The transcriptional role of c-Myc in prostate cancer

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I am so clever that sometimes I don't understand a single word of what I am saying.

Oscar Wilde

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Preface

List of papers included in this thesis

Paper I

Meta-analysis of prostate cancer gene expression data identifies a novel discriminatory signature enriched for glycosylating enzymes.

Barfeld SJ, East P, Zuber V, Mills IG.

BMC Med Genomics. 2014 Dec 31;7(1):513.

Paper II

Myc-dependent purine biosynthesis affects nucleolar stress and therapy response in prostate cancer

Barfeld SJ, Fazli L, Persson M, Marjavaara L, Urbanucci A, Kaukoniemi KM, Rennie PS, Ceder Y, Chabes A, Visakorpi T, Mills IG.

Oncotarget. 2015 May 20;6(14):12587-602.

Paper III

Overexpression of c-Myc antagonises transcriptional output of the androgen receptor in prostate cancer

Barfeld SJ, Urbanucci A, Fazli L, Rennie PS, De Marzo AM, Yegnasubramanian V, Mills IG.

Manuscript

Supplementary Paper IV (Review)

Androgen-regulated metabolism and biosynthesis in prostate cancer.

Barfeld SJ, Itkonen HM, Urbanucci A, Mills IG.

Endocr Relat Cancer. 2014 Aug;21(4):T57-66.

Supplementary Paper V (Methods Chapter)

Mapping protein-DNA interactions using ChIP-exo and Illumina-based sequencing

Barfeld SJ, Mills IG.

Methods Mol Biol. (under review)

Additional publications not included in this thesis

Modulation of intracellular calcium homeostasis blocks autophagosome formation.
Engedal N, Torgersen ML, Guldvik IJ, **Barfeld SJ**, Bakula D, Sætre F, Hagen LK, Patterson JB, Proikas-Cezanne T, Seglen PO, Simonsen A, Mills IG.
Autophagy. 2013 Oct;9(10):1475-90.

Chromatin relaxation is a feature of advanced prostate cancer
Alfonso Urbanucci, **Stefan J. Barfeld**, Ville Kytölä, Daniel Vodák, Liisa Sjöblom, Teemu Tolonen, Sarah Minner, Christoph Burdelski, Kati K Kivinummi, Donald J. Vander Griend, Eivind Hovig, Stefan Knapp, Teuvo LJ Tammela, Matti Nykter, Tapio Visakorpi, and Ian G. Mills
Manuscript

Abbreviations

ADT	androgen-deprivation therapy
AR	androgen receptor
BCR	biochemical recurrence
BPH	benign prostatic hyperplasia
ChIP qPCR	ChIP quantitative PCR
ChIP-seq	chromatin immunoprecipitation coupled with high-throughput sequencing
CRPC	castration-resistant prostate cancer
CZ	central zone
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ER	estrogen receptor
ER	endoplasmic reticulum
GPCR	G-protein-coupled receptors
GWAS	genome-wide association study
HAT	histone acetyltransferase
HBP	hexosamine biosynthetic pathway
HGPIN	high-grade PIN
HTS	high throughput sequencing
IHC	immunohistochemistry
ISUP	international society of urological pathology
kDa	kilodalton
KDM	lysine demethylase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LOH	loss of heterozygosity
miRNA	micro RNA
ml	millilitre
MPA	mycophenolic acid
ng	nanogram
NR	nuclear receptor
PCa	prostate cancer
PCR	polymerase chain reaction
PCR	polymerase chain reaction
PI3K	phosphoinositide-3-kinase
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PSA	prostate-specific antigen
PZ	peripheral zone
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RTK	receptor tyrosine kinases
SHBG	steroid hormone binding globulin
SNP	single nucleotide polymorphism
T	testosterone
TCGA-PRAD	The Cancer Genome Atlas – Prostate Adenocarcinoma
TF	transcription factor
TNM	tumour-node-metastasis
TURP	transurethral resection of the prostate
TZ	transitional zone
UDP-GlcNAc	UDP-N-acetylglucosamine

Glossary of gene and protein symbols

Gene	Protein	Name
AR	AR	androgen receptor
BRCA2	BRCA2	breast cancer 2, early onset
CDK9	P-TEFb	positive transcription elongation factor b
CDKN1B	p27	cyclin-dependent kinase inhibitor 1B
CHD1	CHD1	chromodomain helicase DNA binding protein 1
CREBBP	CBP	CREB binding protein
DNMT3A	DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
EIF4E	eIF4E	eukaryotic translation initiation factor 4E
EP300	p300	E1A binding protein p300
EP400	p400	E1A binding protein p400
ERG	ERG	ETS-related gene
ETV1/5	ETV1/5	ETS variant 1/5
EZH2	EZH2	enhancer of zeste homolog 2
FOXA1	FOXA1	forkhead box A1
FOXO1/3	FOXO1/3	forkhead box O1/O3
FOXP1/3	FOXP1/3	forkhead box P1/P3
GABPA	GAPBα	GA binding protein transcription factor, alpha subunit 60kDa
GSTP1	GSTP1	glutathione S-transferase pi 1
HMGN2P46	HMGN2P46	High Mobility Group Nucleosomal Binding Domain 2 Pseudogene 46
HOXB13	HOXB13	homeobox B13
IMPDH2	IMPDH2	inosine-5'-monophosphate dehydrogenase 2
KAT2A	GCN5	histone acetyltransferase GCN5
KAT5	TIP60	K(lysine) acetyltransferase 5
KLK3	PSA	prostate-specific antigen
KMT2D/C	MLL2/3	histone-lysine N-methyltransferase 2D/2C
MAD	MXD1	MAX dimerization protein 1
MAX	MAX	MYC associated factor X
MNT	MNT	MAX network transcription repressor
MTOR	mTORC1	mammalian target of rapamycin complex I
MYC	c-Myc/MYC	v-myc avian myelocytomatosis viral oncogene homolog
NCOA2	NCoA-2	nuclear receptor coactivator 2
NCOR2	NCOR2	nuclear receptor corepressor 2
NKX3-1	NKX3.1	NK3 homeobox 1
PAICS	PAICS	phosphoribosylaminoimidazole carboxylase
PIK3CA	p110α	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PTEN	PTEN	phosphatase and tensin homolog
PTSG1	PTSG1	prostaglandin-endoperoxide synthase 1
RB1	Rb1	retinoblastoma 1
RFX6	RFX6	regulatory factor X, 6
RUVBL1	TIP48	RuvB-like AAA ATPase 1
RUVBL2	TIP49	RuvB-like AAA ATPase 2
SLC45A3	SLC45A3	solute carrier family 45, member 3
SPOP	SPOP	speckle-type POZ protein
TMPRSS2	TMPRSS2	transmembrane protease, serine 2
TP53	p53	tumour protein 53
TRRAP	TRRAP	transformation/transcription domain-associated protein
TSC2	TSC2	tuberous sclerosis protein 2

Abstract

Prostate cancer is the most frequently diagnosed cancer in men and poses a serious health threat to our aging society. Decades of research have improved detection and treatment options, and have led to a significant increase in life expectancy. However, the development of ultimately fatal castration-resistant prostate cancer still occurs frequently and treatment options are limited. The androgen receptor plays a crucial role in prostate cancer at all stages of the disease and thus constitutes the main drug target. Recent advances in molecular techniques, however, have uncovered other transcription factors that are commonly overexpressed and contribute to prostate cancer initiation and progression, and underpin expression profiles that classify the disease. Amongst these factors is c-Myc, which has been studied extensively in a variety of malignancies but its precise molecular function in prostate cancer remains largely elusive as of today.

In this study, we sought to define the biological role of c-Myc in prostate cancer. Similar to other model systems, we found c-Myc to regulate a range of metabolic pathways, including purine biosynthesis. We focused on two enzymes within this pathway, PAICS and IMPDH2, and validated their overexpression in patient samples. Furthermore, we demonstrated the therapeutic potential of IMPDH2 inhibition by repurposing a clinically approved immunosuppressant. Notably, the biological effects of IMPDH2 inhibition included a cellular stress response and the activation of tumour-suppressive microRNAs. Next, we assessed the effects of c-Myc overexpression on androgen receptor chromatin occupancy and transcriptional output. We found that the androgen receptor and c-Myc share a substantial amount of target genes and networks, and that c-Myc overexpression antagonises androgen receptor activity. These findings are of utmost interest for the community since dysregulated androgen receptor activity is a major hallmark of prostate cancer.

1. Introduction

The following chapter will provide the reader with a comprehensive yet compact overview of the prostate, its anatomy and physiology as well as its diseases, predominantly prostate cancer. Befittingly, the reader will be introduced to current standards of diagnosis and treatment of the disease before receiving a thorough summary of its molecular principles. These principles include the current molecular model of prostate cancer and the most prevalent hallmarks. The introduction will be concluded with a detailed description of crucial transcriptional networks and their interplay in prostate cancer since the principal idea of functional interactions between transcription factors forms the theoretical basis of this thesis. Naturally, both the androgen receptor and c-Myc will receive special attention throughout this introductory part.

1.1 Prostate anatomy and physiology

The prostate gland is a walnut-shaped structure sitting just below the urinary bladder and in front of the rectum. It surrounds the proximal urethra as it exits the bladder and the ejaculatory duct coming from the seminal vesicles (**Figure 1**). Its purpose is to produce a milky-white fluid, which comprises roughly 30% of the ejaculate during sexual activity. This fluid contains high levels of zinc and citrate, which help to maintain sperm viability, presumably through calcium chelation, and provide an energy source to sustain mobility, respectively (1). High intraprostatic citrate levels are achieved through the accumulation of zinc via elevated levels of members of the zinc transporter family (hZIP) (**Supplementary Paper IV**) (2). Zinc in turn inhibits the citrate-oxidizing m-acotinase enzyme of the citric acid cycle, which leads to a build-up of citrate (3).

Anatomically, the prostate can be divided into three zones, (A) the peripheral zone (PZ) close to the rectum, (B) the central zone (CZ) surrounding the ejaculatory duct and (C) the transition zone (TZ), the innermost section surrounding the urethra (**Figure 1**). All three zones have different embryonic origins and differ vastly in their epithelial and stromal composition, and their susceptibility to prostatic diseases (**Chapter 1.2**) (4).

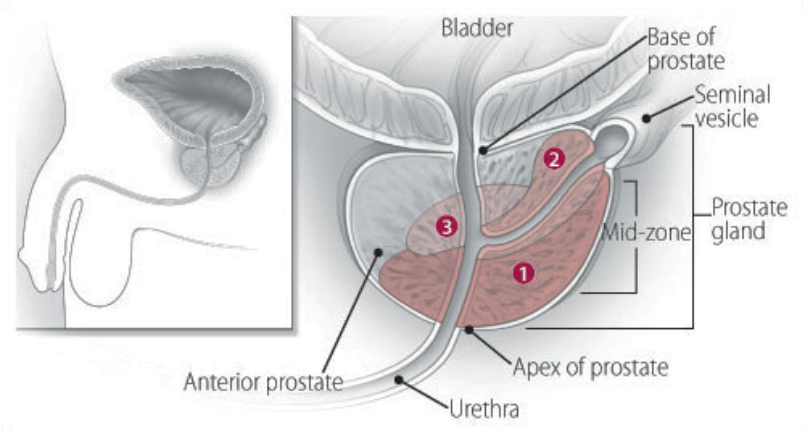

Figure 1: Location of the prostate and anatomy

The prostate gland sits underneath the urinary bladder and next to the seminal vesicles. It surrounds the urethra and the ejaculatory duct and produces a zinc-rich prostatic fluid, which comprises about 30% of the ejaculatory fluid. Anatomically, it can be divided into three main zones (red). (1) Peripheral Zone (2) Central Zone (3) Transition Zone. Taken from (5)

The size of the human prostate varies greatly with age and both its development and function are regulated by male sex hormones, androgens. The most prominent circulating androgen, testosterone, is primarily produced in the testes and exported to the bloodstream (6), where most of it is bound to albumin or Steroid Hormone Binding Globulin (SHBG) (6). Testosterone enters the prostate cells either through transporters or passive diffusion and is converted in the cytoplasm to the more potent dihydrotestosterone (DHT) through the activity of 5-α-reductase (5a-R) (7). DHT in turn binds the ligand-binding domain of the androgen receptor (AR), a ligand-activated transcription factor (TF).

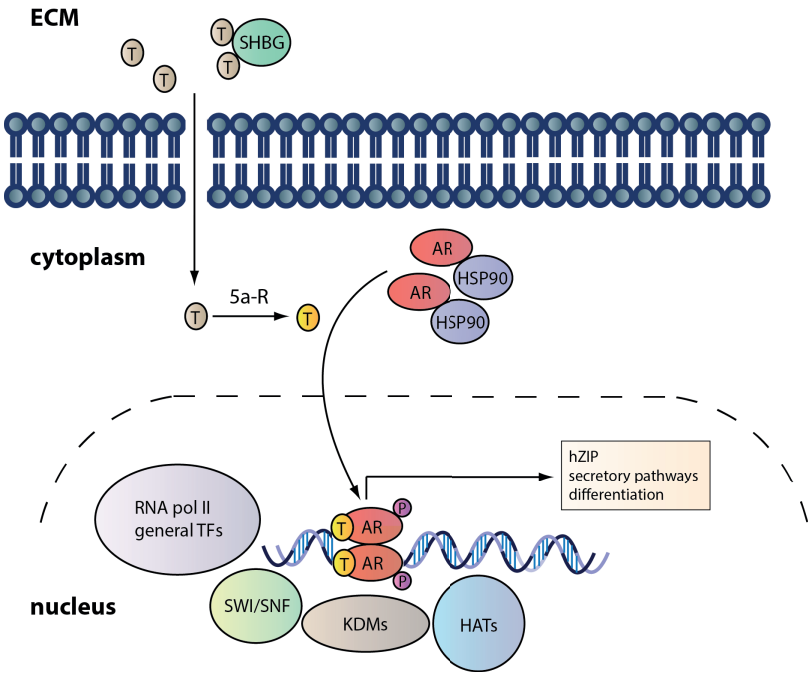

Figure 2. Mechanism of transcriptional regulation by the androgen receptor

Testosterone (T) in the extracellular matrix (ECM) is mainly bound by steroid hormone binding globulin and enters cells both passively through diffusion and with the help of transporters. Once in the cytoplasm, testosterone is converted to the more potent ligand dihydrotestosterone and binds to the AR, which releases the AR the chaperone HSP90. The AR dimerizes, gets phosphorylated and translocates into the nucleus. There it drives the expression of its target genes by recruitment of various transcriptional complexes, including histone acetyltransferases (HATs), lysine demethylases (KDMs), ATP-dependent chromatin modifiers (SWI/SNF) and the general transcription machinery.

Androgen receptor

The androgen receptor (AR) is an approximately 110kDa large nuclear receptor (NR), which is expressed in many cell types throughout the human body (**Supplementary Paper IV**) (8). However, AR levels in the secretory luminal epithelial cells of the prostate are particularly high (9). In absence of a ligand, the AR is bound in the cytoplasm to heat-shock proteins, for example HSP90 (10) (**Figure 2**). Upon ligand binding, the AR is released

from the chaperone, homodimerises and is phosphorylated (11, 12). It then translocates into the nucleus, where it binds androgen-response elements in the genome and initiates transcription of its target genes. Mechanistically, the AR and other NRs modify chromatin structure through the recruitment of chromatin modifiers and remodelling enzymes, such as histone acetylases or demethylases (13-17), and ATP-dependent chromatin modifiers, such as the SWI/SNF complex, to promoter and enhancer regions (18-20) (**Figure 2**). Furthermore, the AR and other NRs also facilitate the recruitment of components of the general transcription machinery, such as RNA polymerase II, to promoter regions (21, 22).

Importantly, the AR is essential for normal prostate development and function. For example, it controls the expression of hZIPs, which in turn increase intracellular zinc and concomitantly citrate levels (**Chapter 1.1**). However, it is also believed that the AR is the main initiator and driver of prostate cancer (PCa) and potentially other prostatic diseases (23).

1.2 Prostatic diseases and prostate cancer

The prostate is the origin of the two most common urological diseases of elderly men, PCa and benign prostatic hyperplasia (BPH). Other conditions that can occur in the prostate are prostatitis, or inflammation of the prostate gland, proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN), both of which are considered precursors of PCa. Due to the prostate's proximity to the reproductive and urinary systems, prostatic diseases often affect urination and sexual function.

1.2.1 Prostatitis

Prostatitis, infection or inflammation of the prostate gland, is the most common prostatic disease in men under 50 with prevalence between 5-9%

(24, 25). In fact, about 15-25% of all men will develop prostatitis at some point in their lives (26). Prostatitis is typically divided into four different subtypes, (A) acute, (B) chronic bacterial, (C) chronic nonbacterial and (D) asymptomatic (27). (A) and (B) are primarily caused by *Escherichia coli* and represent the best characterized but with about 10% of all cases also least common subtypes (28). About 90% of all symptomatic patients are diagnosed with chronic nonbacterial prostatitis (C), which thus far remains poorly understood. Potential triggers include viruses, urine reflux, dietary factors and physical trauma (29). The exact relationship between prostatitis and PCa risk has not yet been elucidated and remains a field of extensive research but it has been suggested that prostatitis may increase the risk for PCa and BPH (25, 27, 30, 31). This is further corroborated by the observation that bacterial prostatitis can exhibit molecular changes similar to PCa (32).

1.2.2 Benign prostatic hyperplasia

The inner part of the prostate (the TZ) often keeps growing with age, thereby exerting pressure on the urethra and causing discomfort and problems with urination. This condition is called benign prostatic hyperplasia (BPH). The TZ of the prostate makes up only 5% of the total volume but 100% of BPH cases emerge from this region (4). It is currently unclear whether BPH increases the risk to develop PCa and multiple publications support both sides of this highly controversial topic (33, 34).
To treat BPH and relieve symptoms, excessive prostate tissue is often removed using transurethral resection of the prostate (TURP). Subsequent pathologic examination of the resected tissue occasionally reveals the presence of PCa.

1.2.3 Prostate cancer and precursors

Proliferation of normal cells is carefully controlled by production and release of growth-promoting factors, which ensure tissue architecture, integrity and function (35). Cancer cells, however, have acquired the means to control their own destiny and proliferate independently.

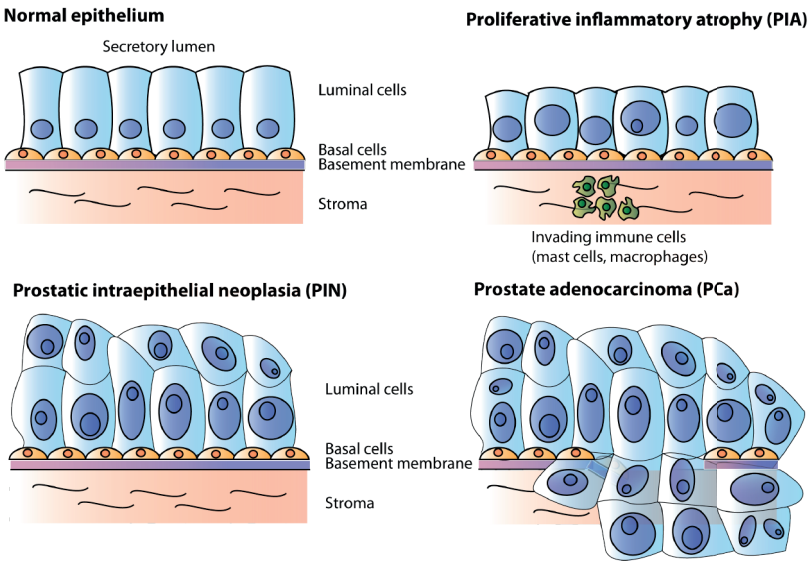

Figure 3. Current model of prostate cancer progression

Prostate cancer (PCa) is thought to develop in a stepwise manner, starting with proliferative inflammatory atrophy (PIA) or prostatic intraepithelial neoplasia (PIN). It then progresses slowly to invasive PCa. Progression is characterised by atrophy, increased proliferation, enlarged nuclei and nucleoli and finally breach of the basement membrane and invasion of the stroma. It is currently thought that both luminal and basal cells can develop cancerous properties.

Several hallmarks of cancer cells have been described and form the current basis of our understanding of the malignancy. These include the abilities to sustain proliferative signalling and to ignore growth suppressing effects, replicative immortality and the capacities to invade and metastasize into other tissues (35). Furthermore, cancer cells need to be able to evade cell death and stimulate angiogenesis to ensure supply with nutrients and oxygen. This classical list of cancer hallmarks has recently been expanded to

give justice to novel insights gained in recent years (36). Thus, the latest model suggests that cancer cells also require the capacity to deregulate cellular metabolism and to avoid detection or destruction by the host's immune system. In addition, genomic instability and mutations as well as tumour-promoting inflammation are now seen as two typical characteristics of human tumours. Initially, PCa is a hormone-dependent cancer driven by androgens and the activity of the AR. It is often indolent, i.e. asymptomatic and barely growing but it can also be aggressive and fast growing. It is thought to develop in a step-wise manner, starting with proliferative inflammatory atrophy (PIA) or prostatic intraepithelial neoplasia (PIN) and subsequent slow progression to invasive adenocarcinoma (**Figure 3**).

Proliferative inflammatory atrophy

PIA is closely associated with chronic inflammation and describes a frequently observed lesion in prostate biopsies characterized by chronic inflammatory cell infiltrates, such as mast cells and macrophages, and atrophic glandular structures, mainly in the PZ of the prostate (37, 38) (**Figure 3**). The affected epithelial luminal cells exhibit enlarged nuclei, increased proliferation and a reduced apoptotic rate (39). This hypothesis is further corroborated by the observation that invading immune cells have been shown to stimulate the formation of cancer in various animal models, albeit not PCa. This is presumably mediated by secreted cytokines, such as tumour-necrosis factor alpha (TNF-α) (38, 40, 41). Due to its predominant localization in the PZ, PIA has been hypothesized to be a precursor lesion of prostatic intraepithelial neoplasia (PIN) or PCa (37).

Prostatic intraepithelial neoplasia

PIN is a precancerous lesion in which some luminal cells of the prostate epithelium start to look and behave abnormally. They exhibit enlarged nuclei and nucleoli, and increased abnormal proliferation (42, 43).

Importantly though, these changes only affect luminal cells; the basement membrane, which forms the lining of the epithelium, remains intact (**Figure 3**).

PIN itself is usually asymptomatic but considered a precursor of PCa. It is often discovered in biopsies taken when PCa is suspected, and it harbours many of the genetic alterations present in PCa (**Chapter 1.5.1)**. However, it does not yet represent an invasive carcinoma. Historically, PIN was subdivided into three groups, I, II and III, which were classified as low grade (I) and high grade (II and III) PIN. Because low grade PIN does not have any prognostic value, PIN is nowadays often used as a synonym for high grade PIN (HGPIN). Clinically, various studies have shown that patients with HGPIN have an increased risk of a subsequent PCa diagnosis although this area remains highly controversial since subsequent studies reported otherwise (44-47). Just as PCa, PIN is most likely to occur in the PZ of the prostate.

Prostate cancer

The diagnosis changes from PIN to PCa once the uncontrolled proliferation of epithelial cells penetrates the basement membrane and cells invade the stroma (**Figure 3**) (48). Eventually, the tumour might grow large enough to invade surrounding tissues and organs, such as the seminal vesicles, the lymph nodes or the rectum. It might also spread to distant organs via the bloodstream and form metastases. The most common metastatic sites in PCa are bone (90%), lungs (46%) and liver (25%) (49).

Approximately 70% of all prostate cancers originate in the PZ and about 25% in the TZ. Interestingly, cancers of the CZ are rather uncommon and comprise only about 5% of all PCa (4). In contrast to many other epithelial cancers, such as breast cancer, there are hardly any distinguishable histopathological subtypes in PCa. The vast majority (>90%) of PCas are adenocarcinomas, i.e. cancers of the glandular epithelial cells. Other rare cancer types include ductal adenocarcinomas (originate in the prostatic

duct), squamous cell carcinomas (originate in the flat cells covering the prostate gland), sarcomas (originates in prostatic muscle cells) or small cell carcinomas (a type of neuroendocrine tumour). The exact cell type of origin of PCa in the glandular epithelium still remains controversial and there is evidence that both basal and luminal cells can give rise to PCa (**Figure 3**) (50-53). It is thought that luminal-cell-containing tumours can evolve from basal-cell-induced cancers, potentially explaining the lack of basal cell markers in patient tumours (54).

1.3 Epidemiology of prostate cancer

PCa is the most common cancer in men and the second most common cause of cancer-related death in men (55). According to the American Cancer Society, more than 240,000 men were diagnosed with PCa in 2011 in the US and more than 33,000 men died of it (55). Worldwide, the numbers for new diagnoses and deaths in 2012 were 1.11 million and 300,000, respectively (56). Currently, the lifetime risk, i.e. the risk of a newborn child to develop PCa at some point in their life is approximately 14% (1 in 7) (56). Norway has one of the highest incidences of PCa worldwide (129.7 age-standardised rate per 100,000 people) with 4,919 new diagnoses and 1,006 deaths in 2012 (57, 58). Strikingly, Northern European countries, such as Norway, Sweden or Finland, appear to have particularly high PCa incidence and mortality rates (59). In general, PCa is a disease of the elderly in the developed world; the average age at diagnosis is 66 and about 60% of men diagnosed are 65 or older (56). However, about 10% of all PCa cases are diagnosed in men under the age of 55 (60). These early onset cancers are generally more aggressive and have a higher mortality rate than men diagnosed at older age, except those over the age of 80 (60). It has been postulated that these early onset cases have a strong genetic component and these men could benefit from risk loci screening (60).

Risk factors

Several endogenous and exogenous risk factors are suspected to contribute to the development and/or progression of PCa (**Table 1**). When it comes to endogenous factors, age is by far the most significant risk factor for PCa. The vast majority of patients diagnosed with PCa are in their sixties with an average age of 66 at diagnosis (56). Another strong link exists between race and PCa: in the UK, black men are, depending on age group, 1.1 to 3.4 more likely than white men to develop PCa (61). In the US, both their risk to develop and their risk to die from PCa are significantly higher in comparison to white men (62). Although other factors, such as socio-economic status or demographic characteristics, certainly play a major role, they are not sufficient to explain this disparity in its entirety (63). Furthermore, obesity and high levels of Insulin-like growth factor 1 (IGF-1) have been shown to increase PCa risk and risk of death from PCa (64-67). Another risk factor is family history; a man whose father and/or brother has or had PCa is approximately 2-3 times more likely to develop PCa himself (68, 69). The risk of early onset PCa is associated with family history and these patients are also more likely to carry a larger number of genetic variants than older men who develop PCa (60, 70).

Various genome-wide association studies (GWAS) have linked a host of genomic loci to PCa risk (71-74). The underlying biological mechanisms, however, remain to be elucidated for most of them, as they predominantly lie in gene-free regions of the genome. A notable exception is the recent discovery of a single-nucleotide polymorphism (SNP) in a risk-associated allele on chromosome 6q22, which the authors could link to an in increase in HOXB13 binding to the enhancer region of RFX6 (75, 76). Strikingly, a germ line mutation in the HOXB13 gene itself has been reported to increase the risk for PCa and the prevalence of this mutation was highest in Sweden and Finland (77, 78). Germ line mutations in the BRCA2 gene, which dramatically escalate the risk of breast and ovarian cancer have also been

shown to increase the risk for PCa approximately 7-fold (79). In total, about 5-9% of all PCas are estimated to be familial, i.e. linked to genes and family history (80).

Table 1. Summary of prostate cancer risk factors

Risk factor(s)	Prostate cancer risk	References
Endogenous factors		
Age	Increased with age	(56)
Race	Increased in black men	(61, 63)
Family history of PCa	Increased	(68, 69)
Obesity/High IFG-1 levels	Increased	(64-67)
Genetic factors		
BRCA2	Early-onset risk increased when mutated	(79)
HOXB13	Increased when mutated	(77, 78)
various SNPs	Both	(71-76)
Exogenous factors		
Calcium, folate, cadmium	Increased (limited evidence)	(81-85)
Arsenic, pesticides	Increased (limited evidence)	(86-88)
Lycopenes, selenium	Decreased (limited evidence)	(89-92)

Neither the International Agency for Research on Cancer (IARC), nor the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) have thus far classified any exogenous factor as having 'sufficient' or 'convincing' evidence to promote PCa risk (93, 94). Several potential risk factors, however, are considered to have 'limited' or 'probable' evidence. These include but are not limited to dietary components (calcium, folate, cadmium), occupational exposures (arsenic, pesticides), testosterone supplements and ionizing radiation. On the other hand, factors that might decrease the risk for PCa but lack 'sufficient' or 'convincing' evidence include physical activity and dietary components (lycopenes, selenium) (93, 94).

1.4 Prostate cancer diagnosis and treatment

In more than 65% of newly diagnosed cases, PCa is asymptomatic (95). Some early stage patients, however, experience varying symptoms, including lower back pain, difficulty urinating or bloody urine (hematuria). In later stages, PCa often causes bone pain in the vertebrae or pelvis due to metastatic spread. When PCa is suspected, only a biopsy can confirm or refute the diagnosis but often less invasive methods are used to gather additional information, including Prostate-specific antigen (PSA) testing, digital rectal exam (DRE) or prostate imaging. Most patients screened for PCa are diagnosed with localised disease and only few patients present metastatic disease upon initial diagnosis (96).

1.4.1 Diagnosis parameters

Clinical PCa staging is usually conducted using the TNM (Tumour-Node-Metastasis) system devised by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) in 1992 (97). The system is constantly being revised and the most current version originates from 2010 (98). If radical prostatectomy is performed during treatment, the initial clinical stage might be corrected after a thorough examination of the removed tissue. This so-called pathologic staging is likely to be more accurate than the initial clinical assessment, as it is based on first-hand impressions of isolated tissue rather than biopsy samples. Both staging procedures use the same categories but T1-stage is only used in clinical staging.

In addition to the TNM system, which is also used in other cancers, two prostate-specific criteria, PSA levels and Gleason grading, determine the clinical stage grouping of PCa. These five parameters will be described in more detailed in the following.

Tumour stage

Tumour stage or T-stage is used to describe the tumour's extend. The following main categories and subcategories are currently being used (**Table 2**) (97).

Table 2: Tumour stage categories

TX		Tumour stage could not be assessed
T0		No evidence of primary tumour
T1		Tumour cannot be felt by DRE or detected by ultrasound
	T1a	Cancer was accidentally found during a TURP procedure and represented less than 5% of the resected tissue
	T1b	Cancer was accidentally found during a TURP procedure and represented more than 5% of the resected tissue
	T1c	Cancer was found by needle biopsy, which was performed due to increased PSA levels
T2		Tumour is confined to the prostate
	T2a	Tumour involves a maximum of 50% of a single lobe
	T2b	Tumour involves more than 50% of a single lobe but not the other
	T2c	Tumour involves both lobes
T3		Tumour extends through the prostate capsule
	T3a	Tumour extends outside the prostate but does not involve the seminal vesicles
	T3b	Tumour extends to the seminal vesicles
T4		Tumour is fixed or invades adjacent structures, such as external sphincter, rectum, bladder, levator muscles or pelvic wall

Node stage

Node stage or N-stage is used to describe whether the tumour has spread to nearby lymph nodes. The following categories are currently being used (**Table 3**) (97).

Table 3: Node stage categories

NX	Nearby lymph nodes were not assessed
N0	Tumour has not spread to nearby lymph nodes
N1	Tumour has spread to nearby lymph nodes

Metastasis stage

Metastasis stage or M-stage is used to describe the tumour's extend to distant tissues and organs. The following main categories and subcategories are currently being used (**Table 4**) (97).

Table 4: Metastasis stage categories

M0		No distant metastases could be detected
M1		Distant metastases are present
	M1a	Metastases in non-regional lymph nodes
	M1b	Bone metastases
	M1c	Other metastases with or without bone involvement

Prostate-specific antigen

Prostate-specific antigen (PSA) is a member of the kallikrein subgroup of serine proteases and the AR tightly controls its expression. It is almost exclusively expressed in secretory epithelial cells in the PZ of the prostate, where it is rendered inactive by high intracellular zinc concentrations (99). Upon ejaculation, PSA in the prostatic fluid is mixed with sperm and activated in the slightly acidic vaginal environment, where lower zinc concentrations are prevalent. Once active, PSA digests the main component of the sperm-entrapping coagulate, the seminal plasma motility inhibitor precursor/semenogelin I (SPMIP/SgI), which leads to the release of motile sperm cells (100, 101).

In patients with a healthy, normal prostate, PSA is confined to prostate cells and sperm, and thus blood levels are low. Its levels, however, positively correlate with the patient's age and size of his prostate (102). Furthermore, race-specific differences have been reported (103). Therefore, normal age- and race-specific reference values range from 0 to approximately 7ng per ml of blood. Prostatic diseases, including BPH and PCa, often disrupt the integrity of the basal cell layer and basement membrane (**Figure 4**), which

leads to a leakage of PSA into the bloodstream (104). Consequently, assessing blood levels of PSA in combination with DRE was initially thought to be of value as a biomarker for the early detection of asymptomatic PCa. However, not every PCa patient has high PSA blood levels and conversely not every patient with a high PSA level has PCa for PSA levels are also elevated in other prostatic diseases (105). Furthermore, PSA blood levels are also influenced by other factors, such as recent DRE or obesity (106-108). Hence, in recent years routine PSA screening has become less popular since benefits for patients remain questionable, and overdiagnosis and overtreatment occur frequently (109). PSA, however, is still routinely used to measure progression in PCa patients after initial treatment as rising PSA values indicate biochemical recurrence (BCR) and potentially treatment failure (**Chapter 1.4.5**).

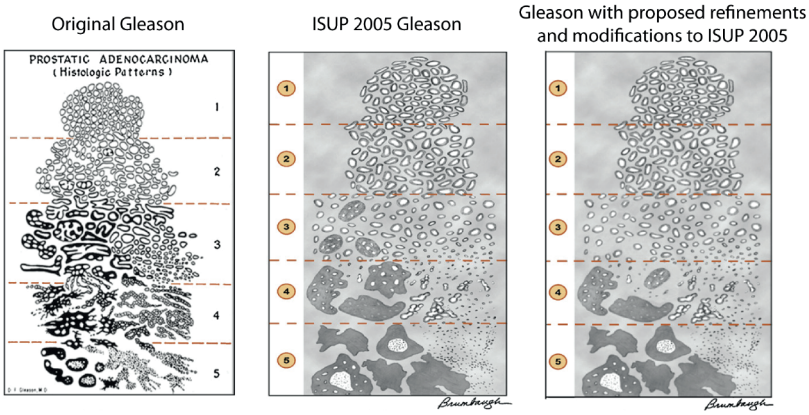

Figure 4. Original Gleason scoring system and recent modifications

(Left) The original scoring devised by Donal Gleason in 1966. (Centre) Modifications introduced after the 2005 meeting of the International Society of Urological Pathology (ISUP) (Right) Recent modifications proposed by Jonathan I. Epstein and colleagues in 2010. Modified from (110)

Gleason grading

Donald Gleason first described the Gleason grading system in 1966. It underwent major revisions in 2005 and 2010 (111, 112), but still remains a valuable tool for PCa diagnosis and prognosis (113) (**Figure 4**).
Basically, a pathologist examines prostate specimen derived from a biopsy or radical prostatectomy under a microscope and assesses the architectural patterns of the gland. The pathologist then assigns a score from 1 to 5 to the two most prevalent patterns in the specimen, based on the level of cell differentiation and the presence of cribriform structures (**Figure 4**). Although the grading system ranges from 1 to 5, pathologists practically do not use 1 and 2. Both assigned scores are combined and result in a total Gleason score ranging from 6 to 10. In principal, the higher the combined Gleason score, the worse the prognosis for the patient.

1.4.2 Staging

Based on the five parameters PSA level, Gleason score and TNM stage, patients are sorted into one of currently five stages (**Table 5**), which help doctors and patients to make appropriate treatment decisions.

Table 5: Prostate cancer stages defined by TNM, Gleason and PSA

Stage		T	N	M	Gleason	PSA
I		T1a-c	N0	M0	≤6	<10
		T2a	N0	M0	≤6	<10
		T1-2a	N0	M0	X	X
II	**A**	T1a-c	N0	M0	7	<20
		T1a-c	N0	M0	≤6	≥10&<20
		T2a	N0	M0	7	<20
	B	T2b	N0	M0	≤7	<20
		T2b	N0	M0	X	X
		T2c	N0	M0	Any	Any
		T1-2	N0	M0	≥8	Any
		T1-2	N0	M0	Any	≥20
III		T3	N0	M0	Any	Any
IV		T4	N0	M0	Any	Any
		Any	N1	M0	Any	Any
		Any	Any	M1	Any	Any

1.4.3 Treatment

First and foremost, the patient and his doctor have to decide whether a treatment is advisable at all. Many prostate tumours are asymptomatic and slow growing or even indolent. Furthermore, the advanced age or deteriorated health of many patients might make treatment undesirable or impossible. Hence, a careful assessment of the patient's individual situation is necessary to identify indolent or aggressive cancers to avoid overtreatment and unnecessary suffering. To aid doctors and patients in their decision, several risk assessment methods have been devised over the years to predict PCa specific mortality on the basis of pre-treatment risk. Two major approaches are nowadays widely used, the D'Amico Risk Stratification (114), and the University of California, San Francisco Cancer of the Prostate Risk Assessment (UCSF-CAPRA) score (115). Both methods use a variety of clinical parameters, including PSA, Gleason score and tumour stage. In addition, the UCSF-CAPRA score includes age and '% of biopsy cores positive' (115).

Once PCa has been diagnosed and staged, several principal treatment options are available (based on the current recommendations by the American Cancer Society) (116).

Stage I cancers are often very small and therefore rarely require treatment. Thus, active surveillance and regular follow-up to monitor the tumour's development is the commonly chosen approach in these cases. Sometimes, however, radical prostatectomy or radiation therapy might be suitable options.

Stage II cancers are larger than stage I but are still confined to the prostate. They are, however, more likely to spread to lymph nodes or other organs. As with stage I cancers, active surveillance and, where appropriate, radical prostatectomy or radiation therapy are commonly recommended treatment strategies.

Stage III cancers have expanded beyond the prostate and thus radical prostatectomy (often with removal of the surrounding lymph nodes), radiation therapy and hormonal therapy, such as androgen-deprivation therapy (ADT), are common approaches. ADT aims at disrupting the AR, a critical TF in PCa (**Chapter 1.1.1**). Stage III cancers have a higher probability of relapsing after treatment than lower stage cancers.

Treatment options for stage IV cancers include the abovementioned therapy options, classic chemotherapy, experimental clinical trials and, if all other options fail, palliative care.

1.4.4 Prognosis

Prognosis of PCa is generally favourable due to the slow growth of most early stage prostate tumours and the advanced age of most patients at diagnosis. Furthermore, due to extensive PSA testing, PCa is usually detected early and most patients screened for PCa are diagnosed with localised disease (96). Thus, the relative cancer-specific 5- and 10-year

survival rates are higher than 90% when all PCa stages are included (117, 118).

Usually, however, the five different stages are subgrouped into three different groups, each having different prognoses. Group 1 or localised disease includes stages I and II (A and B), group 2 describes locally advanced disease (Stage III and non-metastatic stage IV cancers) and group 3 describes metastatic PCa (stage IV with metastases) (117).

The 5-year survival rate for patients presented with low-grade cancers (group 1) at diagnosis is about 98%. For advanced localised cancers (group 2), this drops to approximately 70% and patients with metastatic disease (group 3) have a 5-year survival rate of approximately 30% (118). However, these values are dramatically influenced by time of diagnosis, individual background and chosen treatment options.

1.4.5 Castration-resistant prostate cancer

Regardless of what kind of treatment is chosen by the patient, PSA blood levels usually drop significantly post-treatment, as the tumour is surgically removed (radical prostatectomy) or goes into remission (radiotherapy/ADT). Subsequently, patients receive regular follow-up and PSA levels are routinely measured to detect potential relapse (**Figure 5**). An increase in post-treatment PSA levels indicates BCR but currently no clear consensus definition of a clinically relevant increase exists. Depending on initial tumour stage and treatment, approximately 15-35% of PCa patients will develop BCR within ten years (119-121). The standard of care for these patients is ADT, albeit the appropriate timing (early/late) for treatment remains controversial (122-125).

Initially, most patients with BCR respond to ADT to varying extent but ultimately the cancer becomes resistant, a stage called castration-resistant prostate cancer (CRPC) or metastatic CRPC (mCRPC). Approximately 30% of

patients with BCR will develop mCRPC within 8 years of BCR (119). Over 90% of CRPC patients display bone metastases but liver, lung and adrenal gland metastases are also observed (49, 126, 127). CRPC is a fatal disease and the average overall survival is less than 1.5 years but varies significantly with different metastatic sites (128). Although a variety of treatments for CRPC have been approved in recent years and treatments might be beneficial in individual cases, no curative treatment for CRPC is currently available (128). This underlines the importance of finding new treatments for CRPC.

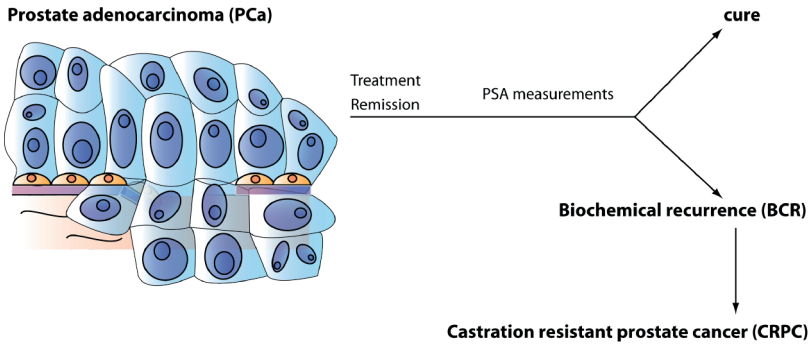

Figure 5: The development of castration resistant prostate cancer

Successful treatment of localised prostate cancer, usually by radical prostatectomy or radiation therapy leads to remission of the tumour and a drop in Prostate-Specific-Antigen (PSA) levels. Afterwards, patients receive regular follow-up and PSA measurements to assess treatment efficacy and potential relapse. A rise in post-treatment PSA, which occurs in about 15-35% of patients, is called BCR. Within 8 years of BCR, approximately 30% of patients will develop metastatic castration resistant prostate cancer, which has an average survival of less than 1.5 years.

The following treatment options are taken from the guidelines for the treatment of CRPC published by the European and American Urological Associations (EAU and AUA, respectively) (127, 129). Current first- and second-line treatments of CRPC include the autologous vaccine Sipuleucel-T (not approved in Europe), the anti-mitotic chemotherapeutics Docetaxel and Cabazitaxel, the immunosuppressant Prednisone, the androgen-biosynthesis-inhibitor Abiraterone acetate and the anti-androgen Enzalutamide (MDV3100) (127, 129). In addition, treatments targeting the

highly prevalent bone metastases are also implemented. These include the monoclonal antibody Denosumab, the bisphosphonate Zoledronate and the radiopharmaceutical radium-223 (127, 129-131). However, survival remains disappointing despite large therapeutic randomised controlled trials, such as the Systemic Therapy in Advancing or Metastatic Prostate Cancer (STAMPEDE) trial, which included a large variety of treatment approaches for mCRPC (132, 133).

1.5 Molecular biology of prostate cancer

On a molecular level, the exact causes and mechanisms of progression of PCa and CRPC remain largely elusive as of today. Its hormone-dependency, however, has been known for decades and has thus been at the centre of extensive research. Historically, Charles Huggins was the first to describe the benefits of castration and ADT for metastatic PCa in the 1940s (134, 135). This ground-breaking discovery, for which he was later awarded the Nobel Prize Physiology or Medicine in 1966, marked a new era of PCa treatment and research. In the late 1960s, the AR was discovered and briefly afterwards the first chemical anti-androgen that prevented the binding of DHT to the AR, Cyproterone acetate, hit the market (136-138). Ever since, PCa research has been focused on the AR and improving ADT, and several new anti-androgens have been developed. However, while treatment of PCa and concomitantly life expectancy have improved significantly since the early days, CRPC still remains a poorly understood and fatal disease.

In recent years, advances in molecular techniques have led to the discovery of a whole array of thus far unknown molecular alterations in PCa and CRPC, opening up new diagnostic and therapeutic avenues. Through extensive research, many of these alterations have been attributed to various precursors and stages of PCa (**Figure 6**), and the current status of knowledge will be summarised in the following chapter.

1.5.1 Molecular model

The current molecular model of PCa proposes that early insults, such as aging, infection and hereditary factors, afflict parts of the prostate, causing DNA damage, oxidative stress, telomere shortening, cell injury and death, and potentially invasion of immune cells (PIA) (38, 139). It has been proposed that this 'field effect' affects a certain proportion of the prostate and some epithelial cells respond to these insults with atrophy, increased proliferation (downregulation of PTEN, NKX3.1 and p27) and stress-response (upregulation of GSTP1 or PTGS1). In a subset of these cells, GSTP1 is repressed through promoter methylation. GSTP1 repression and NKX3.1 downregulation as well as other factors are thought to affect the efficacy of DNA damage repair (specifically double strand break repair) and thus increase the chance of illegitimate recombination and chromosomal rearrangements (140), which are frequently observed in PIN and PCa. Importantly, double-strand breaks are normal events occurring in AR-mediated transcription (141, 142). The emerging chromosomal losses, gains and rearrangements include PTEN and NKX3.1 LOH, 8q24 amplification and multiple gene fusions (e.g. TMPRSS2-ERG) (**Figure 6**). Interestingly, the 8q24 locus contains the TF MYC, which has been shown to promote genomic instability and this could further increase the frequency of chromosomal rearrangements (143).

These rearrangements induce changes in expression patterns and activity spectra of TFs and chromatin remodelers. Thus, the AR is exposed to a range of novel or altered interactors and cofactors, which are thought to influence its transcriptome and interactome (144) (**Figure 7**). This results in the transcription of different genes, thus changing the cellular composition of proteins, altering the cell's identity and inducing transformation (144). Furthermore, loss of cell cycle control (p53 mutations and RB1 loss) occurs frequently in PCa, leading to increased proliferation and tumour growth. Late stage cancer and CPRC are dominated by AR-related mutations and

amplifications, which increase the sensitivity of the AR, render it sensitive to other ligands, produce ligand-independent splice variants of the AR or provide cancer cells with the ability to produce DHT in an autocrine manner to sustain cell proliferation (145-150).

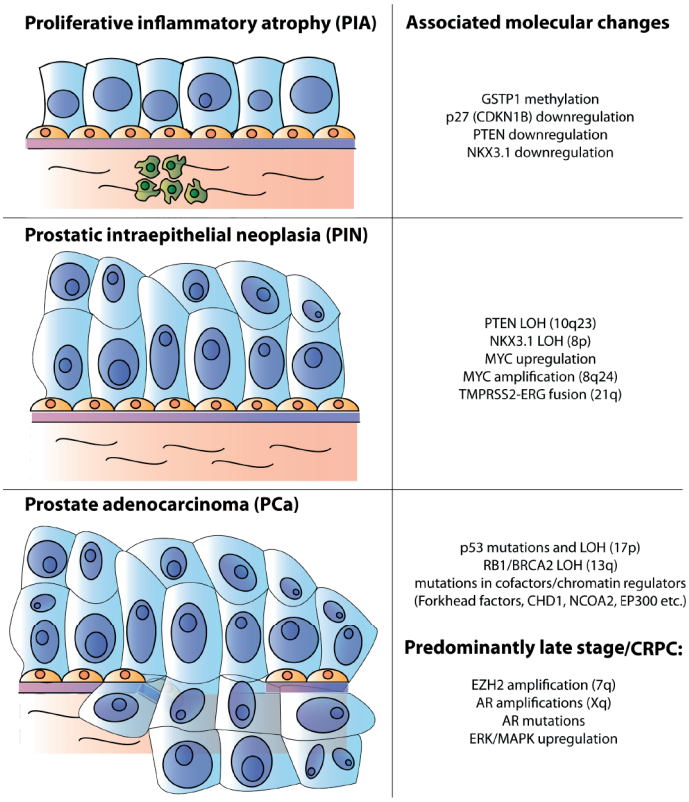

Figure 6. Molecular model of prostate cancer and its precursors

Proliferative inflammatory atrophy (PIA) is characterized by inflammatory stresses in epithelial cells, presumably caused by invading immune cells. In combination with other factors, such as age, diet or genetic predisposition, this leads to atrophy and increased proliferation through the downregulation of p27, PTEN and NKX3.1 and methylation of the GSTP1 promoter. These alterations are thought to impair a host of cellular processes, amongst others DNA damage repair. Inaccurate double strand break repair leads to illegitimate recombination and genomic instability. Thus, chromosome and gene amplification (e.g. 8q24/MYC), loss of heterozygocity (e.g. PTEN and NKX3.1) and gene fusions (e.g. TMPRSS2-ERG) occur frequently and are thought to drive the neoplastic phenotype, resulting in prostatic intraepithelial neoplasia (PIN) and prostate cancer (PCa). In PCa, loss of cell cycle control (p53, Rb1) further drives proliferation. In later stages and CRPC, mutations and amplifications of the androgen receptor (AR), which are thought to maintain the transcriptional activity of the transcription factor (TF), are highly prevalent.

Notably and consistent with the 'field effect' hypothesis, it has been shown that non-cancerous cells of the prostate often harbour a subset of genomic alterations of their cancerous counterparts (151). Furthermore, several studies have shown that 60-90% of localised PCas are multifocal (152-155), i.e. two or more tumours are present in a single prostate. These tumours are spatially separate and usually clonally distinct, i.e. have different Gleason scores, different stages and harbour different molecular and genetic alterations (156).

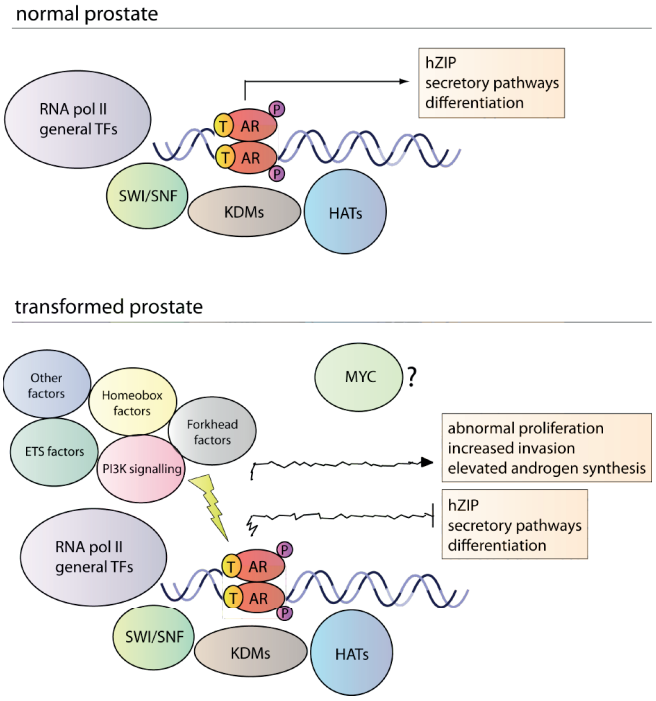

Figure 7. Transcriptional control by the AR in normal and transformed prostate cells

In normal prostate cells, the AR drives a transcriptional program resulting in differentiation and the accumulation of zinc and citrate. It drives expression through interaction with and recruitment of chromatin remodelling complexes (SWI/SNF), lysine demethylases (KDMs), histone acetyltransferases (HATs) and RNA polymerase II in combination with general TFs. In transformed cells, however, the AR is exposed to and interacts with a different range of TFs and co-regulators, leading to altered transcriptional output. These factors include but are not limited to ETS, homeobox and forkhead factors, and altered PI3K signalling. The altered transcriptional output results in abnormal proliferation, increased invasion and elevated androgen synthesis. The impact of MYC on AR signalling in prostate cancer has not been studied extensively yet.

Two principal theories about the origins of multifocality exist, (1) multiclonality of the initial disease, i.e. the tumours were different to begin with or (2) clonal evolution from the initial disease, i.e. the previously identical tumours evolved in different directions (157). This area of research remains controversial but undoubtedly, multifocality and heterogeneity are highly prevalent characteristics of PCa.

In contrast to the local tumour, however, PCa metastases have been shown to be of monoclonal origin and maintain the unique genetic signature of the parental cancer cell (158, 159). Surprisingly, it was recently shown that the lethal cell clone of a deceased patient did not originate from the large, high-grade primary tumour or a lymph node metastasis, but rather unexpectedly from a smaller, low-grade cancer focus in the primary tumour (160). This interesting case illustrates the challenges multifocality and heterogeneity impose on diagnosis and treatment of PCa.

1.5.2 Clinically relevant molecular hallmarks

Despite being a highly heterogeneous and multifocal disease, a host of molecular hallmarks are particularly prevalent in PCa and its precursors PIA and PIN. As mentioned in **Chapter 1.5.1**, chromosome aberrations are thought to promote the development of PCa and thus many of the frequently observed alterations fall into this category (**Table 6**). Furthermore, activating and suppressing mutations are also regularly observed. Mechanistically, the most common changes can be divided into five categories, (1) Signalling pathways, (2) Cell cycle control, (3) Transcription factors, (4) Transcriptional cofactors and chromatin regulators and (5) Others. The most common alterations are summarized in the following table (**Table 6**) (161, 162). Interestingly and in contrast to other cancers, such as colorectal or kidney cancer (163), PCa appears to be largely

devoid of highly recurrent somatic mutations with the notable exception of p53 mutations in mCRPC (164).

Table 6: Overview of clinically relevant molecular hallmarks of PCa

Gene/protein	Alteration(s)	Earliest stage	References
I. Signalling pathways			
PTEN	downregulation, LOH (10q23)	PIA, PCa	(164-169)
PIK3CA	amplification, mutation	PIN?, PCa	(164, 165, 170, 171)
MAPK/ERK1/2	early decrease, late increase	PIN, CRPC	(172-174)
II. Cell cycle control			
RB1/BRCA2	LOH (13q)	PCa	(175-178)
CDKN1B (p27)	downregulation, loss (12p12)	PIA	(37, 179)
III. Transcription factors			
TP53	mutation, LOH (17p)	PCa	(165-168, 180-182)
MYC	amplification (8q24), overexpression	PIN	(164, 183-186)
AR	amplification (Xq), overexpression, mutations	CRPC	(147, 148, 164, 168, 187, 188)
ERG/ETV1/ETV5	gene fusion (TMPRSS2, 21q etc)	PIN/PCa	(164, 189-192)
NKX3.1	downregulation, LOH (8p)	PIA/PIN	(193-195)
IV. Cofactors and chromatin regulators			
NCOA2	mutation, amplification (8q)	PCa	(161, 166, 167, 181)
EP300	mutation	PCa	(161, 166, 167, 181)
NCOR2	mutation	PCa	(161, 166, 167, 181)
FOXA1/O1/O3/P1	mutation, loss (3p, 6q, 13q)	PCa	(164-167)
MLL2/3	mutation	PCa	(161, 166, 167, 181)
CHD1	mutation, loss (5q21)	PCa	(161, 166, 167, 196, 197)
EZH2	amplification (7q), overexpression	CRPC	(164, 198, 199)
V. Others			
GSTP1	promoter methylation (11q13)	PIA/PIN	(200)
SPOP	mutation	PCa	(156, 164, 165, 181, 197)

Undoubtedly, TFs, transcriptional cofactors and chromatin modifiers are highly abundant in this list. Thus, understanding transcriptional regulation and the interplay between these factors are of utmost importance to improve PCa detection, classification and treatment.

1.5.3 Transcriptional networks and their interplay

PCa is a hormone-dependent cancer driven by androgens and the AR. Thus, most therapeutic interventions aim at perturbing AR activity and lowering its transcriptional output. Recent advances in molecular techniques, however, have uncovered a multitude of cofactors and interactors, which influence AR-mediated transcriptional control in PCa and CRPC, and also interact with each other (**Figure 7** and **Figure 8**).

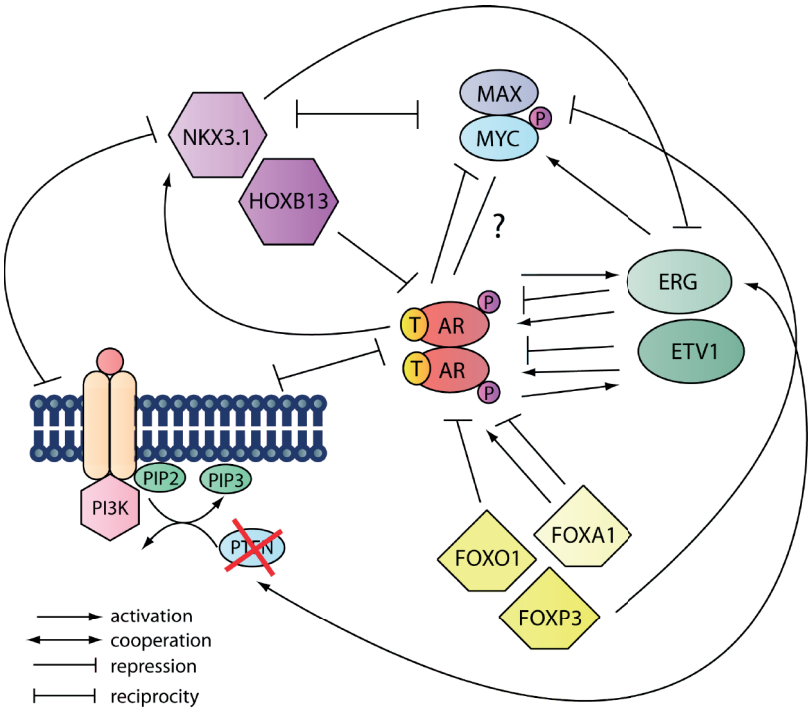

Figure 8. Interplay of transcription factors, chromatin modifiers and signalling pathways

Various publications have begun to uncover a sophisticated network of TFs, chromatin modifiers and signalling pathways in PCa. Examples of reported interactions between commonly altered components of the cellular machinery are highlighted with single-headed arrows (symbolizes activation), blunt arrows (repression), double-headed arrows (cooperation) or double-headed blunt arrows (reciprocal feedback). See text for details and references.

Understanding the impact these interactors have on AR activity and transcriptional output will be crucial for a deeper understanding of the disease and will ultimately produce better treatment options. To this end, researchers have made extensive use of chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) and gene expression analysis using expression arrays or more recently high-throughput RNA-sequencing (RNA-seq). These techniques have proven valuable in defining binding properties and activity spectra of the AR and other TFs, and chromatin regulators. Befittingly, we make use of these techniques in Paper III of this thesis.

Androgen receptor signalling and its modulation

The AR is a ligand-activated TF essential for normal prostate development and function (**Chapter 1.1.1**) but it is also the main initiator and driver of PCa. Extensive unbiased approaches have defined the transcriptional network of the AR in various PCa cell lines and more recently clinical samples (201-203). Through these experiments, it has been shown that the AR directly controls metabolism and cell cycle genes to fuel anabolic metabolism resulting in increased proliferation (202).

It has been postulated that excessive ADT may lead to CRPC, which is ultimately fatal. Strikingly, most CRPCs still express a functional AR network, which is immune to current AR-perturbing therapies. The activity of the AR is presumably maintained through a range of mutations, including point mutations and gene amplifications (146, 147, 187). Furthermore, alternative splicing events can lead to the production of AR variants without a ligand-binding domain (148, 188). These variants are thought to be constitutively active, even in the absence of a ligand. In addition, PCa cells in CRPC patients have been shown to produce hormones in an autocrine manner, thereby providing fuel for the AR (145, 150). This facilitated the development of Abiraterone acetate, which inhibits CYP17A1, an enzyme in

the androgen biosynthetic pathway. Finally, it has been proposed that AR signalling can also be cross-activated by other growth factors, such as epidermal growth factor (EGF) (204-206).

It has been hypothesized that the abovementioned alterations in AR signalling affect AR-mediated transcription and result in a different transcriptome, which drives the disease (**Figure 7**). For example, it has been shown that AR chromatin occupancy is more intimately associated with promoter regions in late state CRPC than in normal prostate tissue or localised disease, and that AR regulates a different set of target genes (203). In recent years, various other TFs and signalling networks have been shown to interact with and influence the AR, and the most important ones will be discussed in the following.

ETS transcription factors

In 2005, highly prevalent fusions between the 5'-UTR of androgen-regulated transmembrane serine protease TMPRSS2 and two members of the ETS family of TFs, ERG and ETV1 was described (189). Other fusions, such as ETV1 and ETV5 with SLC45A3, or ETV1 with HMGN2P46, were found soon thereafter (207, 208). All of these fusions put the respective ETS TF under the transcriptional control of the AR. The clinical incidence of the most frequent fusion, TMPRSS2-ERG, exceeds 50% and was initially thought to be associated with an aggressive, invasive subtype of PCa and shorter time to BCR (191, 209, 210). Subsequent studies, however, failed to show a prognostic value (211, 212), or even reported TMPRSS2-ERG to be a predictor of favourable outcome (213, 214).

Mechanistically, the TMPRSS2-ERG fusion is thought to repress the differentiation programs of cells into luminal and neuroendocrine types (215, 216). Furthermore, ETS-fusion TFs have been shown to influence the activity of the AR, albeit with differing effects and depending on the identity of the ETS factor. ERG has been shown to repress AR signalling by inhibiting

AR expression and direct binding to the receptor at specific genomic loci (201, 217). In a mouse model with a homozygous PTEN deletion, however, ERG markedly amplified AR chromatin binding and signalling (218). This remarkable difference stresses the importance of context-specificity and the need to carefully assess the composition and activity of transcriptional networks and signalling pathways. In the same models, ETV1 has been shown to amplify AR signalling and confer an aggressive phenotype (217, 218). In a different model, however, ETV1 has been shown to antagonize AR action (219), another example of context-specificity. GABPα, another member of the ETS family commonly overexpressed in PCa, has been shown to modulate the expression of a subset of AR target genes, to mediate an aggressive phenotype and to modulate sensitivity to AR antagonists (220).
In addition to their interplay with the AR, ETS family members have also been shown to interact with other TFs and signalling networks (**Figure 8**). Specifically, TMPRSS2-ERG has been shown to cooperate with the PI3K signalling pathway (221), which is intriguing since a negative regulator of this pathway, PTEN, is frequently lost in PCa. Furthermore, the loss of the tumour suppressor NKX3.1, which occurs early in prostate carcinogenesis (**Chapter 1.5.1**), has been shown to promote the fusion of TMPRSS2 and ERG (222). Lastly, TMPRSS2-ERG drives the expression of MYC with concomitant repression of epithelial differentiation genes (216).

Forkhead transcription factors

The superfamily of forkhead TFs (FOX) contains more than a 100 distinct but structurally related members, classified into 18 subfamilies, such as A, O and P (223, 224). The defining feature of this protein family is the forkhead domain, a winged helix DNA binding domain (225). Members of this family are often termed ‘pioneer factors’ for their ability to bind compacted chromatin and make these regions accessible for other chromatin binding factors (226). Mutations in and loss of members of this family, specifically

FOXA1, FOXO1, FOXO3 and FOXP1, are commonly observed in PCa, with an incidence of approximately 3-9% (165, 166). Especially one member, FOXA1, has been extensively studied in breast cancer, another hormone-dependent cancer. There, FOXA1 is essential for proliferation and normal AR and estrogen receptor (ER) alpha activities (227, 228), and its overexpression is associated with favourable outcome (229, 230).

In PCa, however, FOXA1 is overexpressed in metastatic PCa and CRPC and associated with poor prognosis (231-233). Mechanistically, FOXA1 has been shown to influence AR chromatin binding and signalling (**Figure 8**). Specifically, FOXA1 downregulation triggered a massive reprogramming of the AR, both assisting and antagonizing its activity at certain loci (233, 234). In another setting, FOXA1 overexpression facilitated and reprogrammed AR chromatin binding and increased sensitivity to DHT-induced proliferation (235). Additionally, FOXA1 has been reported to act as a cofactor of AR in a model of CRPC, facilitating androgen-independence and cell cycle progression (236).

On the other hand, another member of the forkhead family, FOXO1, is frequently lost in PCa and thus considered a candidate tumour suppressor (237). Mechanistically, it has been shown to mediate PTEN-induced repression of AR activity, both of full length and splice-variants (238-240). Furthermore, Docetaxel- and Cabazitaxel-induced inhibition of the AR in CRPC patients is thought to be dependent on FOXO1 (241). Another example of the forkhead family, FOXP3, is also a candidate tumour suppressor frequently downregulated in PCa, and has been reported to transcriptionally repress the oncogene MYC (**Figure 8**) (242).

Homeobox-containing proteins

Several homeobox-containing TFs have been implicated in prostate function as well as PCa initation and progression. Especially NKX3.1 plays a vital role in normal prostate development and function (243, 244), albeit

other functionally redundant members of the NK family appear to be able to compensate for NKX3.1 loss (245). During development, its initial appearance precedes that of the AR, although its maintenance requires AR activity (246, 247). Expression of NKX3.1 is lost in many cases of PIA and up to 85% of PIN through LOH (8p21) and potentially other mechanisms (193, 248-250). In addition, loss of expression correlates with tumour progression (195). Interestingly, elevated NKX3.1 expression has been observed in BPH, which further highlights the profound differences between BPH and precursors of PCa (251).

In mouse models, loss of NKX3.1 is sufficient to induce PIN but not PCa (252, 253). Furthermore, it has been shown that inflammatory cytokines are able to suppress the expression of NKX3.1 via phosphorylation and ubiquitinylation (254). Mechanistically, NKX3.1 controls the expression of anti-and pro-oxidative enzymes (255). Additionally, NKX3.1 has been shown to interact with topoisomerase I and this interaction enhances DNA repair (140, 256). Thus, loss of NKX3.1 might impair this critical process and lead to an increase in illegitimate recombination and genomic instability (222), which are prominent features of PIN and early PCa. It has been postulated that NKX3.1 is a driver of differentiation and a haploinsufficient tumour suppressor that acts as a gatekeeper gene for PCa initiation (139).

As mentioned above, NKX3.1 knockout induces PIN but not PCa. Loss of NKX3.1 function, however, cooperated with loss of PTEN, resulting in a synergistic activation of Akt and accelerated onset of PIN and early stages of PCa (**Figure 8**) (257). Furthermore, PTEN loss has been reported to suppress NKX3.1 expression and conversely, NKX3.1 restoration inhibited Akt and AR signalling (258). Strikingly, AR drives the expression of NKX3.1 and a reciprocal feedback regulation between AR and PI3K/Akt signalling has been reported (see section on **Phosphoinositide-3-kinase signalling** below**)**. These findings suggest a highly dynamic interplay between AR, NKX3.1 and the PI3K/Akt signalling pathway. Both NKX3.1 and the oncogene MYC are located on chromosome 8, which is frequently altered in

PCa (165-167). Strikingly, an inverse relationship between NKX3.1 and MYC has been reported, and both TFs have been shown to share a subset of their target genes (259). MYC overexpression reduces NKX3.1 levels and conversely, NKX3.1 can oppose MYC transcriptional activity (**Figure 8**) (259, 260).

Another homeobox-containing TF, HOXB13, is exclusively expressed in AR-positive prostate cells and has been reported to suppress AR activity and reduce intracellular zinc levels (261, 262). Germline mutations in this gene have been shown to increase the risk for PCa (77).

Phosphoinositide-3-kinase signalling

The PI3K/Akt/mTOR signalling pathway is one of the most consistently altered pathways in human cancers and is known to regulate metabolism, inflammation, cell survival, motility and cancer progression (263). Phosphoinositid-3-kinases (PI3K) and specifically class I PI3K are a class of protein kinases, which can be activated by a multitude of signals, including phosphorylated receptor tyrosine kinases (RTK) and G protein-coupled receptors (GPCR) (264). Upon activation, PI3K phosphorylates Phosphoinositid-4,5-diphosphate $PI(4,5)P_2$ to $PI(3,4,5)P_3$, which serves as a docking site for downstream kinases, such as Akt. Once bound to $PI(3,4,5)P_3$, Akt gets phosphorylated by PDK1. This phosphorylation turns on the catalytic domain of Akt and allows it to activate the mammalian target of rapamycin complex (mTORC1) via phosphorylation of proline-rich Akt substrate of 40kDa (PRAS40) and tuberous sclerosis protein 2 (TSC2). The activated mTORC1 complex drives a range of cellular processes, such as protein synthesis and cell survival through a range of downstream effectors, including ribosomal S6 kinase (S6K1) and eIF4E-binding proteins (**Figure 9**). PI3K signalling is negatively regulated by the tumour suppressor phosphatase and tensin homolog (PTEN). This phosphatase

dephosphorylates $PI(3,4,5)P_3$ back to $PI(4,5)P_2$, which inactivates the pathway (265, 266).

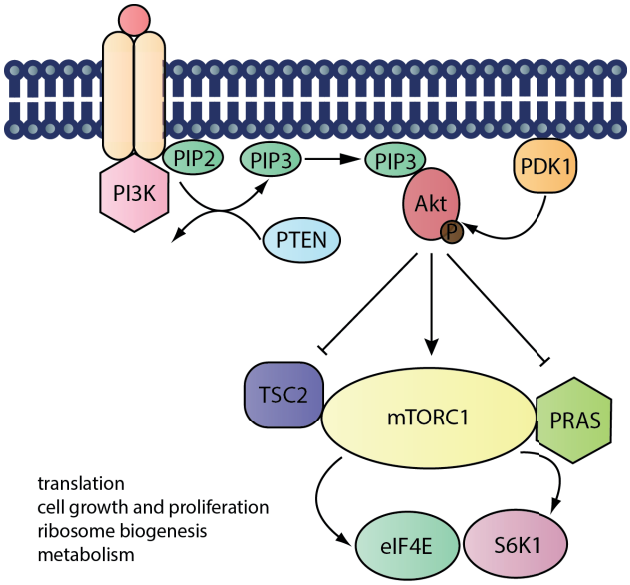

Figure 9. The Phosphoinositid-3-kinase signalling pathway

The Phosphoinositid-3-kinase (PI3K) signalling pathway drives key cellular processes, such as translation, cell growth, proliferation, ribosome biogenesis and various metabolic pathways. A variety of receptor tyrosine kinases (RTK) or G-protein couple receptors (GPCR) can activate PI3K, which in turn phosphorylates PIP2 to PIP3. PIP3 serves as a docking station for Akt, which is phosphorylated by PDK1. The now active Akt phosphorylates a range of downstream targets, including tuberous sclerosis protein 2 (TSC2) and proline-rich Akt substrate of 40kDa (PRAS40). This leads to activation of the mammalian target of rapamycin complex (mTORC1) complex, which in turn drives the abovementioned key cellular processes through a range of targets, including eIF4E-binding proteins (eIF4E) and ribosomal S6 kinase (S6K1).

In PCa, the two most commonly altered components of this pathway are PIK3CA, the gene for PI3K, and the negative regulator PTEN. It is estimated that this pathway is upregulated in about 25-70% of all PCa, and alterations are more common in metastatic disease (161, 168). PI3KC is amplified and mutated in approximately 25-30% of PCa (165, 171), and PTEN loss and inactivating mutations occur in about 45-50% (165, 166, 169, 267). Interestingly, these two are in general mutually exclusive.

The PI3K/Akt signalling pathway has been shown to interact with the AR and influence AR transcriptional activity. Specifically, a reciprocal feedback

regulation of the AR and the PI3K/Akt signalling pathway could be demonstrated in various murine and human models of PCa (268-270). PTEN loss and concomitant upregulation of the PI3K/Akt pathway reduced AR levels and transcriptional output. Conversely, inhibition or deletion of the AR activated Akt signalling (268, 269). Strikingly, combined pharmacological inhibition of PI3K and AR potently reduced tumour burden in a PTEN-loss mouse model (269).

c-Myc

The TF V-myc avian myelocytomatosis viral oncogene homolog or short c-Myc (MYC) is part of the myc family of TFs, which comprises at least the four members c-Myc, N-Myc, L-Myc and S-Myc. It was first described in 1981 as the mediator of avian leucosis virus (ALV)-induced lymphoma (271). Subsequently, it has been reported that reciprocal fusions between MYC and either the immunoglobulin heavy, lambda or kappa loci are strongly associated with non-Hodgkin's lymphomas (272-274). These fusions are thought to deregulate the expression of MYC and thus, the mutant transcriptome of MYC has been extensively studied in lymphomas (275-277). The MYC-containing 8q24 locus has also been reported to be amplified or rearranged in a range of other cancers and cancer cell lines, including colorectal, lung and cervical (278-281). Thus, genome-wide MYC binding properties have been described for these cell lines and others through the ENCODE initiative (282), however, with the striking exemption of PCa. MYC is also thought to play a vital role in embryogenesis and stem cell maintenance (283). Thus, MYC function in embryonic stem cells (ESC) has been characterised comprehensively (284, 285). In addition, MYC was one of the four TFs originally used to reprogram terminally differentiated cells back to induced pluripotent stem cells (iPS) (286), which further stresses its importance in stem cell maintenance.

Mechanistically, MYC acts as a heterodimeric TF, requiring the presence of its partner protein MAX (287). The assembled MYC-MAX complex binds

consensus DNA sequences in the genome, so-called E-boxes with a canonical sequence CACGTG, and initiates the transcription of its target genes (288). The MYC-MAX complex is predominantly assembled in proliferating cells. In resting or differentiated cells, however, MAX often binds to inhibitory partners, such as MAD or MNT, and these complexes repress transcription (289-291) (**Figure 10**). It has recently been proposed that MYC might act as a non-linear and universal amplifier of virtually all genes in lymphocytes and ESC (292, 293). Contradictory to these findings, however, it has been shown that MYC is also involved in transcriptional repression, which has been proposed to work through the interaction of the MYC/MAX complex with MIZ1 (294-297) (**Figure 10**).

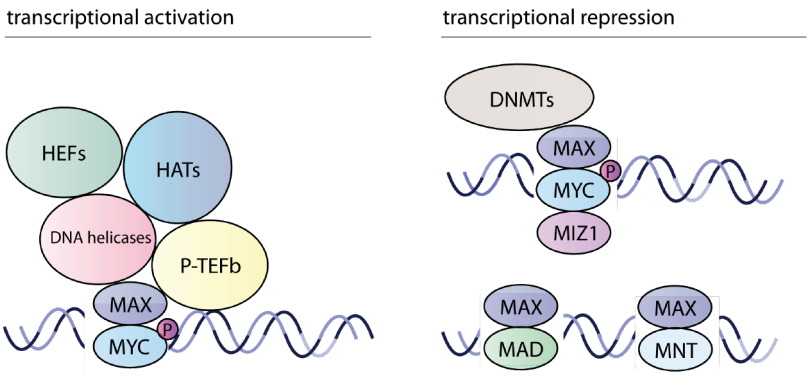

Figure 10. Transcriptional activation and repression by MYC

MYC is both involved in transcriptional activation and repression, and both processes require a distinct set of binding partners and cofactors. When activating transcription, MYC is bound to its partner protein MAX and generally located at gene promoters. The complex recruits and binds a subset of cofactors, including histone exchange factors (HEFs, e.g. p400), histone acetyltransferases (HATs, e.g. CBP or TIP60), DNA helicases (e.g. TIP48 or TIP49) and elongation factors (e.g. P-TEFb). MYC's binding partner MAX, however, can also bind to MAD or MNT, and these complexes have been shown to inhibit transcription. Furthermore, the assembled MYC/MAX complex can bind to MIZ1 and elicit repressive functions this way, presumably through the recruitment of DNA methylases (DNMTs), such as DNMT3a.

Both transcriptional activation and repression are achieved through the recruitment of distinct protein complexes. These include histone acetyltransferase complexes (containing P300, TIP60, GCN5, CBP and adapters, such as TRRAP) (298-303), histone exchange factors (P400) (304),

DNA helicases (TIP48, TIP49) (305, 306), TF elongation complexes (p-TEFb) (307, 308), and DNA-methyltransferases (DNMT3a) (309) (**Figure 10**). Interestingly and rather unusual for a TF, MYC is apparently not involved in RNA polymerase II recruitment and formation of the pre-initiation complex (310-312), but rather in the regulation of transcriptional pause release (313).

As mentioned above, MYC binding profiles and transcriptional networks have been extensively studied in a variety of model systems. Despite great differences between cellular models, a core MYC-regulated network of cell cycle control, anabolic metabolism and biomass accumulation has emerged in recent years (**Supplementary Paper IV**) (314). This core network includes genes essential for cell cycle control, ribosomal components and metabolism (315-318). Interestingly, these are also functions controlled by the AR in PCa (202). Strikingly, however, the studies focusing on MYC function in PCa are limited to studies confirming MYC's involvements in ribosome biogenesis and glutaminolysis (319, 320), and most recently its impact on lipid metabolism (321). Hence, our understanding of MYC's exact contribution to PCa initiation and progression remains limited.

Clinically, the chromosome locus containing MYC (8q24) has been reported to be amplified in up to 40% of PIN and potentially PIA (183, 322), making it an early event in prostate carcinogenesis, which predicts poor outcome (323, 324). Although the MYC gene in 8q24 is not always amplified itself, enhancer elements in commonly amplified upstream regions have been shown to increase MYC expression (325). Furthermore, elevated MYC protein levels are highly prevalent in PIN precursor lesions and thus constitute an early alteration in PCa (186). However, a clear correlation between 8q24 gain and MYC mRNA and/or protein levels could not always be shown (186), which highlights an important characteristic of MYC regulation. For example, there have been various reports of transcriptional regulation of MYC in PCa models (216, 242). In addition, post-transcriptional (326, 327), and translational (328, 329) mechanisms have been shown to

affect MYC levels in other model systems and these mechanisms might also act in PCa. Furthermore, post-translational modifications, such as phosphorylation and glycosylation, have been reported. For example, it has been postulated in mouse fibroblasts and mammary cells that phosphorylation at serine residues 62 and 71, or threonine 58 increases MYC's transcriptional activity and might alter binding specificity (330, 331). In prostate models, it has been shown that phosphorylation at serine 373 impairs MYC activity and that glycosylation influences stability of the protein (332, 333).

In normal prostatic tissue, ectopic MYC overexpression immortalises cells, facilitates castrate-resistant growth and induces PIN and early-stage PCa in mouse models (260, 334). In this mouse model, MYC overexpression also repressed NKX3.1 (260), and conversely, MYC's transcriptional activity is repressed by NKX3.1 (259). PTEN loss has been shown to drive MYC expression in cooperation with the MAPK signalling pathway (335). MYC expression is increased through TMPRSS2-ERG activity (216), and interestingly repressed by AR (336). In breast cancer cells, the AR has been reported to drive the expression of MYC, which in turn amplifies the transcriptional output of the AR (337). Notably, however, in PCa the effect of altered MYC expression on AR activity has thus far not been elucidated. Merely one review suggested that MYC knockdown might increase the expression of AR target genes (338).

Challenges and opportunities

As can be seen from the previous chapters, PCa is a highly complex and heterogeneous disease driven by a multitude of genomic alterations. Paradoxically, however, the focus of therapeutic intervention lies almost exclusively on the AR and AR signalling. Thus, studying the interplay of intracellular signalling pathways and transcriptional networks beyond the AR bears great potential for a better understanding of the disease and is likely to result in new treatment options. For example, this could happen

through repurposing of biomarkers or drugs used to treat other cancers, where these TFs have been defined as the main drivers. Defining the dominant TFs and networks in a given tumour will help to predict the activity of target pathways and concomitantly sensitivity to inhibitors (personalised medicine). With this in mind, the focus of this work will be to define the role of MYC in PCa and its influence on AR activity.

However, there is a range of fundamental challenges that researchers need to overcome. As mentioned above, the gold standard for defining transcriptional networks of a given factor is a combination of ChIP-seq and gene expression analysis (**Chapter 1.5.3**). This requires the ability to alter the activity of the factor in question in a reliable and optimally absolute manner, i.e. on/off state. For nuclear hormone receptors, such as the AR or ER, this can been achieved through hormone-ablation and reintroduction. Other TFs, however, cannot be switched on and off this easily and this includes MYC. In addition, integrating both types of data in order to attribute changes in mRNA levels to changes in TF binding can often be challenging. Various TFs, such as MYC, have been reported to bind preferentially to proximal promoters and in this case, a direct regulation of a given target gene by the TF can generally be inferred. Other TFs, however, including the AR and the ER bind preferentially to intergenic and intronic regions, often several kilobases away from any gene. To tackle this issue, a variety of chromosome conformation capture methods have been developed (3C, 4C and 5C) to demonstrate the influence of distant TF binding on gene expression (339-341). However, these methods are experimentally demanding.

Furthermore, changes in mRNA levels of a given target gene do not always predict changes in protein levels or activity since other factors, such as post-translational modifications or protein half-life, influence these, too. Lastly, cell lines are often poor representations of *in vivo* tumours and thus, insights derived from these models need to be validated in a clinical setting. One approach to tackle this confounding challenge of PCa research has been the

development of three-dimensional organoid cultures, which more closely resemble the *in vivo* architecture of the prostate gland (342, 343). However, these models still lack the microenvironment and immune system component of patient tumours. Prostate tumours are often multifocal and heterogeneous, and are likely to contain a variety of cell types, including epithelial, stromal and immune cells. Cancer genomic approaches and transcriptional analyses usually use bulk tumour samples containing several thousand cells and this heterogeneity complicates analysis even further. Even if the majority of cells in a given tumour respond to a specific drug, this does not guarantee therapy response since it might be a previously undetected subclone that results in a lethal phenotype.

This concludes the introductory part of this work. Based on previous research, this study set out with three distinct goals, which shall be described in more detail in the following section.

2. Aims of the study

Defining transcriptional networks and especially transcriptional interplay will be crucial for the development of a more profound understanding of PCa and will ultimately lead to better detection and treatment options. Based on the current status of knowledge and technical expertise, this study aimed to tackle three distinct yet intimately connected issues (**Figure 11**).

Part I (**1**) was purely a bioinformatics project and strived to assess the potential and significance of older gene expression datasets for current approaches (**Figure 11**). Array and sequencing platforms as well as technical principles have evolved and changed vastly over the past years. Thus, evaluating the biological durability of previously published datasets and results is a crucial factor in transcriptomics. We aimed to analyse older bulk expression data and develop a strategy to limit the impact of tumour heterogeneity and poorly defined cellular composition on subsequent analysis. Furthermore, experimental follow-up of postulated molecular concepts has often proven difficult due to their considerable sizes. Hence, we sought to develop a strategy to trim and refine complex transcriptomics data to make it more accessible to experimental and clinical follow-up.

Part II (**2**) and III (**3**) were closely connected and will make use of similar techniques and datasets (**Figure 11**). Using unbiased approaches, we aimed to define the transcriptional network of MYC in PCa. To this end, we modulated MYC activity by overexpression and created several ChIP-seq and gene expression datasets. These included ChIP-seq data for AR, MYC and various active and repressive histone marks (H3K4me1/3, H3K27me3/ac), and complementing expression array data at various timepoints. We then wanted to compare these data to and combine it with previously published experimental datasets and publicly available clinical data. The questions we sought to answer with this approach were as follows.

A. Which gene networks/pathways are controlled by MYC in PCa but independent of the AR? Can we identify a single MYC-driven pathway that, if inhibited, improves AR targeted therapies?

B. What is the genome-wide relationship between the AR and MYC? Are there overlapping genomic regions/gene networks/pathways in PCa shared by MYC and the AR? If yes, does modulating MYC levels influence their expression?

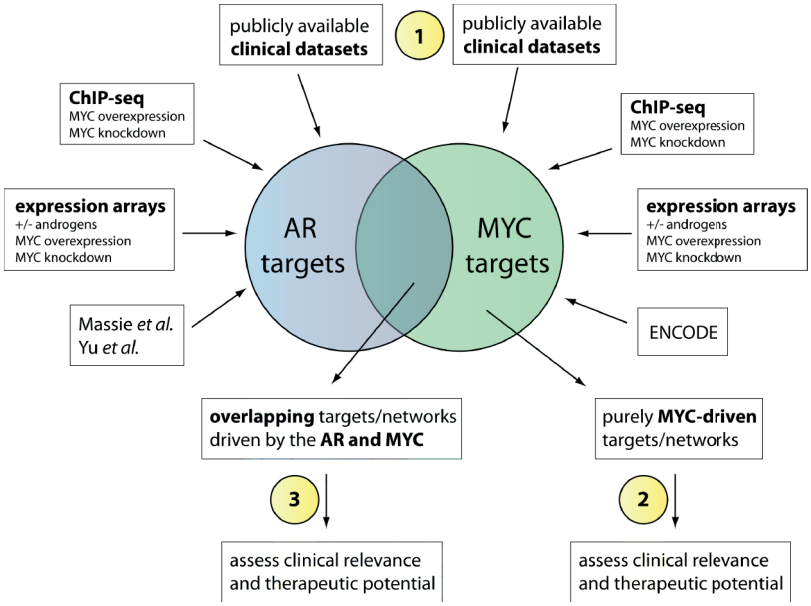

Figure 11. Aims of this study

The aims for this study were threefold and marked with yellow circles. Part 1 was purely bioinformatics and independent of any wet lab work. Using an array of previously published clinical PCa datasets, the goal was to develop a novel approach to analyse and interpret these highly heterogeneous datasets. Sequencing techniques and their interpretation have developed a lot over the past years and the aim was to evaluate whether information derived from older datasets still holds up in newer datasets generated with more sophisticated methods. Part 2 focused on establishing a thorough definition of MYC-driven gene networks in PCa cells. ChIP-seq and expression arrays under MYC-manipulating conditions (overexpression and knockdown) were generated to define MYC's transcriptional networks and combined with datasets from the ENCODE initiative and publicly available clinical datasets. Part 3 made use of the previously established MYC-network and assessed overlaps with the AR-driven transcriptome. Both self-generated ChIP-seq and expression array datasets for AR under MYC-manipulating conditions and previous publications (Massie *et al.* and Yu *et al.*) as well as publicly available clinical datasets were used. Both purely MYC-driven and overlapping networks were be individually assessed for their clinical relevance and therapeutic potential.

We then selected an interesting subset of these networks/pathways and assessed their clinical relevance in patient cohorts. Furthermore, we sought to evaluate their therapeutic potential by repurposing existing drugs in a variety of preclinical models (**Figure 11**).

3. Summaries of included papers

The three main papers included in this work will be briefly summarized. For more details, please refer to the original papers attached at the end of this thesis.

3.1 Paper I

Meta-analysis of prostate cancer gene expression data identifies a novel discriminatory signature enriched for glycosylating enzymes
Barfeld SJ, East P, Zuber V, Mills IG.
BMC Med Genomics. 2014 Dec 31;7(1):513.

In this study, we performed a meta-analysis of several clinical expression array and RNA-seq datasets. Most importantly, we sought to identify robust gene signatures that were statistically significant in both datasets generated in whole tissue, and laser-capture dissected material. If we were to find such signatures, we wanted to develop a strategy to refine and compare them to a point that would allow comfortable experimental or clinical validation. Typical sizes of clinically utilised gene expression signatures are currently in the range of 20-30 genes.
We started with a relatively old and small whole tissue dataset (Varambally *et al.*), and initially defined differentially expressed genes in BPH versus localised, and localised versus metastatic disease patients. We then applied a novel bioinformatics approach (Pearson correlation combined with hierarchical clustering using the Ward agglomerative method) to these differentially expressed genes and constructed a gene co-expression network. This approach created four large gene signatures consisting of several hundreds of genes each.

Next, we applied these signatures to two other datasets, a larger whole tissue (Ramaswamy *et al.*) and a laser capture microdissected dataset (Tomlins *et al.*). We asked whether our four large gene signatures or any of their sub-signatures could discriminate between patient sample groups (ranging from PIN to hormone-refractory CRPC) in the three datasets. We continued with the smallest sub-signature that was capable of discriminating between benign tissue and localised PCa in all three datasets. This signature consisted of 71 genes and the majority of the genes within were downregulated in PCa compared to benign tissue. Strikingly, MYC was among the few genes upregulated in PCa. We validated the expression patterns of the signature in two newer datasets - The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) and Taylor *et al.* - and found it to be consistent with the previous three sets.

Most biomarkers currently in use or under evaluation are overexpressed transcripts. Since the co-expression signature only contained few overexpressed genes, we went back to the originally defined differentially expressed genes in the Varambally *et al.* dataset, but only focused on overexpressed transcripts this time. In total, there were 97 overexpressed genes, which we refined using the Oncomine online compendium of expression array data. After applying a stringent threshold, we continued with a set of 33 genes that were consistently overexpressed among a broad range of PCa datasets. This list contained genes with a well-established role in cancer, such as MYC and ERG, but was clearly dominated by one metabolic pathway, O-glycan biosynthesis. Finally, we applied this signature to another dataset (Grasso *et al.*) and assessed its ability to discriminate between benign tissue, localised PCa and metastatic PCa. We found that the signature outperforms the discriminatory potential of AR, ERG and PSA (KLK3) in all comparisons.

3.2 Paper II

Myc-dependent purine biosynthesis affects nucleolar stress and therapy response in prostate cancer

Barfeld SJ, Fazli L, Persson M, Marjavaara L, Urbanucci A, Kaukoniemi KM, Rennie PS, Ceder Y, Chabes A, Visakorpi T, Mills IG.

Oncotarget. 2015 May 20;6(14):12587-602.

Our goal for this study was to use a global and unbiased approach to define MYC-regulated transcriptional networks in PCa. We combined *in vitro* and *in vivo* expression data, using an inducible MYC-overexpressing cell line model (LNCaP MYC), and co-expression analysis of clinical expression data (Taylor *et al.*), respectively. Both of our approaches revealed strikingly similar pathway enrichments, indicating that MYC-regulated genes are highly conserved between tissues and cell lines. The most significantly enriched pathways were ribosome biogenesis and several metabolic pathways, including purine, pyrimidine and amino acid metabolism.
We decided to focus on the compact purine *de novo* biosynthesis pathway and confirmed its direct regulation by MYC in PCa cell lines using qRT-PCR and ChIP. Strikingly, the AR was not involved in the transcriptional regulation of the genes in the pathway.
Next, we confirmed the clinical relevance of purine *de novo* biosynthesis. We interrogated the Oncomine online compendium of expression array data and focused on the two most consistently overexpressed genes in the pathway, PAICS and IMPDH2. We validated their overexpression at the mRNA and protein levels in two independent patient cohorts using clinical qRT-PCR and IHC, respectively.
Subsequently, we applied siRNA-mediated knockdown to determine, whether PAICS or IMPDH2 were essential for PCa cell growth. Whilst PCa cells were largely unaffected by PAICS knockdown, IMPDH2 knockdown significantly impaired PCa cell growth. We went on to treat PCa cells with a

clinically approved IMPDH2 inhibitor, mycophenolic acid (MPA), and observed a dose-dependent reduction in cell viability. Furthermore, we discovered additive effects of IMPDH2 knockdown and MPA with currently prescribed anti-androgens and androgen synthesis inhibitors. We confirmed the specificity of MPA and reported that IMPDH2 inhibition induces nucleolar stress, activation of p53 and tumour-suppressive miRNAs, and concomitantly a feedback downregulation of the regulator of the pathway, MYC.

3.3 Paper III

Overexpression of c-Myc antagonises transcriptional output of the androgen receptor in prostate cancer

Barfeld SJ, Urbanucci A, Fazli L, Rennie PS, Yegnasubramanian V, de Marzo AM, Mills IG.

Manuscript

For this study, we performed extensive ChIP-seq and gene expression analyses for AR, MYC and various active and repressive histone marks to characterize the impact of MYC overexpression in LNCaP MYC cells. We showed that AR and MYC co-occupy a substantial number of genomic loci amounting to approximately 25% of all AR and 30% of all MYC binding sites. MYC overexpression, however, did not significantly alter the AR binding profile or potency. Interestingly, the AR/MYC co-occupied sites resembled typical AR enhancer binding sites as they were largely intronic or intergenic, and exhibited high levels of histone marks characteristic for enhancer regions. Notably, the forkhead factor and pioneering factor, FOXA1 appeared to be highly enriched at AR/MYC overlapping binding sites.

Next we assessed the effect of MYC overexpression on androgen-induced gene expression. Surprisingly, we found that MYC mainly antagonised AR

signalling, which was in contrast to a recent breast cancer study, in which it amplified it. These findings were corroborated by the observation that MYC overexpression reduced the amount of active histone marks characteristic for enhancers (H3K4me1) and increased the number of repressive H3K27me3 marks. Furthermore, siRNA-mediated depletion of MYC led to de-repression of AR target genes.

Subsequently, we integrated both our ChIP-seq and expression array data and found that AR target genes that were antagonized by MYC were enriched for AR/MYC overlapping peaks in their vicinity. We validated our ChIP-seq and microarray predictions using ChIP qPCR and qRT-PCR, respectively. In addition, we also showed that the protein levels of multiple antagonized AR targets are significantly reduced. We then moved on to validate the antagonistic relationship of MYC and two AR target genes, KLK3 and GNMT, in an established patient cohort consisting of patients with BPH, localized PCa and CRPC. Strikingly, we found that with increasing levels of MYC protein, both KLK3 and GNMT expression decreased.

4. Methodological considerations

The most widely applied methods in the included publications and their advantages and disadvantages as well as potential alternatives will be briefly discussed in this section.

4.1 *In vitro* cell line models

In vitro cell line models are common tools for scientists and although they share many characteristics of patient tumours and are a useful starting point for PCa research, they have several inherent flaws that limit their translational potential. For example, cell lines are usually grown on plastic dishes using a defined composite medium, which hardly resembles the complex three-dimensional nature of an *in vivo* tumour and its microenvironment.

In our studies, we mainly worked on two widely used androgen-responsive and AR-expressing PCa cell lines, the LNCaP and VCaP lines. The LNCaP line was derived from a metastatic lesion to the supraclavicular lymph node of a Caucasian CRPC patient (344, 345). It has been extensively characterised and remains one of the most commonly used in vitro model systems for PCa research. Importantly for the analysis of transcriptional networks, LNCaP harbour an ETV1 fusion and PTEN LOH (161). VCaP cells were derived from a metastatic lesion to a lumbar vertebral body of a patient with CRPC (346). These cells contain a TMPRSS2-ERG fusion, one of the most frequent alterations in PCa (**Chapter 1.5.3**). The LNCaP-abl subline used in Paper II was created by culturing the parental LNCaP line in androgen-depleted medium for 87 passages and exhibits Lastly, we worked on a transgenic inducible MYC-overexpressing LNCaP derivative, termed LNCaP MYC. This cell line was previously published (346), and we performed extensive

timecourses to optimize our experimental conditions (see supplementary data in Paper II).

4.2 siRNA-mediated knockdown

To assess the relevance and function of a variety of genes, we reduced the expression of these genes in cell lines using small interfering RNAs (siRNAs). The underlying principle of RNA interference was first described by Fire *et al.* in 1998 and later awarded with the Nobel Prize in Physiology or Medicine (347). siRNAs were first described in detail in 1999 and shortly thereafter utilized in cell culture experiments to knockdown the expression of target genes (348, 349). In principal, cells are transfected with double-stranded siRNA molecules, which are processed by a variety of cellular proteins, including Dicer, Argonaute proteins, RISC, Drosha, TRBP and PACT (350). This results in degradation or block of translation of the target mRNAs and concomitant reduction in protein levels.

Naturally, the technique comes with a range of possible drawbacks. Most importantly, siRNAs may bind unspecifically, which might reduce the expression of non-target genes. To overcome this, appropriate control experiments were used (non-targeting control siRNAs), and whenever possible, at least two different siRNAs targeting the same gene were used (IMPDH2 in Paper II). Another issue is time point selection since mRNA and protein levels do not necessarily correlate, and are influenced by a range of factors, including post-translational modifications and protein half-life. Thus, we always validated knockdown efficacy at both mRNA and protein level (MYC, IMPDH2, PAICS in Paper II) after conducting time courses.

4.3 RNA isolation and gene expression analysis

Global gene expression analysis can be performed in a variety of ways. Most commonly, expression arrays or RNA-seq are being used to determine the expression status of a broad range of genes in an unbiased manner. Both require high quality RNA and thus we used Qiagen's RNeasy kit, which is widely used for these applications. Furthermore, RNA integrity was always validated using Agilent's Bioanalyzer system and spectrophoto-/fluorometric methods where applicable (NanoDrop or Qubit systems). Preparation of biotin-labelled cDNA for hybridization on expression arrays is largely standardized and was performed by the genomics core. For expression arrays, various platforms exist and these include but are not limited to Affymetrix, Agilent and Illumina. Both probe sequences and genome coverage differ between the platforms, which regularly leads to issues when trying to compare datasets from different platforms. Thus, not all genes were covered in all platforms analysed in Paper I. In Paper II and III, we used Illumina Human HT-12 Expression BeadChips microarrays to create our own datasets.

Expression array analysis

Downstream analysis of expression array data requires extensive bioinformatics knowledge but due to the advanced nature of the technique, analysis pipelines are largely standardised. However, authors have used different algorithms to define differentially expressed genes or alternatively varying levels of fold change since no obligatory consensus exists. A range of third-party open-source software has enabled wet-lab scientists to perform these analyses themselves. For example, we used the freely available open source software J-Express (http://jexpress.bioinfo.no/site/), which allowed for a broad range of commonly used analysis procedures, such as standard normalization commands, fold change analysis and

hierarchical clustering (351). Other freely available software used included Cluster 3.0 (352), web-based Venn diagram creators (e.g. http://bioinfogp.cnb.csic.es/tools/venny/) and various functional annotation tools, such as DAVID or genecodis (353, 354). Furthermore, unbiased gene set enrichment analysis (GSEA) without prior data processing was performed using a web-based tool created by the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp) (355). In general, we always made sure to validate our microarray predictions using qRT-PCR.

4.4 Chromatin immunoprecipitation

ChIP is a technique commonly used to map protein-DNA interactions and was first described in 1988 (356). Briefly, protein-DNA complexes are fixed using a suitable crosslinker, e.g. formaldehyde. After quenching, washing and cell lysis, cellular chromatin is sheared with an ultrasonic shearing device to an appropriate size range (usually 200 to 500bp). Subsequently, the fragmented chromatin is precipicated with the antibodies of choice, which are usually pre-bound to magnetic or agarose beads. The next day, bead-antibody-protein-DNA complexes are washed and crosslinking is reversed. Precipitated DNA sequences are validated after DNA clean-up using either qRT-PCR, microarrays or high-throughput sequencing (ChIP-seq) (357).

The technique has several potential pitfalls and issues researchers must be aware of. First and foremost, appropriate choice of antibody is crucial. Naturally, integrity and abundance of precipitated chromatin highly depends on antibody quality. Thus, only properly validated ChIP-grade antibodies, which had been experimentally validated in other studies, were used in our ChIP reactions in Papers II and III. Furthermore, we always included suitable controls, such as non-specific IgGs and total input control, to establish background signals. In terms of materials, magnetic beads are superior over their agarose/sepharose counterparts regarding background

and handling, and virtually all research groups exclusively use those. Both crosslinking and sonication times require optimization and optimal time points vary between factors and cell lines. Therefore, we always ran optimization experiments for these conditions.

When ChIP qPCR was used to validate precipitated DNA sequences, we made sure to use appropriate positive and negative genomic control regions. These were based on previously published datasets or datasets from the ENCODE consortium, which are publicly available, for example through the UCSC genome browser (http://genome.ucsc.edu/). We always included melting curves for the primer pairs to ensure specific amplification. Data presentation for ChIP qPCR is not standardized but displaying the data as either '% of input' using the formula 2^(ct input-ct ChIP) or 'fold over IgG' using 2^(ct IP-ct IgG) are commonly used approaches.

ChIP-seq and data analysis

ChIP-seq requires the precipitated material to be processed to sequencing libraries compatible with the desired sequencing platform (usually Illumina or SOLiD based systems). During library preparation, the DNA is end repaired, ligated to barcoded sequencing adapters and amplified by PCR prior to HTS. Although this process has largely been standardized (Illumina TruSeq kits), it is nonetheless crucial to optimize the amount of PCR cycles to avoid overamplification and concomitant underrepresented libraries.

For data analysis in Paper III, we combined the expertise of bioinformatics collaborators and the open-source web-based framework Galaxy in its various instances. Galaxy provides wet-lab scientists without programming knowledge with a means to perform their own basic and advanced analyses of ChIP-seq data (358, 359). Data analysis is complicated by the lack of a clear consensus analysis pipeline or publication requirements. Furthermore, the amount of quantitative information that can be derived from direct comparisons between treatment conditions in ChIP-seq experiments is still under debate. This is because multiple steps in the library preparation

process, such as PCR amplification or clustering, might mask biological differences. Recently, various approaches including novel bioinformatics normalisation steps and spike-in strategies have been developed to improve the analyses procedure (360-362). However, no clear consensus strategy currently exists and thus we tried to limit the conclusions drawn from our quantitative analyses performed in Paper III.

The ENCODE consortium has attempted to introduce a gold standard for the publication of ChIP-seq data to increase reproducibility and data quality but so far no obligatory consensus has been established (363). In general, we adhered to the ENCODE guidelines and always performed and present overlaps of our datasets with previously published sets to increase confidence in data quality and analysis procedure (Paper III). Lastly, experimental validation of bioinformatics predictions using ChIP qPCR was always included for a broad range of loci.

In Paper III, we also included a recently published modification of the original ChIP-seq protocol, termed ChIP-exo (**Supplementary Paper V**) (364). ChIP-exo incorporates two exonuclease digestion steps and on-bead library preparation and these steps are thought to narrow peak width and reduce hands-on time during library preparation, respectively. A more in depth comparison of ChIP-seq and ChIP-exo, and a detailed experimental procedure can be found in the appendix (**Supplementary Paper V)**.

4.5 Clinical samples

Tumour heterogeneity and multifocality, as well as cellular composition and integrity of the samples are confounding challenges when operating on patient-derived specimens and these need to be carefully addressed. Due to the translational nature of our research, all three papers included in this thesis made use of clinical samples. In Paper I we focused on previously published clinical expression data derived from patient tumours. Since this

paper is a meta-analysis of previously published datasets, it was not in our power to control sample quality and integrity. In Papers II and III, however, we used qRT-PCR and IHC on our own patient cohorts.

Immunohistochemistry

Paper II evaluated the protein levels of PAICS and IMPDH2 in a cohort consisting of a total of 194 PCa specimens obtained from the Vancouver Prostate Center. This patient cohort has previously been used in various other publications (202, 365), and thus our confidence in sample quality and integrity was high. More information on antibody validation and quality control can be found in the material and methods section of Paper II. In Paper III, we validated the expression of MYC, KLK3 and GNMT in the same patient cohort and more information can be found in the supplemental section of this paper.

Clinical real-time PCR

In Paper II, we performed qRT-PCR on a total of 55 clinical samples derived from patients with BPH (15), localised PCa (27) and CRPC (13). This patient cohort has also been used in numerous publications (366-368), and thus we were assured of its quality and integrity. The exon-spanning primers targeting PAICS and IMPDH2 used in the paper were carefully assessed in their specificity using melting curves and gel electrophoresis.

5. Discussion

In this chapter, the work conducted in the three summarised papers will be discussed and put into context. For a more detailed discussion of the individual results, the reader is advised to consult the discussion sections of the respective papers.

A novel co-expression approach reveals gene signatures capable of clustering patient samples

In Paper I, Meta-analysis of prostate cancer gene expression data identifies a novel discriminatory signature enriched for glycosylating enzymes, we performed extensive meta-analyses of several publicly available gene expression datasets (369-371).

Using a novel co-expression approach, we defined a compact gene signature (71 genes) that was capable of subclustering patient samples and validated it in two additional cohorts (167, 372). Interestingly, the signature mainly consisted of genes downregulated in PCa relative to BPH and was enriched for genes involved in smooth muscle contraction and focal adhesion. This high frequency of downregulated genes in epithelial tumour tissue relative to controls has previously been reported (373). Furthermore, numerous myosin components were present among the downregulated genes. It has been shown that PCa progression depletes stromal components from the tissue and this might explain this observation (374). Strikingly, MYC was among the only four genes that were significantly overexpressed in this signature.

Enzymes involved in glycosylation are highly upregulated within the co-expression gene signatures

From a biomarker perspective, genes that are downregulated during the course of a disease are less attractive than upregulated ones. Thus, we shifted our focus to 33 genes that were consistently upregulated in our co-expression analyses and the Oncomine database (375). Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment revealed glycosylation to be the most significantly enriched pathway with four enzymes involved in glycosylation contributing to the enrichment.

Glycosylation describes an enzyme-catalysed reaction in which glycans, sugar molecules of varying complexity, are attached to other molecular structures, most commonly proteins or lipids. Depending on composition, this creates a variety of different molecules, including glycoproteins, proteoglycans and glycolipids (376). Glycosylation influences protein folding and stability, cell-cell interactions, and proteoglycans are a crucial part of the ECM. The two most abundant forms of glycosylation are N-linked, where the glycan is attached to a nitrogen atom of asparagine, and O-linked, where the glycan is attached to an oxygen atom of serine or threonine (377, 378).

Notably, UAP1 is the final enzyme of a metabolic pathway called hexosamine biosynthetic pathway (HBP), which produces UDP-N-acetylglucosamine (UDP-GlcNAc) (379). UDP-GlcNAc is the starting molecule for more complex glycans that are subsequently attached to target proteins via N-linked and O-linked glycosylation in the endoplasmic reticulum (ER) and the Golgi apparatus. The HBP has been described as an integration point for multiple metabolic pathways, including glycolysis, glutaminolysis and nucleotide biosynthesis. Hence, the activity levels of the HBP might reflect the overall energy status of a given cell. Strikingly, UAP1 has recently been reported to be overexpressed early in PCa and to protect against inhibitors of N-linked glycosylation (380). The high prevalence of

glycosylation enzymes in our signature merits future investigation into this exceedingly interesting metabolic pathway.

A compact molecular signature containing MYC discriminates between benign cases, localised and metastatic prostate cancer

To validate the discriminatory potential of our 33 gene signature, we applied it to a total of three different datasets (Taylor *et al.*, TCGA-PRAD and Grasso *et al.*) and assessed its ability to cluster patients into different disease stages. We compared the performance of our signature to the classical PCa classifiers AR, KLK3 and ERG. Strikingly, our signature discriminated between BPH, localised PCa and metastatic samples with excellent sensitivity and specificity as reflected in area under the curve (AUC) values ranging from 0.95 to 0.99. No individual gene was able to discriminate with such a high sensitivity and specificity and this highlights the translational potential of multigene signatures. Notably, various multigene signatures have recently been developed and applied in diagnostics to improve patient stratification and treatment decisions (e.g. GenomeDx Decipher) (381-383). Intriguingly, multiple genes in our signature have been postulated as PCa biomarkers in blood or urine. These include ERG, AMACR, CRISP3, GDF15, TDRD1 (384, 385), and most prominently PCA3, which is already in clinical use (386).
Interestingly, out of the 33 genes in our signature, five were TFs – SIM2, DLX1, HOXC6, ERG and MYC. Increased levels of SIM2 have recently been reported as a novel marker of aggressive PCa (387). DLX1 is part of a candidate gene panel for the early diagnosis of PCa and strikingly, this panel also includes HOXC6 (388). Furthermore, other homeobox factors, such as NKX3.1 or HOXB13 have well-established roles in PCa (**1.5.3**). ERG, which is frequently fused to the androgen-regulated gene TMPRSS2, has been studied extensively in PCa (**1.5.3**). Intriguingly, this was not the case for MYC, which will be the focus of the next parts of this discussion.

MYC directly controls purine *de novo* biosynthesis and other metabolic pathways in prostate cancer cells

MYC was consistently overexpressed in a broad range of datasets used in Paper I and part of our 33 gene signature. However, surprisingly little was known about the biological consequences of elevated MYC levels in PCa. Thus, we went on to define MYC's transcriptional networks in an unbiased manner. Our approach, which resulted in Paper II, was twofold. Firstly, we used a recently published inducible MYC-overexpression derivative of the LNCaP cell line, termed LNCaP MYC, and gene expression microarrays after short periods (5h and 12h) of MYC overexpression (389). Secondly, we revisited the Taylor *et al.* dataset and performed an *in silico* analysis using a similar strategy as in Paper I to define genes co-expressed with MYC in CRPC patients (167).

Notably, both approaches revealed a strikingly similar pathway enrichment pattern enriched for biosynthetic processes, including ribosome biogenesis and various metabolic pathways, such as purine and pyrimidine biosynthesis. Interestingly, our findings were similar to previous reports from other models systems, such as lymphomas or embryonic stem cells (**Supplementary Paper IV**) (390, 391), suggesting that MYC is a driver of biomass accumulation and controls a core set of genes and networks, regardless of cell type. In PCa, MYC had previously been reported to drive ribosome biogenesis in PCa (320), and thus we focused on the second most significantly enriched pathway in both approaches, purine *de novo* biosynthesis.

This pathway consists of six enzymes that catalyse the conversion from phosphoribosyl pyrophosphate (PRPP) to inosine-5'-monophosphate (IMP), which in turn is converted to adenosine-5'-monophosphate (AMP) or xanthosine-5'-monophosphate (XMP) via the activity of ADSS/ADSSL1 or IMPDH1/2, respectively. XMP in turn is converted to guanosine-5'-monophosphate (GTP) (392). The *de novo* purine biosynthesis can be

regarded a supplemental metabolic pathway fuelling core cellular processes, such as DNA replication, transcription and energy budgeting. Non-dividing and differentiated cells generally rely on the purine salvage pathway for their purine needs and the *de novo* biosynthetic pathway is thought to be active only in dividing cells, such as cancer or stem cells (392). The compactness and potential specificity for rapidly dividing cells made the pathway an extremely attractive target to focus on. Furthermore, revisiting the Oncomine database revealed two members of this pathway, IMPDH2 and PAICS, to be consistently overexpressed among 16 other published clinical datasets (375).

We then moved on to validate our *in silico* predictions in two PCa cell lines and found MYC to directly regulate five enzymes of the *de novo* biosynthesis (PPAT, GART, PFAS, PAICS and ADSL) plus one IMP-converting enzyme (IMPDH2). These enzymes responded to MYC overexpression and knockdown with increased and decreased mRNA and protein levels, respectively. Furthermore, they exhibited binding of MYC to their respective promoters as shown by ChIP qPCR experiments, which suggested a direct regulation. This strategy has been used before to confirm MYC's role as a direct regulator of nucleotide biosynthesis in Burkitt's lymphoma cells and of ribosome biogenesis in PCa cells (276, 320). Notably, the genes appeared to be independent of AR activity since androgen treatment did not alter mRNA or protein levels.

PAICS and IMPDH2 are overexpressed in prostate cancer patients

Our *in vitro* approach and the Oncomine database suggested that the *de novo* purine biosynthetic pathway was a clinically relevant metabolic pathway in PCa. To confirm these *in vitro* predictions in patients, we assessed the mRNA and protein levels of the two most consistently overexpressed enzymes in the pathway, PAICS and IMPDH2, in two different patient cohorts. Both cohorts had been used previously to assess the clinical

relevance of a variety of genes/proteins, including ZWINT1, FEN1, CAMKK2 and TAF1 (202, 365, 368). We found PAICS to be upregulated at the mRNA level in CRPC and at the protein level both in localised PCa and CRPC. This was the first time PAICS expression was assessed in any cancer and our study suggested a strong potential for PAICS as a biomarker. SAICAR, the intermediate of the *de novo* purine biosynthetic pathway produced by PAICS has previously been implicated in the regulation of pyruvate kinase isoform M2, a potentially crucial enzyme for cancer cell growth in glucose-limited conditions, which are commonly observed in solid tumours (393). Similarly, IMPDH2 mRNA and protein levels were elevated in CRPC and thus suggested potential for IMPDH2 as a PCa biomarker for late stage disease. The biomarker potential of IMPDH2 had previously been reported in colorectal cancer but our study was the first to assess IMPDH2 expression in PCa (394).

Inhibition of IMPDH2 reduces proliferation and leads to nucleolar stress, p53 activation and induction of MYC-targeting miRNAs

Next we determined whether PAICS or IMPDH2 were essential for PCa cell proliferation. Using siRNA-mediated knockdown, we observed a significant decrease in cell proliferation when IMPDH2 but not PAICS expression was reduced. Intriguingly, an uncompetitive inhibitor for IMPDH2, mycophenolic acid (MPA), is a clinically approved immunosuppressant (395), and we observed a dose-dependent reduction in cell viability in PCa cell lines. Interestingly, combinatory treatment with an established anti-androgen (Enzalutamide/MDV3100) or an androgen biosynthesis inhibitor (Abiraterone) resulted in additive effects. This further strengthened the AR-independence of this metabolic pathway and merits further investigation into the clinical potential of MPA for the treatment of late stage PCa. This is particularly intriguing since several governmental agencies have started to collaborate with pharmaceutical companies to loosen intellectual property

restrictions with the goal of repurposing existing drugs for new diseases (396). The main advantage of this strategy is that the existing drug has already passed most basic clinical tests regarding general toxicity and other issues that commonly lead to failure during drug development. Examples of repositioned drugs currently in clinical trials for PCa treatment include Nelfinavir (originally an HIV drug) and Digoxin (originally developed for cardiac diseases) (397). MPA, however, due to its advanced age is no longer protected by any intellectual property restrictions and is a relatively cheap drug. Thus, the motivation for a clinical trial involving a combination treatment of MPA and AR-targeting drugs would be to lower the necessary doses of expensive AR-targeting drugs. This could dramatically reduce treatment costs and thus reduce the financial burden for the healthcare system.

Mechanistically, we showed that MPA treatment reduced intracellular GTP levels but did not significantly alter the levels of other nucleotides. Furthermore, adding guanosine rescued the anti-proliferative effect and confirmed the specificity of MPA. Depleting cells of GTP has a range of physiological effects due to the wide-spread functions of nucleotides and particularly the nucleolus has been shown to be extraordinarily sensitive to GTP shortages. The nucleolar protein nucleostemin, also called guanine nucleotide-binding protein-like 3 (GNL3), is a positive regulator of cell proliferation and expressed in a variety of cancer and stem cells (398). GTP depletion leads to rapid degradation of GNL3 and nucleolar stress, and we were able to confirm these effects in PCa cell lines (399). Notably, coping with cellular stress is a hallmark of cancer cells (36), and being able to trigger a massive stress response in tumour cells might improve drug response in patients. Nucleolar stress has a multitude of downstream effects and amongst others, it triggers p53 stabilisation and concomitant cell cycle arrest, both of which we observed in PCa cells (400, 401). Strikingly, MPA treatment also led to a feedback downregulation of MYC, which at least in part appears to be mediated by the MYC-targeting microRNAs (miRs) 34b

and 145, which have previously been shown to reduce MYC levels in other PCa models (402, 403). In conclusion, MYC directly controls purine *de novo* biosynthesis and the conversion of IMP to XMP/GMP in PCa cell lines. Inhibition of IMPDH2, an IMP-converting enzyme inhibits proliferation, leads to nucleolar stress and sensitises cells to androgen-deprivation therapy. Notably, it also led to a feedback downregulation of MYC. This is particularly intriguing since MYC is overexpressed in a substantial proportion of PCa patients, and even after decades of research, no MYC inhibitor has been clinically approved yet. Thus, targeting MYC-dependent pathways instead might help to diminish the contribution of the TF to the malignancies and serve as a patient stratifier. A recent study has shown that approximately 90% of CRPC cases harbour 'clinically actionable' molecular alterations, such as PI3K pathway, AR signalling or cell cycle control (164). However, the immediate clinical impact of inhibiting these molecular alterations in CRPC patients has been rather underwhelming so far. Including MYC-targeted therapies might significantly improve these numbers.

MYC and AR share a substantial amount of overlapping binding sites

After having established that MYC regulates a range of core metabolic processes in PCa cells that are independent of AR activity, we aimed to define the relationship between AR and MYC and evaluate whether both TFs share common targets or gene networks. It was recently shown in apocrine breast cancer cells that MYC levels were increased by androgen treatment and that MYC in turn amplified AR activity and transcriptional output in a positive feedback manner (337). To our surprise, however, androgen treatment of PCa cells did not increase but significantly decreased MYC levels and thus suggested a different relationship between these two TFs in PCa cells.

As previously reported, MYC overexpression induced androgen-independent growth of our LNCaP cell line model, which we used in Paper II

(260, 334, 389). Thus, we sought to investigate the role of ectopic overexpression of MYC on AR activity in LNCaP MYC cells. Firstly, we defined the global MYC and AR binding profiles using ChIP-exo after androgen treatment with or without MYC overexpression. ChIP-exo is a modification of the traditional ChIP-seq approach and incorporates two novel exonuclease reactions to narrow peak widths and refine signal to noise ratio (364). The overlaps between our AR and MYC datasets and previously published datasets in PCa and other cell lines ranged were substantial and gave us confidence in our experimental approach and analysis pipeline. We found that MYC overexpression did not significantly alter MYC or AR binding profiles, which might, however, be due to nature of ChIP-seq and its restrictions in terms of quantitative analyses (362). MYC overexpression did not redistribute MYC or AR binding sites and motif enrichments were similar in both conditions. Interestingly, MYC and AR shared 11,857 binding sites, which amounted to approximately 25% of all AR and 30% of all MYC sites. These sites were largely intergenic and intronic, and were highly enriched for FOXA1 binding (approximately 55%), which we confirmed by overlapping our sites with a previously published dataset for FOXA1 (404). This is the first time an overlap between MYC and FOXA1 has been reported and suggests the existence of a larger transcriptional complex containing AR, MYC and FOXA1. Furthermore, the average peak height of these AR/MYC overlapping peaks was significantly higher than for all AR peaks and this suggested that AR/MYC overlapping peaks were high-affinity binding sites for the AR.

MYC overexpression alters global H3K4me1 and H3K27me3 levels

MYC has recently been shown to correlate inversely with H3K27me3 levels, which in turn correlate with differentiation (405). Thus, we sought to evaluate the effect of MYC overexpression on global H3K27me3 levels using ChIP-seq in our model. In addition, we included three other histone marks in

our study, H3K4me1, H3K4me3 and H3K27ac, which serve as markers for active enhancers and promoters (406). As with our ChIP-exo data for the AR and MYC, we compared our datasets to previously published datasets and observed substantial overlaps ranging from 45-100%. Upon MYC overexpression, the most striking differences were observed for H3K4me1, where increased MYC levels reduced the total number of peaks by approximately 20%, and for H3K27me3, where MYC overexpression increased the number by roughly 30%. This suggested that ectopically elevated MYC levels decreased the amount of active enhancers (H3K4me1) and increase the amount of condensed chromatin regions (H3K27me3) (406).

Integration of our histone ChIP-seq data with the AR and MYC ChIP-exo datasets revealed that AR sites were predominantly associated with enhancer-like features (high H3K4me1 and H3K27ac) and MYC sites with those of active promoters (high H3K4me3 and H3K27ac). Strikingly, the AR/MYC overlapping sites resembled pure AR sites in their histone modification pattern (high H3K4me1 and H3K27ac) and neither AR nor MYC sites were significantly associated with H3K27me3.

Overexpression of MYC antagonises AR-mediated transcription

As mentioned above, MYC has been shown to amplify transcriptional output of AR-regulated genes in breast cancer cell lines (337). To evaluate the effect of MYC overexpression on AR-mediated transcriptional regulation in our model, we performed gene expression analysis under similar conditions as our ChIP-seq experiments (androgen stimulation alone or together with MYC overexpression). We used unbiased gene set enrichment analysis (GSEA) and found, as expected, a classic androgen signature (Nelson *et al.*) to be the top-upregulated gene set upon androgen stimulation (407). Upon MYC overexpression, we observed a range of typical MYC target signatures among the top-upregulated gene sets and this

confirmed the validity of our model system (408). Intriguingly, the top-downregulated gene set when MYC was overexpressed was the abovementioned androgen signature, suggesting that MYC does not amplify but antagonise AR-mediated gene transcription in PCa cells. This was further corroborated by GSEA of previously published gene expression data that used siRNA-mediated knockdown of MYC (320). Correspondingly, MYC knockdown led to a significant upregulation of the Nelson androgen signature. We then looked at individual genes and found that a substantial amount of androgen-induced genes, roughly 25%, were antagonised by MYC overexpression while only about 1.5% were amplified. We subjected the antagonised genes to pathway analysis using KEGG and gene ontology (GO) and found a variety of metabolic pathways and transcriptional regulatory networks. Strikingly, UDP-N-acetylglucosamine biosynthesis, which we discussed in Paper I was among the pathways. Thus, MYC overexpression resulted in an increased number of condensed chromatin regions (H3K27me3), a decrease in active enhancers (H3K4me1) and primarily antagonised androgen-induced gene transcription. We then integrated our ChIP-seq and expression array datasets and found a significant enrichment of AR/MYC overlapping peaks in the vicinity of antagonised genes, which suggested a direct effect of MYC overexpression on these genes. However, establishing direct effects of distant TF binding on gene expression remains a challenge (**Chapter 1.5.3 – Challenges and opportunities)**.

MYC levels inversely correlate with antagonised AR targets *in vivo*

Our list of antagonised genes included several AR targets that have previously been suggested as putative PCa biomarkers. These included SOCS2 and GNMT, which have both been shown to perform in opposite and contradictory directions in various studies (409-412). We validated the

antagonistic effect of MYC overexpression on these genes in our cell line model using ChIP qPCR, qRT-PCR and western blotting.

Subsequently, we progressed to clinical samples and assessed the protein levels of MYC, KLK3 and GNMT in the same patient cohort we used in Paper II. As predicted by our cell line experiments, staining intensities for KLK3 and GNMT decreased with increasing MYC levels. Our findings suggest that the levels of putative protein biomarkers in biopsy samples depend not only on the activity of the AR but are also influenced by other TFs, such as MYC. The fusion between TMPRSS2 and the ETS TF ERG has been reported to suppress prostate cell differentiation and drive a more stem-like phenotype (215, 216). Likewise, the repressive effect of MYC overexpression on a subset of AR-targets could be interpreted as a similar process, especially since MYC has been shown to drive stem-cell like phenotypes and control the balance between differentiation and self-renewal in different models (283, 413). This could help to explain the somehow contradictory findings regarding several AR-regulated biomarkers, such as SOCS or GNMT (409-412). Thus, thorough characterization of the dominant transcriptional networks in a given tumour might help to improve the accuracy of promising biomarkers and treatment stratifiers.

6. Future perspectives

In Paper I, we identified a compact 33 gene signature that was capable of discriminating between BPH, localised PCa and metastatic PCa in several publicly available datasets. Since this was a meta-analysis of existing datasets, we did not produce any new data from previously uncharacterised patients. Naturally, the next step would be to test our signature in a larger clinical setting. Various questions could be asked in such a study, e.g. 1) does our signature predict the need for a repeat biopsy in case of a negative result but elevated PSA levels, analogous to PCA3 (early detection) (386), 2) does our signature predict post-operative BCR or metastasis (prognostic/recurrence), 3) is it able to distinguish between tissue isolated from BPH, primary PCa or metastatic PCa (diagnostic) or 4) if performed on biopsy samples, does our signature help to predict treatment efficacy (predictive)? A prominent example of an RNA-based gene signature is the 22 biomarker test GenomeDx Decipher that has recently been tested in a range of larger cohorts with so far promising results (381-383). In addition, a variety of other diagnostic/prognostic/predictive tests are emerging, e.g. Prolaris Myriad and Oncotype DX, which are FDA-approved tools to distinguish between indolent and aggressive disease (414). Notably, multiple genes in our signature (e.g. ERG, AMACR, CRISP3, GDF15, TDRD1, PCA3) have also been applied to assess PCa risk or stratify patients using other biological fluids, such as blood or urine (384-386). It will be interesting to evaluate the biomarker potential of the remaining genes in our signature in these fluids.

Paper II focused on the *de novo* purine biosynthesis and identified the IMP-converting enzyme IMPDH2 as a potential drug target in PCa. However, we merely focused on *in vitro* models in our study (PCa cell lines) and did not progress to preclinical models, such as xenografts or transgenic mouse models. Thus, a next step would be to assess the efficacy of MPA in

xenografted LNCaP or VCaP, preferably in a castrate setting since IMPDH2 levels in our patient cohorts were elevated in CRPC but not significantly in localised disease. Even more intriguing would be transgenic mouse models driven by MYC overexpression, such as hiMYC mice (415). Given these experiments were to be successful, progression to clinical trials would be an option. Since MPA is a clinically approved immunosuppressant (395), initial toxicity tests could be omitted and this is a major advantage of drug repositioning (**Chapter 5**). Patient selection should be based on MYC expression since this would likely predict the activity of the pathway in the patient's tumour. Strikingly, the current standard of care for CRPC includes a combination treatment of an androgen-synthesis inhibitor (Abiraterone) with an immunosuppressant (Prednisone) (127, 129). This suggests that targeting the immune component of PCa might be beneficial for patients and thus MPA could have great potential as a treatment alternative.

In Paper III we elucidated the antagonistic relationship between MYC and the AR. MYC overexpression counteracted androgen-induced gene transcription and led to the downregulation of a subset of AR target genes. We also validated this antagonistic effect of MYC on the expression of the two AR targets KLK3 and GNMT in patient samples using IHC. However, our IHC data is merely derived from parallel sections of the same tumour and represents an average score. For a more thorough analysis, double-staining and more detailed scoring on a cell-by-cell basis would be necessary to strengthen the message. Furthermore, making use of novel *in situ* RNA hybridization technologies, such as RNAscope (416), could help to validate the antagonistic regulation at the mRNA level. There have been contradicting reports regarding the prognostic properties of GNMT expression with one study claiming high GNMT levels to be a marker of poor outcome (411), and one study showing the opposite (412). Our findings suggest that this controversy might actually be explained by the relative levels of MYC in the respective tumour samples. Consequently, validation of this hypothesis by assessing MYC and GNMT in a larger cohort would be of

great interest. Furthermore, our list of MYC antagonised genes contained various proteins involved in cellular signalling pathways (e.g. ERRFI1 and EGFR signalling, and SOCS2 and JAK/STAT signalling). Her2/EGFR signalling is a principal target in breast cancer treatment and targeting this pathway has been postulated for the treatment of PCa (417, 418). Similarly, the inflammatory JAK/STAT signalling pathway has been shown to play a role in PCa and inhibition might be a viable treatment option in the future (419). The relative levels of MYC might serve as a surrogate marker for the activity of these pathways and concomitantly predict treatment efficacy.

Overall, this thesis has elucidated the transcriptional role of MYC in PCa and unveiled the effects of clinically relevant levels of MYC overexpression on AR activity. Further studies will need to assess the translational potential of these findings by using better *in vivo* models and undertaking larger scale studies.

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