the regulation of S6K1 and 4E-BP1 activities. The activation by TGF-β of a pathway that leads directly to increased protein synthesis stands in contrast with the changes in gene transcription through the Smad pathway.

Thus, in addition to changes in gene expression, TGF-β signaling through mTOR leads to the enhanced translation of proteins that contribute to the behavior of cells that undergo EMT. Accordingly, studies using rapamycin have implicated mTOR in the regulation of collagen synthesis. A characterization of the relative changes in protein levels that are independent of changes in gene expression and can be blocked by rapamycin will provide insight into the contribution of mTOR signaling to the cell's response to TGF-β.

Therelationships of SMAD and other key genes is summarized in the following Figure.



Now there are a set of differing SMADs as noted by Moustakas et al who state:

Functionally, Smads fall into three subfamilies:

(i) receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, Smad8), which become phosphorylated by the type I receptors;

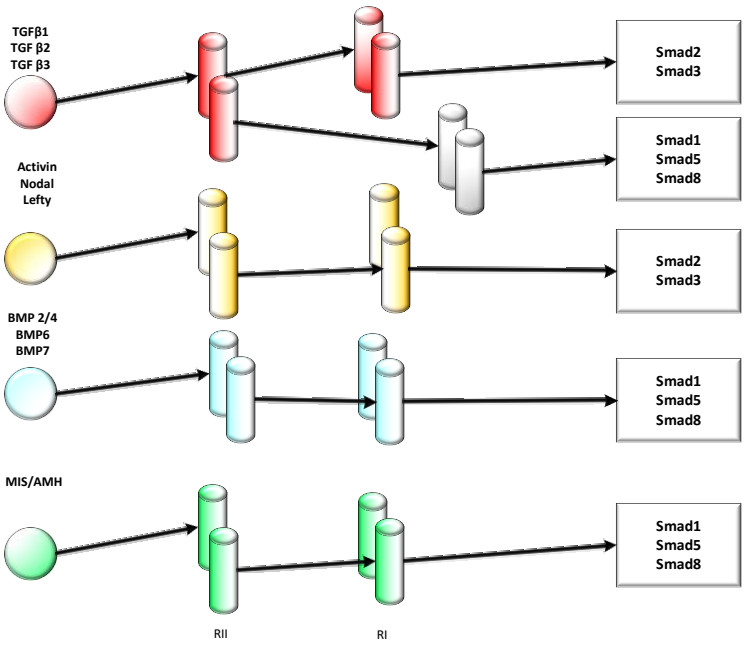
(ii) common mediator Smads (Co-Smads: Smad4), which oligomerise with activated R-Smads; and

(iii) inhibitory Smads (I-Smads: Smad6 and Smad7), which are induced by TGF-b family members. The latter exert a negative feedback effect by competing with RSmads for receptor interaction and by marking the receptors for degradation.

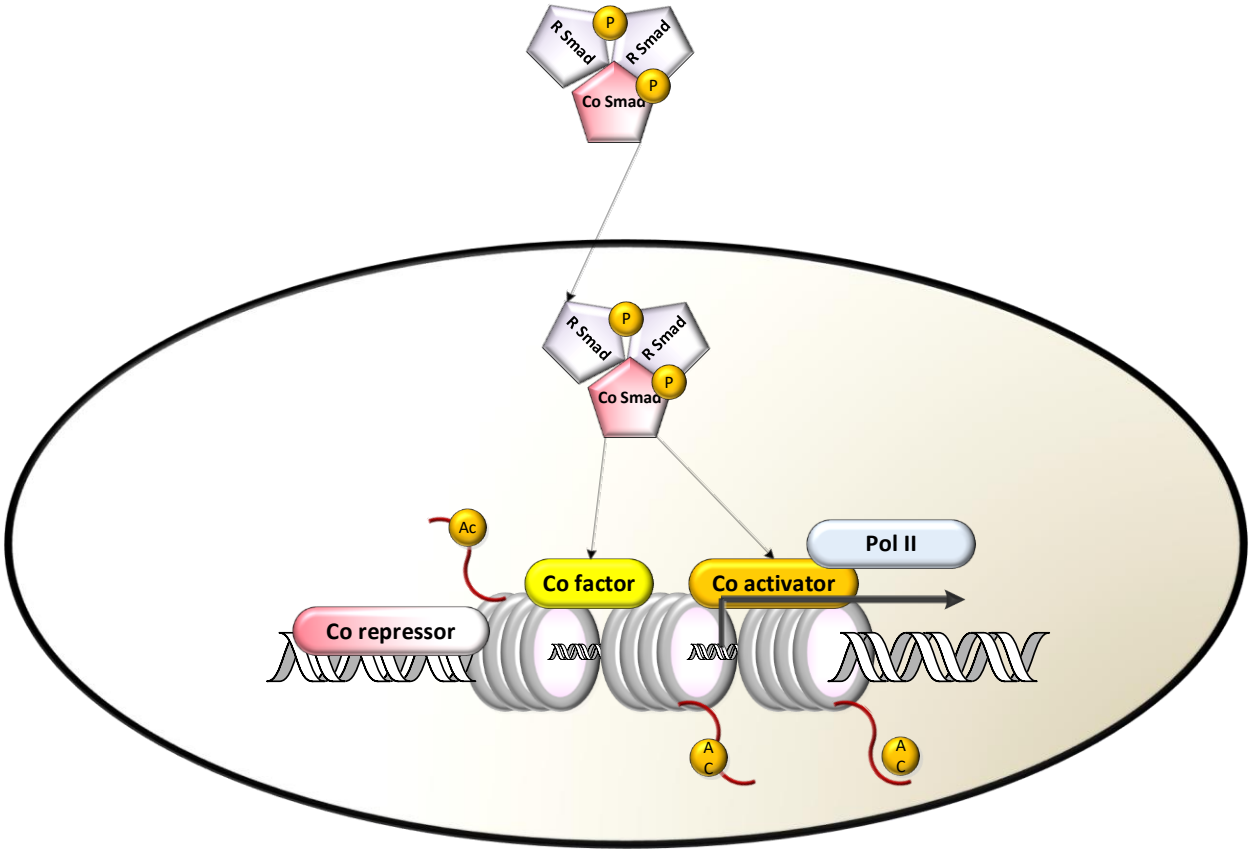
We shall see that TGF and its elements react with all but SMAD4 which is internal to the cell and operates in conjunction with the other SMADs.

3.4.2 SMAD Functioning

We now detail some of the specific SMAD functioning which we referred to above. SMAD plays a role in many of the pathways seen above. We see R Smads, Co Smads and I Smads. The Figure below shows the action in the cell cytoplasm. There is a ligand binding a phosphorylation and then a binding with a Co Smad.



This amalgam then enters the nucleus and acts upon the DNS which is still wrapped in histones and via co-factors and polymerase can result in the expressing of the targeted gene.



3.4.3 Superfamily

Finally, we summarize the SMAD family as a means to best understand its interactions. We use the Figures as modified from Fang and Derynck. First the families of the various SMADs. The authors note:

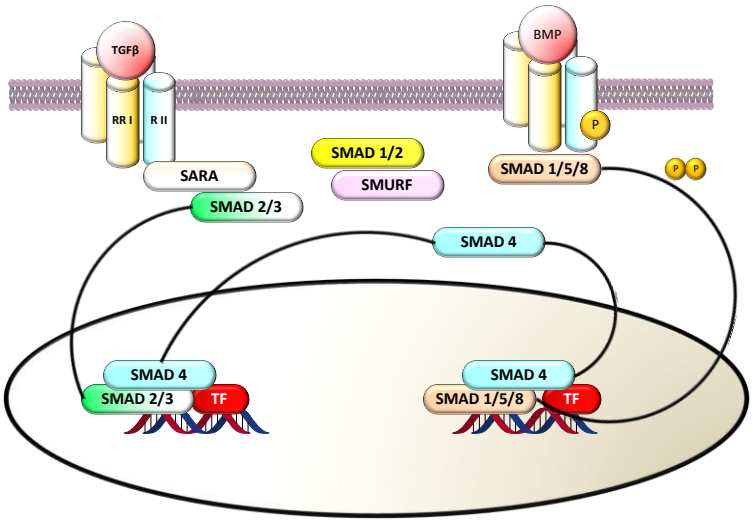
The TGF-β family comprises many structurally related differentiation factors that act through a heteromeric receptor complex at the cell surface and an intracellular signal transducing Smad complex. The receptor complex consists of two type II and two type I transmembrane serine/threonine kinases. Upon phosphorylation by the receptors, Smad complexes translocate into the nucleus, where they cooperate with sequence-specific transcription factors to regulate gene expression.

The vertebrate genome encodes many ligands, fewer type II and type I receptors, and only a few Smads. In contrast to the perceived simplicity of the signal transduction mechanism with few Smads, the cellular responses to TGF-β ligands are complex and context dependent. This raises the question of how the specificity of the ligand-induced signaling is achieved. We review the molecular basis for the specificity and versatility of signaling by the many ligands through this conceptually simple signal transduction mechanism.

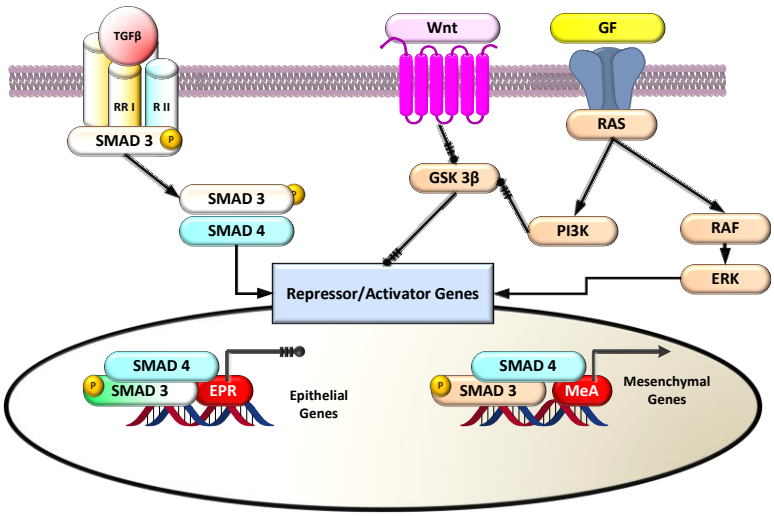
Namely the TGF receptors are a type I and type II each in a dimer configuration and the TFG ligand attaches to the receptor complex. We have shown this above. But now the specific TGF as a ligand activates a different SMAD pathway activity. We can summarize these in some detail in the following Table. Note that there are interactions with RII and RI complexes as shown in the Figure. There are multiple TGF ligands and in turn multiple SMAD reactions.

Ligand	*R II*	*R I*	*SMAD Activation*
TGF-β1 **TGF-β2** **TGF-β3**	TβRII	TβRI	Smad2 Smad3
TGF-β1 **TGF-β2** **TGF-β3**	TβRII	ALK1	Smad1 Smad5 Smad8
Activin **Nodal** **Lefty**	ActRII ActRIIB	ACTRIB ALK7	Smad 2 Smad 3
BMP 2/4 **BMP 6** **BMP 7**	BMPRII BMPRIIB	BMPRIA BMPRIB	Smad1 Smad5 Smad8
MIS/AMH	MISRII	ALK2	Smad1 Smad5 Smad8

The specific actions can be combined as we show below. Here we show both TGFβ1 and BMP each on their own receptors and thus activation separate SMADs and in turn the SMADs entering the nucleus and assisting as transcription factor adjuncts in gene expression.

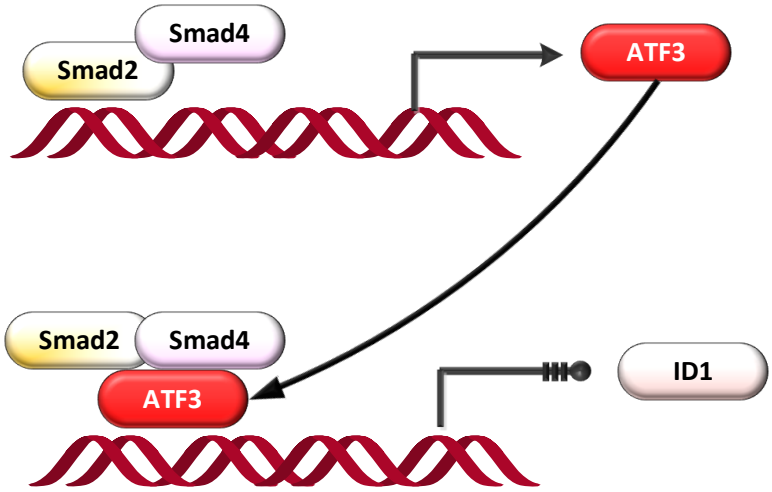


From Fuge et al we also have a detailed description of other ligand and receptors and their actions on other pathway elements and in turn on Smads.:

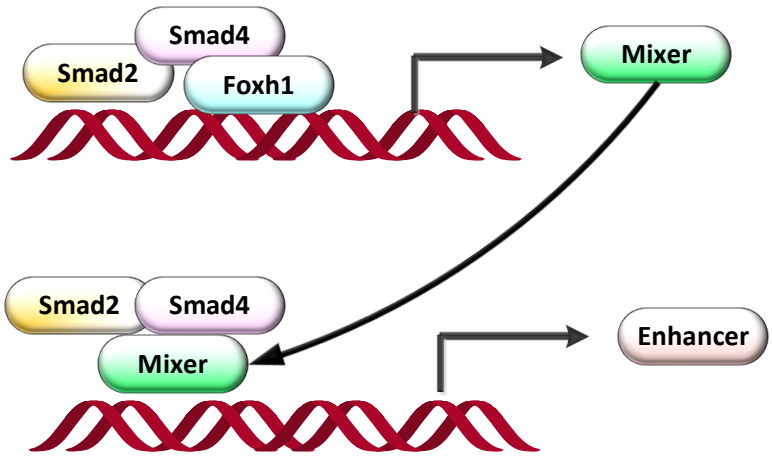


3.4.4 Actions

SMADs can effect a multiplicity of actions. Below from Hill we show a self-enabling (negative) control:



The next is the self-enabling (positive) control.



SMAD genes seems to be playing a significant intermediary roles in the processes effected by the previous sets.

4 PROCEDURE AND PERFORMANCE

We now present a simple overview of the protocols which are available.

4.1 PROCEDURE

There has been a development of a significant number of chromatin conformations procedures developed over the past twenty years. The initial one is the 3C approach which we will focus on, From Crutchley et al we have a detailed list of and explanations of many of the available testing procedures. They are summarized below:

Technique	Genomic resolution/scale/throughput
3C	High resolution Small genomic domains Low throughput
4C	High resolution Genomic environment surrounding a given region Low throughput
5C	High resolution Genome scale High throughput
6C	High resolution Genome-wide contacts associated with a given protein Intermediate throughput
3C-Loop	High resolution Genome-wide contacts associated with a given protein Low throughput
Hi-C	High resolution (proportional to sequencing depth) Genome-wide High throughput
ChlA-PET	High resolution Genome-wide contacts associated with a given protein (proportional to sequencing depth) High throughput
DNA-FISH	Low resolution Genome-wide Low throughput
RNA-TRAP	Intermediate resolution Genomic environment surrounding a given gene Low throughput

4.1.1 3C

The oldest test is 3C. From Crutchly et al:

Chromosome conformation capture was initially developed to study the complete conformation of a chromosome in yeast. 3C is now used as a standard research tool to analyze the organization of complex genomic domains and investigate the relationship between genome architecture and gene expression.

3C can be divided into five experimental steps.

The first step in conventional 3C is to chemically fix cells.

This step captures interactions between DNA regions by crosslinking chromatin-bound histones and other associated proteins such as transcription factors. Thus, chemical fixation produces a snapshot of the 3D chromatin architecture in vivo. Chemical fixation is a common step in all techniques currently used to study genome organization. ***Although unavoidable, it is important***

to note that this step may still introduce artifacts that will be carried over in between approaches.

The second step of 3C consists of digesting the genomic DNA with enzymes.

Enzymatic digestion of chemically fixed chromatin releases DNA fragments that were crosslinked as a result of their physical proximity in the nuclear space.

The third 3C step involves ligation of crosslinked DNA fragments. The DNA is ligated under conditions favoring intramolecular ligation of crosslinked fragments and minimizes random ligation.

During the fourth step of 3C, the DNA is purified to remove all proteins and other contaminants.

The resulting 3C library features pair-wise ligation products between DNA segments that were close to each other in the nuclear space regardless of their linear distance along the genome. The relative abundance of these ligation products is inversely proportional to the original 3D distance separating DNA segments and can therefore be used to reconstruct the spatial organization of the genome in vivo.

The final 3C step consists of measuring the relative abundance of individual ligation products in the library.

3C library products are usually quantified by PCR amplification of ligation junctions and agarose gel detection. ***Alternatively, ligation junctions can be measured by TaqMan quantitative PCR or by melting curve analysis.*** *A major caveat of 3C and 3C-based technologies is that* ***it generates datasets from cell populations and therefore features averaged interaction frequencies derived from various cell cycle states.***

Thus, these technologies yield averaged structural models rather than true structures. Although these models can be noisy, they remain useful to identify changes between cell states

4.1.2 6C

The authors continue:

The combined chromosome conformation capture ChIP cloning (6C) technique is also derived from 3C and is an immediate extension of the 3C-Loop approach.

6C was developed to identify cis or trans long-range DNA interactions mediated by specific proteins without prior knowledge of the regions involved.

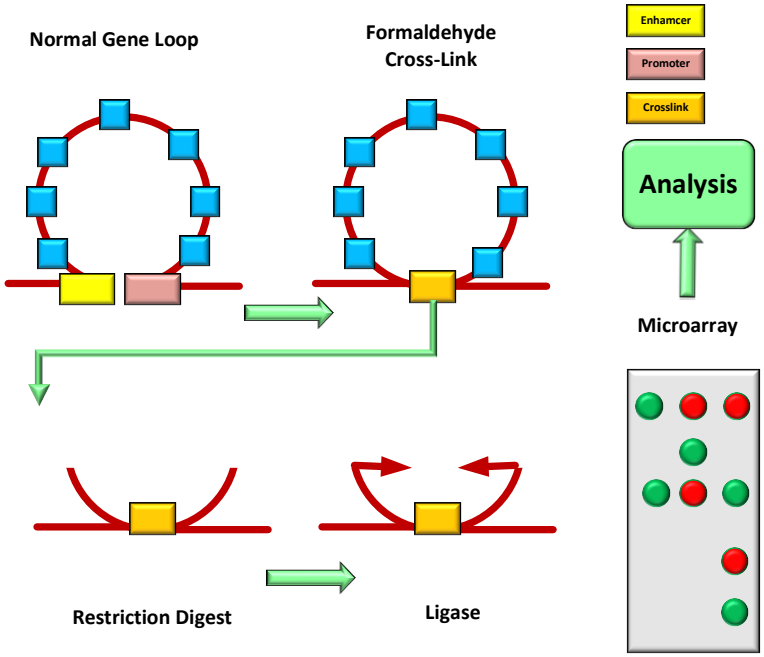
As such, the 6C protocol is identical to 3C-Loop until the library purification step, but then uses a different approach to analyze libraries. During 6C, ligation products are first cloned into vectors rather than analyzed individually by PCR. Individual clones are then amplified and

characterized by restriction digest analysis to identify those containing more than one DNA fragment. Clones with two or more fragments are then sequenced from both ends of the cloning vector to identify interacting sequences.

Although 6C does not quantify DNA contacts like 3C-Loop, the combined cloning/ sequencing shotgun approach qualitatively identifies long-range DNA interactions mediated by specific proteins. The development of 3C technology by Dekker et al. in 2002 prompted the aggressive expansion of alternative 3C-derived approaches to study high-resolution genome organization in vivo. These methods share similar protocols each with advantages and limitations but none are altogether genome-wide, quantitative, high throughput and applicable for ab initio contact identification. However, two state-of-the-art technologies developed over the past year fulfill these criteria. These techniques are called chromatin interaction analysis with paired-end tags (ChIA-PET) and Hi-C.

4.2 Data Analysis Issues

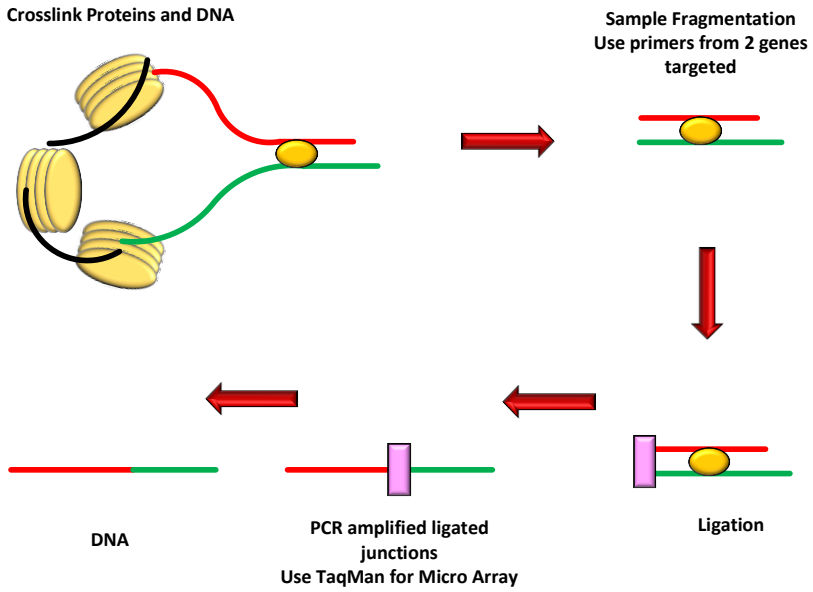
We now provide a high level overview of the principle techniques employed in identifying chromatin conformations in the case of a PCa diagnosis. From Alshaker et al we have the flow of the generalized process shown graphically below.



The blue blocks are histones and the loop is DNA. The process allows for the extraction of selected gene sequences based on the restriction enzyme. The result is processed on a micro array to see which segments are expressed in PCa and then a detailed analysis is performed to assess the choice of segments to maximize performance. However the above is lacking in some fundamental detail.

Below we show a 3C design. We have a set of histones and two separate strands of DNA, perhaps from two chromosomes, wrapped around a protein such as an enhancer or promoter. This co-location is a classic example of what we see in chromatin confirmation. The process flow as shown. We first remove the two strands by classic restriction enzymes. That separates the two gene strips yet still bound to the protein.

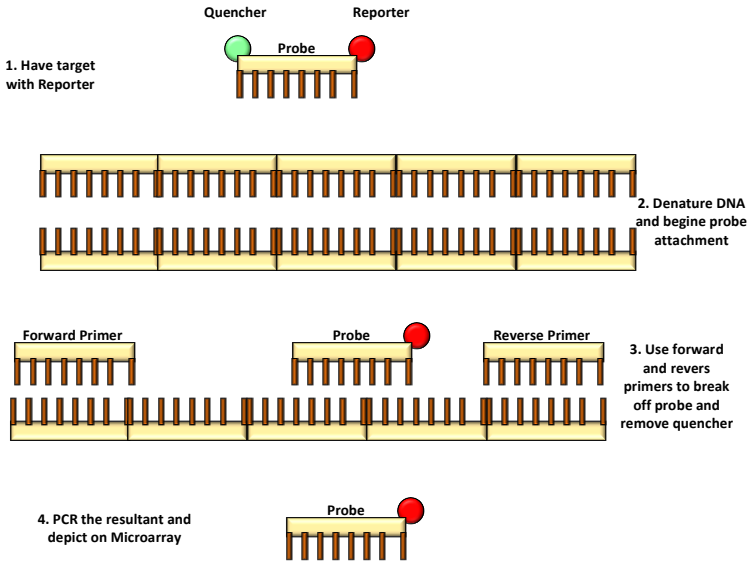
We then ligate the two by another protein and remove the first one. The resulting set of DNA are now joined, the proteins removed and PCR applied to amplify.



See Illumina DNA SEQUENCING METHODS COLLECTION, www.illumina.com/science/sequencing-method-explorer.html

Having a set of these amplified DNA we can now use a TaqMan approach of labelling the sequences for use in a Microarray[24]. We show an example of this below.

[24] See Hofmann and Clokie



The net result will be a microarray with multiple inputs from various patients with PCa and across rows will be evidence of presence or absence of the putative gene pairs.

The final result is passed through some form of pattern recognition identifier or classifier for diagnostic accuracy[25]. The details of the classifier and its algorithm are lacking in what we have found in the literature.

[25] See https://www.researchgate.net/publication/344565580_Correlation_vs_Causation_The_Perils_of_AI We examine such classifiers using various AI approaches.

5 OBSERVATIONS

We now examine several observations flowing from the above discussion.

5.1 DETAILS ON METHODOLOGY OF SELECTION

There are published protocols for many CCS approaches. However the protocol at the focal point of this report has limited exposure. The EpiSwitch approach starts with a massive number of genes and then selects down to a small number that separate patients with/without PCa. It employs a 3C type approach with a TaqMan measurement followed by a proprietary selection process. Since it is proprietary one cannot attempt to replicate it. Then the use of continuous PSA rather than a select threshold value alleges yields excellent results.

5.2 PATHWAYS, INITIATION, AND AGGRESSIVENESS

Genomic alterations have been the normal approach to assessing the complexity of the malignancy. Have PTEN or p53 been lost, are there gene fusions? Methylation has also become a methodology for gene expression alteration. To provide a significant therapeutic we must have more than just a good estimate on the presence of PCa.

5.3 CLINICAL VALIDATION IN LARGER GROUPS

The number of patients in the pool for EpiSwitch is quite small and for the results to be valid we need a substantially larger base. Thus how large a sample is necessary? Furthermore what are the bases for an improper classification? Has a positive test merely shown a cancer in some other organ, making the PSA component irrelevant?

5.4 USE OF MULTIPLE TESTS

There is the question of the return on multiple tests. The tests discussed herein use the standard PSA plus the genes they have identified. One may ask if the genes are optimum, would three be fine would seven be better. The objective in diagnosis is to safely avoid a biopsy.

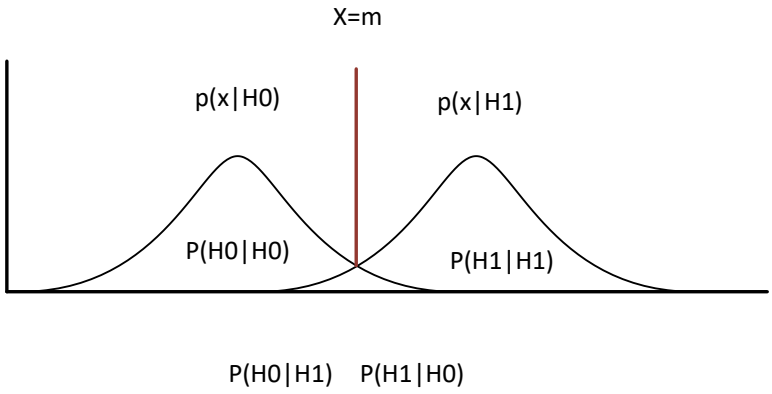
5.5 RISK VS RETURN OF BIOPSY VS TESTING

Biopsy of the prostate can result in morbidity. It also does not present a fully dispositive result. For example the resulting Gleason score may often be under-estimated. Surgical removal of the prostate is generally the only way to assess the state of the cancer. It allows for morphological and genetic profiling and the latter may very well present therapeutic focused options. Thus adding more tests may just focus biopsy results.

5.6 SENSITIVITY, SPECIFICITY AND TESTS

Recall that sensitivity is the probability that a patient is said to have PCa given that they actually have it and specificity is the probability that a we say patient does not have PCa when they

actually do not have PCa. The graphic below demonstrates this concept. As the distance between the peaks decreases then both measures decrease. As the variance increases, spreading out the curve, we see a similar effect.



Thus to achieve his sensitivity and specificity we need a wide distance between the metrics and as well a high level of certainty, or low variance.

Generally the more independent variable used for testing the better the overall performance.

5.7 WHY THESE GENES: WHAT DO THEY DO?

We have examined the genes the approach selects. The question; why these genes? Is really more than just running 3C on thousands a picking the top 5-6. As we noted each of these genes has a functional role. If that is the case then can we remediate the problem that is impeding their functioning.

5.8 IS THERE REALLY ANY DNA THERE AND FROM WHERE?

One challenge in obtaining DNA or even other more dispositive markers is to be able to assert its origin. Namely, we can extract cfDNA in the blood, along with many other things, but we cannot assert its source.

5.9 ARE THERE BASES FOR THERAPEUTICS?

If chromatin conformation issues are principal actors in cancer initiation and growth then are there possible therapeutics that may ensure from this understanding? If as is asserted these are genes and epigenetic factors leading to malignancies then what can be done to neutralize them? At this stage this is still a question with what appears to have little to go on.

5.10 WHAT ARE THE INTERACTIONS?

The selection of targets seems to be fortuitous. It does not appear to have been selected in some well-established holistic manner based on gene interactions. It would be useful, as we have

attempted to do herein, to understand why the gene targets are really targets and that there are or are not underlying controller genes. Furthermore this approach shows no regard for such things as the tumor microenvironment and immune system responses.

5.11 What is the impact of Extrachromosomal DNA?

What we have been discussing thus far is all occurring in the nucleus and interacting with the area of a chromosome or even inter-chromosome. But recent work has demonstrated that extrachromosomal DNA, ecDNA, has a controlling impact as well[26]. As NCI has noted[27]:

Extrachromosomal circular DNAs (ecDNAs), or particles of DNA existing outside the autosomal genome, were discovered in the 1960s and more recently have been implicated in cancer development. EcDNAs frequently occur across many cancer types and often in high copy numbers. The oncogenes they carry are thought to be highly expressed compared to copy number-matched linear DNA. Cancers carrying ecDNAs are also associated with shorter survival for patients. … Extrachromosomal circular DNA elements are pieces of DNA that have broken off the linear chromosomes and circularized. There are two types: small 100 bp – 10 kb elements that can be found in many different cell types in the body, with unknown function. And then there are larger (50 kb – 5 Mb) oncogenic elements, which are only detected in cancer cells and carry genes known to activate cancer cells. These oncogenic ecDNAs are found in ~15% of newly diagnosed cancer. … These are methods used to map genome-wide, long-range chromatin interactions between regulatory elements. ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag sequencing) is a method we developed to reveal the general spatial chromatin organization and to identify chromatin interactions associated with specific proteins. The resulting paired sequences from ChiA-PET tell us about the connectivity between different genomic regions and the 3D organization of the chromatin. While informative, ChIA-PET is limited in that it is only telling us about pairwise interactions aggregated from bulk cells—we don't know whether or not those pairs are occurring together within a single complex. So we have developed the ChIA-Drop (Chromatin Interaction Analysis by Droplet sequencing) chromatin interaction method to identify the combinations of chromatin interactions that occur within a single complex. … Our goal was to determine the spatial chromatin organization and chromatin interactions of ecDNAs in general. Given the circular structure of ecDNAs, we anticipated that they would exhibit unique spatial patterns. We uncovered very high levels of chromosome connectivity and transcriptional activity: the ecDNAs exhibit a pattern of dense and widespread chromatin interactions with actively transcribed genes and regulatory elements that reside both within the ecDNA (in cis) and on the chromosomes (in trans). We reasoned that this is because the small size of ecDNAs allow them to move freely amongst the chromosomes. Such mobility could enable ecDNAs to interact with genes residing on chromosomes. Moreover, the interaction sites on the ecDNAs exhibited key characteristics of super-enhancers (SEs), which are known to exert a unique regulatory influence that could promote tumorigenesis. … With its mobility and potentially high copy numbers, ecDNAs can potentially transverse the nucleus, and function as trans-acting, mobile transcriptional enhancers, establishing extensive chromosomal

[26] https://news.cancerresearchuk.org/2023/02/20/how-ecdna-drives-cancer-evolution/

[27] https://www.cancer.gov/about-nci/organization/ccg/blog/2022/interview-ecdna

interactions and driving transcription of specific chromosomal genes. Thus ecDNAs may be a very powerful mechanism to promote cellular fitness. … We observed a sharp elevation of nTIFs in ecDNA regions compared to the interaction frequencies of their corresponding native chromosomal regions in cells without ecDNAs. These interactions were specifically enriched with chromosomal promoters. Chromosomal genes whose promoters interact with ecDNAs were expressed at significantly higher levels.

There has recently been a great deal of investigation of this area[28]. Thus an investigation combining these areas appears to present a significant opportunity.

[28] See Wang et al, Zhao et al, Zuo et al, Kim et al, and Li et al

6 REFERENCES

1. Adamo and Ladomery, The oncogene ERG: a key factor in prostate cancer, Oncogene (2016) 35, 403–414
2. Adhikary et al, Circular PVT1: an oncogenic non-coding RNA with emerging clinical importance, Jrl Clin Path 2019.
3. Albany et al, Epigenetics in Prostate Cancer, Prostate Cancer Volume 2011, Article ID 580318, 12 pages, 2011
4. Allis, C. et al, Epigenetics, Cold Spring Press (Cold Spring Harbor, NY), 2007.
5. Almeida et al, Chromatin conformation regulates the coordination between DNA replication and transcription, Nature Communications , (2018) 9:1590
6. Alpha et al, Actin Cytoskeleton in Cancer Progression and Metastasis - Part A , International Review of Cell and Molecular Biology, 2020
7. Alshaker et al, Chromatin conformation changes in peripheral blood can detect prostate cancer and stratify disease risk groups, J Transl Med (2021) 19:46
8. Anastasiadou, E., F. Slack, Malicious exosomes, Science, 19 December 2014 Science Sciencemag.Org Vol 346 Issue 6216, Pp 1459-1460.
9. Armstrong et al, Chromatin Deregulation in Cancer, CSHL Press, 2017
10. Ayala GE, Dai H, Powell M, et al. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin Cancer Res 2008; 14: 7593-603.
11. Baker, M. Long noncoding RNAs: the search for function, Nature Methods, VOL.8 NO.5, May 2011, p| 379.
12. Baylin, S., Understanding DNA Methylation and Epigenetic Gene Silencing in Cancer, 2004 Current Therapeutics Inc.
13. Bazett-Jones and Dellaire, The Functional Nucleus, Springer, 2016
14. Bejar, R., et al, Clinical Effect of Point Mutations in Myelodysplastic Syndromes, NEJM, June, 2011.
15. Beltran, et al, Molecular characterization of neuroendocrine Prostate cancer and identification of new Drug Targets, November 2011, Cancer Discovery, p 487
16. Berghe W., Epigenetic impact of dietary polyphenols in cancer chemoprevention: Lifelong remodeling of our epigenomes, Pharmacological Research 65 (2012) 565– 576
17. Berman-Booty and Knudsen, Models of neuroendocrine prostate cancer, Bioscientifica, 2015
18. Boltsis et al, Chromatin Conformation in Development and Disease, Frontiers in Cell and Developmental Biology, 1 August 2021, Volume 9, Article 723859
19. Boumber, Y., et al, Epigenetics in Cancer: What's the Future?, ONCOLOGY. Vol. 25 No. 3 March 16, 2011

20. Braadland et al, Low beta(2)-adrenergic receptor level may promote development of castration resistant prostate cancer and altered steroid metabolism. OncoTarget, 7(2): 1878-1894, 2015

21. Braicu, C., et al, Exosomes as divine messengers: are they the Hermes of modern molecular oncology?, Cell Death and Differentiation (2015) 22, 34–45

22. Brayman et al, MUC1: A multifunctional cell surface component of reproductive tissue epithelia, Reproductive Biology and Endocrinology 2004, 2:4

23. Brena, R., et al, Mining Methylation for Early Detection of Common Cancers, PLOS Medicine December 2006, Volume 3, Issue 12|.

24. Brooks, C., W. Gu, How does SIRT1 affect metabolism, senescence and cancer?, Nature Reviews Cancer 9, 123-128 (February 2009)

25. Brooks, J., et al, CG Island Methylation Changes Near the GSTP1 Gene in Prostatic Intraepithelial Neoplasia, Can Epid 1998, pp 531-536.Donkena, K., et al, Oxidative Stress and DNA Methylation in Prostate Cancer, Ob & Gyn Internat 2010, pp .

26. Brower, V., Unravelling the cancer code, Nature, March, 2011.

27. Burotto et al, The MAPK pathway across different malignancies: A new perspective, Cancer. 2014 November 15; 120(22): 3446–3456

28. Cabili et al, (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev. 25,1915-1927.

29. Cantley et al, Signal Transduction, CSHL, 2013

30. Chang et al, Joint Effect of HSD3B1 and HSD3B2 Genes Is Associated with Hereditary and Sporadic Prostate Cancer Susceptibility, CANCER RESEARCH 62, 1784–1789, March 15, 2002

31. Chen and Ayala, Innervating Prostate Cancer, NEJM 15 Feb 2018.

32. Chen et al, Genetic and epigenetic regulation of the organic cation transporter 3, SLC22A3, The Pharmacogenomics Journal volume 13, pages 110–120 (2013)

33. Connolly et al, Complexities of TGF-β Targeted Cancer Therapy, Int Jrl Bio Sci, 2012; 8(7):964-978.

34. Crane and Berx, Regulatory networks defining EMT during cancer initiation and progression, Nature Reviews Cancer 13,97-110 (2013)

35. Croce, C., Causes and consequences of microRNA dysregulation in cancer, Nature RE REVIEWS, Genetics, 704, October 2009, VOL 10.

36. Crutchley et al, Chromatin conformation signatures: ideal human disease biomarkers?, Biomarkers Med. (2010) 4(4), 611–629

37. da Silva, H., et al, Dissecting Major Signaling Pathways throughout the Development of Prostate Cancer, Hindawi Publishing Corporation Prostate Cancer Volume 2013, Article ID 920612, 23 pages

38. Das, P., R. Singal, DNA Methylation and Cancer, Jrl Clin Onco, Nov 2004.

39. DeCraene and Berx, Regulatory networks defining EMT during cancer initiation and progression, Nature Reviews Cancer volume 13, pages 97–110 (2013)

40. Derrien et al, (2012). The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 22, 1775-1789.

41. Derynck et al, TGF-β signaling in tumor suppression and cancer progression, Nature Genetics 29, 117–129 (2001)

42. DeVita, Hellman, and Rosenberg, Cancer: Principles & Practice of Oncology, 12th Ed, Wolters Kluwer (New York) 2023.

43. Di Croce, L., et al, Methyltransferase Recruitment and DNA Hypermethylation of Target Promoters by an Oncogenic Transcription Factor, Science Vol 295 8 February 2002.

44. Di Sante, G., et al, Loss of Sirt1 Promotes Prostatic Intraepithelial Neoplasia, Reduces Mitophagy, and Delays Park2 Translocation to Mitochondria

45. Dittmer, The Biology of the Ets1 Proto-Oncogene, Molecular Cancer volume 2, Article number: 29 (2003)

46. Dominy, J., et al, The Deacetylase Sirt6 Activates the Acetyltransferase GCN5 and Suppresses Hepatic Gluconeogenesis, Molecular Cell 48, 900–913, December 28, 2012

47. Donkena K., et al, Oxidative Stress and DNA Methylation in Prostate Cancer, Obs & Gyn Int 2010, pp.

48. Ehrlich, M., DNA methylation in cancer: too much, but also too little, Oncogene (2002) 21, 5400 – 5413.

49. Ellis and Loda, Advanced neuroendocrine prostate tumors regress to stemness, PNAS, November 24, 2015, vol. 112, no. 4

50. Esteller, M., Epigenetics in Cancer, NEJM, March 2008.

51. Feinberg, A., et al, The epigenetic progenitor origin of human cancer, Nature Reviews, Genetics Volume 7, January 2006.

52. Feldman and Feldman, The development of androgen-independent prostate cancer, Nature Reviews , Cancer Volume 1 , October 2001 , 35

53. Feng and Derynck, Specificity and Versatility in TGF-β Signaling Through Smads, Annu. Rev. Cell Dev. Biol. 2005. 21: 659-93

54. Fuxe et al, Transcriptional crosstalk between TGF-β and stem cell pathways in tumor cell invasion, Cell Cycle 9:12, 2363-2374; June 15, 2010

55. Gal-Yam, E., et al, Cancer Epigenetics: Modifications, Screening, and Therapy, Annu. Rev. Med. 2008. 59:267–80.

56. Goldstein, A., et al, Identification of a Cell of Origin for Human Prostate Cancer, Science, Vol 329, July 2010, p 568.

57. Gonzalgo, M., et al, Prostate Cancer Detection by GSTP1 Methylation Analysis of Post biopsy Urine Specimen, Clin Can Res, 2003, pp 2673-2677.

58. Goudarzi, The recent insights into the function of ACAT1: A possible anti-cancer therapeutic target, Life Sciences Volume 232, 1 September 2019, 116592

59. Greenberg, P, et al, Revised International Prognostic Scoring System for Myelodysplastic Syndromes, Blood, 20 September 2012 Volume 120, Number 12.

60. Griffith, D., Targeting aggressive prostate cancer, Eureka, http://www.eurekalert.org/pub_releases/2013-08/uoc--tap081213.php , 14 August 2013.

61. Grigore et al, Prostate cancer and neuroendocrine differentiation: more neuronal, less endocrine?, Frontiers in Oncology, March 2015, Volume 5, Article 37

62. Guatente, L., Sirtuins, Aging, and Medicine, NEJM, Vol 364 June 2011

63. Gudgeon et al, The role of macrophage scavenger receptor 1 (MSR1) in inflammatory disorders and cancer, Front. Immunol. 13:1012002, 2022

64. Gutman, M., J, Rinn, Modular regulatory principles of large non-coding RNAs, Nature, V 462, 14 Feb 2012.

65. Hagege et al, Quantitative analysis of chromosome conformation capture assays (3C-qPCR), Vol.2 No.7, 2007, Nature Protocols

66. Hall et al, The Prospective Study of Epigenetic Regulatory Profiles in Sport and Exercise Monitored Through Chromosome Conformation Signatures, Genes 2020, 11, 905

67. Han et al, 3C and 3C-based techniques: the powerful tools for spatial genome organization Deciphering, Molecular Cytogenetics (2018) 11:21

68. Hassler, M., G. Egger, Epigenomics of cancer e emerging new concepts, Biochimie 94 (2012) 2219e2230.

69. Hayakawa Y, Wang TC. Nerves switch on angiogenic metabolism. Science 2017; 358: 305-6.

70. Heinlein and Chang, Androgen Receptor in Prostate Cancer, Endocrine Reviews 25(2):276–308, 2004

71. Herman, J., S. Baylin, Gene Silencing in Cancer in Association with Promoter Hypermethylation, NEJM 2003, pp 2042-2034.

72. Heslop, H., et al, Immunotherapy of Hematologic Malignancy, Hematology 2003.

73. Hill, Transcriptional Control by the SMADs, Cold Spring Harb Perspect Biol, 2016

74. Hines, Z., et al, The cAMP/PKA Pathway Rapidly Activates SIRT1 to Promote Fatty Acid Oxidation Independently of Changes in NAD+, Molecular Cell 44, 851–863, December 23, 2011

75. Hofmann and Clokie, Principles and Techniques of Biochemistry and Molecular Biology, Cambridge, 2018

76. Hu et al, Neuroendocrine differentiation in prostate cancer: a mechanism of radioresistance and treatment failure, Frontiers in Oncology, Genitourinary Oncology April 2015, Volume 5, Article 90

77. Huarte, The emerging role of lncRNA in cancer, Nature Medicine 21 2015

78. Introna, M., et al, Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study, Haematologica 2007; 92:952-959.

79. Issa, J., M. Hagop, Targeting DNA Methylation, Clin Cancer Res 2009; V 15: pp 3938-3946.

80. Issa, J-P., Epigenetic Changes in the Myelodysplastic Syndrome, Hematol Oncol Clin North Am. Author manuscript; available in PMC 2011 April 1.

81. Jacobsen, S., Gene Silencing: Maintaining Methylation Patterns, Curr Bio 1999, pp 617-619.

82. Jia, H., Osak, M., Bogu, G.K., Stanton, L.W., Johnson, R., and Lipovich, L. (2010). Genome-wide computational identification and manual annotation of human long noncoding RNA genes. RNA 16, 1478-1487.

83. Jiang et al, ETS proto-oncogene 1 modulates PTP1B expression to participate in high glucose-mediated endothelial inflammation, Acta Biochim Biophys Sin 2022, 54(4): 565–573

84. Jiang, J., et al, Cytokine-induced killer cells promote antitumor immunity, Journal of Translational Medicine 2013, 11:83.

85. Jones, P., M. Gonzalgo, Altered DNA methylation and genome instability: A new pathway to cancer?, Proc. Natl. Acad. Sci. USA Vol. 94, pp. 2103–2105, March 1997

86. Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., Thomas, K., Presser, A., Bernstein, B.E., van Oudenaarden, A., et al. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc. Natl. Acad. Sci. USA 106, 11667-11672.

87. Khanppnavar et al, Structural basis of organic cation transporter-3 inhibition, Nature Communications | (2022) 13:6714

88. Kim et al, Extrachromosomal DNA is associated with oncogene amplification and poor outcome across multiple cancers, Nat Genet. 2020 September ; 52(9): 891–897.

89. Kim, H., et al, Antitumor activity of cytokine-induced killer cells against human lung cancer, International Immunopharmacology 7 (2007) 1802–1807.

90. Kish et al, The Expression of Proto-Oncogene ETS-Related Gene (ERG) Plays a Central Role in the Oncogenic Mechanism Involved in the Development and Progression of Prostate Cancer, Int. J. Mol. Sci. 2022, 23, 4772

91. Kornienko, A., et al, Gene regulation by the act of long non-coding RNA transcription, BMC Biology 2013, 11:59.

92. Kovalchuk, I., O. Kovalchuk, Epigenetics in Health and Disease, FT Press (Upper Saddle River, NJ) 2012.

93. Kovalchuk, I., O., Kovalchuk, Epigenetics, FT Press (Upper Saddle River, NJ) 2012.

94. Kubiczkova et al, TGF-β – an excellent servant but a bad master, Journal of Translational Medicine 2012, 10:183

95. Labbe, D., et al, Role of Diet in Prostate Cancer, the Epigenetic Link, Oncogene, 2014.

96. Laird, P., R. Jaenisch, DNA Methylation and Cancer, Human Molecular Genetics, Vol 3, 1487-95, 1999.

97. Lam, E., et al, FOXO Transcription Factors, Biochem Soc Trans 2006, pp 722-726.

98. Lamouille and Derynck, Cell size and invasion in TGF-β–induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway, The Journal of Cell Biology, Vol. 178, No. 3, July 30, 2007 437–451

99. Lee, Epigenetic Regulation by Long Noncoding RNAs, Science Vol 338 14 December 2012

100. Lefkowitz, G., et al, Follow Up Interval Prostate Biopsy of High Grade Prostatic Intraepithelial Neoplasia is Associated with High Likelihood of Prostate Cancer Independent of Change in Prostate Specific Antigen Levels, Journal of Urology, Vol 168, Oct 2002.

101. Li et al, Long noncoding RNAs in prostate cancer: mechanisms and applications, Molecular & Cellular Oncology 1:3, e963469; October 1, 2014

102. Li et al, Molecular aspects of prostate cancer with neuroendocrine differentiation, Chin J Cancer Res. 2016 Feb; 28(1): 122–129

103. Li et al, Pioneering insights of extrachromosomal DNA (ecDNA) generation, action and its implications for cancer therapy, International Journal of Biological Sciences, 2022; 18(10): 4006-4025.

104. Li, Q., Selective anticancer strategies via intervention of the death pathways relevant to cell transformation, Cell Death and Differentiation (2008) 15, 1197–1210.

105. Liang et al, A novel long non-coding RNA-PRLB acts as a tumor promoter through regulating miR- 4766-5p/SIRT1 axis in breast cancer, Cell Death and Disease, 2018

106. Linn, Y., K. Hui, Cytokine-Induced NK-Like T Cells: From Bench to Bedside, Journal of Biomedicine and Biotechnology, Volume 2010.

107. Liz and Esteller, lncRNAs and microRNAs with a role in cancer development, Biochimica et Biophysica Acta (2015)

108. Long et al, Structure of nevanimibe-bound tetrameric human ACAT1, Nature, Vol 581, 21 May 2020, 339

109. Lu et al, Impact of beta-blockers on prostate cancer mortality: a meta-analysis of 16,825 patients, OncoTargets and Therapy 2015:8 985–990

110. Luchtel et al, ETS1 is a novel transcriptional regulator of adult T-cell leukemia/lymphoma of North American descent, Blood Advances, Lymphoid Neoplasia| October 13, 2022

111. Luczak, M., P. Jagodzinski, The role of DNA methylation in cancer development, Folia Histochemica Et Cytobiologica Vol. 44, No. 3, 2006 Pp. 143-154.

112. Magnon C, Hall SJ, Lin J, et al. Autonomic nerve development contributes to prostate cancer progression. Science 2013; 341: 1236361.

113. Mariathasan et al, TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells, Nature, Jan 2018

114. McCabe, M., et al, Cancer DNA Methylation: Molecular Mechanisms and Clinical Implications, Clinical Cancer Res 3927 2009;15(12) June 15, 2009.

115. Melo et al, Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis, Cancer Cell, Volume 26, Issue 5, p707–721, 10 November 2014.

116. Miranda, T., P. Jones, DNA Methylation: The Nuts and Bolts of Repression, J. Cell. Physiol. 213: 384–390, 2007.

117. Mizuno, S., et al, Expression of DNA methyltransferases DNMT1,3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia, Blood, 1 March 2001 Z Volume 97, Number 5.

118. Montparler, R., Cancer epigenetics, Oncogene (2003) 22, 6479–6483.

119. Morikawa et al, Genome-wide mechanisms of Smad binding, Oncogene (2013) 32, 1609–1615

120. Morikawa et al, TGF-b and the TGF-b Family: Context-Dependent Roles in Cell and Tissue Physiology, Cold Spring Harb Perspect Biol 2016;8:a021873

121. Moustakas et al, Smad regulation in TGF-b signal transduction, Journal of Cell Science 114, 4359-4369 (2001)

122. Mydlo and Godec, Prostate Cancer, 2nd Ed, Academic (Waltham MA) 2016.

123. O'Shaughnessy, J, et al, Treatment and Prevention of Intraepithelial Neoplasia: An Important Target for Accelerated New Agent Development, Clinical Cancer Research, Vol. 8, 314–346, February 2002.

124. Ogoshi, K., et al, Genome-wide profiling of DNA methylation in human cancer cells, Genomics (2011).

125. Oksuz et al, Systematic evaluation of chromosome conformation capture assays, Nature Methods, VOL 18, September 2021, 1046–1055

126. Orom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Notredame, C., Huang, Q., et al. (2010). Long noncoding RNAs with enhancer-like function in human cells. Cell 143, 46-58.

127. Pakasama, P, et al, Ex vivo generation of cytokine-induced killer cells (CD3+ CD56+) from post-stem cell transplant pediatric patients against autologous–Epstein–Barr virus–transformed lymphoblastoid cell lines, Pediatr Transplantation 2007: 11: 511–517.

128. Pali, S., K. Robertson, Epigenetic Control of Tumor Suppression, Critical Reviews™ in Eukaryotic Gene Expression, 17(4):295–316 (2007).

129. Pardini and Calin, MicroRNAs and Long Non-Coding RNAs and Their Hormone-Like Activities in Cancer, Cancers 2019, 11, 378

130. Parimi et al, Neuroendocrine differentiation of prostate cancer: a review, Am J Clin Exp Urol 2014;2(4):273-285

131. Pastell, R., M. Nevalainen, Prostate Cancer, Humana (Totowa) 2008.

132. Pchejetski et al, Circulating Chromosome Conformation Signatures Significantly Enhance PSA Positive Predicting Value and Overall Accuracy for Prostate Cancer Detection, Cancers 2023, 15, 821.

133. Pekarik, V., et al, Prostate Cancer, miRNAs, Metallothioneins and Resistance to Cytostatic Drugs, Current Medicinal Chemistry, 2013, 20, 534-544

134. Peter W. Laird, P., R. Jaenisch, DNA Methylation and Cancer, Human Molecular Genetics, Vol 3, 1487-95, 1994. http://hmg.oxfordjournals.org/content/3/suppl_1/1487.abstract?sid=0ef56eee-6b89-4808-9ded-2004a353783f

135. Pievani, A. et al, Dual functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity, Blood, Prepublished online August 5, 2011.

136. Powell, M., et al, Disruption of a Sirt1 -Dependent Autophagy Checkpoint in the Prostate Results in Prostatic Intraepithelial Neoplasia Lesion Formation, Cancer Res Published OnlineFirst December 28, 2010.

137. Prensner and Chinnaiyan, The Emergence of linRNA in Cancer Biology, October 2011 Cancer Discovery | 391

138. Qi et al, Siah2-Dependent Concerted Activity of HIF and FoxA2 Regulates Formation of Neuroendocrine Phenotype and Neuroendocrine Prostate Tumors, Cancer Cell 18, 23–38, July 13, 2010

139. Rinn, J., H. Chang, Genome Regulation by Long Noncoding RNAs, Annu. Rev. Biochem. 2012. 81:145–66.

140. Robertson, K., A. Wolffe, DNA Methylation in Health and Disease, Nat Rev Gen 2000 pp 11-19.

141. Robertson, K., DNA methylation, methyltransferases, and cancer, Oncogene (2001) 20, 3139-3155.

142. Roy, S., et al, Inhibition of PI3K/AKT and MAPK/ERK pathways causes Activation of FOXO Transcription Factor, Jrl Mol Sig 2010, pp 1-13.

143. Ruan et al, SIRT1 contributes to neuroendocrine differentiation of prostate cancer, Oncotarget, 2018, Vol. 9, (No. 2), pp: 2002-2016

144. Sakai et al, Long Noncoding RNA ELIT-1 Acts as a Smad3 Cofactor to Facilitate TGFβ/Smad Signaling and Promote Epithelial–Mesenchymal Transition, Cancer Research, Mol Cell Bio, June 2019

145. Sangiolo, D., Cytokine Induced Killer Cells as Promising Immunotherapy for Solid Tumors, Journal of Cancer 2011, 2.

146. Schmitt and Chang, Long RNAs wire up cancer growth, Nature 2013

147. Shackelford, D., R. Shaw, The LKB1–AMPK pathway: metabolism and growth control in tumour suppression, Nature Reviews, Cancer Volume 9, August 2009, 563

148. Shen and Rubin, Prostate Cancer, CSHL Press, 2019

149. Shi X, Sun M, Liu H, Yao Y, Song Y. (2013) Long non-coding RNAs: A new frontier in the study of human diseases. Cancer Letters, August 2013.

150. Sigova, A.A., Mullen, A.C., Molinie, B., Gupta, S., Orlando, D.A., Guenther, M.G., Almada, A.E., Lin, C., Sharp, P.A., Giallourakis, C.C., and Young, R.A. (2013). Divergent transcription of long noncoding RNA/mRNA gene pairs in embryonic stem cells. Proc. Natl. Acad. Sci. USA 110, 2876-2881.

151. Singh and Settleman, EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer, Oncogene (2010) 29, 4741–4751

152. Singh et al, Death Associated Protein Kinase 1 (DAPK1): A Regulator of Apoptosis and Autophagy, Frontiers in Molecular Neuroscience, www.frontiersin.org , 1 June 2016| Volume 9 Article 46

153. Steensel, Chromatin: constructing the big picture, The EMBO Journal (2011) 30, 1885–1895

154. Strathdee, G., R., Brown, Aberrant DNA Methylation in Cancer; Potential Clinical Interventions, Exp Rev Mol Med, 2002, pp 1-17.

155. Sun et al, Neuroendocrine differentiation in prostate cancer, Am J Transl Res 2009;1(2):148-162

156. Sun M, Kraus WL. (2013) Minireview: Long Non-Coding RNAs: New "Links" Between Gene Expression and Cellular Outcomes in Endocrinology. Mol Endocrinol, August 2013.

157. Taberlay, P., P. Jones, DNA Methylation and Cancer, Epigenetics and Disease, Progress in Drug Research 67, 2011.

158. Tang et al, Bacon: a comprehensive computational benchmarking framework for evaluating targeted chromatin conformation capture specific methodologies, Genome Biology, 2022

159. Tefferi, A., J. Vardiman, Myelodysplastic Syndromes, N Engl J Med 2009; V 361: pp 1872-85.

160. Teng, G., F Papavasiliou, Long Noncoding RNAs: Implications for Antigen Receptor Diversification, Advances in Immunology, Volume 104, 2009.

161. Thomsen, M., et al, Loss of JUNB/AP-1 promotes invasive prostate cancer, Cell Death & Differentiation , (19 December 2014) doi:10.1038/cdd.2014.213.

162. Ting et al. dentification of microRNA-98 as a Therapeutic Target Inhibiting Prostate Cancer Growth and a Biomarker Induced by Vitamin D, The Journal Of Biological Chemistry Vol. 288, No. 1, Pp. 1–9, January 4, 2013

163. Tiwari et al, nonapoptotic role for CASP2/caspase 2, Modulation of autophagy, Autophagy 10:6, 1054–1070; June 2014

164. Tollefsbol, T., Epigenetics in Human Disease, Academic (New York) 2012.

165. Uemura et al, Novel 5α-steroid reductase (SRD5A3, type-3) is overexpressed in hormone-refractory prostate cancer, Cancer Sci, January 2008, vol. 99, no. 1, 81–86

166. Ungefroren, TGF-β Signaling in Cancer: Control by Negative Regulators and Crosstalk with Proinflammatory and Fibrogenic Pathways, Cancers 2019, 11, 384

167. van der Heide, L., et al, The Ins and Outs of FOXO Shuttling, Biochem Jrl 2004, pp 297-309.

168. Vasiljevic, N , et al, Association between DNA methylation of HSPB1 and death in low Gleason score prostate cancer, Prostate Cancer and Prostatic Disease (2013) 16, 35–40.

169. Vely, F., E., Vivier, Natural Killer Cell Receptor Signaling Pathway, Sci. STKE 2005 (292),

170. Vogelstein and Kinzler, Cancer genes and the pathways they control, Nature Medicine Volume 10 Number 8 August 2004

171. Wade and Kyprianou, Profiling Prostate Cancer Therapeutic Resistance, Int Jrl Mol Sci, 2018 V 19, No 904

172. Wang et al, Extrachromosomal circular DNA: a new potential role in cancer progression, J Transl Med (2021) 19:257

173. Wang et al, LncRNA SNHG6 promotes proliferation, invasion and migration in colorectal cancer cells by activating TGF-β/Smad signaling pathway via targeting UPF1 and inducing EMT via regulation of ZEB1, Int Jrl Med Sci 2019; 16(1): 51-59.

174. Washeti, S., et al, Computational analysis of noncoding RNAs, WIREs RNA 2012. doi: 10.1002/wrna.1134.

175. Watson, J, F. Crick, Molecular Structure of Nucleic Acids, Nature, pp 737-738, April 25, 1953.

176. Weinberg, R., The Biology of Cancer, 2nd Ed, Garland (New York) 2013.

177. Wellner et al, The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs, Nature Cell Biology volume 11, pages 1487–1495 (2009)

178. Wilusz et al, Long noncoding RNAs: functional surprises from the RNA world, Genes & Development 23:1494–1504 2009

179. Wu, L. et al, long non-coding RNAs: versatile master regulators of gene expression and crucial players in cancer, Am J Transl Res 2012; 4(2):127-150.

180. Xiao et al, ETS1 acts as a regulator of human healthy aging via decreasing ribosomal activity, Science, April 2022

181. Xie et al, Potential Regulatory Roles of MicroRNAs and Long Noncoding RNAs in Anticancer Therapies, Mol Therapy Nucleic Acids Vol 13 Dec 2018

182. Yang and Weinberg, Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis, Developmental Cell 14, June 2008

183. Yang et al, lncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs, Nature, 29 Aug 2013

184. Ye, Y., et al, Progressive Chromatin Repression and Promoter Methylation of CTNNA1 Associated with Advanced Myeloid Malignancies, Cancer Res Nov 2009.

185. Zahalka AH, Arnal-Estapé A, Maryanovich M, et al. Adrenergic nerves activate an angio-metabolic switch in prostate cancer. Science 2017; 358: 321-6.

186. Zamai, L. et al, NK Cells and Cancer, J Immunol 2007; 178:4011-4016.

187. Zhao et al, Extrachromosomal circular DNA: Current status and future prospects, eLife 2022;11:e81412

188. Zilberman, D., The Evolving Functions of DNA Methylation, Curr Opin in Plt Bio, 2008, pp 554-559.

189. Zuo et al, Extrachromosomal Circular DNA (eccDNA): From Chaos to Function, Frontiers in Cell and Developmental Biology | www.frontiersin.org January 2022 | Volume 9 | Article 792555

7 INDEX