Chromatin reprogramming as an adaptation mechanism in advanced prostate cancer

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Summary

Tumor evolution is based on the ability to constantly mutate and activate different pathways under the selective pressure of targeted therapies. Epigenetic alterations including those of the chromatin structure are associated with tumor initiation, progression, and drug resistance. Many cancers, including prostate cancer, present enlarged nuclei and chromatin appears altered and irregular. These phenotypic changes are likely to result from epigenetic dysregulation. High-throughput sequencing applied to bulk samples and now to single cells has made it possible to study these processes in unprecedented detail. It is therefore timely to review the impact of chromatin relaxation and increased DNA accessibility on prostate cancer growth and drug resistance, and their effects on gene expression. In particular, we focus on the contribution of chromatin-associated proteins such as the bromodomain-containing proteins to chromatin relaxation. We discuss the consequence of this for androgen receptor transcriptional activity and briefly summarize wider gain-of-function effects on other oncogenic transcription factors and implications for more effective prostate cancer treatment.

Key words: castration resistant prostate cancer; chromatin structure; bromodomain; glucocorticoid receptor; androgen receptor; c-MYC, histone acetylation, BRD4

Words count: 11,168

Introduction

Prostate cancer (PC) is the most common cancer in North American and European men. Despite recent decrease in the mortality rate in the Nordic countries (Kvale, et al. 2017), PC represents the second leading cause of cancer-related death in Norway (Center, et al.).

Treatment for clinically localized PC tumors mainly involves radical prostatectomy (RP) or radiation therapy. For men with advanced and/or metastatic disease, however, treatments targeting androgen signaling remain the cornerstone intervention strategy. Androgen deprivation therapy (ADT), which lowers patient serum testosterone levels and thereby limits ligand-mediated androgen receptor (AR) activity, is initially effective in most tumors due to their androgen dependence. Unfortunately, ADT is associated with a near inevitable recurrence into castration-resistant prostate cancer (CRPC), which is ultimately lethal. Antiandrogens such as enzalutamide and apalutamide, and drugs targeting hormone synthesis, such as abiraterone, have offered a survival benefit for men with CRPC. Like for ADT however, resistance towards these drugs is predictable, and can manifest as distinct molecular disease subtypes with varying dependency on the AR signaling axis (Bluemn, et al. 2017; Culig 2017).

The AR is a transcription factor (TF) that senses androgens levels (McEwan 2004) and mediates essential signaling required for both prostate gland development, maintenance and PC progression (Kim and Ryan 2012). Upon ligation of androgens, the AR translocates to the nucleus where it binds to specific genomic regions (AR binding sites; ARBSs) containing androgen responsive elements (AREs). This drives the expression of so-called AR target genes. AR target gene transcriptional regulation is associated with extensive chromatin remodeling, which includes alteration of histone modifications (Wang, et al. 2018a). The chromatin packs DNA, histones (organized as octamers, collectively forming the nucleosomes), and other chromatin-associated proteins in a dynamic structure within the nucleus of cells. As the chromatin structure dictates the accessibility of the genome, it allows cell-type specific transcription. Unsurprisingly, chromatin structure regulation contributes greatly to cell differentiation and preservation of cell identity, and chromatin deregulation is associated with many diseases, including PC (Ruggero, et al. 2018).

The fact that CRPCs often show clinical responses upon treatments targeting the AR signaling axis indicates that AR activity remains important to sustain growth of these tumors (Rehman and Rosenberg 2012). Although the emergence of CRPC has been imputed to several mechanisms (reviewed in (Waltering, et al. 2012; (Watson, et al. 2015), mechanisms involving the AR and its signaling axis are considered fundamental. Supporting the importance of AR, large-scale sequencing studies on clinical material has shown that AR is overexpressed or altered in more than 90% of advanced CRPCs (Barbieri, et al. 2012; Grasso, et al. 2012; Robinson, et al. 2015b; Taylor, et al. 2010). These studies have also highlighted a plethora of alterations associated with PC progression and therapy resistance, including multiple chromatin- and histone-modifying genes (Barbieri et al. 2012; Grasso et al. 2012; Robinson et al. 2015b). Importantly, genomic alterations associated with chromatin remodeling-associated genes are enriched in therapy resistant tumors (Robinson et

al. 2015b), suggesting that chromatin remodeling represents an adaptation mechanism that enables PC progression and therapy resistance.

Macroscopically, cancer initiation, including PC oncogenesis, is associated with alterations of the chromatin structure and density. Together with the observation of alterations in the tissue architecture of transformed prostate glands, one of the first major acknowledged criteria for pathological evaluation and diagnosis of PC was the presence of nuclear and nucleolar enlargements observed nearly 70 years ago (Totten, et al. 1953). This latter histological feature is still uniformly accepted (Humphrey 2007). In particular, different nuclear morphometric descriptors have been shown to be able to predict occurrence of distant metastasis and death in PC patients with biochemical recurrence after RP (Khan, et al. 2003). More recently, visualization of chromatin in tumor cell nuclei by image texture analysis have also been used to predict PC patient outcomes (Hveem, et al. 2016; Kleppe, et al. 2018).

Aside from imaging techniques, epigenomic assays such as chromatin immunoprecipitation followed by hybridization to arrays (ChIP-chip), sequencing (ChIP-seq), or simply PCR (ChIP-qPCR) (Johnson, et al. 2007; O'Neill and Turner 1996), have been used to analyze chromatin structures. More recent technical advances including formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) (Song, et al. 2011), assay for transposase-accessible chromatin for sequencing (ATAC-seq; (Buenrostro, et al. 2013)), chromatin interaction analysis by paired-end tag sequencing (ChIA-PET), and chromatin conformation capture (3C, 4C and 5C; (Fullwood and Ruan 2009)) have opened for a better understanding of higher-order chromatin structural alterations during cancer initiation and progression (Figure 1). Studies on chromatin structure and dynamics in PC have mainly revolved around understanding the mechanism by which the nuclear testosterone/dihydrotestosterone-ligated AR binds to the chromatin and modulates target gene transcription. Altered chromatin binding patterns of AR or other TFs, together with alterations of the chromatin structure, are increasingly appreciated as oncogenic drivers also in PC (Corces, et al. 2018; Makova and Hardison 2015; Sharma, et al. 2013; Stelloo, et al. 2015; Taipale 2018; Urbanucci, et al. 2017).

Importantly, the AR cistrome, which is the repertoire of ARBSs within the cells, has been shown to be extensively reprogrammed during PC initiation (Pomerantz, et al. 2015) and progression (Sharma et al. 2013). In this context “reprogramming” relates to the altered pattern of ARBSs that is different in normal epithelial cell and in PC cells. More generally, the mechanisms by which TF activation, re-activation, and reprogramming are occurring in PC are incompletely understood, but considerable evidence point at epigenetic alterations, including changes in the chromatin structure, as an oncogenic process, which alters the cistromes of active TFs.

It is apt that the activity of chromatin associated proteins, their abundance and stoichiometry will have an effect on chromatin structure and its global degree of relaxation, thereby dictating the accessibility of TFs to bind the genome. The set of accessible elements in the genome is associated with the cell transcriptional program and it is therefore defined at least in part by the chromatin structure. In this context, reprogramming of the chromatin structure is a remodeling of the chromatin that alters the patterns of open and closed

chromatin altering the set of accessible elements in the genome, suggesting that alterations to chromatin structure spanning genes' regulatory elements are likely to impact on the transcriptional output.

In this review, we highlight the importance of alterations in chromatin structure and remodeling processes that are able to confer PC plasticity and facilitate the emergence of drug resistance to AR-targeted therapies. Although multiple chromatin reader proteins and remodelers exist, we emphasize here the impact of bromodomain-containing proteins (BRDs), as BRD inhibitors are in clinical development for PC patients.

Chromatin relaxation is a feature of prostate cancer

The term "chromatin relaxation" relates to the process in which the chromatin changes to a more open conformation and allows genes that otherwise are sterically restricted from being transcribed to become transcriptionally active. This process happens through chromatin remodeling that allows for binding of highly specific TFs to genes' regulatory elements (enhancers and/or promoters). Therefore, chromatin remodeler proteins and chromatin-associated proteins are key regulators of both gene transcription and chromatin structure. These proteins open or close the structure of the tightly packed chromatin by modulating the make-up of the histone tails with covalent modifications such as acetylation, methylation, and ubiquitylation that are commonly defined histone post-translational modifications (PTMs). Chromatin writers add PTMs, while erasers remove them. The consequential change in histone charge can induce local chromatin opening, which evicts nucleosomes from the chromatin, unwinds negatively charged DNA, and exposes regulatory elements on the DNA to binding of TFs and assembly of the transcriptional machinery. Thus, chromatin relaxation renders the chromatin transcriptionally permissive. Conversely, chromatin remodeling can repress transcription by wrapping the DNA more tightly around newly introduced nucleosomes and consequently tightening the chromatin structure, thereby preventing TFs binding (reviewed in (Lee and Young 2013)). Most of the chromatin remodeling is mediated by chromatin readers, which recognize histone PTMs. A number of reader domains have been identified with affinity for different PTMs, such as methylation (e.g. PHD [plant homeodomain], chromo [chromatin organization modifier], Tudor, MBT [Malignant Brain Tumor]) or acetylation (e.g. BRDs) (Yun, et al. 2011).

An example suggesting that chromatin of cells in fast progressing PCs may be reprogrammed and in a more relaxed conformation than their benign counterparts comes from immunohistochemical studies of the trimethylation of histone 3 lysine 27 (H3K27me3). H3K27me3 is a polycomb heterochromatin marker and is widely known to be associated with chromatin compaction and transcriptional repression of proximal genes. Analyses of H3K27me3 protein levels by quantitative immunohistochemistry (IHC) in BPH, pre-malignant prostate intra-epithelial neoplasia, primary PC, and CRPC have shown an inverse correlation with worsening disease, in which high-grade tumors show the lowest levels of H3K27me3 (Pellakuru, et al. 2012; Xu, et al. 2012). Interestingly, IHC quantification of the levels of H3K9me2, another mark associated with transcriptional repression, was also found to be associated with disease outcome, with lower levels predicting poorer prognosis in prostate and other cancers (Seligson, et al. 2009).

Multiple mechanisms that alter the charge of histones and that are associated with increased chromatin opening and rate of transcription are well characterized. IHC analysis of global levels of mono-, di- and trimethylated H3K4 (H3K4me1/2/3), which are marks of active transcription, and acetylated H3K18 (H3K18ac), which marks TSS in genes active or poised for transcription, have been shown to be independent predictors of recurrence in PC patients (Ellinger, et al. 2010; Seligson, et al. 2005; Zhou, et al. 2010).

Global levels of H3 and H4 acetylation have also been investigated in nonmalignant prostate tissue and various stages of PC including clinically localized PCs and advanced CRPCs. Interestingly, CRPC tumors showed lower levels of histone acetylation than localized tumors in one study by Ellinger et al., (Ellinger et al. 2010), but the number of normal tissues stained was low and only one tenth of the number of PCs. Seligson et al., highlight a global increase of histone acetylation with disease stage and percentage of proliferating cells, albeit with inter-individual variability in staining intensities (Seligson, et al. 2005), which may also explain the results obtained by Ellinger and colleagues.

Acetylation of many other lysines in the histone tails, such as H3K9ac or H3K27ac, is catalyzed by histone acetyltransferases (HATs), and these also are generally associated with chromatin relaxation and transcriptional activity (Dancy and Cole 2015). These HATs, including p300/CREB-binding protein (CBP), are often overexpressed in PC and associated with poor outcomes (Comuzzi, et al. 2004; Dancy and Cole 2015; Debes, et al. 2003). A recent study also suggested that global increases in histone acetylation could be a mechanism of chemoresistance in PC (Xu, et al. 2018).

Collectively, IHC studies of histone modifications suggest that global histone modification expression pattern goes hand in hand with tumor progression and therapy resistance. Moreover, the global increase of marks associated with active transcription and open chromatin, and concomitant loss of repressive marks such as H3K27me3 during disease progression falls in line with increasingly decondensed (relaxed/permissive) chromatin observed during cancer progression (Timp and Feinberg 2013).

Chromatin condensation, leading to transcriptional repression, can be catalyzed by the ATP-dependent SWI/SNF, ISWI, Mi2/NuRD families of proteins. These proteins function by promoting nucleosome formation and DNA re-packing, and are key regulators of cellular proliferation. Upon functional loss of SWI/SNF, transcription of proliferation-associated genes is turned on (Längst and Manelyte 2015). Importantly, these proteins are often lost or mutated in CRPC (Grasso et al. 2012; Medina and Sanchez-Cespedes 2008), indicating that the loss of ATP-dependent nucleosome formation and subsequent chromatin decondensation may give a selective advantage conferring therapy resistance.

Recently, using ATAC-seq, the landscape of open chromatin was profiled in over 20 human tumor types (Corces et al. 2018; Taipale 2018). The study by Corces and colleagues revealed cancer type-specific enrichment of DNA binding motifs for TFs that indeed are known to be active in the respective cancer types. This included, for instance, the microphthalmia-associated transcription factor (MITF), which is important in melanoma,and the AR in PC (Corces et al. 2018; Taipale 2018). These findings represent an indication that

chromatin in open conformation is able to drive tumor growth by allowing key TFs binding. Moreover, specifically, the capacity of AR in driving PC is tightly linked to the degree by which the AR is able to access the genome.

By employing ChIP-seq in clinical samples, Sharma and colleagues previously reported that AR binding to chromatin is enhanced in CRPC tissue compared to that of primary PC or benign prostate hyperplasia (BPH) (Sharma et al. 2013). Comparing ARBSs in PC from RP specimens and normal adjacent tissue, Pomerantz et al., have also reported that the genome-wide set of ARBSs is extensively and consistently reprogrammed during prostate tumorigenesis (Pomerantz et al. 2015). As the AR requires permissive open chromatin to bind to its target elements on the DNA, Stelloo et al., and we have investigated whether the chromatin structure of CRPC specimens is more relaxed than that of primary PC or BPH (Stelloo et al. 2015; Urbanucci et al. 2017). In both studies, FAIRE-seq was applied to clinical specimens of benign prostate tissue, and tumor specimens from primary untreated PC, locally recurrent and metastatic CRPC. CRPC specimens had the highest number of both genomic sites showing chromatin in open conformation and sequenced reads at these sites (Stelloo et al. 2015; Urbanucci et al. 2017), indicating that the number of cells displaying chromatin in open conformation was also increased with disease stage, as illustrated in Figure 2.

An integrative analysis of chromatin structures, methylation and transcriptomes in patient samples, revealed that open chromatin proximal to gene transcriptional start sites (TSSs) was positively correlated with expression of those genes, while DNA methylation within 1 kb and 5 kb around the genes' TSSs were instead negatively correlated with gene expression (Urbanucci et al. 2017). This reinforces the notion that gene transcription is dictated by the chromatin structure and is in agreement with previous studies showing local DNA methylation to negatively correlate with transcript abundances (reviewed in (Cedar and Bergman 2012)). By integrating chromatin structural information and transcriptomic data, gene expression patterns have been found to correlate with genes proximal open chromatin and negatively correlate with TSS methylation in BPH, primary PCs, and CRPC specimens (Urbanucci et al. 2017). The consistency of these correlations across different disease stages is supportive of other studies showing occurrence of epigenetic deregulation both during tumor initiation and progression to therapy resistance (Perry, et al. 2010; Ruggero et al. 2018).

Interestingly, patterns of chromatin in open conformation were on average similar in BPH and primary tumor specimens while they appeared different in CRPC specimens (Urbanucci et al. 2017). This suggests that extensive chromatin reprogramming occurs during emergence of therapy resistance, and pinpoints a more marked role of chromatin remodeling in the emergence of CRPC rather than in PC development. By inter-patient sample analyses, we observed that the core set of genomic regions in open conformation were very similar in both benign tissue and primary PC tumors. In CRPC samples, on the other hand, we observed a large variation in inter-patient samples (Urbanucci et al. 2017). Collectively, it seems plausible that selective and/or adaptive remodeling events occur mainly upon treatment challenge, and that these events are predominantly stochastic.

Chromatin remodeling events can alter cells' transcriptional state, leading to a higher probability of permitting transcription of key genes involved in cancer growth and drug resistance (Sur and Taipale 2016). Pomerantz and colleagues exemplified this phenomenon in PC tumorigenesis where they identified FOXA1 and HOXB13 colocalizing within the reprogrammed AR cistrome (Pomerantz et al. 2015). Forced expression of *FOXA1* and *HOXB13* into an immortalized prostate epithelial cell line reprogrammed the AR cistrome to resemble that of a clinical prostate tumor (Pomerantz et al. 2015), which functionally links these specific TFs to ARBSs reprogramming. Therefore, chromatin remodeling triggered by pioneer factors such as FOXA1 or HOXB13 that allow increased and reprogrammed binding of TFs such as the AR, and the increased accessibility of the DNA given by a more relaxed chromatin in advanced PC, may help to explain the increased rate of transcription observed in CRPC compared to primary tumors (Latonen, et al. 2018; Robinson et al. 2015b; Sharma et al. 2013; Taylor et al. 2010; Ylipaa, et al. 2015); a phenomenon that has been attributed historically to the increased levels of AR in these tumors. By high-throughput mass spectrometry proteomic profiling, Latonen et al., showed that the discrepancies in protein profiles versus the matched transcriptional output disease stage-wise were greater in CRPC than in primary PC. From this it can be inferred that the increased transcriptional dosage observed in CRPC does not translate directly into corresponding proteins. Latonen et al., also identified a group of miRNA-protein pairs that were found to be negatively correlated (Latonen et al. 2018). This implies that buffer regulatory mechanisms should be actively "getting rid" of transcriptional (e.g. by miRNAs) and translational (e.g. the unfolded protein response and autophagy) byproducts of the escalating overproductive transcriptional.

Finally, multiple genomic alterations occur upon therapeutic challenge as a means for the tumor cells to adapt to the exerted pressure and to alleviate their addiction towards the drug-targeted pathways. The notion that an open chromatin structure may increasingly permit these alterations, such as structural variations, including gene rearrangements, copy number alterations and genomic breakpoints, has prompted studies associating these events with chromatin structure in PC. DNA breakpoints were recently found to be associated with open and transcriptionally active chromatin in PC (Gerhauser, et al. 2018). Through deep sequencing-based genomics analyses of early- and late-onset primary PCs, it was earlier shown that whereas structural rearrangements were stochastic in late onset PC (i.e. increasingly likely with increasing age), the rearrangements were associated with ARBSs in early onset PC (Weischenfeldt, et al. 2013). More recently, a breakpoints analysis revealed an increased rate of DNA double-strand breaks in functionally active chromatin regions (Gerhauser et al. 2018). As androgen signaling has been shown to induce DNA damage which can facilitate genetic rearrangements, e.g. between the *TMPRSS2* and the *ERG* genes (Haffner, et al. 2010; Mani, et al. 2009), it is therefore conceivable that increased chromatin accessibility creates more opportunities for random structural rearrangements likely to contribute to PC development and progression to CRPC. Accordingly, a recent study by Quigley and colleagues discovered tandem duplications associated with notoriously open chromatin structures at multiple enhancers near *AR*, *MYC*, and *FOXA1* by deep whole-genome analysis of 101 CRPC metastases. Intriguingly, 80% of the cases showed local amplification of the enhancer proximal to *AR*, which correlated with increased *AR* transcription (Quigley, et al. 2018).

Taken together, these studies show that chromatin relaxation is a feature of PC, and that chromatin opening is associated with increased gene transcription and reprogramming of the global transcriptional output through aberrant TFs binding and increased rate of DNA structural variants.

The androgen receptor drives chromatin relaxation as an oncogenic feed-forward process

The AR signaling modulates gene transcription during embryonic development and maturation of the healthy prostate, and is overexpressed in PC leading to transcriptional reprogramming which promotes disease progression (Matsumoto, et al. 2013). More than a decade ago the group of Charles Sawyers demonstrated that AR overexpression alone is able to drive PC cells to castration resistance (Chen, et al. 2004).

Interestingly, consequences of activation or reactivation of TFs have been extensively studied with the Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) in the induction of pluripotent stem cells from adult human fibroblasts, and it is apt that this process is associated with considerable epigenetic reprogramming (Schmidt and Plath 2012; Takahashi, et al. 2007). The role of these TFs in PC have been reviewed in (Ruggero et al. 2018). In PC, reprogramming of normal human epithelial prostate tissue to a lethal neuroendocrine cancer lineage has proven successful by forcing the expression of TFs such as c-MYC or N-MYC in combination with myristoylated AKT1 (a partial mimic of *PTEN* loss) (Park, et al. 2018). This experiment proves that overexpression of TFs in cancer is a common mechanism of cell plasticity to lead to drug resistance and tumor progression.

Several studies now suggest that also the AR is implicated in shaping the chromatin structure by modifying the activity of epigenetic factors (Takayama. 2018). Through transcriptomic profiling of isogenic AR-overexpressing CRPC cell line models ("mimicking" adenocarcinoma-CRPC) and LuCaP PDXs with different AR expression levels (Jalava, et al. 2012; Urbanucci, et al. 2012; Urbanucci, et al. 2013; Urbanucci, et al. 2008; Waltering, et al. 2009; Waltering, et al. 2011), it was shown that high AR levels associated with increased expression of androgen responsive genes and AR coregulators.

Many AR coregulators have been described and many AR coactivators are overexpressed in primary PC and CRPC (Heemers and Tindall 2007; Linja, et al. 2004; Liu, et al. 2017). Interestingly, we showed that a number of the AR coregulators were AR-regulated, and that enhanced expression of a subset of these coregulators was observed in castration-challenged PC cells ectopically overexpressing AR (Urbanucci et al. 2008). Among the androgen regulated coregulators identified were Amplified in breast cancer 1 (AIB1) and CREB-binding protein (CBP), both HATs which have been shown to increase nuclear receptors' activities and are implicated in mechanisms of drug resistance (Chang and Wu 2012; Culig 2016; Jin, et al. 2017).

Other coregulators of AR, such as Lysine-specific histone demethylase 1A (LSD1), have been shown to have a reprogrammed activity in CRPCs, where it is also highly expressed (Liang, et al. 2017; Sehrawat, et al. 2018). Importantly, LSD1 has been shown to be one of the responsible factors activating the over-expression of *AR* in castration-challenged PC cells (Cai, et al. 2011).

Of note, several of the AR-upregulated AR coactivators, including the mentioned CBP/p300 and SRC1, have been shown to exert chromatin remodeling functions through e.g. histone modifications (Bannister and Kouzarides 2011), thus hinting that AR overexpression may increase the likelihood of further oncogenic events by up regulating chromatin-associated proteins.

In two independent preclinical AR-overexpression model systems, one of which was isogenic and therefore more independent of confounding factors (Waltering et al. 2009), we demonstrated that androgen treatment in AR overexpressing cells led to enhanced AR recruitment with faster kinetics (Urbanucci, et al. 2011; Urbanucci et al. 2012). Increased H3K9 acetylation in nucleosomes flanking ARBSs was found in the isogenic AR-overexpressing cell line models in key genes regulatory regions such as enhancers and promoters (Urbanucci et al. 2011). Interestingly, these ARBSs appeared deprived of nucleosomes (Urbanucci et al. 2011). This indicated that AR overexpression might seed further AR recruitment at ARBSs through increasing chromatin permissiveness. Corroborative of this, we have shown by ChIP-seq that high AR expression was associated with an increased number of ARBSs and intensity of AR binding to the chromatin (Urbanucci et al. 2012).

These observations were later confirmed using FAIRE-seq, as AR overexpression drove genome-wide chromatin relaxation in two independent cell line models, concomitant with increased permissiveness to ARBSs (Urbanucci et al. 2017). We found that high levels of AR were associated with increased number of chromatin sites in open conformation and higher number of sequenced reads at these sites (Urbanucci et al. 2017), indicating that the number of cells displaying chromatin in open conformation was also increased in AR overexpressing cells. The addition of androgens affected primarily increased opening at ARBSs (Urbanucci et al. 2017) suggesting an AR-mediated feed forward loop increasing chromatin opening at these sites. This study supports the notion that ligand-mediated, AR-driven chromatin remodeling in the context of the AR-overexpression may confer transcriptional permissiveness at ARBSs (Urbanucci et al. 2017). This would represent a positive feedback loop in which the AR promotes chromatin remodeling which in turn permits the AR to more tightly bind to ARBS-containing chromatin regions.

Historically, the first studies on how AR drives target gene transcription utilized ChIP-qPCR to investigate the loading of AR, RNA Pol II and AR coactivators onto the prostate-specific antigen (PSA/*KLK3*) regulatory regions (Kang, et al. 2004; Kang, et al. 2002). Later on, multiple studies have used ChIP-chip and ChIP-seq to map AR binding onto chromatin in cell line models and tissue samples (Massie, et al. 2011; Pomerantz et al. 2015; Sahu, et al. 2011; Sharma et al. 2013; Urbanucci et al. 2012; Wang, et al. 2009; Yu, et al. 2010), revealing that AR activity is hijacked or reprogrammed in PC to respond to oncogenic insults and activate oncogenic transcriptional programs (reviewed in (Mills 2014)).

The molecular events leading to the aberrant AR binding pattern onto chromatin in therapy-challenged PC tumors can be attributed to several interconnected factors, possibly depending on the administered intervention strategy: (i) Overexpression of the AR protein that increases the abundance of the protein located into the nucleus and the probability that the AR binds the chromatin (Jia, et al. 2006; Massie et al.

2011; Sharma et al. 2013; Stelloo et al. 2015; Urbanucci et al. 2011; Urbanucci et al. 2012; Wang et al. 2009; Yu et al. 2010); (ii) alterations of the activity of proteins that enable binding of AR to the chromatin (pioneer factors) by triggering the recruitment of chromatin remodelers (Jia, et al. 2008; Lupien, et al. 2008; Pomerantz et al. 2015; Robinson, et al. 2014; Sahu et al. 2011; Zhao, et al. 2016); (iii) alterations in the composition of the proteins within the AR transcriptional complex which also include a number of co-regulatory proteins (Chen, et al. 2013; Heemers and Tindall 2007; Jariwala, et al. 2009; Jia et al. 2008; Kang et al. 2004; Kotaja, et al. 2002; Liu et al. 2017; Rytinki, et al. 2011; Stelloo, et al. 2017); and (iv) alterations in the chromatin structure and composition which renders it more permissive toward AR binding (Andreu-Vieyra, et al. 2011; He, et al. 2010; He, et al. 2012; Jia et al. 2006; Stelloo et al. 2015; Tewari, et al. 2012; Urbanucci et al. 2017; Yu et al. 2010).

The AR preferentially binds to nucleosome-deprived regions with access to regulatory elements (Jia et al. 2008), suggesting that preceding chromatin remodeling and e.g. pioneer factor binding may be necessary to permit AR binding to otherwise transcriptionally restricted AREs: In ARBS-containing regulatory regions (primarily enhancers) proximal to specific AR target genes, the chromatin is open even in absence of AR binding (Andreu-Vieyra et al. 2011; He, et al. 2018). The reason for the pre-determination of these sites is still partly unclear, although many factors have been identified to cooperate in order to maintain a permissive chromatin structure to enable AR binding, such as GATA2 and FOXA1 (Figure 3) (Andreu-Vieyra et al. 2011; He et al. 2010). GATA2 is an important mediator of androgen signaling within the hierarchical binding of other transcriptional regulators responsible for AR activity (Jia et al. 2008; Rodriguez-Bravo, et al. 2017; Wang, et al. 2007), and has been shown to act downstream of FOXA1 in modulating AR binding to chromatin (Zhao et al. 2016). FOXA1 has been further characterized as a pioneer factor for characterizing the AR and estrogen receptor (ER) cistromes in both prostate and breast cancer (Lupien et al. 2008; Robinson, et al. 2011; Sahu et al. 2011; Wang, et al. 2011; Zhang, et al. 2011). More studies are needed to understand how FOXA1 is regulated. However, recently, a study by Wang and colleagues showed that in breast cancer cells the activity of FOXA1 can be modulated by multiple kinases, and that the cell cycle control kinase CDK1 may directly phosphorylate FOXA1 (Wang, et al. 2018b).

Tewari and colleagues showed using DNase-seq that the AR not only binds to pre-docked open chromatin, but is able to induce chromatin remodeling events which alters the accessibility of chromatin (Tewari et al. 2012). The identified regions of increased chromatin accessibility were enriched with ARBSs, and these regions were associated with increased H3 acetylation and enhanced transcription of AR-regulated genes (Tewari et al. 2012). He and colleagues proposed a model in which AR binding to chromatin favors the eviction of local nucleosomes (He et al. 2012). This was later confirmed by Taberlay and colleagues (Taberlay, et al. 2014). Although it remains elusive how this putative nucleosome eviction takes place, AR-interacting proteins with chromatin remodeling functions in the transcriptional subcomplexes are likely to play a role in such remodeling events (Stelloo et al. 2017).

Supportive of an indirect role of AR binding-mediated chromatin remodeling, remodeling proteins FOXA1 and HOXB13 are known to co-localize with AR subcomplex on the chromatin (Stelloo et al. 2017). FOXA1 has been shown to recruit chromatin-remodeling complexes such as the MLL complex to deposit H3K4 mono- and dimethylation at histones flanking gene regulatory regions (Jozwik, et al. 2016). However, the sole activity of FOXA1 cannot explain how the AR is able to open chromatin, as, paradoxically, knocking down FOXA1 in PC and breast cancer cells increases the number of ARBS (Robinson et al. 2011; Sahu et al. 2011; Wang et al. 2011). Moreover, overexpressing FOXA1 in PC cells leads to novel ARBSs, but at locations different from the *de novo* AR binding sites identified upon FOXA1 knockdown (Robinson et al. 2014). In stark contrast to the reprogramming functions of FOXA1 on the AR cistrome, FOXA1 is required for ER to bind chromatin, and FOXA1 loss abrogated the capacity of the ER to bind chromatin in breast cancer cells (Hurtado, et al. 2011). This implies that FOXA1's pioneering activity on different TFs is mediated by other factors. HOXB13 might be one such pioneer TF (Pomerantz et al. 2015), but its role in reprogramming the AR cistrome in PC, and possibly in breast cancer, has not been clearly characterized. In PC, AR target genes important for driving emergence of castration resistance, such as ubiquitin conjugating enzyme E2 C (*UBE2C*), have been shown to be overexpressed upon FOXA1 recruitment through PI3K/AKT phosphorylated MED1, collectively favoring looping between its promoter and distant regulatory regions (Chen, et al. 2011). This indicates that there are a number of factors that pioneer and mediate AR transcriptional output.

Levels of AR variants lacking the LBD were shown to be increased in specimens from CRPC patients (Antonarakis, et al. 2014; Sharp, et al. 2019; Watson, et al. 2010) and were shown to contribute to resistance to enzalutamide and abiraterone (Sharp et al. 2019). Interestingly, evidence of a distinct ligand-independent chromatin binding profile of constitutively active AR splice variants (Chen, et al. 2018; Lu, et al. 2015) could be the result of the chromatin being incidentally more relaxed in CRPC. Moreover, recently, Chen and colleagues also showed that HOXB13 directly interacts and pioneers binding of one of the most abundant AR splice variant, AR-V7, thereby suggesting cooperation in up-regulating target oncogenes (Chen et al. 2018).

Given the increased chromatin relaxation observed in CRPCs compared to primary PC tumors, it is apt that mechanisms leading to enhanced transcription are possibly dependent on the increased chromatin opening at newly activated enhancers. Accordingly, the group of Susan Clark showed that a variant of Histone H2A (H2A), namely H2A.Zac (H2A.Z), is involved in exposure of packed and "unbound" enhancers; a process that leads to AR binding to these "neo-enhancers" (Valdes-Mora, et al. 2017). H2A.Z is predominantly found at promoters, however, and has been shown to be important in maintenance of poised bivalent promoters in stem cells (Rudnizky, et al. 2016; Surface, et al. 2016). In particular, mono-ubiquitylated H2A.Z competes with BRD2, which promotes nucleosome eviction and chromatin opening, thus illustrating an antagonistic relationship between the two (Surface et al. 2016). Valdez-Mora et al. showed that acetylated H2A.Z is absent in nucleosomes of closed/inactive chromatin at both distal enhancers and proximal promoters to ensure appropriate oncogenic silencing in normal cells (Valdes-Mora, et al. 2017). However, in PC cells,

H2A.Z-nucleosomes were present at new regulatory elements, promoting a poised local chromatin conformation. H2A.Z acetylation was associated with the formation of nucleosome-deprived regions and a loss of DNA methylation at both enhancers and promoters, priming these new sites for gene transcription upon androgen stimulation. Supporting the relevance and oncogenic properties of H2A.Z, immunohistochemical staining of acetylated H2A.Z has been shown to be increased in PC and associated with poor prognosis (Valdes-Mora et al. 2017). This body of work shows that that PC initiation and progression is associated with increased local chromatin opening which leads to increased AR binding and it is in line with AR overexpression driving increased chromatin opening in advanced PC.

Collectively, present evidence show that AR overexpression associates with increased expression of AR target genes and AR coregulators, many of which favor chromatin remodeling and are upregulated in lethal CRPC. This transcriptional deregulation, in turn, favors chromatin relaxation through nucleosome eviction and is likely to drive PC progression by promoting stemness properties and plasticity in a oncogenic feed-forward process.

Chromatin relaxation drives PC progression by altering the patterns of transcription factor binding to the chromatin

Although substantial progress is being made to understand the mechanisms and players involved in chromatin reprogramming in PC, the underlying mechanisms driving higher chromatin disorganization in cancers, including PC, are largely unknown. It is established that the chromosome conformation inside the nuclear envelope favors engagement of highly interactive chromatin substructures of approximately 1 Mb called topologically associated domains (TADs) (Yaffe and Tanay 2011). Reconfiguration and alterations of these domains have been shown in PC cells to be enriched with regulatory elements such as enhancers, promoters and insulators, and associated with alterations in gene expression (Taberlay, et al. 2016). Boundaries of TADs have been shown to be dependent on CTCF in the sense that CTCF is able to mark chromatin regions within active and inactive TADs, and loss of CTCF can highly deregulate not only the chromatin conformation but also transcription of genes within these TADs (Ghirlando and Felsenfeld 2016).

Several groups have shown that newly generated TAD boundaries delineated by transcriptional repressor CTCF are acquired during prostate carcinogenesis (Taberlay et al. 2016; Taslim, et al. 2012). Fiorito et al., have previously shown in breast cancer cells that the presence of CTCF at enhancer regions results in modulation of oestrogen-induced gene transcription by preventing ER chromatin binding and by hindering the formation of additional enhancer-promoter looping (Fiorito, et al. 2016). Depletion of CTCF facilitates the expression of ER target genes associated with cell division and increases the rate of breast cancer cell proliferation. Fiorito et al., have also shown that CTCF mediates contact of the regulatory regions to the nuclear lamina (Fiorito et al. 2016). This process was regulated by oestrogens, which altered the chromatin structure interfering with enhancer-promoters loop formation (Fiorito et al. 2016). Like in breast cancer, a role of CTCF in mediating hormone-dependent gene transcription has been shown in PC: Taslim and colleagues found that subsets of androgen-responsive genes were significantly enriched within the same

CTCF blocks, suggesting that CTCF is implicated in regulation of a subset of distally located androgen-responsive genes (Taslim et al. 2012) which are potentially involved in prostate carcinogenesis (Guo, et al. 2018; Taslim et al. 2012). Collectively, these studies show that the higher-order chromatin conformation is interconnected with local chromatin relaxation and interfere with gene regulation which may have implications for PC development and progression.

Interestingly, performing extensive motif enrichment analysis of open chromatin regions in PC cell lines and clinical specimens of BPH, primary PCs and CRPCs, we found that CTCF-like motifs were the top enriched motifs in both clinical specimens and cell lines, followed by ETS-like motifs (Urbanucci et al. 2017). Of note, both CTCF and ETS-like motifs were equally enriched in BPH as well as in primary PCs and CRPCs, supporting the notion that these TFs could be implicated in early tumorigenesis rather than progression and CRPC development. ETS rearrangements have been in fact characterized as an early event in PC (Weischenfeldt et al. 2013), while the role of CTCF in PC oncogenesis remains elusive. As opposed to CTCF-like and ETS-like motifs, c-MYC DNA binding motifs were exclusively enriched in open chromatin regions found in CRPC samples (Urbanucci et al. 2017), which is in agreement with several studies suggesting that, although c-MYC activity may be responsible for tumorigenesis, MYC oncogenic activation is a late event in PC progression and is involved in CRPC emergence (Ahmadiyeh, et al. 2010; Gurel, et al. 2008; Hawksworth, et al. 2010; Koh, et al. 2010; Nupponen, et al. 1998). Other TF motifs were also enriched in open chromatin regions of CRPC specimens, including glucocorticoid receptor (GR) motifs (Urbanucci et al. 2017), which is in agreement with recent data showing its reactivation in CRPC (Arora, et al. 2013; Culig 2017; Isikbay, et al. 2014; Kroon, et al. 2016; Puhr, et al. 2018).

Although the chromatin binding of these TFs has not been profiled in clinical samples, the expression profiles and transcriptional activity of these TFs have been found to differ between CRPC subtypes with variable dependency on AR signaling. In the following section, we detail evidence collected in cell models that associate them with PC development, progression and emergence of AR-negative CRPC subtypes (Figure 4).

c-MYC

c-MYC is overexpressed in a subset of PCs and c-MYC overexpression in primary PC is associated with biochemical recurrence following RP (Hawksworth et al. 2010). Mechanistically, the overexpression of TFs such as AR and c-MYC results from pressure put upon PC cells to survive and sustain growth in androgen-deprived environments, as is the case in patients undergoing ADT or androgen blockade (Ni, et al. 2013; Waltering et al. 2009). Importantly, overexpression of c-MYC has been shown to confer androgen-independent growth in PC cells (Bernard, et al. 2003). We confirmed these findings using an isogenic LNCaP cell-based model with enforced inducible *c-MYC* overexpression (Barfeld, et al. 2017). Using ChIP-exo sequencing, a variant of the ChIP-seq protocol that utilizes exonucleases for improved resolution of TFs binding sites(Rhee and Pugh 2012), we further investigated the interplay of c-MYC with AR on chromatin and the transcriptional output in the context of c-MYC overexpression (Barfeld et al. 2017). Overexpression

of c-MYC partially reprogrammed AR chromatin occupancy, although the binding of c-MYC itself was not substantially altered. Interestingly, c-MYC overexpression was accompanied by altered distribution of histone marks, most notably H3K4me1 and H3K27me3. This is consistent with previous findings showing that c-MYC expression is inversely correlated with global protein expression of H3K27me3 in PC (Pellakuru et al. 2012). More recently, Kieffer-Kwon and colleagues showed that c-MYC activation was essential for chromatin opening and decompaction during B cell activation (Kieffer-Kwon, et al.), which is in agreement with the above-mentioned studies. We also found that c-MYC overexpression triggers DNA damage in LNCaP cells independently of AR signaling being activated or not (Barfeld et al. 2017). DNA damage leads to dislocation of nucleosomes from the point of DNA damage, and chromatin remodeling is an integral part of the DNA damage response process (Audia and Campbell 2016). Cellular levels of histones drop 20–40% in response to DNA damage which is accompanied by chromatin decompaction and increased DNA fiber flexibility (Hauer, et al. 2017). This suggests that, similar to AR overexpression, c-MYC overexpression in CRPC may equally be able to mediate chromatin reprogramming.

By performing interactome profiling (RIME: rapid immunoprecipitation mass spectrometry of endogenous proteins) for both AR and MYC, we found that a great part of TFs or coregulators interacting with both MYC and AR were indeed implicated in DNA damage response (Barfeld et al. 2017), thus supporting the role of both AR and MYC in controlling DNA damage response. We also found that c-MYC and the AR co-occupied a substantial number of binding sites in PC cells and these exhibited enhancer-like characteristics. We performed motif enrichment analysis of the AR and c-MYC ChIP-seq datasets and retrieved FOXA1 as one of the top enriched motifs in both. Therefore, it is possible that FOXA1 may pioneer opening at these sites in conditions in which e.g. MYC is overexpressed. Under these conditions, MYC could have an increased chance to bind to chromatin sites pre-docked for AR by FOXA1. However, immunoprecipitation between MYC and AR from independent RIME experiments did not show direct interaction between MYC and AR, nor FOXA1 interacting with c-MYC (Barfeld et al. 2017). Previous studies in breast cancer cells have shown that MYC regulates androgen signaling via a context-specific activation of AR in which MYC is able to co-opt the functions of other TFs to coordinate differential gene expression programs in a cell-type dependent manner (Ni et al. 2013). However, in the same study, a direct interaction between MYC and AR was not demonstrated (Ni et al. 2013). Furthermore, unlike in apocrine breast cancer in which c-MYC is thought to be an amplifier of AR-driven gene transcription (Ni et al. 2013), we found in our study in PC that the AR-c-MYC interplay was largely antagonistic (Barfeld et al. 2017).

Taken together, these studies of the interplay between c-MYC and AR activity suggest that different therapeutic approaches may impose different selective utilization of survival and drug resistance pathways depending on the hormonal environment and chromatin structure of the tissue.

Steroid receptors and other transcription factors

Binding of steroid receptors, such as AR, ER, GR, and progesterone receptor (PR) to chromatin, are dynamic processes in which binding has been shown to occur in cycles of “touch and go” to the regulatory regions of

target genes (Carlberg and Seuter 2010). Proteasomal activity towards the AR has also been proposed to play a role in the context of AR binding to chromatin (Kang et al. 2004; Kang et al. 2002). We showed that AR overexpression altered the dynamics of the AR binding to chromatin (Urbanucci et al. 2011). More recently, the group of Gordon Hager has shown using microscopic techniques how the binding of steroid receptors can be divided into long- and short-lived events that lead to transcription of target genes. A great part of the unliganded/unstimulated steroid receptors may diffuse into the nucleus of the cells, from which a proportion of them can in fact ligate chromatin (Paakinaho, et al. 2017). It is therefore possible to speculate that unliganded receptor binding events may occur on permissive chromatin in open conformation, and that this can lead to aberrant activation of oncogenic transcription if key binding sites reside in open conformation. This is a plausible scenario in CRPCs with AR overexpression, in which the excess of the receptor in a low-androgen micromilieu is translocated into the nucleus. Concordantly, a recent report has shown that constitutively active AR variants (AR-Vs) can bind to open chromatin and promote abiraterone-resistant growth (He et al. 2018).

The DNA binding domains of GR, PR, and AR are highly similar, with nearly identical residues involved in contacting DNA and high similarity of their dimerization interfaces (Claessens, et al. 2013). DNA motifs bound by these steroid receptors are also similar, but for the AR it has been demonstrated that the DNA sequence of the response elements (the DNA binding motif) is not as stringent as for other steroid receptors and it is a special feature of the AR chromatin binding that sets it apart from other steroid receptors such as e.g. the GR (Sahu, et al. 2014).

Steroid receptors interaction with the chromatin seems to be a very specific process in physiological condition (reviewed in(Pihlajamaa, et al. 2015)), which may reflect a tightly organized chromatin structure allowing only specific chromatin binding events. However, in the context of deregulated chromatin structure as in advanced PC, the functional steps that follow steroid receptors activation leading to e.g. AR binding to the chromatin can be influenced by many highly variable and context-specific factors discussed previously. The same pioneer factors and coregulators can interact with several steroid receptors, and multiple receptors can bind to the same cis-elements on chromatin. These processes ensure distinct tissue- and cancer-type specific gene expression profiles. An open chromatin environment that permits TFs binding creates also some ground for TFs to compete for chromatin binding. Interestingly the competition for the chromatin binding between these TFs is less well studied, but an intrinsic interplay has been shown for steroid-receptors specifically (reviewed in (Pihlajamaa et al. 2015)). Therefore, overexpression of one or more specific TFs, or overexpression of the repertoire of coregulators and pioneer factors, can result in deregulated cistromes and transcriptome reprogramming in cancer cells as a result of competitive binding.

Gene transcriptional activation can occur by the cooperative action of AR with other TFs such as ETS or HOXB13 bound to DNA at adjacent sites (Ratnam, et al. 2013). It is not clear in this context whether the AR would act as cofactor or dictate TF binding. In our previous study, more than three-fold higher number of open chromatin sites was found in CRPC compared to primary PC or BPH (Urbanucci et al. 2017).

Therefore, the increased open chromatin observed in CRPCs creates additional possibilities for other TFs to bind chromatin and increases the likelihood for activation of oncogenic transcriptional programs. For example, we have shown that a core of ARBSs are conserved during all phases of the cell cycle, but other ARBSs are deputed to drive a transcriptional program specific in each cell cycle phase (McNair, et al. 2017). Deregulation of these AR binding dynamics in the context of AR overexpression pushes toward faster cell cycle, as demonstrated by studies of PC transcriptomics (Waltering et al. 2009) and by the fact that the composition of androgen-responsive genes changes during disease progression (Lee, et al. 2013).

An example of TFs re-activated and overexpressed in CRPC that mediate resistance to therapy is the GR (Isikbay et al. 2014; Puhr et al. 2018). FOXA1 depletion leads to an increased chromatin binding of AR and decreased GR binding in PC models (Sahu et al. 2011), which confirms a context-dependent pioneering function of FOXA1, but also potentially explains lowered expression of GR in a subtype of primary tumors expressing low levels of FOXA1. Shah and colleagues found that *GR* polycomb-mediated silencing in primary PC was due to an ARBS at the upstream enhancer of the *GR* gene. Re-expression of GR in ADT resistant tumors was mediated by the activity of BRD4, a BRD, member of the subgroup of proteins called bromodomain and extraterminal (BET) proteins (Reviewed in (Urbanucci and Mills 2017)). Inhibition of BRD4, using a BET inhibitor (BETi) was able to restore sensitivity to enzalutamide in these tumors (Shah, et al. 2017). BRD4 is also a HAT that evicts nucleosomes from chromatin (Devaiah, et al. 2016). Shah and colleagues also demonstrated that GR overexpression-mediated antiandrogen resistance is dependent on BRDs (Shah et al. 2017), which, in this context, provides indirect evidence for increased chromatin accessibility in these tumors.

These studies supports the idea that in a open chromatin environment, TFs can be interchangeably usable for CRPCs to adapt transcription to cellular stress, disease treatment, and that dedifferentiation and stemness can be a product of such TFs interchangeability in advanced tumors.

Transcription factor binding and chromatin in neuroendocrine prostate cancer

With the clinical implementation of novel AR-directed therapies (e.g., abiraterone and enzalutamide) for patients with metastatic CRPC, the prevalence of AR-negative CRPC variants has increased (Aggarwal, et al. 2018; Beltran, et al. 2016; Bluemn et al. 2017). These therapy-resistant CRPC subtypes generally show low dependence on AR signaling, a different transcriptome and mutational landscape, and are anticipated to become more prevalent with more widespread use and implementation of novel AR-targeted therapies. CRPC is normally defined as adenocarcinoma in the sense that harbors the typical features of epithelial differentiation with expression of luminal genes and are frequently still reliant on sustained AR signaling. Treatment-related neuroendocrine CRPCs (t-NEPCs), on the other hand, are emerging subtypes of CRPC characterized by stem cell/basal like features, neuroendocrine differentiation, and are frequently AR-negative (Ellis and Loda 2015).

The chromatin structure of t-NEPCs has not yet been extensively studied, and it will be intriguing to understand whether the increased chromatin opening observed in CRPC is maintained or even enhanced in t-NEPC and how this influences the activity of characterized TFs in this PC subtype.

t-NEPCs have been reported to harbor alterations in *RB1* and *TP53* more frequently than CRPC adenocarcinomas yet are believed to arise through clonal divergent evolution (Beltran et al. 2016). Interestingly, *RB1* loss has been shown to lead to cistrome reprogramming of other TFs in CRPC (McNair, et al. 2018) while concomitant loss of p53 and RB1 was shown to drive upregulation of chromatin modifying factors such as the polycomb repressive complex 2 (PRC2) catalytic subunit enhancer of zeste homolog 2 (EZH2) and SRY (sex determining region Y)-box 2 (SOX2), epigenetic reprogramming, and emergence of t-NEPC (Ku, et al. 2017; Mu, et al. 2017). The Yamanaka factor SOX2 is involved in lineage plasticity and resistance to ADT (Lee, et al. 2018), and was shown to be markedly elevated in two thirds of t-NEPC patient samples in the NEPC WCM 2016 cohort (Beltran et al. 2016).

Also overexpression of N-MYC has been found to promote tumor characteristics reminiscent of clinical t-NEPC, and *N-MYC* is upregulated in clinical t-NEPC tumors (Beltran et al. 2016; Dardenne, et al. 2016; Lee, et al. 2016). Dardenne and colleagues showed that N-MYC overexpression-driven NEPC development in mouse and cell line models was associated with suppression of AR signaling (Dardenne et al. 2016). They also performed ChIP experiments that suggested that N-MYC could bind to enhancer regions in absence of active AR. Interestingly, binding of N-MYC to these AREs was stabilized by DHT supplementation (Dardenne et al. 2016). We recently showed that Aurora kinase A (AURKA), which is commonly overexpressed in AR-negative t-NEPC (Beltran, et al. 2011), is also commonly altered in CRPC (Kivinummi, et al. 2017). Interestingly, AURKA has been shown to interact and stabilize the transcriptional activity of N-MYC in neuroblastoma (Brockmann, et al. 2013), suggesting that binding of N-MYC can occur as a consequence of the activation of different signaling pathways.

N-MYC has been found to complex with and promote the activity of EZH2 (Dardenne et al. 2016). Earlier data supported the notion that EZH2 overexpression drives emergence of CRPC in a PRC2-independent manner, thus independently of its histone methyltransferase activity (Xu et al. 2012). Recently, using a ChIP-seq approach, EZH2 was shown to occupy the *AR* promoter and act as a transcriptional activator for *AR* transcription (Kim, et al. 2018), suggesting that its overexpression in t-NEPCs compared to CRPC adenocarcinomas (Clermont, et al. 2015) may actually be associated also with its increased coactivator-function rather than its function in deposition of the repressive H3K27me3 mark. Clermont and colleagues showed that several histone-modifying enzymes with chromatin remodeling activity, including CBX2 and EZH2, were upregulated in t-NEPCs as compared to CRPC adenocarcinomas (Clermont et al. 2015). Furthermore, they showed that polycomb group proteins with DNA methyltransferase (DNMT) activity were also aberrantly expressed in t-NEPC (Clermont et al. 2015).

Together with evidence that the transcriptomes of t-NEPC subtypes are so intrinsically different from e.g. CRPCs (Beltran et al. 2016; Dardenne et al. 2016; Robinson, et al. 2015a), the above-mentioned studies

suggest that reconfiguration of the TF complexes at the regulatory regions of target genes can drive both PC progression to CRPC, and also the development of t-NEPC. This may possibly explain how some overexpressed TFs such as N-MYC can dominate the transcriptional output of these latter tumor subtypes through chromatin remodeling activity.

Bromodomain-containing proteins and chromatin reprogramming in prostate cancer

BRDs are a family of epigenetic reader proteins, and many BRDs are aberrantly expressed in PC (reviewed in (Urbanucci and Mills 2017)). BRDs are able to recognize acetylated histones, but often have additional chromatin remodeling functions. Moreover, they make out a part of multi-subunit chromatin remodeling complexes. Recent advances in the understanding and appreciation of BRDs in cancer have prompted investigations into whether BRD inhibition can be exploited clinically. In fact, targeting BRDs is currently being evaluated as a major therapeutic strategy in the treatment of blood cancers and solid tumors, including PC (reviewed in (Urbanucci and Mills 2017)).

BRDs have been shown to modulate key transcriptional programs during cancer progression (Fu, et al. 2015). For example, the BRD protein BRG1, encoded by *SMARCA4*, is an ATPase subunit of the SWI/SNF complex that mobilizes nucleosomes (Griffin, et al. 2008; Medina and Sanchez-Cespedes 2008). Ding and colleagues recently showed that increased BRG1 expression in PTEN-deficient PC cells lead to chromatin remodeling into a configuration that drove a protumorigenic transcriptome (Ding, et al. 2018). They employed ATAC-seq in PTEN-deficient 22Rv1 PC cells to show that BRG1 knockdown led to a 60% reduction in open chromatin regions compared to BRG1-intact cells (Ding et al. 2018). They also showed that high BRG1 expression was associated with worse outcomes in PC patients with low *PTEN* expression (Ding et al. 2018). Moreover they demonstrated in preclinical models of *PTEN* knockout mice that PC tumors become addicted to BRG1 expression (Ding et al. 2018). The work by Ding and colleagues suggests that BRG1 may be a promising target in *PTEN*-deficient PCs.

Similar to BRG1, BET BRDs such as BRD2 and BRD4 have been implicated in chromatin remodeling processes. *In vivo* overexpression of BRD4 has been associated with chromatin de-compaction and nucleosome eviction (Devaiah et al. 2016), and BRD4 has been reported to transcriptionally co-activate AR (Asangani, et al. 2014). Similar involvement in nucleosome eviction has been reported for BRD2 (Surface et al. 2016).

BET proteins have previously been shown to be of therapeutic relevance in treatment of CRPCs (Asangani et al. 2014). Having established that the activity of AR coregulators play a role in driving AR-mediated chromatin opening, our group focused on understanding whether BRDs could be responsible for the generalized chromatin opening mediated by AR in CRPC (Urbanucci et al. 2017). Employing FAIRE, we could show that the enhanced local chromatin accessibility in AR-overexpressing cells could be reversed by treatment with sub-toxic concentrations of the bromodomain inhibitor JQ1 (Urbanucci et al. 2017) that predominantly targets BET proteins (Filippakopoulos, et al. 2010). Concomitantly, the most upregulated class of genes after treatment with JQ1 were histone genes and genes encoding chromatin structure-

associated proteins (Urbanucci et al. 2017), which is consistent with the effect of chromatin re-compaction elicited in these cells by the treatment. We selected three key BRDs, namely BRD2, BRD4, and ATPase Family, AAA Domain Containing 2 (ATAD2), for knock-down experiments followed by FAIRE at regulatory regions of AR target genes to test which of these BRDs had the most pronounced impact on local chromatin opening. Knockdown of all three proteins separately influenced chromatin opening at selected loci. However, the effects on local chromatin remodeling following single knockdown seemed to be locus-specific. This suggested that these proteins can act differently on different genomic loci, and that their functions may be redundant or that compensatory mechanisms exist (Urbanucci et al. 2017).

ATAD2 has been shown to be a co-activator of both AR and c-MYC in hormone-responsive human breast and prostate tumors (Ciro, et al. 2009). The role of ATAD2 as a regulator of chromatin dynamics has been extensively studied in yeast (Cattaneo, et al. 2014): It is implicated in chromatin structure maintenance and is capable of reading acetyl modifications on histone residues. Koo and colleagues showed that ATAD2 is highly expressed in replicating PC cells, and ATAD2 expression correlated with the expression of cell cycle and DNA replication genes that have overlapping functions in meiosis and tumor progression (Koo, et al. 2016). Moreover, ATAD2 has been reported to be important in sustaining specific gene expression programmes via regulating chromatin opening in embryonic stem cells (Morozumi, et al. 2016). In particular, Morozumi et al. found that ATAD2 sustained open chromatin states and ATAD2 depletion desensitized cells to Micrococcal nuclease (MNase) treatment. Morozumi et al. also found that histone acetylation guides ATAD2 to chromatin, resulting in an overall increase in chromatin accessibility (Morozumi et al. 2016).

In agreement with a previous study (Zou, et al. 2009), we found that *ATAD2* was regulated by androgens (Urbanucci et al. 2017). In addition, we showed that AR-overexpressing cells expressed higher levels of *ATAD2* in androgen depletion-challenged PC cells (Urbanucci et al. 2017). We identified also *BRD2* as an androgen regulated gene and BRD2 protein levels were elevated in AR-overexpressing cells (Urbanucci et al. 2017). Although BRD4 protein levels were elevated in AR-overexpressing cells, we could not observe a significant transcriptional regulation of *BRD4* by androgens (Urbanucci et al. 2017).

We also investigated the clinical value of the aforementioned BRDs as prognostic biomarkers in independent PC patient cohorts (Urbanucci et al. 2017). We determined that one of the isoforms of BRD4, the BRD4 long isoform, BRD2, and ATAD2 were all overexpressed in CRPC tissues compared to primary tumors. Moreover, high BRD2 expression in primary tumors was associated with shorter PC-specific survival (Urbanucci et al. 2017). More recently, nuclear BRD4 protein expression was confirmed to increase following castration resistance in longitudinally matched tumor samples collected pre- and post-treatment (Welti, et al. 2018). We also found that high expression of ATAD2 was positively associated with biochemical recurrence on a cohort of ten thousand patients (Urbanucci et al. 2017).

These studies demonstrate that BRDs are important tissue biomarkers, which can molecularly define subtypes of PC characterized by high chromatin alterations and responsiveness to BRD-inhibitors.

Asangani and colleagues have demonstrated the efficacy of BETi in reducing viability of PC cells (Asangani et al. 2014), and later they showed that BETi could reduce growth of enzalutamide-resistant PC cells as well (Asangani, et al. 2016). Knockdown of BRD4 had the strongest effect on PC cell viability in our models of AR overexpression (Urbanucci et al. 2017). We also showed that BETi in combination with enzalutamide had an additive inhibitory effect, and that this effect was stronger in AR-overexpressing cells compared to "naïvely" AR-expressing cells. This suggested that PC cells resistant to enzalutamide still rely on mechanisms mediated by both AR and BRDs for their survival. For example, we have reported that several CRPC-associated genes, such as UBE2C, HOXB13, CAMKK2, and AURKA were repressed by JQ1 treatment, and the chromatin at regulatory regions of these genes was re-compacted (Urbanucci et al. 2017). It is still uncertain whether bromodomain activity favors expression of key genes important for enzalutamide resistance, however. AURKA has been identified as an important driver of t-NEPC arising from treatment with novel antiandrogens such as enzalutamide (Mosquera, et al. 2013), and we have shown that it is a target of both BRDs (Urbanucci et al. 2017) and AR (Kivinummi et al. 2017) activity. We have also shown that AURKA was overexpressed in CRPC (Kivinummi et al. 2017), and interestingly, AURKA has been shown to sustain the expression and activity of AR splice variants (Jones, et al. 2017). This suggests that BRD inhibition may still be an effective therapeutic strategy in combination with other agents in t-NEPCs that overexpress AURKA. Although Wyce and colleagues showed that BETi was unable to impact tumor growth in a PDX model displaying NEPC characteristics, (LuCaP 145.2) (Wyce, et al. 2013), the stochasticity of the evolution of these particular classes of tumors and their high heterogeneity (Aggarwal et al. 2018; Lee et al. 2018) suggests that BETi should be evaluated in more preclinical t-NEPC models. Successful identification of the subset of t-NEPC tumors likely to respond to bromodomain inhibition may have large implications for the treatment of this increasingly prevalent PC subtype.

In summary, these data suggests that the increased expression of BRDs in CRPCs may be a driving force for the increased chromatin relaxation observed in these tumors, and consequently for their increased transcriptional plasticity.

Clinical implications

Chromatin deregulation and relaxation result in aberrant transcriptional reprogramming, cell plasticity, and increased chance to activate oncogenic pathways that lead to therapy resistance. The possibility to target a deregulated chromatin structure or, more generally, a deregulated epigenome, should be regarded as a way to tackle the acquired increase in plasticity that renders PC cells able to adapt to different therapeutic approaches. In PC, combination of existing therapies with bromodomain inhibition, and with inhibition of proteasome and autophagy in transcriptionally overdosed PCs could be therapeutically beneficial (Chude and Amaravadi 2017). For example, BETi in combination with drugs such as enzalutamide may also be therapeutically beneficial by reverting the chromatin structure toward a more differentiated state, and clinical trials investigating these strategies are ongoing. It can be speculated that such epigenomic re-differentiation may help in maintaining AR dependency and continued efficacy of AR-targeted therapies while preventing further lineage alterations.

Mechanisms of resistance to BETi have been already reported (Rathert, et al. 2015), and are probably due to compensatory mechanisms still linked to chromatin reprogramming which are capable of activating alternative oncogenic pathways (Pawar, et al. 2018). Therefore, targeting a deregulated chromatin structure with BETi is an attractive therapeutic strategy as it is plausible that chromatin deregulation is a reversible mechanism. In this context, in PC, the epigenetic "fluidity" and tendency of the chromatin to be in relaxed structure could be a liability if targeted intermittently to prolong the duration of the effect and delay the emergence of resistance. This epigenetic "fluidity" can potentially explain the positive results demonstrated by the use of bipolar androgen therapy (BAT) (Teply and Antonarakis 2016; Teply, et al. 2018), intermittent androgen deprivation therapy (Abrahamsson 2017; Hussain, et al. 2016), and, with due precautions, also supra-physiological androgen therapy (Mohammad, et al. 2017).

High androgen levels lead to LSD1/AR-mediated *AR* gene suppression in PC, but castrate levels of androgens leads to upregulation of the AR (Cai et al. 2011; Coutinho, et al. 2016). This fundamental process is at the base of PC addiction to AR signaling. Well-controlled experiments in preclinical models have shown that AR upregulation is the result of adaptive autoregulation of the AR to low androgen levels (Isaacs, et al. 2012). As we have discussed that AR upregulation is associated with increased chromatin deregulation, preventing this step with repeated cycles of androgen deprivation and supplementation, which in fact affects the AR level (Isaacs, et al. 2017), may also delay the emergence of chromatin deregulation and cell plasticity. This can explain why in asymptomatic men with metastatic CRPC, BAT was able to resensitize tumors to enzalutamide treatment in most patients undergoing rechallenge (Teply et al. 2018).

Molecular probes for different BRD targets are now being tested in PC patients for exploiting epigenetic alterations in the clinical setting (Baumgart and Haendler 2017; Fernandez-Salas, et al. 2016; Urbanucci and Mills 2017). Whether selection of patients with high chromatin deregulation will respond better to these therapeutic approaches/regimens remains to be investigated. To this end, the assessment of stratification biomarkers, such as genetic signatures or tissue biomarkers should be evaluated in clinical trials and ultimately clinically implemented (Cieślik and Chinnaiyan 2017).

Others and we have showed that BRDs such as BRD4, BRD2 and ATAD2, are mediators of the increased chromatin accessibility observed in CRPC, and are prognostic tissue markers overexpressed in CRPC (Urbanucci et al. 2017; Welti et al. 2018). Therefore BRDs can be used as readout of an altered epigenome. We have generated BROMO-10, a ten-gene signature that proxies the chromatin remodeling activity and chromatin status in PC tumors (Urbanucci et al. 2017). Thus, BROMO-10 could be used for selecting patients with high AR activity likely to benefit from BET-targeted therapies. BROMO-10 was retrospectively able to identify also intermediate-risk PC (i.e. Gleason score 7) patients with a high risk of early progression (Gerhauser et al. 2018), which indicates that these tumors are likely driven by a "fluid" chromatin structure and can be triggered by therapeutic pressure to progress.

Ultimately, as BET inhibitors have been proven efficacious in a number of other pathologies, its effect on chromatin accessibility should be considered as a major mechanism of action not only in PC, but in a cell-specific manner in diseases of other tissues as well.

Future perspectives

Studies on chromatin structure evolution upon therapeutic pressure are lacking. For example, it remains to be shown whether the chromatin structure is further altered in t-NEPCs as compared to CRPC adenocarcinomas. Although several cohorts contain patients with these disease entities, they lack longitudinal biopsies, and can thus not infer direct proof of tumor evolution as opposed to selection. A genomic study on longitudinal biopsies from tumors before and after t-NEPC emergence is ongoing (Aggarwal et al. 2018) and with the appropriate analytical tools this study could show whether further chromatin relaxation occurs upon lineage plasticity-driven AR-targeted therapy resistance.

Structural variations are found in regions of open chromatin, which include ARBSs (Gerhauser et al. 2018). Overall, PC has a low somatic mutational burden compared to other cancers yet has a tendency towards accumulating structural alterations (Barbieri et al. 2012; Grasso et al. 2012; Zehir, et al. 2017). The most frequent structural alteration is the *TMPRSS2:ERG* gene fusion which can be detected in more than half of clinically localized and metastatic PC cases (Taylor et al. 2010; Tomlins, et al. 2005). Interestingly, this and other fusion genes have been shown to involve androgen regulated genes (Rubin, et al. 2011), suggesting that chromatin structure is involved in inducing proximity between the regulatory regions of the AR-target genes and the fusion partner genes. In this context, one of the key questions that remains to be addressed is whether it will be possible to characterize the earliest tumorigenic chromatin alterations during initiation of PC. This has been done for DNA methylation (Massie, et al. 2017) but to a lesser extent for chromatin structure. Chromatin accessibility has been used to identify binding of TFs and genomic regulatory elements, and it is used together with information on binding of TFs such as AR to prioritize disease-associated single nucleotide polymorphisms (SNPs) that are not within coding regions. We have shown that chromatin regions bound by BRD4 can identify risk SNPs that achieved significance in genome-wide association studies (GWAS) for prostate, breast, and lung cancer in a tissue/disease specific manner (Zuber, et al. 2017). This, together with the evidence that BRDs are upregulated already in primary PCs possibly implies a role of BRDs in early deregulation of chromatin structure and tumor initiation, which should be further explored.

Furthermore, the chromatin structure may reflect the metabolic status of a cell as it depends on the availability of many metabolites in order to maintain the make-up of histone modifications (Schvartzman, et al. 2018). Therefore, it will be increasingly important to understand the link between metabolic perturbations occurring in CRPC and the effects that these elicit on the chromatin structure (Li, et al. 2018). The metabolite addiction to e.g. acetyl groups for HAT activity and transcription in CRPC may ultimately rely on deregulation of metabolic pathways (Kinnaird, et al. 2016) which should be better characterized to understand their effect on chromatin remodeling.

Conclusions

In this review we have collected evidence of the AR overexpression-mediated positive feedback loop that boosts the expression of many chromatin-associated proteins, including BRDs that act to increase the chromatin accessibility of AR and other TFs in CRPCs.

AR overexpression-driven chromatin structural alterations can be thought of as a key determinant feature of PC progression, which leads to activation of several adaptive oncogenic transcriptional responses and drive tumor growth and therapy resistance: a phenomenon of epigenetically driven adaptation to therapeutic pressure.

We are now beginning to understand how the chromatin structure can be modulated to reprogram PC cells. More work is needed to understand how the chromatin structure and the higher order conformation of the chromatin in the nucleus is organized. This knowledge will help us understand and predict events driving PC development and progression. Finally, targeting pathways involved in chromatin reprogramming arises as a compelling strategy for preventing and possibly reverting the therapy-driven increase in plasticity of PC cells.

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Figure legends

Figure 1. Methods for studying the chromatin structure and chromatin-associated proteins. (**a**) Non-immunoprecipitation-based methods for assessing chromatin in open conformation include DNase or MNase. These methods enrich for chromatin sites hypersensitive to enzymatic digestion, and can be used to assess regulatory or nucleosome-free regions, respectively. Regulatory regions within chromatin in open conformation can also be assessed by FAIRE-seq, in which nucleosome-free DNA is isolated with a phenol-chloroform extraction and sequenced. In ATAC-seq, hyperactive transposases allow for insertion of primers into accessible chromatin regions, and the resulting products can be sequenced. (**b**) ChIP-based techniques allow for identification and quantification of regions of DNA bound by either protein (e.g. transcription factors (TFs)), or histone modifications by immunoprecipitation with specific antibodies followed by e.g. sequencing. (**c**) The spatial conformation of higher order chromatin can be studied by chromosomal conformation capture (3C)-based techniques. These methods can assess both intra- and inter-chromosomal interactions between regions of DNA that localize in proximity to one another. 3C is used predominantly for promoter-enhancer interactions and is coupled to qPCR to quantify the products of these interactions. 4C is used to measure the interactions between one specific locus and the rest of the genome simultaneously by coupling 3C to sequencing. 5C requires knowledge of the interacting chromosomal regions, but can map all interactions within a genomic region by ligation of universal primers. ChIA-PET utilizes immunoprecipitation of proteins of interest within conformation-captured chromatin regions, thus utilizing both conformation capture and ChIP technologies. ChIA-PET is therefore used to study specific interacting genomic regions facilitated by binding of particular TFs. (**d**) Finally, at the macroscopic level, immunohistochemistry can be used on formalin-fixed paraffin-embedded tissue or cells to visualize nuclear size and chromatin structures by microscopy. *Abbreviations: Chr = chromosome, H&E = hematoxylin and eosin, Seq = sequencing, TAD = topologically associating domain, TF = transcription factor.*

Figure 2. Chromatin relaxation during prostate cancer oncogenesis and progression. Schematic illustration of progressively open chromatin during following prostate cancer oncogenesis, subsequent acquisition of therapy resistance, and CRPC development.

Figure 3. Mechanism of chromatin remodeling associated with androgen receptor binding to chromatin. (**a**) Androgen responsive elements (AREs) residing in genomic regions with transcriptionally repressive histone marks may not directly permit AR binding. The pioneering TF FOXA1 may bind directly to condensed chromatin near regulatory enhancer elements and facilitate recruitment of coregulators such as CBP/p300 and MLL (**b**), which regulate chromatin opening through their histone acetylase and methyltransferase activities, respectively (**c**). GATA2 may also act as a pioneering factor, and increased acetylation is captured by bromodomain-containing proteins, e.g. BRD4 or ATAD2, which further boost local chromatin opening and exposes sequences recognizable by TFs such as activated AR. (**d** and **e**) PI3K/AKT-phosphorylated MED1 may recognize FOXA1 and promote chromatin looping which increases enhancer-promoter interactions and RNA polymerase-mediated transcription. Additional chromatin

remodelers may be recruited in the cascade, collectively permitting ligand-activated, dimerized AR binding to AREs. *Abbreviations: BRDs = bromodomain-containing proteins.*

Figure 4. Proposed model for acquisition of plasticity and therapy resistance involving chromatin reprogramming in prostate cancer. (**a**) In androgen deprivation therapy (ADT)-naïve, primary PC, the androgen receptor (AR)-target genes, including *KLK3* (PSA) and other bromodomain-containing proteins (BRDs)-dependent genes are transcribed to maintain growth and survival of the tumor. In this context, gene transcription is mediated mainly by AR binding to defined regions of permissive chromatin, which facilitates recruitment of proteins required for transcriptional initiation. Upon treatment with AR-targeted therapies, events involving chromatin relaxation which facilitates emergence of castration resistant prostate cancers (CRPCs) occur. (**b** and **c**) Events including AR overexpression are found in the majority of CRPCs, and can lead to enhanced expression and/or activity of AR coactivators, further promoting AR-signaling and increasing BRD activity. In turn, this enhances the degree of chromatin relaxation, and promiscuous binding of activated or re-activated TFs such as e.g. glucocorticoid receptor (GR). (**d**) The scenario in which c-MYC overexpression leads to frequent c-MYC binding events which promotes transcriptional reprogramming in concert with AR. (**e**) Continued suppression of AR signaling may confer lineage plasticity and therapy evasion through e.g. reactivation of N-MYC and N-MYC mediated cell reprogramming. N-MYC-related reprogramming may involve epigenetic silencing through recruitment of the polycomb protein EZH2, or enhanced transcription of genes involved in promoting stem cell and/or basal-cell like features, which can alleviate AR-dependence and thereby drive progression to treatment-related neuroendocrine CRPCs (t-NEPC) and other AR-low/null subtypes of CRPCs.

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