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Abstract: Several oncogenic factors have been involved in prostate cancer progression. However, therapeutic approaches still focus on suppression of androgen receptor (AR) signaling. In fact, whereas the full-length AR incorporates a ligand-binding domain, which has become a drug target for competitive inhibitors, other transcription factors often do not have tractable binding pockets that aid drug development. Consequently drug development efforts have turned to transcription co-regulators, often chromatin-modifying enzymes or factors that bind to epigenetic modifications to chromatin. Bromodomain (BRD)-containing proteins fall into the latter category and significant progress has been made in developing small molecule inhibitors that target a particular subgroup of BRD-containing proteins known as the Bromodomain and extra-terminal (BET) family proteins. These inhibitors have proven particularly effective in inactivating c-Myc in lymphoma but more recently members of the BET family have also been identified as AR-interacting proteins raising the prospect of using these inhibitors as an alternative strategy for targeting AR-driven cancers. In this review we will provide an overview of BRD-containing proteins and the potential for exploiting them as biomarkers and drug targets in prostate cancer.

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14th April 2017

Dear Professor Claessens and Professor Visakorpi,

We would like to thank you and the reviewers for the helpful feedback on our submission and the opportunity to present a revised version of our review article entitled “**Bromodomain-containing proteins in prostate cancer**”.

All the textual changes are highlighted in red and we attach a point-to-point rebuttal letter to the reviewers’ comments. We hope that our manuscript is now acceptable for publication.

Yours sincerely,

Alfonso Urbanucci and Ian Mills

Response to Reviewers

We would like to thank the reviewers for the helpful feedback on our submission. We have now addressed every comment and welcome the opportunity to submit a revised manuscript.
We will now proceed to answer the comments in detail.

Reviewer #2: This is well written review article. I have only one major request. Table summarizing BRD proteins, their functions and relations to prostate cancer would be a good addition to the review.

Answer: We thank the reviewer for the helpful feedback. As suggested, we have inserted this as table 1.

A minor comment:

- abstract, 6th line, word "now" probably should be "not" ie "AR is not alone..."

Answer: we have erased the sentence to shorten and focus the abstract.

Reviewer #3: This is a very interesting, relatively novel field in prostate cancer biology and hence I expect this review to have a high impact. Unfortunately, the readability of the text is not Always optimal.

Answer: We thank the reviewer for the feedback. The manuscript has now gone language revision and we hope we improved readability.

I would suggest to change some phrases:

1. the start of the abstract is too general, please omit the first +/- 10 lines

Answer: We have now shortened the start of abstract and focused it more.

2. introduction, cut 2nd sentence in two: ...is complex. Histone ...

Answer: This is now done.

3. page 2 line 10: ... inhibitors which have are been ...

Answer: We have corrected this.

4. page 2 sentence starting at line 14 as well as the next one are just too long

Answer: We have now rephrased and split the sentences.

5. page 3 last 4 lines, please rephrase

Answer: This is now done.

6. page 4 line 2 proteins take mediate ... and the next sentence is too complex. Please also add a reference here at end of paragraph.

Answer: this has now been done.

7. page 4, line 14 needs a reference; please rephrase ...reducing therapies side effects.

Answer: this has now been done.

8. page 5: second paragraph: ...to be preclinical efficacy... trials data-> trial data ...; please rephrase sentence starting at end of page 5 and the next sentence

Answer: this has now been done.

9. page 6 add ref after ...abiraterone acetate. and after ...other than c-MYC. please rephrase the last sentence starting If gene expression reflects the on-off ... and add a ref.

Answer: this has now been done.

10. page 7, first sentence of new section ...contribute to this in prostate cancer... please define 'this' and add a reference; also after ... typical of prostate cancer. Please specify ... can be accounted for by this class of protein.

Answer: We rephrased sentences in the whole paragraph and we hope that this is now clearer. We also added a reference to the newly introduced table one which contains information to the potential contribution of BRD activity on chromatin in PC cells.

11. page 8, ... in these datasets ... please specify; add reference to sentence on ...two chemically diverse inhibitors has been reported ...

Answer: We specified that we were referring to the TCGA and Trento/Cornell datasets. We also rephrased the last sentence of the paragraph to specify that we were referring to the inhibitors we talked about earlier on.

12. page 9, middle ... chemical probes targeting like GSK... you mean ...targeting ATAD2...?

Answer: We have now corrected this.

13. First sentence of last paragraph on page 9 is contraction of two, please resolve.

Answer: The sentence has been split.

14. page 10 2nd sentence (using the ...) and last sentence of 1st paragraph (Phentotypically...) are too long.

Answer: These sentences have been rephrased.

15. page 10 the paragraph 'in summary unlike ... Maybe put second sentence first

Answer: We have now rephrased the paragraph. We now use this paragraph as conclusion of the paper.

16. page 11 please correct this section. Also the sentence ... In other cancers (leukemia... would fit better in another section.

Answer: we have corrected this section and titled it “future challenges”. We hope that it is now more focused. Moreover we added a paragraph on the involvement of several BRD-containing proteins in DNA damage response.

Bromodomain-containing proteins in prostate cancer

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Word count: 4649 excluding references

Abstract

Several oncogenic factors have been involved in prostate cancer progression. However, therapeutic approaches still focus on suppression of androgen receptor (AR) signaling. In fact, whereas the full-length AR incorporates a ligand-binding domain, which has become a drug target for competitive inhibitors, other transcription factors often do not have tractable binding pockets that aid drug development. Consequently drug development efforts have turned to transcription co-regulators, often chromatin-modifying enzymes or factors that bind to epigenetic modifications to chromatin. Bromodomain (BRD)-containing proteins fall into the latter category and significant progress has been made in developing small molecule inhibitors that target a particular subgroup of BRD-containing proteins known as the Bromodomain and extra-terminal (BET) family proteins. These inhibitors have proven particularly effective in inactivating c-Myc in lymphoma but more recently members of the BET family have also been identified as AR-interacting proteins raising the prospect of using these inhibitors as an alternative strategy for targeting AR-driven cancers. In this review we will provide an overview of BRD-containing proteins and the potential for exploiting them as biomarkers and drug targets in prostate cancer.

Keywords

Prostate cancer; BRD BET bromodomain and extra-terminal domain; BRD BET inhibitor; chromatin structure remodeling; transcriptional co-activator; androgen receptor.

Abbreviations

TF: transcription factor; HAT: histone acetyltransferase; HDAC: histone deacetylase; BRD: bromodomain; BET: bromodomain and extra-terminal; NEPC: neuroendocrine prostate cancer; DDR: DNA damage Response.

Introduction

Epigenetics can be defined as a heritable pattern of post-translational modifications leading to the emergence of new phenotypes that are not encoded within genomic DNA sequence (Graca, Pereira-Silva, Henrique et al., 2016). The epigenetic code is made of several chromatin modifications that can be found on histones in different combinations. Histone acetylation is one example of these modifications, which is most significantly linked to regions of open chromatin accessible to DNA and RNA polymerases. Acetylation levels on histones are dysregulated in cancers and this reflects changes in the expression and/or activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Deregulation of HDACs changes the relative expression of oncogenes, tumour suppressors and subsets of microRNAs in many cancer types. This has motivated the development of HDAC inhibitors as oncological agents (Marrocco-Tallarigo, Centenera, Scher et al., 2009, Anne, Sammartino, Barginear et al., 2013). Epigenetic readers such as bromodomain (BRD)-containing proteins offer an alternative target class to disrupt pro-tumorigenic transcription arising from epigenetic modifications (Filippakopoulos and Knapp, 2014). BRD inhibitors that specifically target a subset of BRD-containing proteins, BET (bromodomain and extra-terminal) proteins have been developed. These

inhibitors impact on gene expression programmes by altering how protein complexes utilize histone acetyl marks(Filippakopoulos and Knapp, 2014). In particular, the effect of BRD inhibitors is focused on cell growth and resistance to apoptosis in cancer cells and on inflammatory signaling in immune cells(Muller, Filippakopoulos and Knapp, 2011,Bachu, Dey and Ozato, 2016). The use of such inhibitors has helped to understand the cell/tissue-specific functions that these proteins perform. The main functional role of BET proteins is to promote transcriptional elongation/efficiency. Consequently the somewhat cell type-specific effects on transcription observed speak of the interplay between these proteins and transcription factors (TFs) driving cellular specialization and tumorigenesis (Di Micco, Fontanals-Cirera, Low et al., 2014,Liu, Xu, He et al., 2014).

Prostate cancer, the AR signaling axis, chromatin, and bromodomain-containing proteins

Prostate cancer (PC) is a heterogeneous disease and the etiology is still not completely known. From the molecular point of view, the main focus of research has always been the androgen receptor (AR), which is thought to be an important driver of the disease(Mills, 2014). In recent years a number of drugs such as Enzalutamide and Abiraterone acetate have received clinical approval due to their impact on AR signaling, acting as a potent antagonist to androgen binding to the ligand-binding domain and as a potent inhibitor of androgen synthesis respectively(Teply and Antonarakis, 2016). Each drug has enhanced the median survival of patients with lethal castrate-resistant metastatic disease by months and in the case of some individuals by years. In other cases however responses have been significantly less impressive due to a range of resistance mechanisms that include the expression of AR

splice variants that lack a ligand-binding domain (for example AR-V7)(Ciccarese, Santoni, Brunelli et al., 2016), AR copy number amplification(Visakorpi, Hyytinen, Koivisto et al., 1995) and over-expression and the evolution of a dependency on other oncogenic signaling pathways driven by phosphatidyl inositol 3-kinase/Akt (PI3-kinase/Akt) signaling, receptor tyrosine kinases or indeed other oncogenic TFs such as c-Myc or N-Myc (Beltran, Prandi, Mosquera et al., 2016, Robinson, Van Allen, Wu et al., 2015, Dardenne, Beltran, Benelli et al., 2016, Barfeld, Urbanucci, Itkonen et al.). Regardless of the oncogenic driver however transcriptional dysregulation is fundamental to cellular transformation and cancer progression. Changes in transcriptional profiles are used as cancer biomarkers and are in effect readouts of oncogenic signaling and changes that have occurred within the nucleus to both the accessibility of DNA sequences and the activity of TFs and co-regulators(Luo and Dean, 1999). DNA packed into nucleosomes forms the simplest and most immediate chromatin structure. Such chromatin structure is the ultimate determinant of the transcriptional output, which therefore exerts regulatory functions. In fact the recurring chromatin modifications at the nucleosomes associated with transcriptional changes have been defined as the “histone code”. Through these mechanisms, chromatin modifications and their alterations, have an indirect effect on cell identity and cancer development (Audia and Campbell, 2016). However, several other chromatin-associated proteins mediate transcriptional regulation. TFs and the basal transcription machinery are direct effectors in transcriptions, while chromatin writers and erasers modify chromatin landscapes (Speranzini, Pilotto, Sixma et al., 2016, Zhang, Cooper and Brockdorff, 2015). Another class of chromatin-associated proteins is made of chromatin readers such as BRD-containing proteins. This class of proteins is overlapping with the above-mentioned other classes of chromatin-

associated proteins as they recognize acetylated histones and perform a wide range of downstream functions. BRD-containing proteins can also present other domains and be TFs or transcriptional co-regulators and can act as primary recruiting factors for the assembly of larger protein complexes. They may also perform catalytic activities such as methyltransferase, ATP-dependent chromatin-remodeling, HAT and helicase(Fujisawa and Filippakopoulos, 2017) (**Table 1**). Therefore, BRD-containing proteins are transcriptional regulators that initiate chromatin re-shaping in order to prepare for transcription.

Targeting bromodomain-containing proteins –BRD4 and associated therapeutics

Current therapies aim to shut down the signaling associated with the androgen/AR axes(Teply and Antonarakis, 2016). One of the drawbacks of this approach is that complete shutdown of the pathway has proven difficult to achieve, with little benefit for the patients, in terms of overall survival to the so called castration resistant prostate cancer (CRPC) that still relies on androgens, and therefore on the AR to progress. Forms of neuroendocrine prostate cancers (NEPCs) have been described, which may also represent clonal selection following treatment with next generation anti-androgens such as Enzalutamide (Vlachostergios, Puca and Beltran, 2017). Therefore the current therapeutic strategy to reduce patients' side effects and limit the selection of resistant clones is to identify druggable downstream targets of AR that mediate its oncogenic effect. However, only a few have been found that associate with disease outcome(Urbanucci, Waltering, Mills et al., 2013). Moreover it is not known how the AR switches from being the main driver of physiological healthy prostate cells differentiation, to being the main driver of PC cells growth and resistance to many therapeutic approaches. These clinical challenges have motivated a

renewed focus on targeting **co-activators** supporting the transcriptional activity of not merely the AR but other important oncogenic **TFs** (Culig and Santer, 2014, Rohira and Lonard, 2017). Thanks to the pioneering work of Arul Chinnayian's laboratory, two **BRD-containing proteins** were shown to be involved in PC progression to **CRPC** (Asangani, Dommeti, Wang et al., 2014). In particular, both BRD4 (Asangani et al., 2014) and lysine methyltransferase 2A (**KMT2A**, **alias** MLL) (Grasso, Wu, Robinson et al., 2012, Malik, Khan, Asangani et al., 2015) were found to directly interact with the **AR** and act as transcriptional activator. BRD4 can be viewed as a multi-cancer target. It was firstly identified because of its role in NUT midline carcinoma (NMC) (French, Ramirez, Kolmakova et al., 2008), a rare subtype of squamous cell carcinoma characterized by a translocation most often involving the *NUT* gene and BRD4 (Bauer, Mitchell, Strait et al., 2012). NMC typically arises from midline structures of the head, neck and respiratory tract principally the thorax and can be diagnosed in both adults and children. This extremely aggressive disease has a median overall survival of around 6 months and the BRD4 fusion blocks differentiation of NMC cells in part by promoting expression of c-MYC (Grayson, Walsh, Cameron et al., 2014). The use of a BET inhibitor, OTX015/MK-8628, was able to extend overall survival to 18 months in two patients (Stathis, Zucca, Bekradda et al., 2016) and more recently has achieved significant impacts on a second lymphoma, also acting through c-MYC (Gaudio, Tarantelli, Ponzoni et al., 2016).

BET inhibitors have shown pre-clinical efficacy in CRPC but as yet no trial data are available (Table 2). In pre-clinical models *in vitro* and *in vivo* most of the studies used JQ1, the first inhibitor developed for BET proteins. JQ1 is believed to act predominantly on BRD4, BRD2, BRD3, and, BRDT (Filippakopoulos, Qi, Picaud et al., 2010), and have been shown to block proliferation, induce apoptosis, and repress

expression of anti-apoptotic factors principally in AR-positive PC cell-lines whilst also reducing transcription of AR target genes (Asangani, Wilder-Romans, Dommeti et al., 2016). AR recruitment to genes' regulatory regions in the DNA is impacted by JQ1 treatment to the same extent as using the AR antagonist Enzalutamide (Asangani et al., 2016). Importantly however the mode-of-action of JQ1 does not require the AR ligand-binding domain to be expressed. JQ1 disrupts the interaction between BRD4 and the N-terminal domain of AR, and limits AR recruitment to DNA (Asangani et al., 2014). In fact JQ1 is able to reduce the levels of AR variant 7 (ARv7), an AR splice variant that is constitutively active because it lacks the ligand-binding domain. Alternative splicing form of the AR that lack the ligand-binding domain are over-expressed in patients that become refractory to Enzalutamide or the androgen synthesis inhibitor, Abiraterone acetate (Coutinho, Day, Tilley et al., 2016). As such ARv7 is regarded as one of the main drivers of resistance to androgen deprivation therapy. Moreover in mouse studies JQ1 does not affect normal prostate growth or testosterone levels even whilst inhibiting the growth of CRPC tumours. Significantly BET inhibitors display enhanced efficacy when combined with Enzalutamide *in vivo* (Asangani et al., 2016). These findings have provided a strong pre-clinical basis to trial BET inhibitors in patients with CRPC. Moreover, these and other findings proved that BET-proteins activity is cell context-dependent (Fujisawa and Filippakopoulos, 2017), and in cancer such activity supports many oncogenic TFs (Loven, Hoke, Lin et al., 2013), not only c-MYC expression as initially thought (Delmore, Issa, Lemieux et al., 2011). That being so it is also important to further explore the mechanism-of-action of JQ1 and other BET inhibitors.

We and other groups have previously shown that acetylation of histone H3 and H4 is increased in the regulatory regions governing the expression of AR target genes and

AR binding sites (Urbanucci, Marttila, Janne et al., 2012, Berman, Frenkel, Coetzee et al., 2010). We have also shown that acetylation of histone H3 is increased in AR-over-expressing cell-lines (Urbanucci et al., 2012). Consequently a further plausible mechanistic role for BRD-containing proteins including BRD4 may be to maintain chromatin in an open and acetylated form even in conditions of androgen deprivation therapy. The TFs and transcriptional co-regulators on-off rate on the chromatin impacts on gene expression. Therefore, the maintenance of chromatin opening may be sufficient to elevate transcriptional output by prolonging the dwell time of TFs at accessible regions of the DNA. Others and we investigated the chromatin structure in PC and confirmed that chromatin opening is enhanced and reprogrammed in CRPC compared to primary PC or benign tissue ((Stelloo, Nevedomskaya, van der Poel et al., 2015) and data not show; Urbanucci *et al.*, in submission). This would partially explain why in AR over-expressing PC cell-lines and CRPC specimens others and we observed an increased chromatin binding by the AR (Sharma, Massie, Ramos-Montoya et al., 2013, Massie, Lynch, Ramos-Montoya et al., 2011, Sahu, Laakso, Ovaska et al., 2011, Urbanucci, Sahu, Seppala et al., 2012) while genes transcription was enhanced (Waltering, Porkka, Jalava et al., 2011, Waltering, Helenius, Sahu et al., 2009, Urbanucci, Waltering, Suikki et al., 2008, Taylor, Schultz, Hieronymus et al., 2010, Tewari, Yardimci, Shibata et al., 2012).

Bromodomain-containing proteins – beyond BRD4 and associated drugs

The potential for other BRD-containing proteins to contribute to key chromatin-related processes and alterations in PC, beyond BRD4, is underscored by the fact that almost half of the over 40 known BRD-containing proteins have been implicated in PC (Table 1). An evaluation of recent PC sequencing datasets – example being The

Cancer Genome Atlas (TCGA)(Cancer Genome Atlas Research, 2015) and the Trento/Cornell neuroendocrine CRPC study showed that differential expression of these genes, with a low-rate of autosomal somatic mutation, is observed in 78% of TCGA primary PC cases and 81% of CRPC and NEPC (Figure 1A and B). Only 5 samples did not show evidence of alterations in any of the 42 genes queried in the advanced tumours. We also evaluated the frequency of alterations 42 BRD-containing proteins genes in a larger panel of PC datasets (Figure 2). This pattern of dysregulation, predominantly at the level of gene expression and copy-number in low frequency of autosomal somatic mutations, is typical of PC (Grasso et al., 2012).

The ability to robustly identify molecular subtypes rather than the incident rate of those subtypes is the key to beginning stratified approaches to treatment – alongside of course the availability of specific drugs and a biological understanding of function capable of informing the development of surrogate response biomarkers. Encouragingly in recent years, inhibitor development programs targeting acetyl-lysine interaction domains of the BRD-containing proteins family have been quite successful and have led to the generation of a comprehensive set of potent and selective inhibitors including several compounds targeting BRDs present in remodeling complexes. These now extend to bromodomain adjacent to zinc finger domain 2A/B (BAZ2A/B), ATPase family, AAA domain containing 2 (ATAD2) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2/4 (SMARCA2/4) inhibitors(Ferri, Petosa and McKenna, 2016). Of these, BAZ2A and ATAD2 are copy-number amplified and over-expressed in the TCGA and Trento/Cornell datasets and consequently it is worthwhile reviewing the status of these as drug targets (Figure 1A and B).

BAZ2-ICR and GSK2801 are examples of tool compounds **that** inhibit the interaction of BAZ2A/B with chromatin (Drouin, McGrath, Vidler et al., 2015, Chen, Chaikuad, Bamborough et al., 2016, Jones, Hamana, Nezu et al., 2000). They provide alternative scaffolds to target these proteins. In addition, a negative control compound chemically related to GSK2801 presents further reassurance that the observed biological effects are indeed due to targeting the **BRDs** of BAZ2A/B. BAZ2A/B **are** proteins of the nucleolar remodeling complex (NoRC) complex (Chen et al., 2016). The family member BAZ2A associates with ATPase SNF2h to form the NoRC (Guettg, Lienemann, Sirri et al., 2010). NoRC associates with the regulation of the expression of noncoding RNAs and establishes a heterochromatic structure at centromeres and telomeres, a function that has been **predominantly** linked to BAZ2A (Guettg et al., 2010). BAZ2A is required to maintain the growth of **PC** cells and its high expression levels associates with the recurrence of **PC** (Gu, Frommel, Oakes et al., 2015). **The use of these two** chemically diverse inhibitors **BAZ2-ICR and GSK2801** has not yet been reported and the biological consequences of inhibiting the BAZ2A/B **in prostate and other** cancers remain to be elucidated.

ATAD2, which functions as an ATP-dependent co-regulator of **TFs**, such as c-Myc, estrogen and androgen receptors, and E2F (Zou, Revenko, Li et al., 2007, Hsia, Kalashnikova, Revenko et al., 2010, Duan, Zou, Yang et al., 2013, Zou, Guo, Revenko et al., 2009). ATAD2 is over-expressed in a range of tumours, for example hepatocellular carcinoma, breast and lung cancers and correlates well with a poor prognosis in patients with breast or lung cancer and associates with a distant recurrence (Zhang, Sun, Li et al., 2013, Ciro, Prosperini, Quarto et al., 2009, Hwang, Ha, Bang et al., 2015). We have recently undertaken a large-scale collaborative study with Thorsten Schlomm, Guido Sauter, Sarah Minner and colleagues at the

Eppendorff Hospital in Hamburg, which has confirmed this picture for **PC** too (data not shown; Urbanucci *et al.*, in submission). Recently, potent inhibitors for this interesting **BRD-containing** protein have been reported (Bamborough, Chung, Demont *et al.*, 2016). One example, GSK8814, was tested on the PC cell-line LNCaP but failed to elicit a single-agent anti-proliferative response (Bamborough *et al.*, 2016). Microarray data were generated in this study both for the active probe, GSK8814 and for the less active enantiomer, GSK8815 (GSE78868). Intriguingly these data indicated that treatment with GSK8814 led to increased expression of enzymes in the mevalonate pathway (unpublished observation). This is a pathway that has previously been found to cooperate with Ras and other oncogenes to promote cellular transformation (Clendening, Pandya, Boutros *et al.*, 2010). Cancer stem cells and cell lines exhibit increased cholesterol synthesis through the mevalonate pathway and are often selected for by conventional therapy such as radiotherapy to which they show resistance (Warita, Warita, Beckwitt *et al.*, 2014). Consequently it will clearly be important to elucidate the biological impact of chemical probes like **GSK8814**, targeting **ATAD2**, to appreciate the niche in which they can be used to achieve the most clinical benefit.

SWItch/Sucrose Non-Fermentable (SWI/SNF) complexes exhibit chromatin remodeling activity and components of these complexes are mutated or lost at high frequency in tumours (Sarnowska, Gratkowska, Sacharowski *et al.*, 2016). This remodeling complex has attracted considerable attention in oncology and particularly in hormone-dependent cancer types. SWI/ SNF contain either the catalytic remodeling subunit BRM (SMARCA2) or BRG1 (SMARCA4), which alters chromatin structure in an ATP-dependent manner (Sun, Man, Tan *et al.*, 2015). In turn, this results in an 'open chromatin' competent for interactions with **TFs** (Marshall, Link, Petre-Draviam

et al., 2003, Sun, Tawfik, Gayed et al., 2007). Both catalytic subunits of SWI/SNF contain BRDs, offering an opportunity for inhibitor development. BRG1 is also over-expressed in tumours, including PC and expression levels correlate with tumour progression (Sun et al., 2007). However, targeting the BRD of SMARCA2/4 using the selective inhibitor PFI-3 did not result in cytotoxicity or inhibition of clonogenic growth in SMARCA4-deficient tumours (Vangamudi, Paul, Shah et al., 2015). In contrast, targeting the helicase domain convincingly phenocopied the genetic loss-of-function experiments in lung cancer (Vangamudi et al., 2015). The SWI/SNF complex harbours multiple protein interaction domains that anchor this large remodeling complex to specific chromatin sites marked with histone acetylation (Filippakopoulos, Picaud, Mangos et al., 2012). Hence, PFI-3 does not appear to target BRD-containing proteins that are critical for the recruitment of SWI/SNF to the chromatin as PFI-3 is not able to displace the endogenous SMARCA2/4 from chromatin (Vangamudi et al., 2015), suggesting the role of another interaction domain or a combination of interaction domains for chromatin targeting the SWI/SNF complex. Phenotypically PFI-3 can impact on embryonic and trophoblast stem cell maintenance. Extending these studies to PC will require a more extensive evaluation of the biology of SMARCA2/4 in PC cells. It is intriguing however that if there is a conservation of function across these targets, inhibition of these proteins will impact on pluripotency and its maintenance (Fedorov, Castex, Tallant et al., 2015).

Future challenges

The challenges for PC remain two-fold, the more robust prognostication of the disease and the more effective treatment of progressing disease. BRD-containing proteins inhibition represents an important example of how to expand the current therapeutic

approach from AR to other targets. However, mechanisms of resistance to BET inhibition have already been described in other cancers and involve the activation of Wingless-type MMTV integration site family (Wnt) pathway (Rathert, Roth, Neumann et al., 2015, Fong, Gilan, Lam et al., 2015). This is luckily to restore other factors sustaining a pluripotent chromatin landscape. Paradoxically, BRD-independent activity of BRD4 has been also described (Shu, Lin, He et al., 2016). This is achieved via the association of BRD4 with Mediator 1 (MED1) or the hyperphosphorylation of BRD4 derived from the reduced activity of the serine phosphatase PP2A (Shu et al., 2016). Additional mechanisms described include the acquisition of compensating activating mutations in other signaling pathways such as PI3-kinases (e.g. PIK3CA) (Marcotte, Sayad, Brown et al., 2016). The resulting transcriptional plasticity (Rathert et al., 2015) is also the consequence of re-activation of a suite of oncogenic TFs such as c-MYC (Fong et al., 2015). It is intriguing that similar resistance mechanisms have been described before also in PC in response to androgen deprivation therapy/anti-androgens. Drugs are available to counteract Wnt activation and reciprocal activation of PI3-kinase signaling (Rajan, Sudbery, Villasevil et al., 2014, Carver, Chapinski, Wongvipat et al., 2011). Therefore, one of the primary focuses of future research should be on patients stratification based on treatment-response signatures and new biomarkers able to predict the patients that will benefit from new single agents, or combinatorial treatments. Reassuringly, as with AR-targeted therapies, we will simply have to work harder to monitor these adaptive/resistance responses in pre-clinical and clinical settings and swifter in closing these escape routes as our ability to successfully target BRD-containing proteins will increase. This will possibly delay the evolution of new forms of aggressive treatment-resistant tumours.

Finally, it is very interesting to notice that a subset of BRD-containing proteins are involved in DNA damage response (DDR, **Table 1**) (Gong, Chiu, Cox et al., 2015). For instance an isoform of BRD4, was recently shown to function as an endogenous inhibitor of DDR (Floyd, Pacold, Huang et al., 2013). In addition, a study by Gong and colleagues systematically screened all the known BRD-containing proteins for their nuclear localization upon induced DNA damage. One-third change localization in response to DNA damage. 12 are recruited to DNA damage (KAT2A, KAT2B, p300, BAZ1A, BAZ1B, SMARCA2, SMARCA4, TRIM24, TRIM28, TRIM33, BPTF, and ZMYND8) while 2 (ATAD2 and SP140) disassemble from DNA damage sites (Gong et al., 2015). DDR aims to prevent duplication of DNA lesions and maintains genomic integrity. Specific genes involved in the DDR such as *BRCA1/2* and *P53* are mutated during PC progression, while oncogenic signaling such as Akt and c-Myc are activated (Karanika, Karantanos, Li et al., 2015), and increase DNA damage and genomic instability (Barfeld et al.,Karanika et al., 2015). These events render PC cells particularly sensitive to inhibition of specific DDR pathways such as PARP and Chk1 (Karanika, Karantanos, Li et al., 2017,Wang, Ma, Xiao et al., 2012,Mateo, Carreira, Sandhu et al., 2015).

Only a subset of the BRD-containing proteins has been characterized in PC. Moreover, given their involvement in DDR, the interplay with these stress mechanisms should be investigated further to possibly create opportunities for synthetic lethality or synergistic cytotoxicity that can lead to overcome radioresistance of PC cells or expand therapeutic strategies to enhance radiation-induced lethality.

Conclusions

There is a significant proportion of PCs characterised by dysregulated expression and/or mutations affecting many BRD-containing proteins and we are only just beginning to explore the potential of classes of inhibitors for these proteins and their role in the context of PC. A considerable amount of work needs to be done to optimise the bioavailability and characterise the biological impact and optimal treatment context for these chemical probes/nascent drugs.

Acknowledgments

We thank the referees for their detailed and constructive criticism. We apologise for research that we were not able to cite as a result of space constraints.

Funding

A.U. is supported by the South-East Norway Health Authorities (Helse Sor-Ost grant ID 2014040) at the Oslo University Hospital, and the Norwegian Centre for Molecular Medicine. I.G.M. is supported by the Helse Sør-Øst and by funding from the Norwegian Research Council, the University of Oslo, the Norwegian Cancer Society and by EU FP7 funding. I.G.M. is an Associate Professor at Queen's University Belfast and funded through the PCUK/Movember Centre of Excellence for Prostate Cancer Research. He is a visiting scientist and honorary senior visiting research fellow at Cambridge University.

Figure Legends

Figure 1. Alterations of bromodomain containing proteins in prostate cancer.

cBIO portal visualization of whole exome (somatic mutations and copy number aberrations), and RNA-Seq data of 498 primary prostate cancer samples according to the TCGA provisional data(Grasso et al., 2012) (**A**) and 81 castration resistant adenocarcinoma and castration resistant neuroendocrine prostate cancer samples from “Trento/Cornell/Broad 2016” dataset(Beltran et al., 2016) (**B**) showing transcriptomics and genetic alterations for 42 bromodomain containing proteins genes.

Figure 2. Genetic alterations of bromodomain containing proteins in prostate cancer.

cBIO portal visualization of genetic alterations (mutations and copy number alteration (CNA) data) for a gene set including 42 bromodomain containing proteins genes showing alteration frequency relative to the gene set in the indicated datasets.

Table 1. Function of bromodomain-containing proteins and prostate cancer

BRD-containing protein gene ID(s)	Full name	Function*	Relation to Prostate Cancer
ASH1L	ash1 (absent, small, or homeotic)-like (Drosophila)	Transcriptional activator, methyltransferase	Unknown
ATAD2**	ATPase family, AAA domain containing 2	ATPase, transcriptional co-activator, implicated in chromatin remodeling and DDR	Androgen induced co-activator of both AR (Zou et al., 2009) and c-MYC (Ciro et al., 2009)
ATAD2B	ATPase family, AAA domain containing 2B	ATPase, co-activator, found at replication sites, consistent with a chromatin-related function	Unknown
BAZ1A, BAZ1B	bromodomain adjacent to zinc finger domain, 1	Chromatin assembly and remodeling, implicated in DDR	RUNX2 forms functional complexes with BAZ1B in PC cells DDR (Yang, Quaresma, Nickerson et al., 2015)
BAZ2A**	bromodomain adjacent to zinc finger domain, 2A	Transcriptional repressor, chromatin remodeler	BAZ2A and EZH2 repress frequently silenced genes in CRPC via epigenetic remodeling (Gu et al., 2015)
BAZ2B	bromodomain adjacent to zinc finger domain, 2B	Transcriptional activator, chromatin remodeler	Unknown
BPTF	bromodomain PHD finger transcription factor	TF, transcriptional activator, chromatin remodeler and implicated in DDR	BPTF associated protein of 18 kDa (BAP18) co-activates AR and promote CRPC growth (Sun, Zhong, Wang et al., 2016)
BRD1	bromodomain containing 1	TF, transcriptional activator, part of the MOZ/MORF acetyltransferase complex	Unknown
BRD2, BRD3	bromodomain containing 2, bromodomain containing 3	TF and transcriptional co-regulator interacting with co-activators, co-repressors, and proteins of the SWI/SNF chromatin remodeling complex, probable additional kinase activity	Interacts with and promotes AR activity enabling CRPC cells growth (Asangani et al., 2014), mediate resistance to antiandrogens (Asangani et al., 2016)
BRD4	bromodomain containing 4	TF and transcriptional co-regulator, involved in transcriptional plasticity interacting with multiple cell lineage-specific TFs and chromatin remodeling factors, HAT through which it promotes chromatin de-compaction, involved in transcriptional response to several cellular stress	Interacts with ERG and AR and promotes their activity enabling CRPC cells growth (Asangani et al., 2014, Blee, Liu, Wang et al., 2016), mediate resistance to antiandrogens (Asangani et al., 2016). Involved in PC cells oxidative stress response (Hussong, Borno, Kerick et al., 2014), and susceptibility to PC (Zuber, Bettella, Witte et al., 2017)
BRD7	bromodomain containing 7	Transcriptional repressor, putative tumour suppressor, within the SWI/SNF chromatin remodeling complex, interacts with p53 and is required for p53-dependent oncogene-induced senescence which prevents tumor growth	Represses TRIM24-mediated activation of AR in PC cells (Kikuchi, Okumura, Tsukiyama et al., 2009)
BRD8	bromodomain containing 8	TRAP/TIP60 chromatin remodeling complex component, NRs co-activator	AR co-activator (Jiang, Ruan, Wang et al., 2016)
BRD9	bromodomain containing 9	Transcriptional activator, subunit of the SWI-SNF chromatin-remodeling complex	Unknown
BRDT	bromodomain, testis-specific	TF and transcriptional co-regulator interacting with co-activators, co-repressors, and proteins of the SWI/SNF chromatin remodeling complex, probable additional kinase activity	Unknown
BRPF1 BRPF3	bromodomain and PHD finger containing	Activates components of the MOZ/MORF HAT complexes which function as a transcriptional regulators	Unknown
BRWD1	bromodomain and WD repeat domain containing 1	Transcriptional activator and chromatin remodeling associated with a component of the SWI/SNF complex	Unknown
BRWD3	bromodomain and WD repeat domain containing 3	Chromatin-modifying function involved in JAK/STAT signaling	Unknown
CECR2	cat eye syndrome chromosome region, candidate 2	ATP-dependent chromatin remodeling may additionally play a role in DDR	Unknown
CREBBP**	CREB binding protein	Transcriptional co-activator of many TFs, HAT for histones and non-histones proteins	AR co-activator (Ikonen, Palvimo and Janne, 1997, Fu, Wang, Reutens et al., 2000), androgen induced (Urbanucci et al., 2008), mediates CRPC growth (Comuzzi, Nemes, Schmidt et al., 2004, Culig, 2016)
EP300	E1A binding protein p300	Transcriptional co-activator of many TFs, HAT for histones and non-histones proteins, binds CREB, also co-activator of HIF1A	AR co-activator (Fu et al., 2000), mediates CRPC growth (Debes, Sebo, Lohse et al., 2003)
KAT2A	K(lysine) acetyltransferase 2A	HAT, associates with p300/CBP	Unknown
KAT2B	K(lysine) acetyltransferase 2B	HAT, associates with p300/CBP	AR co-activator (Wang, Yeh, Wu et al., 2001)
KIAA2026	KIAA2026	Uncharacterized	Unknown
KTM2A (MLL)	lysine methyltransferase 2A	Transcriptional activator, methyltransferase	AR co-activator, enables CRPC cells growth (Grasso et al., 2012, Malik et al., 2015)
PBRM1	polybromo 1	Subunit of ATP-dependent chromatin-remodeling complexes, component of complexes necessary for ligand-dependent transcriptional activation by NRs	Mutated in PC (Nickerson, Im, Misner et al., 2013, Berger, Lawrence, Demichelis et al., 2011)
PHIP	pleckstrin homology domain interacting protein	Binds insulin receptor substrate 1 protein and regulates glucose transporter translocation	Unknown
SMARCA2, SMARCA4**	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 or 4	Member of the SWI/SNF family with ATPase and helicase activity to activate gene transcription by modification of chromatin structure	Interact with AR are required for AR activity, and are involved in androgen-dependent PC growth (Marshall et al., 2003, Shen, Powers, Saini et al., 2008, Medina and Sanchez-Cespedes, 2008, Link, Burd, Williams et al., 2005)
SP100, SP110, SP140 SP140L	SP100 nuclear antigen, SP110 nuclear body protein, SP140 nuclear body protein, SP140 nuclear body protein-like	Transcriptional regulators	Unknown
TAF1, TAF1L	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa or 210kDa-like	Participate in basal transcription, serve as co-activators, function in promoter recognition or modify GTFs to facilitate complex assembly and transcription initiation. Have two independent protein kinase domains, HAT that can act as a ubiquitin-activating/conjugating enzyme	AR co-activator (Tavassoli, Wafa, Cheng et al., 2010)
TRIM24**	tripartite motif containing 24	TF and NRs co-activator, implicated in DDR	It is stabilized by mutant SPOP and co-activates AR driving CRPC growth (Groner, Cato, de Tribolet-Hardy et al., 2016)
TRIM28**	tripartite motif containing 28	TF, transcriptional elongation factor, and cofactor	Phosphorylated upon DNA damage in PC cells (Zhang, Yang, Jaamaa et al., 2011); Co-activates AR (Van Tilborgh, Spans, Helsen et al., 2013)
TRIM33	tripartite motif containing 33	TF, transcriptional repressor	Unknown
TRIM66	tripartite motif containing 66	TF, transcriptional regulator	Unknown

ZMYND11	zinc finger, MYND-type containing 11	Transcriptional repressor implicated in viral infection processes	Unknown
ZMYND8	zinc finger, MYND-type containing 8	Transcriptional repressor in complex with histone demethylases; Implicated in DDR	Promotes PC angiogenesis <i>in vivo</i> (Kuroyanagi, Shimada, Zhang et al., 2014)

* Source GeneRIF (Gene Reference into Function) and RefSeq;

** Possible tissue biomarker associated with prostate cancer outcome;

BRD, bromodomain; TF, transcription factor; HAT, histone acetyl transferase; NRs, nuclear receptors; AR, androgen receptor; PC, prostate cancer; CRPC, castration resistant prostate cancer; DDR, DNA damage response; GTFs, general transcription factors.

Table 2. Examples of bromodomain inhibitors in clinical trials

Drug	Target	Cancer	Phase	Trial (accessed clinicaltrials.gov)
OTX015 (MK-8628)	BRD2 and BRD4	NMC, TNBC, <u>CRPC</u> , PDAC, NSCLC	1 (completed)	NCT02259114
OTX015 (MK-8628)	BRD2 and BRD4	GBM	1/2 (terminated)	NCT02296476
OTX015 (MK-8628)	BRD2 and BRD4	NMC TNBC NSCLC <u>CRPC</u>	1 (ongoing, but not recruiting)	NCT02698176
GSK525762	BRD2, BRD3, BRD4, and BRD-T	NMC, SCLC, NSCLC, CRC, NB, <u>CRPC</u> , TNBC, ER+ BC	1 (recruiting)	NCT01587703
GSK2820151	BRD2, BRD3, BRD4, and BRD-T	Advanced solid tumours	1 (recruiting)	NCT02630251
BAY1238097	Preferential <i>in vitro</i> binding to BRD4	Solid tumours and lymphoma	1 (terminated)	NCT02369029
TEN-010 (RO6870810)	BRD2 and BRD4	Advanced solid tumours	1 (recruiting)	NCT01987362
INCB054329	BRD2, BRD3, BRD4 and BRD-T	Advanced solid tumours	1/2 (recruiting)	NCT02431260
ZEN003694	BRD2, BRD3, BRD4 and BRD-T	<u>CRPC</u>	1 (recruiting)	NCT02705469
ZEN003694/Enzalutamide	BRD2, BRD3, BRD4 and BRD-T + AR	<u>CRPC</u>	1 (recruiting)	NCT02711956

ER+ (estrogen receptor positive); BC, breast cancer; CRC, colorectal cancer; CRPC, **castration resistant prostate cancer**; GBM, glioblastoma **multiforme**; NB, neuroblastoma; NMC, non-midline cancer; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma; SCLC, small cell lung cancer; TNBC, triple negative breast cancer.

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Highlights for Urbanucci et al.

Highlights:

- Bromodomain-containing proteins (BRDs) regulate prostate cancer-specific transcriptional programs;
- BRDs are androgen receptor co-activators and/or promote castration resistant growth;
- BRDs genes are frequently altered in advanced prostate cancer cases;
- Effective BRDs inhibitors are tested in clinical trials;
- BRDs-targeted therapies will need better prognostication and stratification of patients

Androgen receptor deregulation drives bromodomain-mediated chromatin alterations in prostate cancer

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Running title: Chromatin relaxation is a feature of prostate cancer

Summary

Global changes in chromatin accessibility may drive cancer progression by reprogramming transcription factor (TF) binding. In addition, epigenetic readers such as bromodomain containing protein 4 (BRD4) have been shown to associate with these TFs and contribute to aggressive cancers including prostate cancer (PC).

Here we show that chromatin accessibility defines castrate-resistant prostate cancer (CRPC). We show that the deregulation of androgen receptor (AR) expression is a driver for chromatin relaxation and that AR/androgen-regulated bromodomain-containing proteins (BRDs) mediate this effect. We also report that BRDs are overexpressed in CRPCs and that ATAD2 and BRD2 have prognostic value. Finally, we have developed gene stratification signature (BROMO-10) for bromodomain response and PC prognostication, to inform current and future trials with drugs targeting these processes.

Targeting bromodomains in selected patients provides a compelling rationale for combination therapy in which BRD-mediated TF binding is enhanced or modified as cancer progresses.

Key words: castration resistant prostate cancer, BROMO-10, chromatin, ATAD2, BRD2, BRD4, androgen receptor, bromodomain inhibitor

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