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INNSBRUCK

**INTERACTION OF ANDROGEN RECEPTOR AND MID1/PP2A/ α 4 COMPLEX AND
IMPLICATIONS ON PROSTATE CANCER PROGRESSION AND THERAPY**

A thesis submitted for obtaining the degree of
Doctor of Philosophy in Molecular Oncology in Innsbruck Medical University

by

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If we knew what it was we were doing, it
would not be called research, would it?

Albert Einstein

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1. Abstract

The MID1 protein is an ubiquitin ligase. The gene encoding MID1 is mutated in a developmental syndrome called Opitz G/BBB. MID1 forms the core of a ribonuclear protein complex including also PP2A and $\alpha 4$ proteins and several mRNAs carrying MIDAS sequences. Until now the studies on MID1 is mostly limited to the Opitz syndrome. AR is a transcription factor commonly aberrated in advanced prostate cancer. Its mRNA has CAG and GGY repeats in its 5' translated region, through which it is associated with the MID1/PP2A/ $\alpha 4$ complex. Based on this data, we hypothesized that the MID1/PP2A/ $\alpha 4$ complex plays a role in the progression of prostate cancer

In our study, we found that the MID1/PP2A/ $\alpha 4$ complex regulates AR in a post-transcriptional way. Interestingly, in a reverse manner, MID1 is an AR target gene and is negatively regulated by androgens.

IHC revealed that MID1 expression in prostate tissue samples has a distinct pattern. MID1 is mainly found in the stromal cells in benign parts of the tissue. In cancerous tissue, expression of MID1 shifted to the epithelial cancer part and increased in correlation with the Gleason score of the tumors. Tumor tissue samples exhibiting a distinct histological pattern called cribriform displayed significantly higher MID1 expression.

Metformin, an anti-diabetic agent, has anti-tumor effects in many types of cancer. Recently, it was reported that it targets the MID1/PP2A/ $\alpha 4$ complex. Therefore, we decided to study the function of the MID1/PP2A/ $\alpha 4$ complex in prostate cancer, in particular its effects on androgen receptor. In our study, metformin showed an anti-proliferative effect in prostate cancer cells and decreased AR protein levels. In the same manner, knock-down of MID1 in prostate cancer cell lines decreased proliferation as well as AR protein level. This anti-tumor effect of metformin was independent of AMPK a key target of the anti-diabetic effects of metformin. As the underlying mechanism of metformin inhibition of prostate cancer cells dissociation of AR mRNA from the MID1/PP2A/ $\alpha 4$ complex was identified.

Our findings revealed that there is a mutual feedback regulation between AR and MID1. Androgen deprivation therapy can disturb this regulation and result in AR upregulation suggesting a role of MID1 in progression of the disease to a therapy-resistant stage.

Metformin targets the MID1/PP2A/ α 4 complex and with that indirectly the AR. Taken together these findings support the use of metformin for the therapy of CRPC.

2. Zusammenfassung

Das Midline 1 (MID1) Protein ist eine Ubiquitin ligase. Das MID1 kodierende Gen ist in einem Opitz G/BBB1 genanntem Entwicklungs-Syndrom mutiert. MID1/PP2A/ α -4-Proteine und mehrere mRNAs, die durch MIDAS Sequenzen charakterisiert sind bilden den Kern eines Ribonukleoproteinkomplexes. Die meisten Studien von MID1 konzentrierten sich bisher auf das Opitz-Syndrom. Der Androgenrezeptor (AR) ist ein Transkriptionsfaktor, der beim fortgeschrittenen Prostatakrebs häufig verändert ist. Seine mRNA besitzt GGY und CAG Wiederholungen in der 5'-translatierten Region, über die sie an dem MID1/PP2A/ α 4 Proteinkomplex binden kann. Basierend auf diesen Daten stellten wir die Hypothese auf, dass der MID1/PP2A/ α 4 Komplex eine Rolle bei der Progression von Prostatakrebs spielen könnte.

In unserer Studie haben wir festgestellt, dass der MID1/PP2A/ α -4-Komplex den Androgenezeptor in einer post-transkriptionellen Weise reguliert. Umgekehrt, ist interessanterweise das MID1 Gen ein Zielen des Androgenrezeptors und wird durch Androgene negativ beeinflusst.

Die Untersuchung der MID1 Expression in Prostata-Gewebeproben ergab ein sehr spezifisches Muster. Im gutartigen Teile des Gewebes ist MID1 häufig in den Stroma Zellen zu finden. Im Tumor verschiebt sich die MID1 Expression zu den Epithelien Krebszellen und steigt mit der Gleason-Score. Tumoranteile mit einem sogenannten cribriformen histologischen Muster zeigen eine deutlich höhere MID1 Expression

Metformin, ein häufig verwendetes Antidiabetikum, zeigte in vielen Arten von Krebs eine anti-Tumor-Wirkung. Kürzlich wurde berichtet, dass Metfomin eine Wirkung auf den MID1/PP2A/ α 4 Komplex hat. Deshalb haben wir die Wirkung von Metformin bei Prostatakrebs, insbesondere die Effekte auf die Interaktion von AR und dem MID1/PP2A/ α 4 Komplex, genauer untersucht.

In unserer Studie zeigen wir eine antiproliferative Wirkung von Metformin bei Prostatakrebs-Zellen und eine Verminderung der Androgenrezeptor Proteinmenge. In gleicher Weise wie Metformin verringert der Knock-down von MID1 die Proliferation und das AR Protein. Diese Anti-Tumor-Wirkung von Metformin ist unabhängig von der AMP-regulierten Kinase,

einem zentralen Zielmolekül der anti-diabetischen Wirkung von Metformin. Als der zugrunde liegende Mechanismus von Metformin konnte die Dissoziation von AR-mRNA aus dem MID1/PP2A/ α -4-Komplex identifiziert werden.

Unsere Ergebnisse zeigen eine gegenseitige Feedback-Regulation zwischen AR und MID1. Androgenentzugtherapie kann diese Regelung stören und im Endergebnis zu einer AR Hochregulation führen, was auf eine Rolle von MID1 beim Fortschreiten der Erkrankung zu einer therapieresistenten Stufe hinausläuft. Metformin zielt auf den MID1/PP2A/ α 4-Komplex und führt damit indirekt zu einer Abnahme des AR Proteins. Zusammengenommen unterstützen diese Ergebnisse die Verwendung von Metformin in der Therapie des kastrationsresistenten Prostatacarzinoms (CRPC).

3. Introduction

3.1. Prostate

The prostate is a walnut-sized, glandular, men-specific organ surrounding the urethra located at the base of bladder. It has a zonal structure. It is composed of the central, the periurethral transition and the peripheral and the anterior fibromuscular stroma zones [1]. The transition zone constitutes 5%-10% of the whole prostate and surrounds the urethra. 20%-25% of the prostate mass is the central zone positioned below the proximal urethral segment. The central zone is positioned in the apical region of the prostate and constitutes 70%-75% of the adult prostate. The fibromuscular stroma is nonglandular and located inside the prostatic capsule (Figure 1) [2].

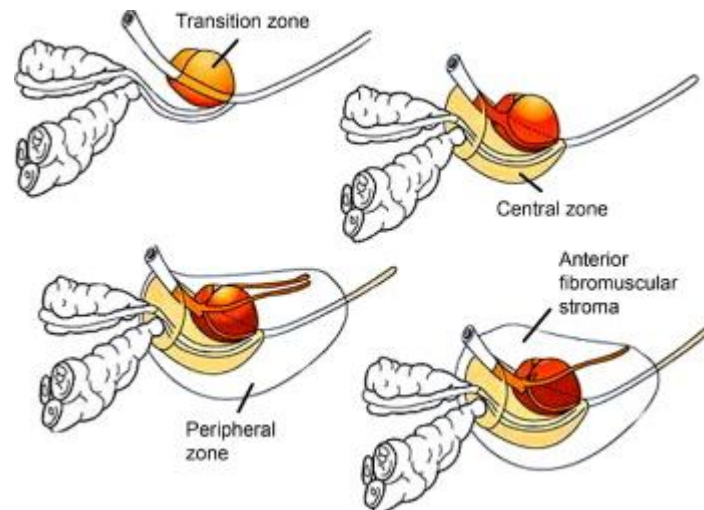


Figure 1: Different zones of the prostate [2].

The normal prostatic epithelium is structured by 3 different cell types. The secretory cells compose the bigger mass and they express AR, cytokeratins 8 and 18, CD57 and PSA. The basal epithelial cells express cytokeratin 5 and 14, CD44 and low levels of AR. Finally, the neuroendocrine cells are androgen independent and express chromogranin A and a variety of peptide hormones [3].

3.2. Prostate Cancer

Prostate cancer is the most common malignancy among men and has the second place for cancer deaths after lung cancer [4].

Prostate cancer is heterogeneous and any distinct histopathological subtypes cannot be correlated with prognosis or therapy response. Acinar adenocarcinoma that expresses AR is the predominant form, ductal adenocarcinoma, mucinous carcinoma and signet ring carcinoma are very rare categories. Neuroendocrine prostate cancer, representing less than 2% is the most aggressive subtype; it expresses the neuroendocrine marker chromogranin A. Because neuroendocrine cancer cells don't express AR, these tumors are hormone ablation therapy resistant. There are several procedures used to detect and diagnose prostate cancer: Digital Rectal Examination (DRE), Trans Rectal Ultrasound (TRUS), PSA blood test and prostate needle biopsy. When the PSA serum level is elevated the patient undergoes needle biopsy to find out if there is cancer in the prostate or not. Pathological grading is based on the Gleason score that determines the degree of cell differentiation. The TNM classification is used to stage prostate cancer. The T stage represents the extent of the primary tumor, the N stage indicates the lymph node metastasis, the M stage describes if there is a distant metastasis. These parameters are used to make therapy decisions and for the management of prostate cancer patients [4].

3.2.1 Initiation and Progression of Prostate Cancer

As in other cancers, prostate cancer is caused by a combination of genetic and epigenetic aberrations in a cell population. Prostate intraepithelial neoplasia (PIN) and atypical small acinar proliferation (ASAP) anomalies are the most likely precursors of prostate cancer.

About 9% of the prostate cancer cases have been estimated to be inherited. However, no single locus has been identified. The idea is that a combination of multiple low risks alleles initiates the disease [3].

The sequence of genetic aberrations causing the initiation and progression of prostate cancer are not well understood. This is probably because of the heterogeneous nature of the disease. Sequencing of the prostate cancer genome revealed that somatic point mutations are relatively rare. Copy number alterations and genomic rearrangements are mostly associated with prostate cancer. The sequence of events is illustrated in Figure 2.

The cytogenetic studies revealed the loss of heterozygosity in 8p12-22. NKX3.1, a homeobox gene with prostate specific expression, was identified as the target tumor suppressor in this region. This aberration is an early event found in 85 % of all prostate cancers [5].

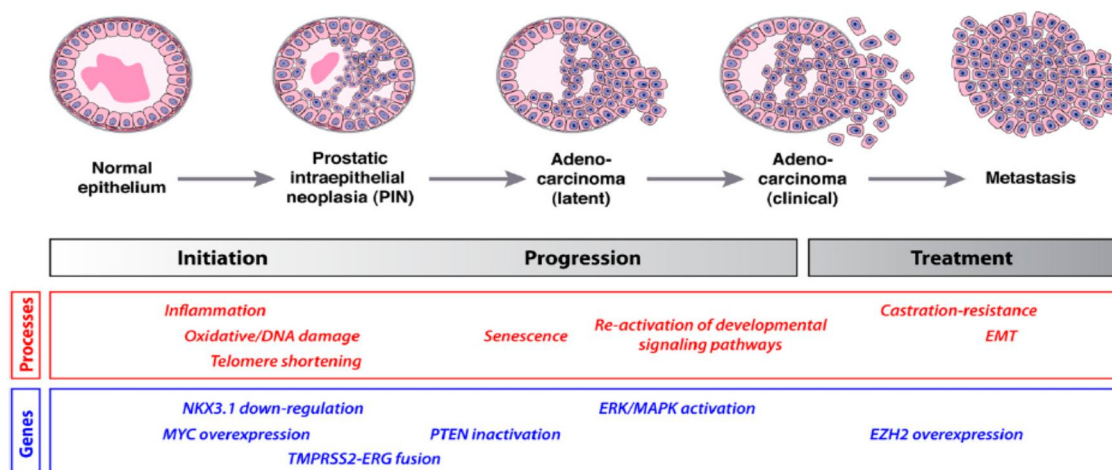


Figure 2: The sequence of events and genetic aberrations leading to prostate cancer initiation and progression [1].

NKX3.1 is expressed as early as the initiation of prostate development from urogenital sinus and it is important for the prostate epithelial differentiation. In the absence of NKX3.1, differentiation is retarded and ductal branching and the production of secretory proteins are impaired. The mouse models revealed that homozygous or heterozygous mutants of Nkx3.1 develop prostate epithelial dysplasia and hyperplasia and intraductal neoplasia (PIN) confirming the tumor suppressor activity of this gene [1].

MYC overexpression is also a determining factor in prostate cancer initiation found upregulated in PIN lesions. Furthermore, the transgenic mice overexpressing MYC develop PIN lesions and progress to an invasive adenocarcinoma sometimes with distant metastases. This points out that MYC is not only important for cancer initiation but also for cancer progression and metastasis.

Copy number loss of the PTEN gene was also determined as an early event in prostate cancer which is evident by frequent deletions (10-70%) of the region 10q23 in prostate cancer. PTEN loss is correlated with progression to aggressive disease and therapy resistance [5].

The chromosomal rearrangements such as translocations, inversions or insertions result in formation of chimeric fusion genes with new or altered activities or deregulation of expression. In prostate cancer, recurrent translocations between ETS transcription factor family genes and the *TMPRSS2* gene were identified. The resulting fusion genes lead to the deregulation of ETS transcription factors due to the fusion to the androgen regulated promoter of the *TMPRSS2* gene. The most common of these fusions is the *TMPRSS2-ERG* gene fusion occurring in about 15% of PIN lesions and 40-70% in localized prostate cancer [6]. It is proposed that these *ERG* rearrangements are not causative for cancer initiation but they are early events and correlated with progression to a clinical disease [7].

The mutation frequencies of common tumor suppressor genes are not high in prostate cancer. Aberrations of the *RB* gene are observed in 20 % of prostate cancer cases. As the disease progresses to an advanced stage this percentage is increased to 50%. *P53* mutations are also very low in primary prostate cancer compared to other cancers but become more frequent in later stages [3].

Some rare mutations identified in prostate cancer are somatic mutations in *CHECK2*, *RAS*, *EGFR*, *KLF6*, *APC*, *PIK3CA*, *FOXA1* genes. The frameshift mutations in the *EPHB2* receptor tyrosine kinase are detected in 10% of prostate cancer cases. The *SPOP* gene, which encodes an ubiquitin ligase is mutated in 6-13% of prostate cancer cases. These are infrequent but recurrent mutations. Although the functional consequences of *SPOP* mutations are not clarified yet, *TMPRSS2*:ETS rearrangements and *SPOP* mutations are mutually exclusive [6, 8].

SPINK1 overexpression was also determined exclusively in *TMPRSS2*: ETS fusions negative prostate cancer cases. Its overexpression was identified in 10% of cancer cases and was associated with an aggressive cancer phenotype [6].

Epigenetic changes also contribute significantly to prostate cancer progression. The hypermethylation of the *GST-P* promoter is the most common epigenetic event. The other genes displaying differential methylation are *p16^{INK4a}*, *O⁶-MGMT*, *E-Cadherin*, *p14^{ARF}*. Moreover, global hypomethylation is commonly observed in metastatic prostate cancer [3].

The androgen receptor (AR) is a key regulator of prostate development and initiation of cancer. It exerts its function differently in normal prostate epithelium and prostate cancer. In normal prostate epithelium, the AR suppresses proliferation and induces differentiation. However, in prostate cancer, AR induces proliferation of the luminal cells and also metastasis. The transgenic mice overexpressing AR develops PIN lesions [1]. Although androgens and AR are crucial for the development of prostate cancer AR aberrations are not early events in prostate cancer progression but they are very important for developing castration resistance. AR overexpression and mutations in the AR gene are common events during progression to castrate resistance under androgen deprivation therapy. They are discussed in more detail in a later chapter (3.5.1).

3.3. The Androgen Receptor

The Androgen receptor (AR) is a member of the steroid receptor family functioning as a transcription factor upon binding and activation by androgen hormones. It has important roles both in normal development and physiology and in disease conditions like androgen insensitivity syndrome, Kennedy disease and prostate cancer.

3.3.1. The AR Gene

The human AR gene is located on Xq11-q12. It spans over 90 kb with 8 exons, 1.1 kb of 5' and 6.8 kb of 3' untranslated regions. The promoter region of the AR gene lacks typical TATA and CAAT sequence motifs however it contains a GC-rich region for binding of the transcription factor Sp-1 and also multiple consensus sequences for general transcription factors like AP-1, CREB or specific transcription factors like ER, GR and for AR itself [9]. A negative regulation of AR is achieved by the transcription factors NF- κ B and NF-1 [10].

3.3.2. The AR mRNA

The full-length AR cDNA was cloned in 1988. Several AR transcript variants have been reported. Most of them encode C-terminally truncated proteins lacking the ligand binding domain (LBD). The role of different splice variants has not been completely elucidated yet, however, it is proposed that they are constitutively active and have a role in prostate cancer progression [11]. The AR splice variant named AR23 encodes a protein with disrupted DNA-binding domain (DBD). ARV7/AR3 are forms lacking the hinge region (HR) and the LBD.

Variants are expressed both in benign and cancerous prostate and they are not involved in the initiation of prostate cancer. However, it is clear that cancer cells having *AR* variants without LBD have a growth advantage under castration and anti-androgen treatment. Because of lack of the LBD, anti-androgens targeting this domain are not effective against these variants. Therefore, these variants are frequently expressed in castrate resistant prostate cancer and metastatic lesions [12]. The roles of different variants in normal epithelium or in prostate development await further studies.

Post-transcriptional Regulation of AR mRNA

The androgen receptor exerts key regulatory functions in male hormone sensitive organs. Therefore, it is important to keep this molecule in homeostasis. It was suggested that there is an autoregulation of the mRNA encoding the AR elicited through its hormone ligand androgen [13, 14]. At the transcription level this can be achieved by two mechanisms a) androgen response elements (ARE) in the AR gene allowing a regulatory feed-back mechanism, or b) the involvement of trans-acting molecules for regulation of the AR mRNA [15, 16]. The AR gene promoter region does not contain any ARE but AREs are located inside the coding region of the AR gene [13, 17, 18]. In coordination with MYC transcription factor binding sites, these AREs regulate the transcription of the AR gene [13] .

A lot of RNA binding proteins (RBPs) have been identified and most of these proteins bind specific sequence motifs in the RNA. AR mRNA 3'UTR has UC rich elements and is bound by human antigen R (HuR) and poly-C binding protein 2 (PCBP2) [15, 16]. Poly-C binding protein 1 was also identified as the negative regulator of AR mRNA translation. However, this effect is independent of 3'UTR of the AR mRNA [19]. AR mRNA contains other sequence motifs like CAG and GGY repeats in its 5' translated region. These sites can also be good candidates for binding of RBPs [20].

3.3.3. AR Protein

The AR is a member of the nuclear receptor superfamily. It exerts its function as a transcription factor in a ligand dependent manner [21]. There are two isoforms of AR; a 110 kDa intact and a 87 kDa N-terminally truncated form [22]. Other C-terminally truncated AR isoforms also have been reported in the literature but it is not clear if these are physiological

isoforms or selection byproducts of cancer progression because of their constitutively active nature.

AR Protein Domains and Their Functions

The AR protein contains several modular domains with different functions as shown in Figure 3:

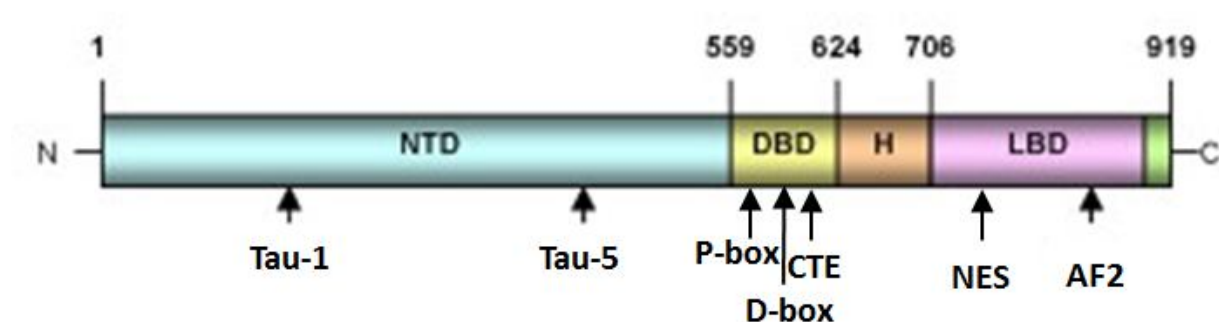


Figure 3: The AR protein domains [22]

DNA binding domain (DBD)

It is the domain responsible for the recognition and binding to AREs in the DNA. It spans amino acids 559-624. This domain is composed of two zinc finger modules and a carboxy terminal extension (CTE) [23]. The first zinc finger contains a stretch of 5 amino acids called P-box. α -helices in the first zinc finger are responsible for specific DNA recognition. For that, a helix enters the major groove of the DNA which contains a hexameric motif for AR binding. AR binds DNA as a dimer. The second zinc finger stabilizes the DNA- AR complex and is also responsible for initiating DNA-dependent dimerization, a function exerted by a 5 amino acid stretch called D-box. The CTE region is also involved in dimerization in concert with the second zinc finger [24, 25]. An AR nuclear localization signal spans the D-box, CTE and the hinge region [23].

Hinge region

This region is located between amino acids 625-676. It is a linker between the DNA binding domain and the ligand binding domain (LBD). Previously the role of this region was not clear and it was only thought as a linker region. However, with recent studies it became clear that it

is involved in nuclear import by binding to importin- α . Furthermore it is important for DNA selectivity and affinity especially for binding to the selective AREs. It is also involved in transactivation by interacting with coregulatory proteins like UBC9, PIAS, SRC, SWI/SNF, p300/pCAF [24] .

Ligand binding domain (LBD)

Androgens, the natural ligands of the AR, bind to the ligand binding domain (LBD) spanning amino acids 677-919. Eleven α -helical sandwiches with a central ligand binding cavity form this domain. The α -helices are numbered 1 to 12 omitting 2 for consistency with the other nuclear receptors. 18 residues of helix 3, 5 and 11 directly contact the bound ligand. The 12th helix serves as a lid and closure of the ligand binding pocket and forms a hydrophobic cleft on the surface. The restructured ligand binding domain is called activation function 2 (AF-2) and forms the docking sites for the coactivator proteins [23, 24]. The coactivator proteins bind to this site and induce further reorganization of the domain conformation. Depending on the presence of different coactivators, the AR adapts distinct conformational states. These also determine the interaction and affinity for DNA response elements and the members of transcription machinery [26]. The unique feature of AF-2 is that it interacts with both the coregulators and the amino terminal domain of the AR itself (N-terminal/C-terminal interaction) [23, 24] . This N/C interaction is induced by the ligand binding in the cytoplasm and it prevents the inappropriate binding of coregulators until the AR is bound to an ARE in the DNA. After binding of the receptor to DNA, the AF-2 domain dissociates from the N-terminal domain and now becomes available to interact with the coregulators for proper enhancement of the transcriptional activity of AR [23]. However, there are studies showing that A/C interaction is necessary even when AR is bound to AREs. This shows that this intramolecular interaction has a dynamic role in different steps of the transactivation function of the AR [24].

The ligand-binding domain also contains a nuclear export signal (NES) in amino acids 742-817. In the presence of a ligand this signal is compromised but in the absence of a ligand the NES overcomes the NLS and facilitates export of the AR from the nucleus [27].

Amino terminal domain

AR amino terminal domain (NTD) spanning amino acids 1-558 has a very flexible, globular conformation that can adapt different conformations in response to cell type, androgen level, post-translational modifications, expression/activity of the binding partners or the chromatin environment [23]. The first 30 amino acids of this domain are important for the interaction with the ligand binding domain [10]. For full transactivation the entire NTD is required and this NTD activity is called activation function 1 (AF-1). There are 2 transactivation units (tau): tau1 (amino acids 100-370) and tau5 (amino acids 360-528) and there are further divisions into AF-1a and AF-1b. Studies with deletion mutants showed that discrete transcription activation domains within the AR NTD can achieve the transcription activation function in different cell types and on different promoters [23].

The AR NTD has two polymorphic repeats. The length of the polymorphic CAG repeat (Poly-Q) varies in the range of 9-36 repeats. Because of the involvement of this repeat in N/C interaction, the variations in the length of repeats also influence AR activity and stability. This region is also involved in the etiology of diseases like degenerative neuromuscular diseases and prostate cancer. A shorter PolyQ tract in the AR causes hyperactivity of the receptor and increased responsiveness to androgens. It was proposed that the increased prostate cancer risk among African-American men compared to Caucasians is attributed to their shorter PolyQ repeat. Beside this inherited shorter AR forms, somatic mutations causing shorter PolyQ repeats were detected in prostate cancer tissues [28]. The second polymorphic region of the NTD is a glycine stretch (Poly-G) in Tau-5. Its length varies between 10-30 residues. Less data is available about the role of this region. However, this region is also involved in N/C interaction and reported as a risk factor for prostate cancer [27].

3.3.4. AR: Mechanism of Action

AR mainly exerts its function by acting as a transcription factor. It controls the transcription of its target genes in an androgen dependent manner. When there is no androgenic stimulation, the AR resides in the cytoplasm bound with a molecular chaperone complex composed of heat shock proteins (HSPs), cytoskeletal proteins and other chaperones [27]. This complex prevents AR protein from aggregation and assures a conformation where the ligand binding pocket is accessible for hormones [29]. The androgenic stimulation causes

gradual dissociation of the AR from these proteins. Ligand binding to the ligand binding domain induces the conformational changes and exposing the nuclear localization signal. This signal is recognized by importin- α and filamin-A and driven by dynein along the cytoskeleton and the AR translocates to the nucleus [27, 30]. Hsp70 plays a role during AR crossing of the intranuclear membrane. Together with YDJ1, a component of the matrix lamina, HSP70 assist AR unfolding and refolding during translocation through the nuclear membrane [29]. After entry to the nucleus, it moves to the subnuclear compartments called the nuclear foci which are active sites for gene transcription. Once AR is inside these foci, it binds the androgen response elements in the promoter or enhancer region or inside its target genes [31]. The classical androgen response element (ARE) half site is 5'-TGTTCT-3' and AR binds to a direct or an inverted repeat of this sequence on DNA. The AR shares the same sequence motifs with other nuclear receptors when it binds inverted repeats but binding to the direct repeats is specific for AR. The selective AREs in opposed half side direction compared to the classical AREs guarantee selective transactivation of AR over other steroid receptors like glucocorticoid, progesterone and mineralocorticoid receptors [24].

Upon DNA binding, the AR forms a homodimer in a DNA dependent manner through its DBD. The first zinc fingers of the DBD of each AR monomer bind one half site of ARE and second zinc fingers of each monomer bind to each other [25]. As a consequence AR binds to its AREs in head-to-head confirmation. Binding of AR to AREs triggers the interaction with the coactivator and the chromatin remodeling machinery. This opens chromatin for the recruitment of the basal transcription machinery and enhances AR-dependent gene expression [32].

When the androgen hormone stimulation is turned off, the AR enters the disactivation loop. HSP90 and p23, two cochaperones, facilitate the disassembly of AR from DNA. CHIP, an E3 ubiquitin ligase, binds to the AR and causes the dissociation of p23. In its unliganded form and devoid of chaperone binding, the AR is eligible for proteasomal degradation. CHIP mediates the degradation of AR to prevent protein aggregation [29].

3.3.5. AR Function in Prostate Development and The Adult Prostate

Basal and luminal epithelial cells together with a small number of neuroendocrine cells forms the epithelial compartment of the prostate. This compartment is separated by a basement

membrane from the surrounding stroma, which regulates the differentiation and the maintenance of the epithelium [33]. The prostate develops from the urogenital sinus in the presence of androgen hormones secreted from the testes. It requires the interaction of Urogenital Sinus Epithelium (UGE) and Urogenital Sinus Mesenchyme (UGM). The AR is initially expressed only in UGM. Therefore AR exerts its function in epithelial ductal morphogenesis, epithelial proliferation and columnar cytodifferentiation in a paracrine manner [34]. This concept is called Andromedin hypothesis and paracrine acting factors are known as andromedins [35]. Androgens initiate their function in the nuclei of stromal cells by binding to the stromal AR. AR induces production and secretion of growth factors like epidermal growth factor [EGF], fibroblast growth factor [FGF], keratinocyte growth factor [KGF] and insulin-like growth factor [IGF] from stromal cells. These factors bind the appropriate surface receptors in the epithelial cells and induce the development of the prostate epithelium [36]. Epithelial AR is not required for proper development of prostate but it is required for expression of AR-dependent secretory proteins. The studies in AR knock-out (ARKO) mice and patients with androgen insensitivity syndromes (AIS) provide information about the role of AR in the development. Patients with AIS have mutations in AR gene. Because of non-functional AR these patients don't have male reproductive organs including the prostate. ARKO mice have the same phenotypic character. Collectively, these studies showed that the AR is essential for prostate development [37, 38].

In the adult prostate, the AR is responsible for production of secretions to form the seminal fluid, which supports male fertility and insemination and protects the lower urinary tract from infections [39]. The AR is expressed both in the epithelial and stromal compartments of the adult prostate. However, like in the developing prostate, the stromal AR is responsible for maintenance of the epithelial cell compartment in a paracrine manner. Epithelial AR has mainly secretory function [33]. Genes encoding main components of the seminal fluid like PSA, kallikrein2, prostatic acid phosphatase are also AR target genes [39]. Conditional knockout models provided valuable information about the cell-specific function of AR. Knockout of the AR in the prostate epithelium has different consequences in different cell types of the adult epithelium. This causes increased proliferation in the adult prostate, however, also apoptosis is increased but this is restricted to the luminal compartment. Differentiation of the epithelium is impaired but branching morphogenesis is not affected.

These findings indicate that the epithelial AR has a growth suppressive function to keep the adult prostate in a growth-quiescent state and stromal AR is mainly responsible for the ductal architecture [40].

3.4. Prostate Cancer Therapy

Early diagnosis of prostate cancer is possible with screening using Prostate Specific Antigen (PSA) serum level. When prostate cancer is diagnosed before metastasis, irradiation, surgery or watchful waiting are the treatment options. In surgery, whole prostate including the cancer is removed by radical prostatectomy. Irradiation is a less radical option aimed only to eradicate the cancerous part of the prostate. Watchful waiting is useful if the disease is not aggressive. However, if the disease is progressive, it is necessary to determine a therapy strategy.

If the cancer is metastasized and advanced, hormone deprivation therapy is the first option. The logic of this therapy is to inhibit the androgen which is the prominent growth hormone for the prostate and prostate cancer. In 1941, Huggins and Hodges established the basis for hormone deprivation therapy by showing the curative effect of surgical castration. Hormone deprivation therapy is applied in several ways. The classical approach is bilateral orchidectomy to suppress testosterone production. However, its use has some drawbacks like it is irreversible and does not allow intermittent therapy. Another option is medical castration by LHRH agonists and antagonists. Individually or combined with medical castration, blocking androgen receptor using competing anti-androgens is the second line hormone deprivation therapy.

According to the 2011 EAU guidelines; hormone deprivation therapy with LHRH agonists is the main treatment for advanced, relapsing prostate cancer. Hormone deprivation therapy may be applied intermittently, delayed or combined with radiation therapy [41]. Therapy strategies are summarized in Table 1.

Sooner or later, the disease progresses to a resistant stage no longer responding to the hormonal therapy (castration resistant prostate cancer CRPC). The therapeutic options for CRPC have been limited with mainly docetaxel chemotherapy. However, very recently,

several novel therapies have been approved and extended the treatment options for advanced prostate cancer patients.

Table 1: Treatment of prostate cancer [44]

Stage	Treatment	Comment
T1a	Watchful waiting	Standard treatment for Gleason score ≤ 6 and 7 adenocarcinomas and <10 -year life expectancy
	Active surveillance	In patients with <10 -year life expectancy, re-staging with TRUS and biopsy is recommended
	Radical prostatectomy	Optional in younger patients with a long life expectancy, especially for Gleason score ≤ 7 adenocarcinomas
	Radiotherapy	Optional in younger patients with a long life expectancy, in particular in poorly differentiated tumours. Higher complication risks after TURP, especially with interstitial radiation
	Hormonal	Not an option
T1b–T2b	Combination	Not an option
	Active surveillance	Treatment option in patients with cT1c–cT2a, PSA < 10 ng/mL, biopsy Gleason score ≤ 6 , ≤ 2 biopsies positive, $\leq 50\%$ cancer involvement of each biopsy Patients with a life expectancy <10 years Patients with a life expectancy >10 years once they are informed about lack of survival data beyond 10 years Patients who do not accept treatment-related complications
T1a–T2c	Radical prostatectomy	Standard treatment for patients with a life expectancy >10 years who accept treatment-related complications
	Radiotherapy	Patients with a life expectancy >10 years who accept treatment-related complications Patients with contraindications for surgery Unfit patients with 5–10 years of life expectancy and poorly differentiated tumours (combined therapy is recommended: see below)
	Brachytherapy	Low-dose rate brachytherapy can be considered for low-risk patients with prostate cancer with a prostate volume ≤ 50 mL and an IPSS ≤ 12
	Hormonal	Symptomatic patients, who need palliation of symptoms, unfit for curative treatment Anti-androgens are associated with a poorer outcome compared to 'active surveillance' and are not recommended
	Combination	For high-risk patients, neoadjuvant hormonal treatment and concomitant hormonal therapy plus radiotherapy results in increased OS
T3–T4	Watchful waiting	Option in asymptomatic patients with T3, well-differentiated and moderately-differentiated tumours, and a life expectancy <10 years who are unfit for local treatment
	Radical prostatectomy	Optional for selected patients with T3a, PSA level of <20 ng/mL, biopsy Gleason score ≤ 8 and a life expectancy >10 years Patients have to be informed that radical prostatectomy is associated with an increased risk of positive surgical margins, unfavourable histology and positive lymph nodes and that, therefore, adjuvant or salvage therapy, e.g. radiation therapy or androgen deprivation might be indicated
	Radiotherapy	T3 with >5 –10 years of life expectancy. Dose escalation of >74 Gy seems to be of benefit. A combination with hormonal therapy can be recommended (see below)
	Hormonal	Symptomatic patients, extensive T3–T4, high PSA level (>25 –50 ng/mL), PSADT < 1 year Patient driven, unfit patients Hormone monotherapy is not an option for patients who are fit enough for radiotherapy
	Combination	OS is improved by concomitant and adjuvant hormonal therapy (3 years) combined with external beam radiation Neoadjuvant hormonal treatment plus radical prostatectomy: no indication
N+, MO	Watchful waiting	Asymptomatic patients. Patient-driven (PSA < 20 –50 ng/mL), PSADT > 12 months. Requires very close follow-up
	Radical prostatectomy	Optional for selected patients with a life expectancy of >10 years a part of a multimodal treatment approach
	Radiotherapy	Optional in selected patients with a life expectancy of >10 years, combined therapy with adjuvant androgen deprivation for 3 years is mandatory
	Hormonal	Standard adjuvant therapy in more than one positive node to radiation therapy or radical prostatectomy as primary local therapy. Hormonal therapy should only be used as monotherapy in patients who are unfit for any type of local therapy
	Combination	No standard option Patient-driven
M+	Watchful waiting	No standard option. May have worse survival/more complications than with immediate hormonal therapy. Requires very close follow-up
	Radical prostatectomy	Not an option
	Radiotherapy	Not an option for curative intent; therapeutic option combined with androgen deprivation for treatment of local cancer-derived symptoms
	Hormonal	Standard option. Mandatory in symptomatic patients

PSADT, PSA doubling time.

3.4.1. Novel Therapies

There are many novel treatment options in clinical use or in clinical trials or in an experimental pre-clinical phase. The novel therapies are summarized in Table 2.

Inhibitors of Androgen biosynthesis: The CYP17 group of enzymes are important for steroid biosynthesis. The anti-fungal drug ketoconazole was the first CYP17 inhibitor in clinical practice. However, its side effects led to the search for more specific and potent CYP17 inhibitors.

Abiraterone acetate is a new generation, oral CYP17A1 inhibitor. It decreases the circulating levels of testosterone specifically targeting the 17, 20 lyase activity of CYP17A1 in testicular, adrenal and prostatic tumor tissues and thus preventing conversion of pregnenolone to DHEA and progesterone to androstenedione. Abiraterone also inhibits de novo androgen production in prostate cancer tissue. Therefore, it is effective against two escape mechanisms in hormonal therapies: adrenal androgens and intratumoral androgen production [42]. It entered the clinical use as a standard second line therapy in men with progressive disease after or before docetaxel-based chemotherapy. Patients treated with abiraterone gain 4 months of OS in mean. The use of this drug earlier in the progression cascade is under investigation [43].

TAK-700 (Oreteronel) shares the similar action of mechanism with abiraterone acetate with a greater potential. It reduces both the testicular and adrenal androgen levels. It is effective in CRPC both in the chemotherapy-naïve and post-docetaxel patients as shown in phase III clinical trials [45].

Table 2: Novel hormonal therapy options in prostate cancer [46]

Abiraterone acetate	Potent and selective inhibitor of CYP17- α -hydroxylase and C17,20-lyase	Phase III studies post- and pre-docetaxel
MDV-3100	AR signaling inhibitor: inhibits androgen-AR binding and nuclear translocation, and blocks AR complex DNA binding	Phase III studies post- and pre-docetaxel
TAK-700	Selective, non-steroidal, small-molecule inhibitor of C17,20-lyase	Phase III studies post- and pre-docetaxel
TOK-001	AR antagonist, AR degrader and a CYP17 lyase inhibitor	Phase I/II (ARMOR1)
SARDs*	Destroy the AR receptor	Preclinical
ARN-509	AR antagonist: inhibits nuclear translocation and DNA binding of the receptor	Phase I/II
Cofactor antagonists	Target coactivator interaction surface--AR antagonists	Preclinical
ODM-201	AR inhibitor, non-steroidal	Phase I/II
EZN-4176	AR mRNA antagonist	Phase I
EPI-001	Inhibits ligand-dependent + ligand-independent transactivation of AR and splice variants	Phase I
AZD-3514	AR downregulator	Phase I

TOK-001 (Galeterone) is not only a CYP17 inhibitor but also antagonizes AR and downregulates AR protein expression. It is also a potent inhibitor of adrenal androgen synthesis. It is in Phase I/II clinical trials in chemo-naïve CRPC patients [46].

Novel anti-androgens: MDV-3100 received attention recently because of its higher affinity to AR than bicalutamide, the standard anti-androgen in clinical use. It is effective also in the presence of amplified AR and doesn't show any agonistic activity. Mechanism of action of MDV-3100 includes not only the inhibition of AR DNA binding but also the inhibition of translocation of AR to the nucleus. In preclinical models, it induced apoptosis and was also effective in bicalutamide resistance models [47]. Survival benefits were observed in several clinical trials compared to the standard therapies both in chemotherapy naïve and post-docetaxel patients [43]. The median survival advantage is 4.8 months [41].

EPI-001 is the only antiandrogen targeting the N-terminal domain of the AR. It is a small molecule that inhibits the transactivation of the AR NTD. It is unique that it can overcome mechanisms of castration resistance such as gain of function mutations in the ligand binding domain or expression of constitutively active splice variants. It is in a preclinical stage and has yet to enter clinical trials [45].

Immunotherapies: Prostate cancer is a good candidate for immunotherapies for several reasons: A) Because of the slow proliferating nature of prostate cancer cells there is enough time for immune response to be developed. B) Immune cells require recognizing some tissue specific antigens for the development of a specific immune response. Tumor specificity is achieved by tumor associated antigens (TAAs). In case of prostate cancer; PSA, Prostate-specific membrane antigen (PSMA) or prostatic acid phosphatase (PAP) are TAAs. C) Prostate gland is poor in terms of immune cells and immune response. Therefore, it is easier to activate prostate specific cytotoxic T-lymphocytes (CTLs). TAA- expressing tumor cells are recognized and killed by activated CTLs. Dendritic cells present the antigens to the T-cells and are crucial for the generation of antitumor CTL responses. Activation of dendritic cells is possible with immunogenic cytokines. For prostate tumor vaccines available for PCa treatment GM-CSF (granulocyte-macrophage colony-stimulating factor) is used for this purpose [48].

Prostvac vaccine is a microbial vector based immunotherapy. It uses vaccinia vector to prime and fowlpox vector to boost the immune system. Vectors express PSA as the tumor antigen and three costimulatory molecules; intercellular adhesion molecule 1, B7-1 and leukocyte function associated antigen to boost immune response (PSA-TRICOM). In response to vaccine, PSA-specific T-cell levels are increased in patients leading to improved survival. PROSTVAC provided 8.5 months overall survival (OS) improvement in CRPC patients [49].

Sipuleucel-T: Sipuleucel-T is a dendritic cell-based immunotherapy to stimulate patients' immune response against cancer cells. Patients' own immune cells are isolated first and activated by human recombinant PAP (prostatic acid phosphatase)/GM-CSF fusion protein and injected back into patients to fight against prostate cancer cells. PAP antigen is recognized by host T-cells leading to T-cell activation and proliferation [50]. Median survival advantage is 4.1 months. It is currently the only immunotherapy approved by FDA for the treatment of asymptomatic or minimally symptomatic metastatic CRPC [48].

GVAX: GVAX is a cell-based immunotherapy in which prostate tumor cell lines PC-3 and LNCaP are modified to secrete GM-CSF. It aims to induce a broad range of prostate tumor specific immune response [49]. However, as a single therapy applied in CRPC patients it was not promising. Combination therapies of GVAX and ipilimumab gave better OS benefits [50].

Ipilimumab: Ipilimumab is an antibody-based immunotherapy. This antibody is directed against CTLA4, which is a cell surface molecule on T-cells that acts for suppression of T-cell activation. Targeting CTLA4 by ipilimumab aims to enhance T-cell responses. Clinical trials have been going on to evaluate the efficiency of this therapy alone or in combination with other immunotherapies in CRPC [49].

3.5. Mechanisms for Development of Castrate Resistance

Two mechanisms were proposed for the emergence of CRPC: A) Adaptation model: This model is similar to the acquired drug resistance. It claims that to adapt to low androgen environment tumor cells acquire additional genetic or epigenetic aberrations. B) Selection model: This model suggests that even during the initiation of cancer there are heterogeneous cell populations inside the tumor. While androgen deprivation is killing androgen dependent

cell types, it does not prevent proliferation of androgen independent cells and these cells become the dominant population in the tumor [51]. The two mechanisms are summarized in Figure 4.

3.5.1. AR Dependent Mechanisms

It is well established that even after castration resistance, AR activity and signaling stay intact. There are studies proving that in castration resistant tumors AR promotes tumor progression in a low androgen hormone environment [52]. This is achieved by several mechanisms:

AR mutations: 10-30 % of castrate resistant tumors have gain of function mutations in the AR gene. The mutations often occur in response to anti-androgen treatments especially to flutamide treatment. The point mutations are found all along the gene but concentrated in the ligand binding domain of the AR. They confer different functions according to their position in AR gene. Mutations in the ligand binding domain constitute 40% of the mutations and allow greater sensitivity to androgens, novel responses to other steroid hormones or growth factors, activation by antagonists such as flutamide or bicalutamide. The T877A mutation found in the LNCaP cell line model is an example for this kind of mutation. Mutations in N-terminal domain constitute 37 % of mutations and confer ligand independent activity and increased recruitment of AR coactivators [53-55].

Recent studies have been investigating splice variants of AR in castrate resistant disease. The most common variation is the truncation of the ligand binding domain which makes AR constitutively active. The 22Rv1 and VCaP cell line models are examples expressing this kind of splice variants. Seven different splice variants were identified in the 22Rv1 cell line. These splice variants were also detected in clinical samples. A truncated AR isoform lacking LBD was found significantly upregulated in CRPC and correlated with the tumor recurrence after radical prostatectomy. The molecular mechanism leading to this differential AR splicing is not clear. One assumption is that they are caused by intragenic rearrangements, which are more common in tumors with AR gene amplifications [56].

AR overexpression: One third of castrate resistant cases have amplification of the AR gene copy number. However, high mRNA or protein levels of AR were reported in 80% of cases

which cannot be explained solely by amplification of the gene. AR autoregulates its own transcription by binding to its gene, a mechanism which can be disrupted during androgen ablation therapy and can cause upregulation of AR mRNA. Another transcriptional regulator of AR is the transcription factor E2F1. In CRPC, the loss of the RB tumor suppressor at both transcriptional and translational level activates E2F1 and upregulates AR expression [51]. There can be also post-transcriptional mechanisms to regulate AR mRNA, however, these mechanisms have not been studied extensively yet [56]. A high AR protein level also allows AR activity at castrate level of androgens, and antiandrogens to act as agonists [54]. LNCaP cells expressing high AR (LNCaP-ARHi) were shown to have 3-5 folds more AR regulated genes than normal LNCaP cell lines. AR regulated genes in LNCaP-ARHi were upregulated by a 10 fold lower concentrations of androgen compared to the concentration required in normal LNCaP cells [56].

AR overexpression is detected also in circulating tumor cells (CTCs) and this information may provide useful information for optimizing therapy strategies[57].

AR coactivators/corepressors: the AR is a transcription factor which requires recruitment of several co-activators to induce transcriptional enhancement. Assembly of the AR-cofactor complex alters the AR conformation and orchestrates the recruitment of the basal transcriptional machinery and of chromatin remodeling enzymes. In castrate resistant disease the activities of these co-activators can be enhanced to induce AR activity in an androgen-scarce environment. SRC1, SRC2, SRC3, p300, ARA70, TIF2 and CBP are some of these factors reported to be involved in castrate resistant disease [58, 59]. TIF2 itself is an androgen regulated gene and during androgen ablation its level goes up. Therefore more TIF2 becomes available to boost AR activation in CRPC. TIF2 is amplified or overexpressed in 37% of metastatic prostate cancer cases [60]. Corepressors also contribute to cancer progression also in several ways: downregulation of the repressor itself, the dismissal of corepressors from the AR complex or the mislocalization of corepressors [58].

Up regulation of androgen production: In normal prostate AR signaling is a paracrine mechanism. Testosterone is produced in the testes (95%) and adrenal gland (5%) and converted to dihydrotestosterone in the prostate. In tumors this paracrine mechanism shifts to an autocrine mechanism and the tumor tissue establishes the production of its own hormones

by turning on endogenous expression of androgen synthetic enzymes or conversion of weaker adrenal androgens to testosterone and dihydrotestosterone. This is for example achieved by changes of the transcriptional program of AR in the castrate resistant stage in which AR increases the transcription of key enzymes of the androgen synthesis pathway such as AKR1C3, CYP17A1, SRD5A1 AND SRD5A2 [58]. The benefit of CYP17A inhibition by abiraterone acetate in castrate resistant disease is a proof for this mechanism [45, 53].

Other signaling pathways: Activation of other signaling pathways enhances AR activity. These pathways include but are not limited to PI3K, Protein kinase A, Ras-Raf-MAP kinase, HER-2/neu pathways. The influence of these pathways on activation of AR is indirect mostly by phosphorylation of coactivators [61]. HER-2/neu is overexpressed in CRPC. This leads to the activation of AR target genes in the absence of androgen. Another pathway important for CRPC is the WNT pathway. β -catenin, an effector molecule in WNT signaling, interacts with AR and modulates its function. Interestingly, AR also acts as a coactivator for β -catenin action in WNT induction of target genes. This cross-talk between AR and WNT pathways plays an important role for the promotion of CRPC in ligand independent manner [62].

3.5.2. AR Independent Mechanisms

In a recent study, it was shown that the suppression of AR pathway causes the upregulation of the PI3K/AKT pathway. The AR target gene FKBP5 is a chaperone for the AKT phosphatase PHLPP. Therefore, when AR signaling is blocked the AR target gene FKBP5 goes down and AKT is phosphorylated and activated [46].

Growth factor pathways such as IGF and their tyrosine kinase receptor cascades trigger proliferative and survival pathways independent of AR in progression to CRPC. However, the mechanisms of bypassing of the AR are not clear [62]. Overexpression of the apoptosis inhibitor BCL-2 is another AR-bypassing mechanism involved in progression of CRPC. BCL-2 overexpression leads anti-apoptotic response and survival of CRPC. IHC studies confirmed the increase in BCL-2 expression in CRPC compared to hormone naïve disease [57].

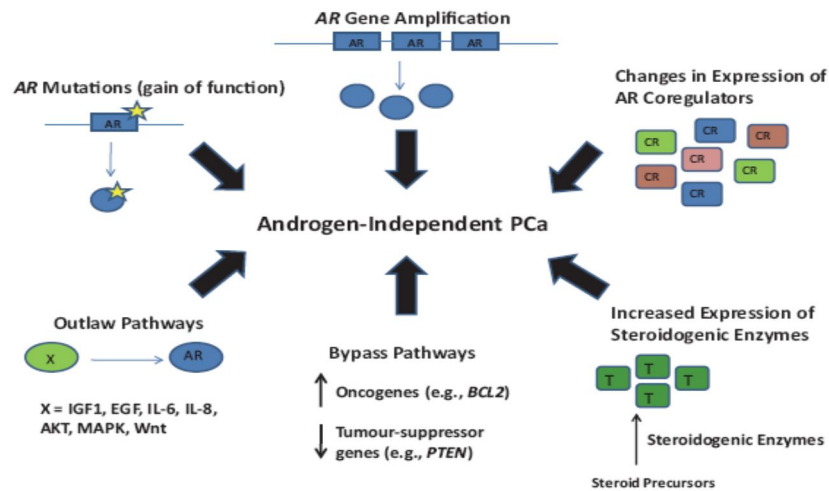


Figure 4 : Mechanisms of progression to CRPC.[62].

3.6. MID1

The Mid1 protein is encoded by a gene mutated in Opitz G/BBB syndrome. This syndrome is characterized by several developmental defects mainly affecting the midline axis and resulting in malformations like clefts of lip, palate and uvula, hypospadias in males, imperforate anus and congenital heart defects [63].

The MID1 gene is composed of 10 exons spanning 385 kb on Xp22. The First exon is non-coding and located in a 250 kb region containing several alternative exons. Usage of different alternative first exons ends up in alternative 5'UTRs and promoters. These alternative promoters show tissue specificity and allow tight control of differential regulation of transcription of MID1. It is also possible that this mechanism guarantees the spatial and time dependent activity in early development [64]. Besides the non-coding first exons there are 14 alternative exons in the coding region giving rise to 63 splice variants. Because of these novel exons some novel stop codons or alternative translation start sites are introduced to produce N-terminally or C-terminally truncated proteins with various lengths. These truncated proteins don't show the same cellular distribution as the full length protein and their functional significances are not known. However, it is evident that these protein variants exert selective binding and affinity for different protein partners of MID1. The splice variants with premature termination codons are destined for non-sense mediated degradation (NMD) [65]. MID1 transcripts have several alternative polyadenylation sites. These act in concert with the alternative 5'UTRs to produce different transcript variants. Among these different

transcripts, a 2 kb transcript was found to be exclusively liver specific. It is expressed both in adult and fetal liver. The MID1 3'UTR contains some conserved elements like cytoplasmic polyadenylation signals or AU rich elements. It was shown that the AU rich elements can bind RNA binding proteins HuR and AUF1 [66]. All these mechanisms point out a tight, tissue specific regulation of MID1 protein expression and function.

The MID1 protein is a member of the TRIM (tripartite motif) family also known as RBCC. They are called in these ways because this family is specified by three motifs: RING, Bbox and coiled coil (CC). 37 members of this protein family were identified. Most of them form homodimers through their CC domain. They are able to form high molecular weight complexes and discrete patterns in the cell. Therefore, trim family proteins are associated with specific compartments like nuclear bodies (PML/TRIM19, TIF1/TRIM24 and RFP/TRIM27), microtubules (TRIM18/MID1, TRIM1/MID2), filaments (TRIM2 and TRIM3), cytoplasmic ribbon like structures (TRIM29), nuclear sticks (TRIM6) and specific chromatin regions (TRIM24, TRIM28, TRIM33) [67].

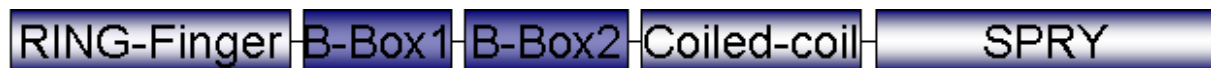


Figure 5: Domain structure of the MID1 protein

MID1 is a 667 amino acid protein with a RING finger motif, two B-boxes (Bbox1 and Bbox2), a coiled coil and a PRY/SPRY (RFP like) domain as shown in Figure 5. It is expressed both in adult and fetal tissues. Kidney, brain and lung show highest expression during fetal development and developing heart doesn't express any MID1. However, heart, placenta and brain are the organs showing highest expression among adult tissues [63]. In mouse like in human early development, it is expressed ubiquitously, in the late stages of development the highest expression is in the central nervous system, in the mucosa of the pharynx, esophagus, trachea and larynx, in the stomach, in the gut and in the urogenital system which are also the organs mostly effected in Opitz patients. MID1 expression is prominent in proliferating cells and in murine development it is expressed in stem cells or non-terminally differentiated cells [68].

All three N-terminal domains, RING, Bbox1 and Bbox2 of MID1 have ubiquitin ligase activity. The RING domain has higher E3 ligase functionality than the Bboxes. These domains act in tandem to amplify E3 ligase activity. Several U2 conjugating enzymes can interact with MID1. MID1 can also autoubiquitinate itself. Poly- or monoubiquitination are possible. $\alpha 4$, the regulatory subunit of protein-phosphatase PP2A is also mono-ubiquitinated by MID1. Interaction of $\alpha 4$ with MID1 and mono-ubiquitination by MID1 ultimately regulates PP2A ubiquitination and degradation [69].

Study of the intracellular localization of the MID1 protein showed that it is a cytoplasmic protein forming filamentous structures. The filamentous structure formed by MID1 is restricted to the cytoplasm, the nuclei is devoid of it. MID1 stabilizes microtubules by co-localizing with tubulin in these filaments but not with actin fibers or intermediate filaments [70]. This association is evident throughout the entire cell cycle. MID1 is associated with the mitotic spindle during mitosis and with midbodies which interact with remains of the polar spindle microtubules during cytokinesis [71]. The analysis with C-terminally mutated MID1, the mutated form commonly found in Opitz patients, showed that C- but not N-terminally affected proteins forms cytoplasmic clumps instead of microtubule bundles [70]. However, the MID1 protein does not have a clear microtubule binding domain. It is found in the cell as 200-250 KD high molecular weight multiprotein complex. MID1 homodimerizes and binds with additional proteins in the microtubules [71].

The coiled coil domain of MID1 is responsible for homodimerization, which is a prerequisite for microtubule binding [72]. $\alpha 4$, the phosphoprotein binding subunit of phosphatase PP2A was the first protein identified as a binding partner of MID1. The B-box 1 (amino acid residues 110-165) of MID1 binds to the C-terminal residues 220-340 of $\alpha 4$ [73, 74]. MID1 recruits cytoplasmic $\alpha 4$ to the microtubules and an excess of $\alpha 4$ is able to dislocate MID1 from microtubules to the cytoplasm [74].

The RING finger family of proteins to which MID1 belongs is known for their ubiquitin ligase activity. The catalytic subunit of PP2A (PP2Ac) was determined as the target of the MID1 ubiquitin ligase activity. Although monoubiquitinated PP2A catalytic subunit was identified in normal fibroblasts it could not be observed in fibroblasts of Opitz syndrome

patients with mutated MID1. As a consequence of this, hypophosphorylation of microtubule-associated proteins was found in Opitz patients as a result of increased PP2A activity [73].

The mTORC1 pathway is one of the pathways downstream of the MID1/PP2A/ α 4 complex. PP2A is involved in complex formation of mTOR and raptor. This became evident from investigations in cells from Opitz syndrome patients showing reduced cap dependent translation, cell size and S6K1 phosphorylation [75].

Protein members of the MID1/PP2A/ α 4 complex were identified by affinity chromatography using a 44 amino acids peptide from the C terminus of α 4 immobilized to a column and electrospray ionisation mass spectrometry protein identification. Consistent with the microtubule binding property of the complex, the tubulin beta-5 chain, a previously identified MID1 interacting partner was found in the complex. An important regulator of protein translation, EF-1 α , was also identified in the complex and its interaction with MID1 was confirmed by yeast two hybrid interaction assays and immunofluorescence. The interaction site was mapped to the PRY/SPRY domain of MID1. Further members of the complex identified are: several 40S ribosomal proteins (S8, S3, 40S ribosomal protein SA (p40)), ribosome and translation-associated proteins, the receptor of activated protein kinase C1 (RACK1), nucleophosmin (NPM), heat-shock cognate 71 kDa protein (Hsc70), heat shock protein HSP90-beta (Hsp90 β), 60 kDa heat shock protein, 30 kDa heat shock protein (CH60), Q subcomponent binding protein (C1qBP) and annexin A2 (ANXA2). As expected from the enrichment of ribosomal and mRNA binding (ANXA1, NPM) proteins in the complex, RNA is also associated with the complex. The mRNAs associated with the complex have specific sequence motif with polyG or polyU rich elements. MID1 mRNA is one of the associated mRNAs pointing to a feedback regulation of MID1 mRNA by MID1 protein itself. Interestingly, MID1 mRNA is not dispersed equally along the microtubules but it shows specific localization to the microtubule-assembly centre. This means that this complex ensures specific localization and compartmentalized translation of specific mRNAs [76]. Furthermore, this complex is dynamic. It moves along the microtubules as is evident by active, bidirectional transport of MID1 along the microtubules with the help of dynein and kinesin motor proteins. The transport of MID1 along microtubules is also dependent on its phosphorylation status. PP2A is involved in this process by keeping the S96 residue of MID1 protein dephosphorylated during transport with the help of the α 4 protein [77]. All these

information points out several important functions of this complex: 1) active transport of proteins and mRNAs back and forth from the cell center to the periphery to establish asymmetry which is an important process for cell function and survival during development. 2) microtubule dependent polarization and migration of neural crest cells which enables the closure of midline structures.

MID1 itself is a phosphoprotein. Although there have been no functional studies about the phosphorylation of MID1, two consensus sites were determined by homology analysis. These are Ser92 and Ser96 within consensus sequences for GSK3 and MAPK/CKI/CKII respectively [72, 74]. Phosphorylation of MID1 by MAPK is important for microtubule association. Increased phosphorylation by MAPK enhances its microtubule binding and dephosphorylation by PP2A reduces binding and induces cytoplasmic clump formation [74].

GLI3 is one of the transcriptional factors in Hedgehog signaling. Its subcellular localization is important because of its transcriptional activity. It is in a functional interaction with MID1/PP2A/ α 4 complex and its subcellular localization is regulated in this way. Although it is not clear if GLI3 is interacting directly with the complex it colocalizes with MID1. Disruption of the MID1 complex by depletion of complex members leads to cytoplasmic retention of GLI3, thus GLI3 is prevented from entering the nucleus and stimulating transcription. These data extend the function of MID1/PP2A/ α 4 complex [78].

3.7. Protein Phosphatase 2A (PP2A)

PP2A phosphatases form a large family of heterotrimeric serine-threonine phosphatases which are essential in eukaryotic cells. The PP2A trimeric core enzyme consists of a catalytic subunit called PP2AC or PPP2C, a structural A subunit called PR65 or PPP2R1 and several regulatory B subunits. PP2AC is encoded by two closely related genes $C\alpha$ and $C\beta$. $C\alpha$ is the dominant form expressed 10 folds higher than $C\beta$. PR65 α and PR65 β encode two distinct genes for the A subunit. PR65 α is the dominantly expressed form. Only 10% of the PP2A holoenzymes contain the B subunit PR65 β . The B subunit regulates both substrate specificity and localization of the holoenzyme. The B subunits are categorized to 4 protein families encoded by 15 different genes, B/B55/PPP2R2, B'/B56/PR61/PPP2R5, B''/ PR72/ PPP2R3 and B'''/ PR93/ SGNA/PR110/Striatin. Different combinations of subunits enable up to 30 possible holoenzymes. Different to the A and B subunits, PP2AC forms heterodimers with

other proteins such as IGBP1/ α -4. When these heterodimers are taken into consideration, there are over 200 distinct complexes of PP2A in a cell. These different combinations allow the existence of different complexes in a tissue or time dependent manners in cells [79-81].

PP2A is well known as a tumor suppressor. However, it is important to notice that different complexes have different functions and substrate specificities. Holoenzymes containing even closely related B subunits may dephosphorylate different sites and achieve opposing functions in the same pathways. Examples are the TGF- β , WNT and MAPK pathways in which PP2A executes both positive and negative regulations. Another dilemma emerges from the fact that a complete loss of catalytic or scaffold subunits is lethal. However, there are inhibiting mutations that lead to tumor promotion [79, 81]. PP2A has the ability to dephosphorylate and regulate hundreds of different proteins in the cell. Some of these proteins inactivated by PP2A are well established tumor promoters. PP2A directly dephosphorylates AKT and thus negatively regulates the PI3K/AKT pathway. It also exerts proapoptotic function by inactivating anti-apoptotic BCL2 or activating the proapoptotic factors BAD and BIM. c-Myc is also a target of PP2A. It is dephosphorylated by PP2A which subsequently leads to ubiquitination and degradation of c-Myc [82, 83].

The PP2A complex has several interaction partners inhibiting its phosphatase activity. SET, CIP2A and SETBP1 are some examples. These physiological inhibitors of PP2A are known for their tumor promoting effects. CIP2A is overexpressed in several malignancies such as prostate, colorectal and hepatocellular tumors and in chronic and acute myeloid leukemia. SET and SETBP1 are overexpressed in acute myeloid leukemia [82].

IGBP1/ α 4 also acts as a negative regulator of PP2A. α 4 binds directly to the catalytic subunit PP2AC and keeps PP2A inactive.

3.8. IGBP1 (α 4)

α 4 is the homolog of yeast TAP42. It is a 42 kD phosphoprotein. C terminal region of the protein is highly disorganized and flexible. It regulates phosphatases like PP2A, PP4 and PP6. α 4 binds directly to the catalytic subunit of PP2A in a constitutive manner. PP2A phosphatase is constructed by the assembly of 3 different subunits: regulatory subunits A and B and catalytic subunit C. The regulatory subunit A functions as a scaffold for recruiting

various regulatory B subunits. $\alpha 4$ binds the C subunit of PP2A (PP2AC) in the absence of an A subunit. The binding site for $\alpha 4$ in PP2AC is a compactly folded α -helical domain between residues 19-165 [84].

The $\alpha 4$ protein is broadly expressed in different tissues. Its expression is essential and embryonic deletion mutants are lethal. The depletion of $\alpha 4$ expression leads to increase in phosphorylation of p53 and c-Jun and induction of p21 expression. These changes ultimately result in induction of intrinsic apoptotic pathways and cell death [85]. Although alteration of the phosphorylation status of p53 and c-Jun points out the involvement of phosphatase activity, it is not clear if this pro-survival role of $\alpha 4$ is dependent on its association with PP2AC resulting in PP2AC inhibition, or an isolated function of its own. Consistent with its pro-survival role, $\alpha 4$ was also reported as an upregulated protein in tumors. The upregulation of $\alpha 4$ was detected in different types of cancer like breast, lung and hepatocellular carcinomas. This upregulation decreases the activity of PP2A. Another important finding was that $\alpha 4$ is a target for microRNA 34b [86].

An additional important function of $\alpha 4$ is the regulation of cell spreading and migration. This function is independent of its pro-survival activity and depends on PP2AC phosphatase activity. Rac1 is the mediator molecule for induction of cell migration by $\alpha 4$. Deletion of $\alpha 4$ results in inactivation of Rac1 [87].

Kong 2009 clarified the role of $\alpha 4$ in regulation of PP2A activity showing that $\alpha 4$ keeps PP2AC in an inactive state until PP2AC recruits the A and B subunits to get ready to execute its function. Although binding of $\alpha 4$ inactivates PP2AC, it also stabilizes PP2AC and protects it from degradation [88].

$\alpha 4$ has the ability to bind MID1 and PP2AC simultaneously. In this manner it facilitates a reciprocal regulation between MID1 and PP2A. It functions as an adaptor molecule for ubiquitination and degradation of PP2AC by MID1 and for dephosphorylation of MID1 by PP2AC. $\alpha 4$ has an Ubiquitin Interaction Moiety (UIM) in its N-terminus and is itself mono-ubiquitinated. UIM in $\alpha 4$ functions as a cap for associated monoubiquitinated form of PP2AC and blocks polyubiquitination of it [89].

3.9. Metformin

Metformin (1,1-dimethylbiguanide), a biguanide derivative, is derived from *Galega officinalis* (French lilac or Goat's rue or Italian Fitch). It is the most commonly prescribed drug for type II diabetic patients. It was introduced to clinical use in the 1950s [90]. Recent studies have shown that it is also useful in some other disease entities like diabetic nephropathy, cardiovascular diseases, polycystic ovary disease, and for the prevention and/or treatment of cancer. It is a very safe and low cost drug. Only a very low incidence of lactic acidosis has been reported as side effect.

3.9.1. Anti-hyperglycemic Action of Metformin

Metformin lowers the blood glucose levels without causing hypoglycemia. It also works as an insulin sensitizer by reducing the insulin resistance. This effect is attributed to metformin's positive effect on insulin receptor expression.

Although the molecular targets of metformin are still a topic of debate the primary function is to decrease hepatic glucose production and to stimulate uptake of glucose in muscle tissue. The inhibitory effect of metformin on gluconeogenesis is explained by the regulation of enzyme activities in the gluconeogenesis pathway. This is most probably mediated by AMP-activated kinase (AMPK) [90] a kinase activated in response to metformin treatment. AMPK is a serine/threonine kinase composed of a catalytic α -subunit and two regulatory subunits β and γ . It works as a fuel gauge sensing the AMP/ATP ratio. In the case of cellular stress such as glucose deprivation, hypoxia, oxidative stress and ischemia the AMP/ATP ratio is increased. Then AMP binds to the regulatory subunit γ , which induces conformational changes that activate the enzyme. This activation requires the phosphorylation of Thr¹⁷² by STK11 (serine/threonine kinase 11)/LKB1 and CAMKK β (Ca²⁺/Calmodulin-dependent protein kinase beta) or TGF- β activated protein kinase 1 (TAK1). Activated AMPK guarantees shutting off of ATP consuming processes such as glucose, lipid and protein synthesis as well as cell growth, and it induces catabolic processes such as fatty acid oxidation and glucose uptake. AMPK achieves this by phosphorylation of key metabolic enzymes and transcription factors. However, activation of AMPK by metformin is most probably indirect through inhibition of the mitochondrial respiratory chain complex I.

Another idea is that metformin bypasses AMPK and gluconeogenesis is suppressed by its inhibition of the mitochondrial respiratory complex I [91] .

3.9.2. Anti-cancer Effect of Metformin

There is epidemiological evidence that Type 2 diabetes (T2D) is associated with increased cancer risk, e.g. breast, colon, kidney and pancreas but not prostate cancer. A recent large cohort study was accomplished in the UK to study cancer mortality among diabetic patients and the effect of different anti-diabetic medications on cancer progression. 112,408 patients diagnosed with a solid tumor for the first time were included in this study. Among these patients 8,392 had T2D. It was concluded that 1) Cancer mortality was increased in patients with diabetes especially in those with breast and prostate cancer but not with lung cancer. When diabetics were categorized according to the medications they took, mortality was increased with insulin and sulfonylurea therapies but decreased with metformin therapy relative to the nondiabetics [92]. In another retrospective large population study in Taiwan, cancer incidence among diabetes patients was explored in 10,491 nondiabetics, 8130 diabetics without any medication, 4327 diabetics with medication other than metformin and 11,390 diabetics with metformin treatment. Cancer incidence increased 2 folds in total in the diabetics. However, the diabetic patients who used metformin as therapy did not have an increased incidence, they even had some benefit [93] .

The retrospective studies correlating diabetes, cancer and metformin use have proven metformin's beneficial anticancer effect. However, a main concern about its use for cancer treatment was the possible (side) effects in nondiabetic cancer patients. Some recent studies on nondiabetic cancer patients and clinical trials are ongoing to address this issue. In addition to the clinical studies, several animal and cell culture studies have been conducted to address the effects of metformin under non-diabetic conditions. Anisimov et. al. used female mice in their study for chronic treatment with metformin starting at different points in the life span. They did not observe any change in serum cholesterol, triglycerides, glucose or insulin levels upon metformin treatment. When started early in the life span metformin delayed first tumor detection by 22-25 % [94] .

3.9.3. Metformin: Mechanism of Action in Cancer

As shown in population studies, metformin therapy reduces the risk of neoplasms. Several mechanisms were proposed as shown in Figure 6. The anti-cancer effect of metformin may simply be explained with the reduced serum insulin and IGF-1 levels because of metformin's anti-glycemic effect. This would suppress the well-established pro-survival and growth effect of insulin and IGF-1 [90]. However, it was proposed that metformin also has a direct anti-proliferative effect. The LKB1/AMPK pathway is a good candidate because it counteracts cell proliferation. It inhibits mTORC1 signaling which subsequently blocks protein synthesis and cellular proliferation by phosphorylating TSC2 (tuberous sclerosis 2) and raptor [95]. On the other hand it was also reported that the mTOR pathway was inhibited by metformin through inhibition of Rag GTPase-mediated mTOR activation without involvement of AMPK [90].

Another important target of AMPK is the de novo fatty acid synthesis pathway. Tumor cells require high levels of de novo fatty acid synthesis for cell proliferation and survival. Activation of AMPK inhibits FAS, a key enzyme of fatty acid synthesis known to be overexpressed in several cancer types [91, 95].

AMPK also phosphorylates insulin receptor substrate 1 (IRS1) upon activation by metformin. This inhibits IGFR1 and the downstream PI3K/AKT and mTORC1 pathways [97].

The wide range of metformin effects and inhibition of the mTOR pathway suggest that metformin acts on the mRNA translation as well. Larsson et. al showed in their study that metformin inhibits the translation of a selective subset of mRNAs. However, there was only a partial overlap with mRNAs repressed by mTOR inhibitors. The metformin-regulated mRNAs constitute a functionally related subset including proliferation and tumor-promoting proteins such as cyclin E2 and ODC1 [98]. This specific subset of mRNAs related to cell cycle also explains that metformin causes cell cycle arrest in G0/G1 and/or apoptosis especially at higher concentration [97].

Figure 6: Possible mechanisms of anti-cancer effects of metformin [96].

3.9.4. Metformin in Prostate Cancer

Population based studies exploring the use of metformin and prostate cancer risk yielded controversial results. The discrepancies between the studies are probably due to the methodological differences.

A large cohort study including 739 prostate cancer and 7,359 control subjects in UK concluded that metformin did not decrease the risk of prostate cancer [100]. Another study in America confirmed this result. 616 patients including 112 diabetic metformin users, 98 diabetic non-metformin users and 406 non-diabetic controls were evaluated after radical prostatectomy. There has been no benefit from metformin use in terms of biochemical recurrence [101]. In contrast, One American study suggested a 44% reduction of prostate cancer risk among metformin users [102].

More consistent are the data with regards to tumor progression and metformin medication. In a very recent clinical study, 157 metformin, 162 diabetic non-metformin and 2582 non-diabetic prostate cancer patients were evaluated. It was shown that metformin use improves Overall Survival (OS), Prostate-cancer Specific Mortality (PCSM), distant metastasis free survival (DMFS) and PSA recurrence free survival (PSA-RFS). More importantly, from 26 metformin users who developed biochemical failure, only 1 patient (4%) went on to develop CRPC compared to 43% among diabetic non-metformin users and 26% among non-diabetics [103]. Another study compared the risk of high grade tumors and time to tumor progression in users of different anti-diabetic medications and non-diabetic prostate cancer patients. The results pointed out that the use of metformin or TZDs (Thiazolidinediones) decreased the risk of high grade tumors and tumor progression while insulin and sulfonylureas increased it [104]. A similar study comparing only users of different anti-diabetic medications obtained a very similar result and reported that metformin and TZDs use improved overall survival compared to insulin use [105].

3.9.5. Mechanism of Action of Metformin in Prostate Cancer

In cellular models of prostate cancer the anti-proliferative action of metformin has been proven in several studies. Iliopoulos et. al used the PC-3 xenograft model to show effectiveness of metformin in combination with doxorubicin. No relapse was observed in any

of the mice in the combined treatment group [106]. Colquhoun et. al showed the effectiveness of metformin both in AR positive and negative prostate cancer cell lines. In LNCaP tumor models they used a combination treatment of metformin and the anti-androgen bicalutamide and found that AR positive and negative cell lines respond differently to metformin treatment. This suggests that AR may be involved in metformin action in these cells [107].

The dominant view about the mechanism of action of metformin in prostate cancer is that it is independent of AMPK activation. Ben Sahra et. al suggested a mechanism through REDD1. In their model metformin bypasses AMPK and inhibits mTOR via REDD1. As REDD1 is regulated by p53 their model requires p53 for metformin action [108].

3.9.6. AMPK in Prostate Cancer

AMPK is not only a main (indirect) target of metformin, as pointed out above, it is also considered as a tumor suppressor because of its role to shut off the energy consuming pathways such as cell proliferation and fatty acid synthesis, which are very crucial for cancer cells to survive. However, its role in prostate cancer is controversial and conclusive data on the tissue expression patterns in different stages of prostate cancer are still lacking. A single study by Park et al. exploring the expression of AMPK in prostate cancer demonstrated elevated AMPK protein in 40 % of prostate cancer tissue samples. Moreover, they showed elevated AMPK and phosphorylated AMPK levels in prostate cancer cell lines as well [109]. In line with this study, Chippa et al. explained that AMPK activation provides an advantage to prostate cancer cells in case of cellular stress because of androgen ablation and lack of growth factors. They showed that chemical or siRNA mediated inhibition of AMPK led to apoptotic cell deaths and this was enhanced by androgen ablation [110]. In prostate cancer CAMKK β is the dominant kinase for phosphorylation and activation of AMPK and it is a target of AR. Due to the central role of the AR in prostate cancer CAMKK β expression is increased in prostate tumors resulting in enhanced activation of AMPK [111]. Taken together these studies suggest that AMPK acts as a survival factor in prostate cancer refuting the initial hypothesis of AMPK as a tumor suppressor.

4. Aim of the Thesis

The uncovering of the genetic defect underlying the Opitz-BB syndrome and the extensive studies of the function of the MID1/PP2A/ α 4 complex have established the crucial role of MID1 for the regulation of the PP2A phosphatase activity and additional functions for specific binding and regulation of mRNAs. Fine-tuning of the phosphorylation-dephosphorylation balance, e.g. by modulation of PP2A activity through MID1 plays a crucial role in cancer and the tumor suppressor role of PP2A and its downregulation in many cancers is well established. Up to now MID1 has not been studied with regard to a role in tumors and there is no report in the literature about the role of MID1 in prostate or in any type of cancer. Also of notice, the most frequent common clinical symptom found in Opitz-BB syndrome patients with MID1 mutations is hypospadias, a malformation of the male urethra. This is a striking link to patients with partial androgen insensitivity syndromes caused by androgen receptor mutations who display the same malformation. This let us speculate about a link between MID1 function and AR activity. This could explain this main developmental defect of Opitz-BB patients, and, in addition, considering the crucial role of AR in prostate cancer, would suggest a role of aberrant MID1 function in prostate cancer. Indeed, prior preliminary experiments revealed an association of AR mRNA with the MID1/PP2A/ α 4 complex.

Based on these leads we planned the first part of the project:

Aim of Project 1: Studying the implication of the MID1/PP2A/ α 4 ribonuclear protein complex for the regulation of AR and the role in prostate cancer

For that transient knock down of MID1 and α 4 in different prostate cancer cell lines were established and the effect on proliferation was studied. During the progression of the project our group established that 1) MID1 is one of the genes negatively regulated by androgens in different prostate cancer cell lines and 2) the androgen receptor binds to the MID1 gene, which has 10 putative AR binding sites (ARBSs) scattered along the gene.

To confirm these findings we used real-time PCR and western blotting to study the mRNA and protein levels of MID1. We constructed reporter plasmids carrying sequences from two of the different ARBSs and confirmed their function as AR targeted regulatory elements In

addition we also established IHC for MID1 and AR in prostate cancer tissue samples and explored their expression pattern and correlation with tumor histology.

During the course of the first project another important finding related to this topic was published; Metformin, a commonly used anti-diabetic drug, was shown to disrupts the MID1/PP2A/ α 4 complex [99]. Concurrently Metformin has attracted enormous attention because of its anti-cancer effect. Based on these new finding we designed the second project:

Aim of Project 2: Studying the mechanism of the anti-cancer effect of metformin in prostate cancer and the role of the MID1/PP2A/ α 4 ribonuclear protein complex for metformin's inhibitory activity in prostate cancer cells.

The anti-proliferative effect of metformin was confirmed in a wide range of prostate cancer cell lines, AR-positive as well as AR negative tumor and benign cell lines. CoIP was established to confirm the interaction of MID1, α 4 and PP2A in prostate cancer cell lines.

PCR analysis of pull-down samples showed the association of AR mRNA with the complex and dissociation of AR mRNA from the complex after metformin treatment. To exclude the possibility that AMPK is mediating the observed metformin effects AMPK inhibitors and activators were employed.

5. Results

My PhD work resulted in 2 manuscripts. They were submitted to peer reviewed internationally reputed journals. The important results of these two projects are presented and discussed in these papers. In the first paper, I shared the first authorship with our collaborator Andrea Köhler.

5.1. Paper 1

Köhler A, Demir Ü, Kickstein E, Krauss S, Aigner J, Aranda-Orgillés B, Karagiannidis A.I., Achmüller C, Bu H, Wunderlich A, Schweiger M.I, Schaefer G, Schweiger S, Klocker H, Schneider R. **A hormone-dependent feedback-loop controls androgen receptor levels by limiting MID1, a novel translation enhancer and promoter of oncogenic signaling.** Submitted to NEOPLASIA.

Ummuhan Demir

Performed all in vitro experiments including knockdowns, androgen binding analysis, reporter gene assays. Involved in planning of the project, data analysis, statistics and manuscript preparation and writing of the first version of the manuscript.

Andrea Köhler

Performed knockdowns, reporter gene assays, overexpressions and protein stability experiments. Involved in planning of the project, data analysis, statistics and manuscript preparation and writing of the first version of the manuscript.

Eva Kickstein, Beatriz Aranda-Orgilles, Sybille Krausse, Johanna Aigner

Performed the Co-IP, RNA-protein binding and mRNA level/stability experiments.

Antonios I. Karagiannidis, Celemens Achmüller

Provided expert guidance in cell culture methods.

Georg Schaffer

Performed IHC analysis.

Huajie Bu, Andrea Wunderlich and Michel R. Schweiger

Performed ChIP-Seq analysis.

Rainer Schneider, Helmut Klocker and Susann Schweiger

Involved in preparation, organization, and collaboration and planning of project, proofreading of the paper.

Running title:

Androgen receptor translation is enhanced by MID1

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Androgen receptor, MID1, translation, prostate cancer, reciprocal regulation

**A hormone-dependent feedback-loop controls androgen
receptor levels by limiting MID1, a novel translation
enhancer and promoter of
oncogenic signaling**

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High androgen receptor (AR) level in primary tumour predicts increased prostate cancer (PCa)-specific mortality. Furthermore, activations of the AR, PI3K, mTOR, NFκB and Hedgehog (Hh) signaling pathways are involved in the fatal development of castration-resistant prostate cancer (CRPCa) during androgen ablation therapy. MID1, a negative regulator of the tumor-suppressor PP2A, is known to promote PI3K, mTOR, NFκB and Hh signaling. Here we show that MID1 also strongly enhances the translation of AR. Modulations of MID1, experimentally in PCa cells or by natural mutations occurring in Opitz-BBB/G patients, directly correlate with AR protein levels. AR on the other hand, in the presence of androgens, exerts a negative feedback loop on MID1 transcription. Thus, androgen withdrawal increases MID1 and concomitantly AR-protein levels. Concurrent promotion of AR-, Akt/mTOR-, NFκB- and Hh-activities by sustained MID1-upregulation during antiandrogen therapy therefore would provides a powerful proliferative scenario for PCa progression toward CRPCa. In line with this, MID1 is significantly over-expressed in PCa in a stage-dependent manner. Thus, MID1 represents a novel, multi-faceted player in PCa and a promising target to treat CRPCa.

Introduction

The androgen receptor (AR) is the key transcription factor regulating androgen-dependent gene expression and is critical for the development and maintenance of a male sexual phenotype. Functional AR signaling is particularly necessary for initiation of prostate development and formation of sexual characteristics during embryogenesis. After development of the prostate, the androgen axis is required to maintain adult prostate function. Survival of the secretory epithelia is dependent on continuous androgen stimulation and this cell type is thought to be transformed in prostate adenocarcinoma [1].

Prostate cancer is the most common malignancy diagnosed in male humans and the second leading cause of male cancer deaths in Western countries [2]. The two most frequently aberrantly activated signaling pathways found in prostate cancer are controlled by the AR and PI3K [3]. However, the AR is the key regulator and oncogenic driver of progression and therapy resistance in prostate cancer. It is up-regulated in late disease stages by gene amplification and other, non-genomic mechanisms [4].

MID1 is a microtubule-associated ubiquitin E3 ligase, which is mutated in the X-linked inherited disorder Opitz BBB/G syndrome (OS). This syndrome is characterized by malformations of the ventral midline along with ocular hypertelorism and hypospadias. The urogenital malformations resemble those of patients with partial androgen insensitivity syndrome [5]. Male sexual development during embryogenesis is particularly dependent on functional androgen signaling, suggesting a link between MID1 and androgen receptor and androgen receptor dysfunction in OS patients would be a good explanation for the development of hypospadias in these patients.

A main function of MID1 and its binding partner alpha4 ($\alpha 4$) is to trigger the degradation of the catalytic subunit of the tumor suppressor phosphatase 2A (PP2A) via the ubiquitin proteasome pathway [6, 7]. PP2A is a cellular master regulator and an important player in the mTOR pathway, opposing mTOR kinase activity and thereby down-regulating proliferation and cell survival. Recently it was shown that MID1, by inhibiting PP2A, also positively modulates inflammatory pathways through the activation of NF κ B [8], a crucial oncogenic factor [9] pivotal for metastasis of PCa [10]. Moreover, PP2A is the principal phosphatase down-regulating cytochrome P450c17 and 17, 20 lyase (CYP17A1) activity, resulting in decreased levels of androgens [11]. CYP17A1 is the target of abiraterone acetate, a recently approved new drug for treatment of castration resistant prostate cancer inhibiting androgen biosynthesis [12]. Furthermore, MID1, via downregulation of microtubule-

associated PP2Ac, has a modulatory effect on another major oncogenic pathway, namely Hedgehog signalling: by promoting the nuclear translocation and activity of the microtubule-associated transcription factor GLI3 that positively regulates the oncogenic cyclin D1 [13].

In OS patients, MID1-mediated regulation of PP2Ac is disrupted, leading to accumulation of PP2A and to hypophosphorylation of its targets, which in turn results in altered protein functions [6, 14]. The increased levels of PP2A in cells of OS patients disrupt the mTOR/Raptor complex and down-regulate mTORC1 signaling, resulting in reduced S6K1 phosphorylation, cell size decrease, and reduced cap-dependent translation [15].

In addition to its function in the regulation of PP2A, MID1, together with the PP2A subunit $\alpha 4$, represent the core of a large microtubule-bound multiprotein complex that associates with active polyribosomes and mRNAs. This complex associates to microtubules and targets specific mRNAs to MID1 mediated translation [16]. Thus, loss of such regulated protein translation could be a novel mechanism underlying the developmental defects observed in Opitz syndrome patients [17]. Involvement of MID1 and also the $\alpha 4$ protein, which links PP2A with the mTOR kinase, suggests a PP2A/mTOR dependent translation mechanism [16, 18]. The sequence motifs that direct the MID1 complex towards specific mRNAs are purine-rich stem-loop structures [18] and include also expanded trinucleotide repeats as found in mutant huntingtin [16].

Moreover, PDPK-1, an important player of PI3K/Akt and mTOR/PP2A signaling is also translationally up-regulated by the MID1 complex [18]. PDPK-1 is the first node in the PI3K signaling output and activates Akt by phosphorylation. Its overexpression and increased gene copy numbers are common events found in cancer [19]. In contrast, down-regulation of PDPK-1 levels inhibits migration and experimental metastasis [20]. In conclusion, these findings identify MID1 as an interesting novel upstream modulator of proliferating pathways and as an important regulatory hub in tumor cells, making it a promising target for anti-cancer drug development.

In a screen of MID1 complex-associated mRNAs we have identified the AR mRNA. In the current study, we examined the role of MID1 in AR signaling and the potential mechanisms by which MID1 may contribute to prostate cancer initiation and/or progression.

RESULTS

The AR mRNA associates with the MID1 complex.

Previously, we have shown that the MID1-protein complex binds mRNAs via purine-rich RNA motifs generating stable secondary structures [17, 18] including expanded CAG repeats as seen in mutant huntingtin [16]. The phenotypic similarities between OS patients and patients with androgen insensitivity syndrome suggest a close connection between the AR and the MID1 protein. Two repeat stretches in the 5' end of the AR mRNA, which are purine-rich structures and form stable hairpins [21], are candidates for being recognized by the MID1 complex.

To test this hypothesis, we first isolated mRNA from HeLa total cell lysate or from co-immunoprecipitates of the MID1 complex using lysates from MID1-FLAG overexpressing cells. RT-PCR was performed using primers for the AR and for two control genes (prefolding 5, thymosin-like 8). A specific PCR product was only obtained in the MID1 pull-down sample, indicating that the AR mRNA is bound by the MID1 complex (Fig. 1A).

To map putative binding sites of the AR mRNA to the MID1 complex, we performed RNA-protein pull-down assays. Similar amounts of five different (Fig. 1B) *in vitro* transcribed, biotinylated AR mRNA fragments were incubated with HeLa cell extracts overexpressing FLAG-MID1. RNAs were purified with streptavidin-coated beads and bound proteins were analyzed by Western Blot using an anti-FLAG antibody. Control experiments with antisense RNA and without RNA respectively, were performed in parallel (Fig. 1C). While there was no binding of MID1 to the 5', 3' or the intermediate region, strong interactions of the MID1 protein with the CAG repeat and the polyGGY-repeat RNA fragments were detected.

AR protein levels and activity depend on MID1.

Next, the question arose, if the MID1 protein is involved in translational control of the AR mRNA. In order to assess the importance of MID1 for AR activity, we performed AR dependent CAT reporter assays. Plasmids incorporating the CAT gene under the control of an AR responsive promoter and wild-type AR were co-expressed with either an empty vector, wild-type MID1 or mutant MID1 in the AR-negative prostate cancer cell line PC-3. AR activity increased up to 5-fold in the presence of MID1 and androgen (R1881). By contrast,

expression of the MID1 mutant del1313TGAC did not enhance AR activity beyond basal levels (Fig. 2A).

To find out if MID1 influences AR activity via elevated protein levels, we co-transfected the same plasmids used for CAT reporter assay in PC-3 cells and analyzed AR protein levels by Western Blot. Increased AR protein levels were detected after co-transfection of wild type MID1 with and without androgen (R1881) addition, but not after co-expression of mutant MID1 (Fig. 2B).

Since MID1 overexpression increased the protein level of AR and because MID1 also binds specifically to the purine-rich repeats regions in AR mRNA (Fig 1C), we tested if, when MID1 is co-expressed, these regions could also increase the expression from a luciferase-reporter when introduced into its 3'UTR. There was a clear increase in luciferase activity in PC3 cells when either the polyCAG or the polyGGY regions of AR mRNA were introduced into the 3'UTR of the luciferase reporter gene. To show that these effects were caused by improved translational efficiency and not due to enhanced transcription/mRNA stability, the luciferase activities were normalized to luciferase mRNA levels.

To determine if loss of MID1 or of its direct binding partner $\alpha 4$ has abrogating effects on endogenous AR protein levels, knockdown experiments were performed in DuCaP cells, a prostate carcinoma cell line expressing high levels of endogenous AR. Consistent with our previous results, siRNA oligonucleotides directed against MID1 or $\alpha 4$ dramatically reduced AR protein levels (Fig 3A). The same could be shown in another AR-positive prostate cancer cell line (LNCaP) and with other AR-specific siRNAs (Fig 3B, Suppl. Fig 1A-C)). Determination of AR-mRNA levels with qPCR during knockdowns in LNCaP cells showed that AR-mRNA levels are not significantly influenced by MID1/ $\alpha 4$ (Suppl. Fig 1D). Furthermore, overexpression of MID1 in LNCaP cells does also not lead to significant changes in AR mRNA levels (Suppl Fig 1E), confirming the impact of the MID1 complex on AR translation rather than on RNA-transcription regulation.

AR mRNA and protein stabilities are not influenced by MID1.

Our data show that MID1/ $\alpha 4$ regulate AR protein levels. Despite having ruled out major transcriptional effects this could still be caused by alterations in AR mRNA or protein stability.

To analyze AR mRNA stability, knockdown of LNCaP cells with MID1, α 4 or non-silencing siRNA oligonucleotides in the presence of the transcription inhibitor actinomycinD showed that the MID1 protein complex had no effects on AR mRNA stability (Fig 4A). Additionally, AR mRNA stability was unaltered in OS patient cells with non-functional MID1, compared to control cells, upon actinomycinD treatment (Fig 4B).

To analyze the influence of the MID1 complex on AR protein stability, AR degradation was quantified by western blot in LNCaP cells that were treated with the translation blocker Cycloheximide after α 4 knockdown (Fig 4C) or after MID1 overexpression in PC3 cells with ectopic expression of AR (Fig 4D). Calculation of AR-protein half-life did not show significant differences in AR-protein stability between control and α 4 knockdowns and MID1-overexpression did not lead to a stabilization of AR-protein in PC3 cells, again corroborating that the MID1/ α 4 protein complex regulates AR protein levels neither by alterations in mRNA levels nor of mRNA or protein stability, but by influencing the translation efficiency of AR mRNA.

Androgen binding is clearly reduced in OS patients.

The impact of MID1 on the levels of AR prompted us to analyse the activities of AR in cells from Opitz BBB/G patients that lack functional MID1. Scatchard Plot analysis in OS infantile primary foreskin fibroblasts allowed determination of maximal specific androgen binding, reflecting AR level. Indeed, androgen-binding capabilities of two OS patients (patient 51, expressing the MID1 A130T mutation and patient 14, carrying a splice site mutation in MID1) compared to age-matched control cells were dramatically reduced (Fig 5A). To confirm the results observed in OS patient cells, AR activity was analyzed by AR-dependent reporter gene assays in PC-3 cells in an analogous experiment as shown in Fig 2A, but transfected with vectors expressing either wild-type MID1 or MID1 harboring the same mutations found in the two respective OS patients. Remarkably, AR activity was diminished in a similar manner as androgen binding in the OS patients, confirming that dysfunctional MID1 results in reduced AR activity (Fig 5B).

Reciprocal feedback regulation of MID1 and AR.

Previous microarray data performed by our group showed MID1 to be negatively regulated by androgens in various AR positive prostate cancer cell lines (unpublished data). To specify

these findings, MID1 protein levels were measured at several time points in the AR-positive prostate cancer cell line DuCaP after treatment with synthetic androgen R1881. MID1 protein levels clearly decreased after 24 h, 48 h, and 72 h (Fig 6A). Next, we tested whether androgen treatment of DuCaP cells also influences MID1 mRNA levels. Consistent with our previous microarray data, MID1 mRNA levels were significantly decreased after 24 h and 48 h (Fig 6B). Considering these results, we hypothesized that MID1 could be feedback regulated by the AR.

To address the potential inhibitory function of the AR on MID1 expression, we performed chromatin immunoprecipitations (ChIP) sequencing using DuCaP cell lysates and found several AR binding sites (AREs) in both the MID1 promoter region and within intron regions (Fig 6C). Two of these MID1-AREs, one in the distal promoter region and one in an intronic region of MID1, were selected for further investigation. Independent ChIP experiments performed upon androgen stimulation resulted in the enrichment of both AR binding sites in the ChIP samples, precipitated using an AR antibody at different time points (Fig 6D).

To confirm our findings, luciferase reporter vectors were constructed including the sequences of these two selected binding sites. Consensus AREs in the two binding sites were identified *in silico* using the online web-based tool Math Inspector. One consensus sequence in the 105 kb distal promoter and two consensus sequences in the intronic region were determined. These consensus sites were mutated using site-directed mutagenesis. DuCaP cells transfected with reporter plasmids containing the wild type MID1-AREs showed increased luciferase activities upon androgen stimulation, whereas transfection of reporter vectors with mutated MID1-AREs abrogated the effect resulting in counts comparable with control levels (Fig 6E), thus confirming the functionality of AR-binding on these two selected regions. Taken together, our results suggest that MID1 is an AR target gene and is negatively regulated by androgens. Given the critical role of AR activation in prostate cancer initiation and progression, negative feedback regulation of MID1 by the AR may be an important and effective way in order to strictly control AR signaling.

MID1 is highly expressed in prostate cancer tissues.

To further address the potential role of MID1 in prostate cancer, we evaluated MID1 and AR expression levels by immunohistochemistry (IHC) in prostate cancer specimens. A statistically significant positive correlation between MID1 and AR expression levels was found (Fig 7A) ($R=0.246$ and 0.263 according to the Pearson and Spearman correlation

analysis, respectively, $p=0.01$). Although the expression pattern of MID1 was heterogeneous, some trends were obvious: in the non-malignant benign parts of the specimens MID1 expression was mainly stromal, consistent with minimal AR expression in these cells (Fig 7C, first panel). MID1 expression was enhanced in tumor areas in correlation with increasing Gleason grade of the tumors (Fig 7B and 7C second panel compared to third panel). MID1 expression level was significantly increased not only in cancer compared to benign but also increased in high compared to low Gleason score tumors (Fig 7B). Notably, high MID1 expression was especially correlated with a histological tumor pattern referred to as cribriform. When the cancer samples were categorized to cribriform and non-cribriform sets, MID1 expression level was significantly higher in the cribriform sample set (Fig 7B). Furthermore, highest expressions levels in cribriform pattern tumors was confirmed with lymph node metastases (Fig 7D), although the number of lymph node samples available for the study was too small for a statistical analysis. Taken together, our results suggest modified MID1 expression in prostate cancer along with high AR expression levels, reflecting one mechanism that contributes to prostate cancer progression.

DISCUSSION

In this study, we identified the MID1 complex as a physiologically important mediator of AR signaling, which acts through the newly identified mechanism of enhancing AR mRNA translation. Many studies have shown that the AR pathway is crucial for prostate cancer progression and that this pathway maintains its role even after androgen depletion and development of resistance to therapy. Several mechanisms were proposed to explain the role of AR in this transition to therapy resistance. Increased expression of AR was reported for a vast majority of prostate cancer tissue samples, achieved through mechanisms such as AR gene amplification, which was found approximately in one third of patient samples [22], or post-transcriptional regulation.

Post-transcriptional regulation of AR mRNA is a mechanism not well described in the literature. The AR mRNA 3'UTR has UC rich regions, which are targeted by RNA binding proteins (RBPs). HuR and poly-C binding protein 2 (PCBP2) were reported to bind the 3'UTR of AR and regulate it posttranscriptionally [23, 24]. In addition, poly-C binding protein 1 (PCBP1) was identified as a negative regulator of AR mRNA translation in a 3'UTR-independent manner [25]. Of note, the AR harbors several repeat regions in its 5'translated region, which are principally also good candidates for the binding of RBPs [26]. However, until now no RBPs have been reported to bind these repeats. To our knowledge, we have here identified the MID1/ α 4 complex as the first positive regulator of AR translation, associating with the AR mRNA through these repeat regions. In a recent publication, it was shown that the MID1 complex associates with purine-rich motifs, named MID1-association sequences (MIDAS). In the presence of the MID1 complex, MIDAS-containing mRNAs are translated more efficiently [18]. Furthermore, in a second paper we show that MID1 enhances the translation of mutant huntingtin mediated by its binding to expanded CAG repeat stretches [16]. While wild type huntingtin mRNA that harbors 20 consecutive CAGs shows only weak binding to the MID1 complex, mutant huntingtin with 51 CAGs shows strong binding resulting in mTOR dependent enhanced translation [16]. The CAG repeat stretch present in the AR mRNA (23 CAGs + 6 adjacent CAGs) is shorter than in mutant huntingtin, but the AR mRNA additionally harbors a repeat with 23 consecutive GGYs that supports binding to the MID1 complex and would finally lead to translation enhancement of AR. Here, we show that the MID1 complex binds to the polyCAG and polyGGY repeat regions of AR mRNA and that these repeats when introduced into the 3'UTR of a reporter gene, can increase the translational efficiency from the reporter. Furthermore, enforced expression of MID1 in PC-3 and DuCaP cells dramatically enhances AR protein levels,

whereas siRNA knockdown of endogenous MID1/alpha4 lowers AR expression to almost the same extent as an siRNA knockdown of AR itself. Notably, neither AR mRNA levels nor mRNA/protein stability were influenced by MID1 to a similar extent, confirming MID1's role as a positive translational regulator.

Mutations in the MID1 gene cause a developmental syndrome called Opitz G/BBB syndrome [27]. Hypospadias is a predominant phenotype of this disease, which is also a common symptom in syndromes of partial androgen receptor defects like Reiffenstein syndrome. This suggests a functional link between MID1 and AR and made us hypothesize that MID1 mutations in Opitz patients might affect AR activity. Indeed, we could show that AR ligand binding activity was decreased to variable degrees in Opitz patients with different MID1 mutations. These findings suggest that the defects in the development of the external male genitalia of Opitz patients are associated with and are probably caused by reduced AR levels caused by compromised MID1-dependent translation of AR mRNA.

Mechanisms that regulate AR levels are intensively studied due to the crucial role of AR in prostate development and maintenance. One mechanism reported in the literature is auto-regulation of AR by androgens. In this setting, AR down-regulates its own transcription by binding putative AREs inside the AR gene when it is bound to androgen [28-31]. In a recent report, the responsible ARE in the AR gene was located to a highly conserved site in the second intron. In response to androgen, liganded AR recruited lysine-specific demethylase 1 (LSD1) to this site to reorganize chromatin structure, leading to activation of a negative feedback to regulate AR activity by directly inhibiting AR transcription [32]. In our study, we propose a novel regulatory loop between MID1 and AR (Fig. 8). While MID1 acts as a translational inducer of AR protein, AR in turn decreases MID1 levels in response to androgen stimulation. Therefore, AR maintains its own levels in equilibrium through MID1. A similar negative regulation between PMEPA1 and AR was proposed by Li et al. via a proteasome-dependent mechanism [31].

We identified several AR binding sites in the MID1 gene by ChIP-Seq analysis. Consistent with current understanding [22], AREs of the MID1 gene are located either within distal promoter regions or in intronic regions. Although we confirmed the functionality of these putative AREs within selected AR binding sites, the negative effect on gene expression, as observed for MID1, could not be shown with the reporter assays used. This indicates the involvement of sequence elements other than AREs or maybe combinations of different AREs that are necessary to assemble an inhibitory AR complex and provide negative

regulation. Commonly, transcription factor binding sites for SP1, AP1, GATA2, Oct1, ETS, FOXA1 are enriched around AREs to fine tune AR regulated transcription [22, 33, 34]. Further studies are obligatory to propose a model in which AR decreases transcription and which other factors are involved in the negative regulation of MID1 with androgen. One possible candidate responsible for AR/androgen-dependent repression of MID1 could be the known AR-co-repressor “small heterodimer partner (SHP)”. Interestingly SHP is involved in several of the pathways that could be correlated with the MIDAS-dependent translational regulations of MID1, such as lipid metabolism, glucose homeostasis, proliferation and migration/angiogenesis [18, 35].

The regulatory impact of MID1 on AR and its possible implications in PCa suggested a role of MID1 expression in development and progression of PCa. Our IHC results with samples of prostate cancer tissues revealed that MID1 is mainly stromal in benign tissue areas. Interestingly, in cancerous tissue the MID1 expression shifted to the epithelial compartment and was excessive in higher-grade tumors and metastatic lesions of prostate cancer. This shift may be due to alterations of the regulatory loop between AR and MID1 which could be correlated with the reported stage-dependent reductions in androgen levels in PCa tissues [36]. It is a well-established fact that during androgen ablation therapy, tumors adapt to low androgen environment by changing AR mediated growth response. MID1 expression is reduced in case of normal androgen levels, whereas androgen ablation relieves the negative feedback on MID1 and leads to increased MID1 levels, which in turn could enhance AR levels via a translational mechanism. This scenario would suggest a role of disrupted MID1-AR equilibrium in the progression of prostate cancer to an androgen ablation therapy resistant stage.

In addition, MID1 seems to positively modulate other important oncogenic players, like PDPK1, which is a crucial kinase in the PI3K pathway and a novel promising target for anticancer drugs – its mRNA harbors a MIDAS motif in its 3'UTR, which triggers MID1-dependent enhanced translation [18]. Notably, PDPK1, besides its oncogenic potential, promotes EMT in cardiac development, a process heavily affected by mutations in MID1 [7]. Furthermore, PDPK1 has an essential role in the promotion of cell migration [37], in part by upregulating MMP14 [38]. As MMP14 also harbours a MIDAS motif, the latter suggests a MID1/PDPK1-driven feed-forward regulation of the expression of this pro-metastatic matrix metalloproteinase. Interestingly, elevated levels of MMP14 have also been observed during PCa progression into androgen independency [39]. The PI3K/PDPK1/AKT pathway is crucial for prostate cancer progression and a recent study showed a reciprocal regulation between

the AR and the AKT pathway. This finding suggests that the failure of androgen ablation therapy might be partially due to the induction of the PI3K/PDPK1/AKT survival pathway in the absence of androgens [3]. Ablation of androgens that increases MID1 levels, would in turn lead to the enhanced translation of PDPK1, the upstream activator of AKT. Interestingly, pharmacologic inhibition of AKT and AR hamper androgen-independent growth of LNCaP cells. Thus, the MID1 complex may be a promising therapeutic target, because it can modulate the major players (PDPK1 and AR) of both proliferation/survival pathways on a translational level in the absence of androgens. Interestingly, the knockdown of AR was reported to lead to the induction of cyclin D1 in prostate cancer cells [40], which would be in line with the effects of microtubule-associated PP2A modulation on cyclin D1 regulation by the sonic hedgehog transcription factor GLI3 [13], as ablation of androgen signaling, according to our results, would lead to an increase of MID1, finally resulting in a decrease of PP2A.

The anti-diabetic drug metformin was recently identified as a disruptor of the MID1 protein complex [41], leading to accumulation of PP2Ac. The growing evidences that metformin treatment is correlated with a reduced incidence of various cancers including prostate cancer would thus be in line with the regulatory role of MID1 on AR protein levels presented here. Furthermore, the observed accumulation of PP2A caused by the disruption of the MID1 complex through metformin, which normally exerts E3 ubiquitin ligase activity towards the catalytic subunit of PP2A to target it for proteasomal degradation, could have a repressive effect on androgen synthesis, as PP2A is a strong negative regulator of cytochrome P450c17 and 17,20 lyase activity, both of which are important co-factors during androgen synthesis. In view of the findings that local androgen synthesis is a major driver of AR in castration resistant prostate cancer this would enhance the contribution of MID1 to tumor progression [42]. Interestingly, cytochrome P450c17 (CYP17A1) activity is also targeted by abiraterone, an approved drug used for the treatment of castration-resistant PCa (Fig. 8). Finally, it was recently reported that metformin negatively regulates NF κ B signalling [43], which would also be in line with its inhibitory impact on the MID1 complex [41], which has recently been shown to promote inflammation via NF κ B activation[8].

Interestingly, the benefits seen with metformin in treatments of polycystic ovary syndrome (PCOS), a condition partially caused by increased androgen signalling [44], could also be explained by its inhibitory effect on MID1, that causes reduction of androgen receptor translation and concomitantly reduction of androgen synthesis via increase of PP2a.

The awareness of MID1 as an essential regulator of a pool of specific mRNAs involved in cancer development and progression is a novel concept. Three translational studies support such a role of the MID1/ α 4/PP2A complex: (i) a study on colorectal cancer, pointing towards MID1 as a metastatic gene and marker for poor survival [45], (ii) a second study, which demonstrated that α 4, the direct binding partner of MID1, is expressed universally in advanced lung adenocarcinomas and that its overexpression is significantly related to outcome [46], (iii) and a third study, which shows that the expression and activity of PP2A is down-regulated in androgen-independent prostate cancer cells [47]. Finally, our present findings and recent reports regarding the crosstalk between MID1/ α 4 and Akt/mTOR, [15, 18, 41], two master regulators of essential pathways in proliferation, development, energy balance and immune responses, as well as MID1's roles in NF κ B activation [8] and promotion of Hedgehog signaling[13] stress the multifaceted nature and therapeutic potential of the MID1 complex.

EXPERIMENTAL PROCEDURES

Cell culture procedures and antibodies

DuCaP and PC-3 cell lines were cultured in RPMI 1640 medium supplemented with 5-10% FBS 1% Glutamax and 1% penicillin/streptomycin (P/S) LNCaP cells were cultured in MCDB-131 medium supplemented with 10% FBS, 10 mM Hepes, 0.45% glucose, 1% sodium-pyruvate and 1% P/S. Cells were maintained at 37°C and 5% CO₂. The concentration of reagents used for in vitro experiments was: synthetic androgen R1881: 1 nM, anti-androgen Bicalutamide: 2.5 µM, Cycloheximide: 80 µg/ml and ActinomycinD: 5 g/ml. For transfection of expression plasmids Attractene (Qiagen) was used for PC-3 cells, Lipofectamine (Invitrogen) for LNCaP and Nanofectin (PAA) for DuCaP and PC-3 cells. Antibodies for western blot, immunoprecipitation and ChIP were obtained from Biogenex (anti-AR mAb), Chemicon (anti-GAPDH mAb), Cell Signaling (3202, anti-AR pAb) and from Millipore (UB 06-680, anti-AR pAb), Stratagene (anti-FLAG) Abcam (anti-a4 pAb) and from Atlas Antibodies (anti-MID1, pAb).

Constructs

Several MID1 variants (wild-type MID1, MID1 Ala130Thr, MID1 del1313TGAT and MID1 IVS8 open reading frames) were inserted into the multiple cloning site of the pCMV-Tag2C vector (Clontech) using EcoRI and HindIII. For investigating the influence of the polyQ and polyG regions of AR mRNA on mRNA translation these region were PCR amplified and inserted into the 3' UTR of the luciferase reporter gene in a pGL3 luciferase reporter plamid (Promega).

For CAT reporter gene assays AR expression vector AR-pSG5, reporter plasmid (ARE)2-TATA-CAT and fill-up plasmid pHRL-Null were used, for Dual-Luciferase Reporter Assays the reporter plasmid was replaced by plasmids constructs of the pGL3 luciferase promoter vector (Promega) and the the renilla firefly expression plasmids pGL4.70 or pGL4.73 were added for normalizing transfection efficiency... Luciferase reporter plasmids containing MID1 105 kb promoter and intronic AR binding sites were constructed by inserting the PCR-amplified AR binding site fragments into the pGL3-promoter vector. The consensus AREs in these AR binding fragments were mutated using the QuickChange II Site-directed Mutagenesis kit (Agilent).

Primers

Primers used for amplification of AR binding site fragments and primers for site-directed mutagenesis

105 kb promoter binding site:

F: 5' AGGAGTGACAGCAATGATTCAGGG'3,

R: 5' AGTGCACACATGTGCCAGACCC'3;

ARE Mutagenesis:

F: 5`CCTTAAGTATGGAGATGAGGTTAGGCCTTGATGTTGCCCTAGGC'3,

R: 5` GCCTAGGGCAACATCAAGGCCTAACCTCATCTCCATACTTAAGG'3.

Intronic binding site:

F: 5'TCTCCAGCCTCCTGGCTCACCTA'3,

R: 5' AGTTGACAAAGCCAGGGTGCCC'3;

ARE1 mutatenesis:

F: 5`TCTCTGGGCTTTTGCCGATGCTAGGCCTTCTGCATGGA'3,

5`TCCATGCAGAAGGCCTAGCATCGGCAAAAGCCCAGAGA'3,

ARE2mutagenesis:

F: 5`TTCTGCATGGAGACTGTAGGCTCACATCCCCTGCTCC'3

R: 5` GGAGCAGGGGATGTGAGCCTACAGTCTTCCATGCAGAA'3

Immunoprecipitation

4x10⁶ HeLa cells overexpressing FLAG-MID1 were lysed in TKM buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM MgCl₂), supplemented with proteinase inhibitors and 1% NP40, incubated for 15 min on ice and passed 6 times through a 27³/₄ gauge needle and centrifuged for 15 min at 12000 x g at 4°C. 4 mg of cytosolic extract were precleared with 50 µl of protein-A/G agarose (Roche) and 10 µg of mouse IgG for 2h at 4°C on a rocking platform. Beads were pelleted and discarded. The supernatant was immunoprecipitated with 50 µl of anti-FLAG M2 affinity gel (Sigma-Aldrich) over night. As control, the IP was carried

out with mouse IgG. Agarose beads were pelleted and washed 3 times with 500 µl TKM buffer supplemented with 0.2% NP40 for 10 min at 4°C. Bound proteins were finally eluted for 1 h with 30 µl of 3x FLAG peptide (5 mg/ml), diluted with 200 µl TKM-buffer and 1 µl RNasin. 50 µl were directly analyzed by Western blotting. From the remaining elution fraction bound RNA was isolated by phenol/chloroform extraction followed by EtOH precipitation. cDNA was synthesized using random primers and subsequently used for RT-PCR with AR (F: CTTCTGCACGAGACTTTGAG, R: CTGAAGGAGTTGCATGGTG), PFDN5 and TMSL8 (negative control) specific primers.

Real-time PCR

To quantify mRNA levels, a one-step real-time PCR method was used, which combines reverse transcription with real-time PCR (Qiagen). Taqman probe-set containing both gene-specific primers and probe for MID1 and housekeeping gene GAPDH were used for amplification and detection on an ABI 7500 Fast PCR machine (Applied Biosystem).

RNA-Protein binding assay

Amplification of different AR mRNA fragments: All forward primers contained the T7 promoter sequence (5'-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3') to allow subsequent in vitro transcription of the PCR product. PCRs were performed under standard conditions using pSG5-AR plasmid as template and gene region-specific primers for the PCR reactions. Amplified transcripts were in vitro transcribed with the RiboMAX™ Large scale RNA production system-T7 (Promega), following the manufacturer's instructions with some modifications. Briefly, 4 µg of purified PCR product was transcribed for 4 h at 37°C in the following reaction mixture: 4 µl T7 transcription buffer, 6 µl rNTPs (25 mM rATP, rGTP, rCTP, 1,6 mM biotin-rUTP, 2,5 mM UTP), 4 µg PCR template, 2 µl RNA polymerase enzyme mix, DEPC-H₂O up to 20 µl. Transcribed RNA was purified and products were kept in nuclease free TE.

RNA-PROTEIN binding assay: 2 µg of biotinylated and purified RNA were incubated with 150 µg of cytosolic protein extract from HeLa cells overexpressing FLAG-MID1 in 450 µl of TKM buffer for 1 hour at 4°C. Subsequently, the mixture was incubated for 2 h at 4°C with 40 µl of 50% slurry of M280 streptavidin coated magnetic beads. Beads were washed 3 times with TKM buffer for 10 min at 4°C. Bound proteins were eluted by boiling the beads in magic

mix (2x: 48% urea, 15 mM Tris-HCl pH 7, 8.7% glycerol, 1% SDS, 143 mM mercaptoethanol) for 10 min at 95°C and analyzed on western blots using the respective antibodies.

Luciferase Reporter gene assays

Reporter gene assays were accomplished according to the Promega Dual Luciferase Reporter gene assay protocol and luciferase activity was measured in a Chameleon V plate reader (Hidex). Cells were transfected with 0.25 µg DNA/well in 24-well format and 0.08 µg DNA/well in 96-well format respectively and kept in medium with 10% charcoal stripped FCS for 24 hours after transfection and treated with 1 nM R1881 for additional 24 hours before luciferase activity was measured. For polyQ/polyG Luciferase Reporter gene assays 0.15 µg reporter vector DNA/well were used to transfect 11,000 PC-3 cells/well in a 96-well format. Cells were kept in RPMI medium with 5% charcoal stripped FCS for 24 hours after transfection and treated with 1 nM R1881 for additional 24 hours.

The polyQ and polyG regions of AR mRNA inserted into the 3' UTR of the luciferase reporter gene were: gb/M23263.1 nt633-nt816 (polyQ) and gb/HM010955.1 nt2448-nt2616 (polyG)

siRNA transfection

Cells were transfected with 20 to 40 nM final concentration of MID1, α 4, AR or control siRNA oligonucleotides using Lipofectamin 2000 or Nanofectin. AR siRNA: 5'-GAAAGCUCCUCGGUAGGUC-3'; α 4-4 siRNA: 5'-UUGAGAUGCCAUAGCAACGAG-3'; MID1-3 siRNA: 5'-GUGUGAUACUAGGAUGCGG-3'; ns siRNA: 5'-AAGAGGCUUGCACAGUGCA-3' (Dharmacon or MWG). After 48 h of incubation the medium was replaced and after 72-120 h cells were harvested and analyzed by western blotting or real-time PCR.

Immunohistochemistry

Paraffin-embedded primary tumor and lymph-node metastasis specimens were obtained from previously untreated patients who had undergone radical prostatectomy at the Department of Urology, Innsbruck Medical University. Use of patients' samples was approved by the ethics committee at the Innsbruck Medical University. Tissue microarrays

(TMAs) were produced from the tumor specimens using a manual tissue arrayer. Immunohistochemistry (IHC) analysis was performed with 5 µm TMA sections employing the Ventana Discovery - XT staining automat (Roche, Mannheim, Germany). Standard CC1 (Tris-borate pH= 7,8, 48 min at 98°C) pre-treatment for antigen retrieval was followed by incubation for 1 h with a polyclonal anti-MID1 antibody or a monoclonal anti-AR antibody diluted in antibody diluent, followed by secondary universal antibody solution for 30 min, staining with DAP map kit and counter staining for 4 min with haematoxylin II bluing reagent (all IHC reagents from Roche). Specificity of staining was controlled by including a control antibody (DAKO). Consecutive sections were HE stained or stained for the tumor marker AMACR and the non-malignant benign gland specific marker P63 for correct assignment of non-malignant and cancer areas. MID1 staining was evaluated and scored by a pathologist (G.S) according to the quick score method and AR staining was evaluated by an automated IHC image acquisition and analysis system (HistoQuest, TissueGnostics). This analysis system uses nuclear counterstaining as a reference and calculates intensity and percentage of specific staining of the target antigen as described previously [48].

Chromatin-immunoprecipitation (ChIP)-coupled deep sequencing and PCR

ChIP was performed as previously described [49]. In brief, DUCaP cells were treated with 1 nM R1881 or control vehicle equivalent for 1 h before formaldehyde crosslinking, DNA sonication and immunoprecipitation with 2 combined AR polyclonal antibodies. Normal rabbit IgG was used as control. The antibody-protein-DNA complex was pulled down, digested and reverse cross-linked. DNA was purified with CHIP DNA kit (Zymo Research, Irvine, CA).

AR antibody precipitated DNA samples were used either for deep sequencing or PCR amplification. Preparation of the libraries and sequencing were performed using the Solexa sequencing platform (Illumina) following the manufacturer's instructions. DNAs were also subjected to amplification by primers listed above.

Bioinformatic analyses

Sequencing data were analyzed as previously described [49]. Illumina analysis pipeline software was applied and bases were called by Bustard and aligned to the unmasked human reference genome (NCBI v36, hg18) using BOWTIE [50]. MACS tool was used to identify

AR-enriched regions in a genome-wide manner [51]. Read-data was visualized using a local installation of the Generic Genome Browser (<http://promotion.molgen.mpg.de/gb2/gbrowse/Human/>).

Statistical Analysis

Student t-test was used to compare two groups. For statistical analysis of AR-IHC, samples were categorized based on histological and pathological parameters. One-way Anova with Posthoc Benforoni or Duncan for categories containing more than 2 groups (benign, cribriform and non-cribriform; benign, Gleason score ≤ 6 and Gleason score >6) and Mann Whitney U test for categories containing 2 groups (benign and cancer) were used for statistical analysis. Pearson and Spearmen correlation analysis were used for studying MID1 and AR correlation. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Error bars denote standard deviations except for box blots where they denote minima and maxima.

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FIGURE LEGENDS

Figure 1. AR mRNA associates with the MID1 complex. **(A)** Agarose gel showing RT-PCR products of AR or of two control genes (prefoldin 5, PFDN5; thymosin like 8, TMSL8). mRNA was isolated either from total cell lysate or after immunoprecipitation of the MID1 complex using lysates from MID1-FLAG overexpressing HeLa cells with an anti-FLAG antibody (IP-FLAG) or with unspecific IgGs (IP-IgG). **(B)** Schematic overview of five different regions on the AR mRNA: 5' region: nucleotides (nt) 212-1224, poly(CAG): nt 1160-1446, intermediate sequence: nt 1390-2445, poly(GGY): nt 2382-2652, 3' region: nt 2593-4123. **(C)** Detection of FLAG-MID1 on western blots analyzing AR mRNA association to MID1. *In vitro* transcribed and biotin-labeled regions of AR mRNA were added to lysates of HeLa cells overexpressing MID1-FLAG. Streptavidin coated magnetic beads were used to isolate the RNA/protein complexes. Whole lysate served as control for presence of MID1 and antisense RNA or no RNA were used as negative controls.

Figure 2. AR protein levels and activity depending on MID1. **(A)** AR-dependent CAT reporter gene assay in presence of different androgen (R1881) concentrations. PC-3 cells were co-transfected with plasmids expressing AR and either wild type MID1, mutated MID1 (del1313TGAT) or empty plasmid as control. CAT activity was measured in counts per minute (cpm) by using the reporter gene construct (ARE)₂TATA-CAT. Error bars in barplots denote standard deviation (n=4). **(B)** Western blot analysis detecting AR and GAPDH proteins after co-transfecting PC-3 cells with AR and either MID1, MID1 1313delTGAT or empty vector as control. Densitometric analysis of respective Western blots from several experiments as indicated. **(C)** Luciferase reporter assay showing that the MID1-binding sites on AR-mRNA are responsible for the MID1-dependent translational enhancement of AR: PC-3 cells were transfected with either control reporter vector or a construct with the polyCAG-region or the polyGGY-region of the AR-mRNA cloned into the 3'UTR of the luciferase reporter gene. Luciferase activity was measured and the values relative to the control and normalized to the luciferase-mRNA levels are shown (n=3).

Figure 3. AR-protein levels in response to MID1 or $\alpha 4$. **(A)** Western blot analysis detecting AR and GAPDH proteins in DuCaP cells after knockdown (kd) of MID1 (n=3) or $\alpha 4$ (n=2)

with siRNAs. Non-silencing siRNA served as control. (B) Analogous experiment with LNCaP cells and other siRNAs.

Figure 4. AR mRNA and protein turnover in response to MID1 or $\alpha 4$. **(A)** Real-Time PCR analysis of AR mRNA stability in LNCaP cells treated with non-silencing (ns), $\alpha 4$ or MID1 specific siRNA oligonucleotides. Levels of AR relative to GAPDH mRNA were plotted against time points after ActinomycinD addition. **(B)** Real-Time PCR analysis of AR mRNA stability in OS patient fibroblast cells (OS17/98) and control cell line (C18/98). Levels of AR relative to GAPDH mRNA was plotted against time points indicating ActinomycinD addition (n=3). **(C)** Western blot analysis detecting AR and GAPDH proteins after knockdown of $\alpha 4$ in LNCaP cells. Samples were taken at indicated time points after cycloheximide addition (n=3). Densitometric analysis of western blots is shown (bottom panels). **(D)** Western blot analysis detecting AR and GAPDH proteins after over-expression of FLAG-MID1 (MID1) in PC-3 cells with ectopically expressed AR. Samples were taken at indicated time points after cycloheximide addition. Densitometric analysis of western blots is shown (bottom panels).

Figure 5. Androgen binding is reduced in OS patient cells. **(A)** Determination of maximal specific androgen binding (Bmax) by Scatchard-Plot analysis in foreskin fibroblast cells of two OS patients (patient 51 and 14) and an age-matched control fibroblast cell line shows that the androgen binding capability in the OS cells is significantly reduced. **(B)** AR-dependent reporter gene assays in PC-3 cells. PC-3 cells were co-transfected with plasmids expressing AR and either wild type MID1 or MID1 mutants corresponding to the mutations in OS patients (p.A130T, IVS8) (n=3).

Figure 6. Reciprocal regulation between MID1 and AR. **(A)** MID1 protein levels in response to androgen and anti-androgen treatment. Western blot analysis (upper part) detecting MID1, AR and GAPDH after treatment with the synthetic androgen R1881 or the androgen antagonist bicalutamide at indicated time points. Lower part: bar-graph of densitometric analysis of respective westernblots from 3 experiments. **(B)** MID1 mRNA levels in response to androgen and anti-androgen treatment. Real-Time PCR analysis of MID1 relative to GAPDH mRNA levels in DuCaP cells after treatment with R1881 or bicalutamide. **(C)** The MID1 gene comprises androgen response elements (AREs) inside its promoter and in

intronic regions. Graphical demonstration of AR binding sites determined by Chromatin Immunoprecipitation (ChIP) coupled with sequencing in the AR positive DuCaP cell line. Differential peaks between control and androgen treated panels indicate AR binding to these regions. Arrows show AR binding sites, circles show binding sites selected for functional validation. **(D)** Confirmation of selected AR binding sites in an independent ChIP sample set. Selected AR binding sites (intronic and 105 kb distal promoter sites) were amplified with specific primers and the enrichment of AR bound fragments was determined by Real-Time PCR in ChIP samples in response to androgen (R1881) or vehicle treatment. **(E)** Functionality of AREs in the MID1 gene. AR-dependent luciferase reporter assays in AR-positive DuCaP cells were performed to assess the function of the 105kb distal promoter and the intronic AR binding sites. Reporter vectors carrying either binding site DNA fragments or the binding site sequences with mutations introduced into the predicted AREs were transfected into DuCaP cells and cells were treated with R1881 or vehicle control for 24h. (n=3).

Figure 7. A prostate cancer tissue microarray containing tissue samples from 94 patients with primary prostate cancer (3 cores of cancer and 1 core of benign for each case) were analyzed for MID1 and AR by IHC. **(A)** Correlation of AR and MID1 immunoreactivity is shown. $R=0.246$ according to Pearson and $R=0.263$ according to Spearman correlation analysis being both significant at $p=0.01$ level. **(B)** MID1 expression pattern in different histological and pathological cancer categories. 79 benign and 88 cancer samples were evaluated and included in the analysis. One-way Anova with Posthoc Bonferroni or Duncan was used for categories containing more than 2 groups and Mann Whitney U test for categories containing only 2 groups (benign and cancer). $*p<0.05$, $**p<0.01$, $***p<0.001$. **(C)** Representative images of AR and MID1 immunohistochemistry in low and high grade cancer. Consecutive sections were double stained for AR or MID1, respectively (brown), and the basal cell marker P63 (dark blue). **(D)** Analogous images of tissue sections from two metastases.

Figure 8.

MID1 is an upstream modulator of proliferation. MID1 enhances protein levels of PDPK-1 and AR, resulting in promotion of PI3K and AR signaling pathways. Moreover, PP2A, which inhibits Akt and mTOR signaling as well as androgen hormone production, similarly as the

approved anti-prostate cancer drug abiraterone, is targeted for degradation by MID1, a process that can be inhibited by metformin. Moreover, MID1 can activate the survival factor NF κ B via inhibition of PP2A. Thus, MID1 drives proliferation by enhancing respective proteins and hormones, however, there is a negative feedback loop between MID1 and AR: MID1 enhances the AR post-transcriptionally, which then in the presence of androgens translocates to the nucleus and, acting as transcription factor, negatively regulates MID1 gene expression. Thus, androgen-ablation therapy could lead to an increase in MID1 which could then promote proliferation paving the way for PCa progression.

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Figure 1.

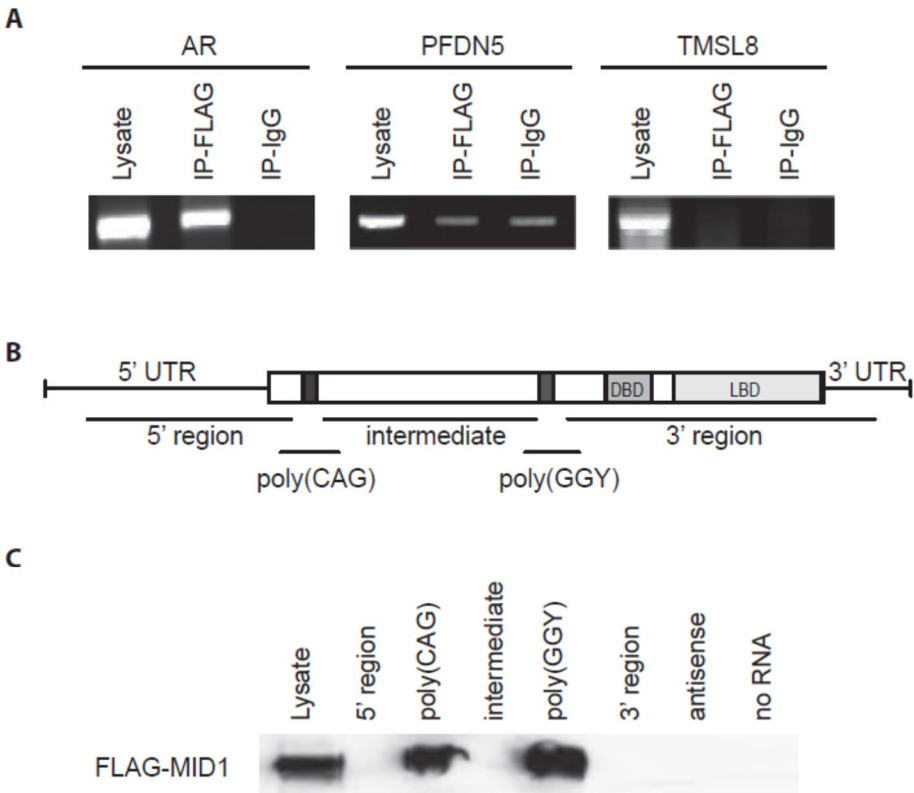
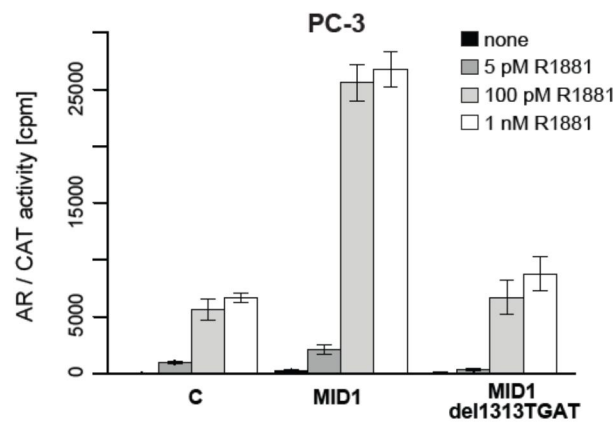
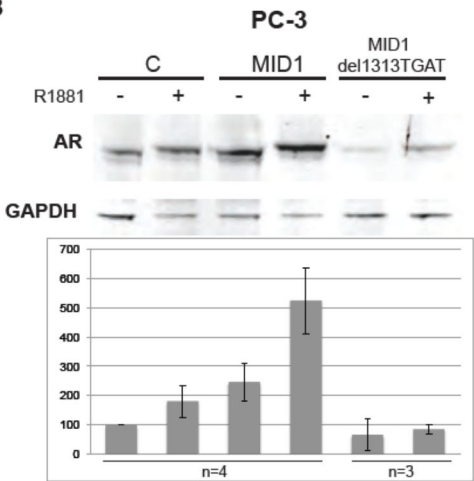


Figure 2.

A



B



C

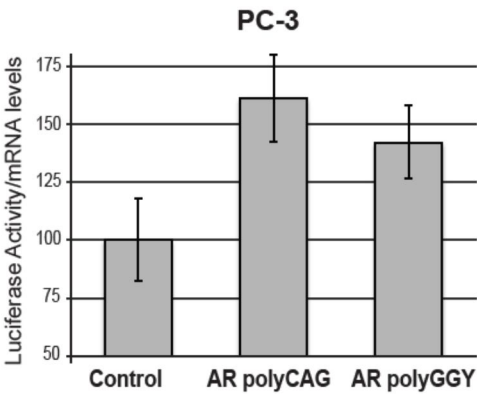


Figure 3.

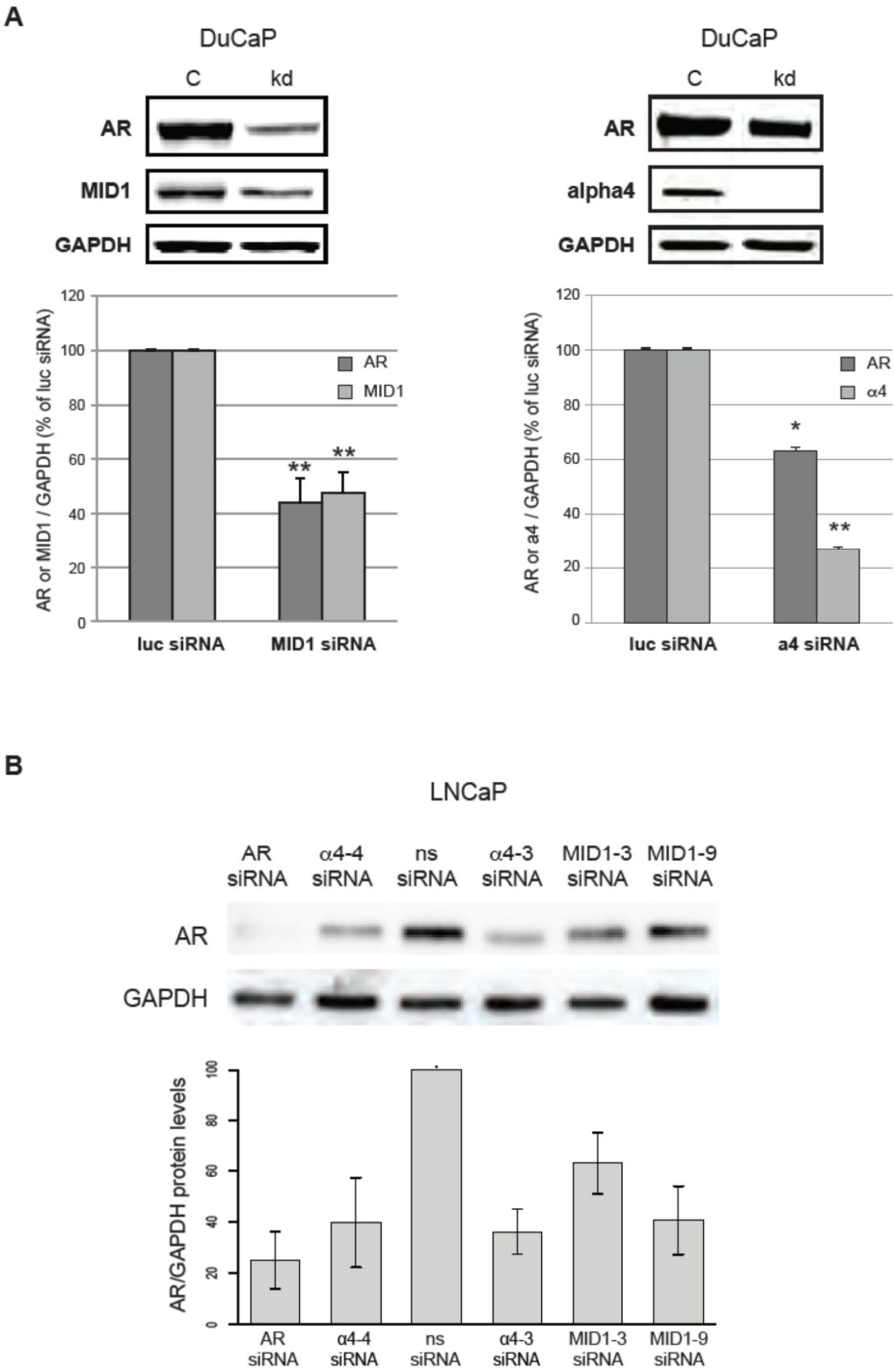


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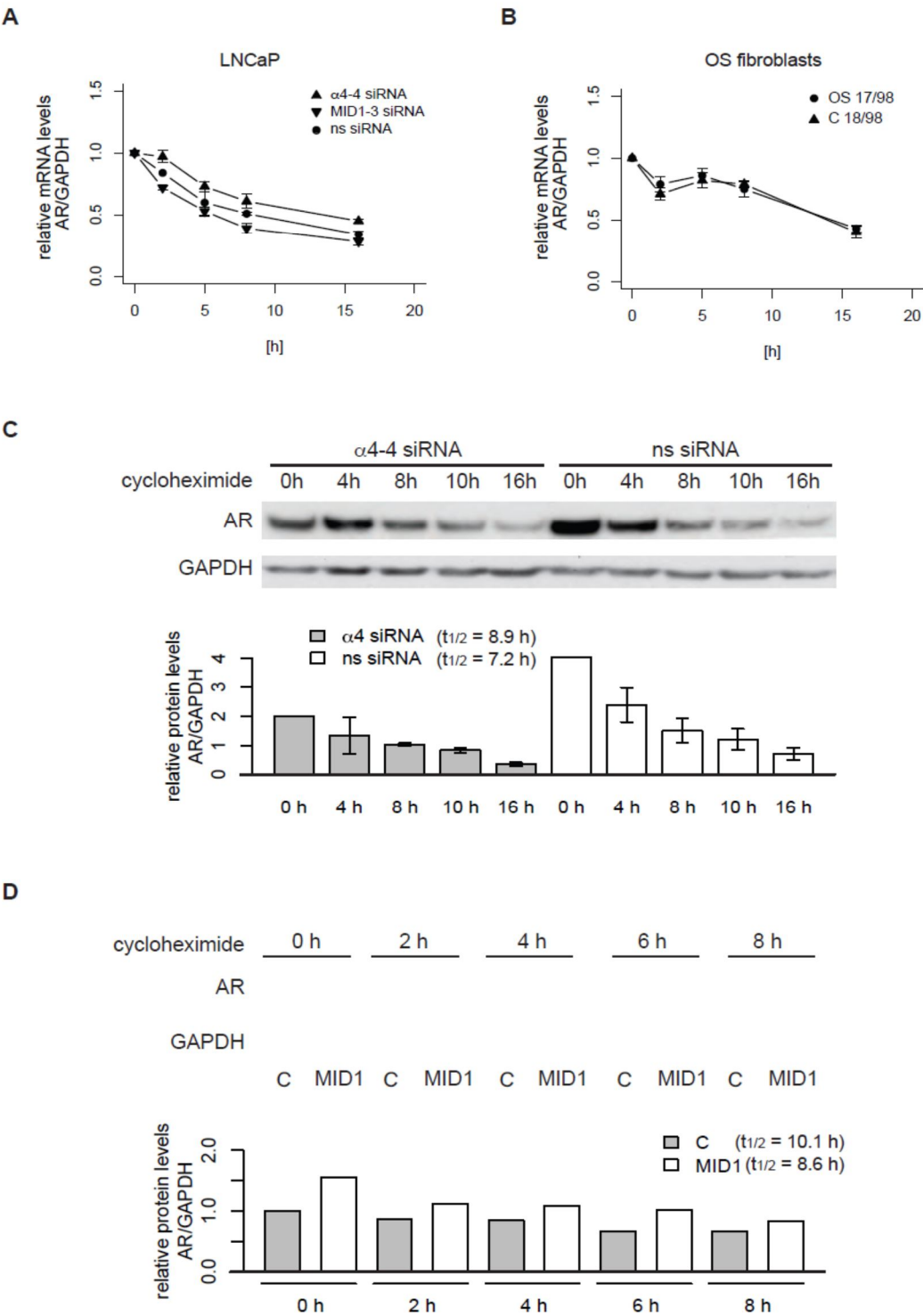


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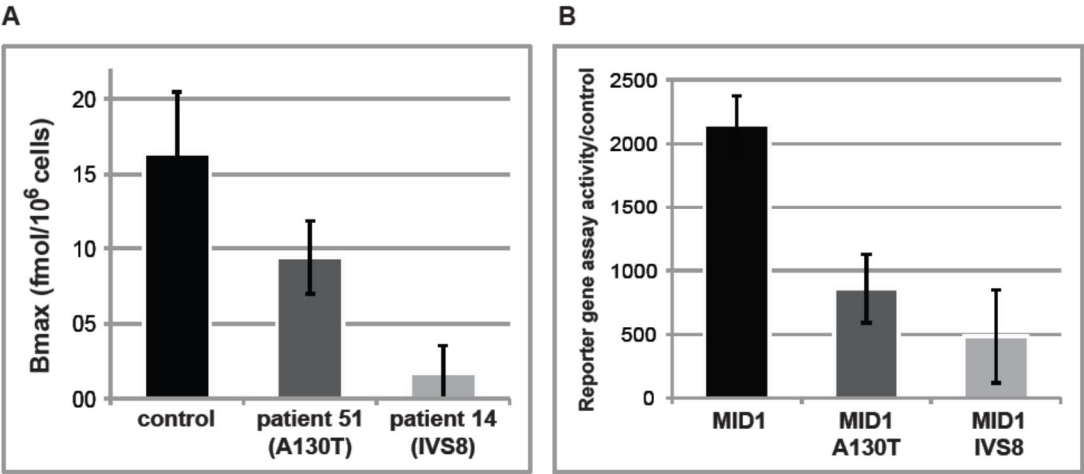


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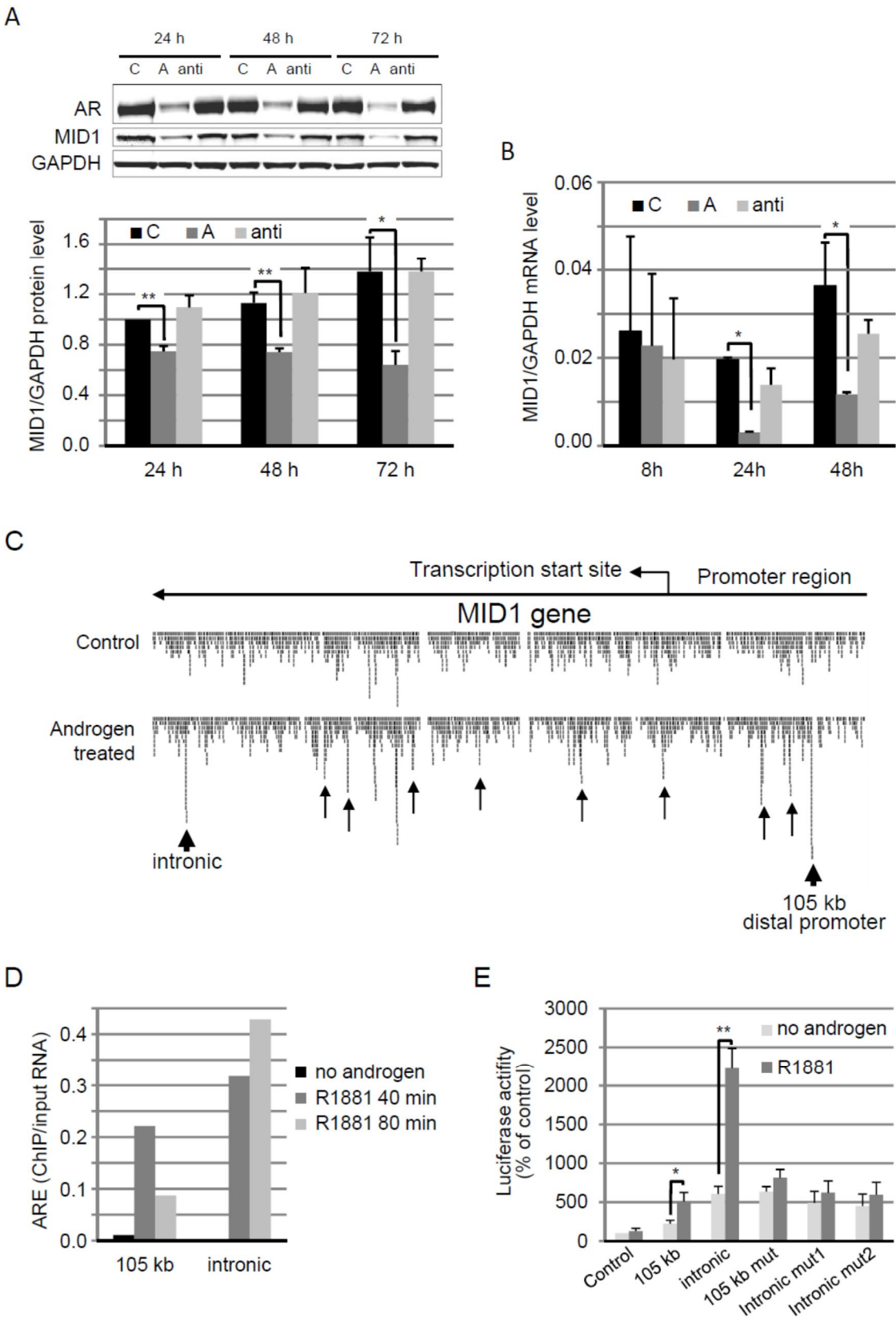


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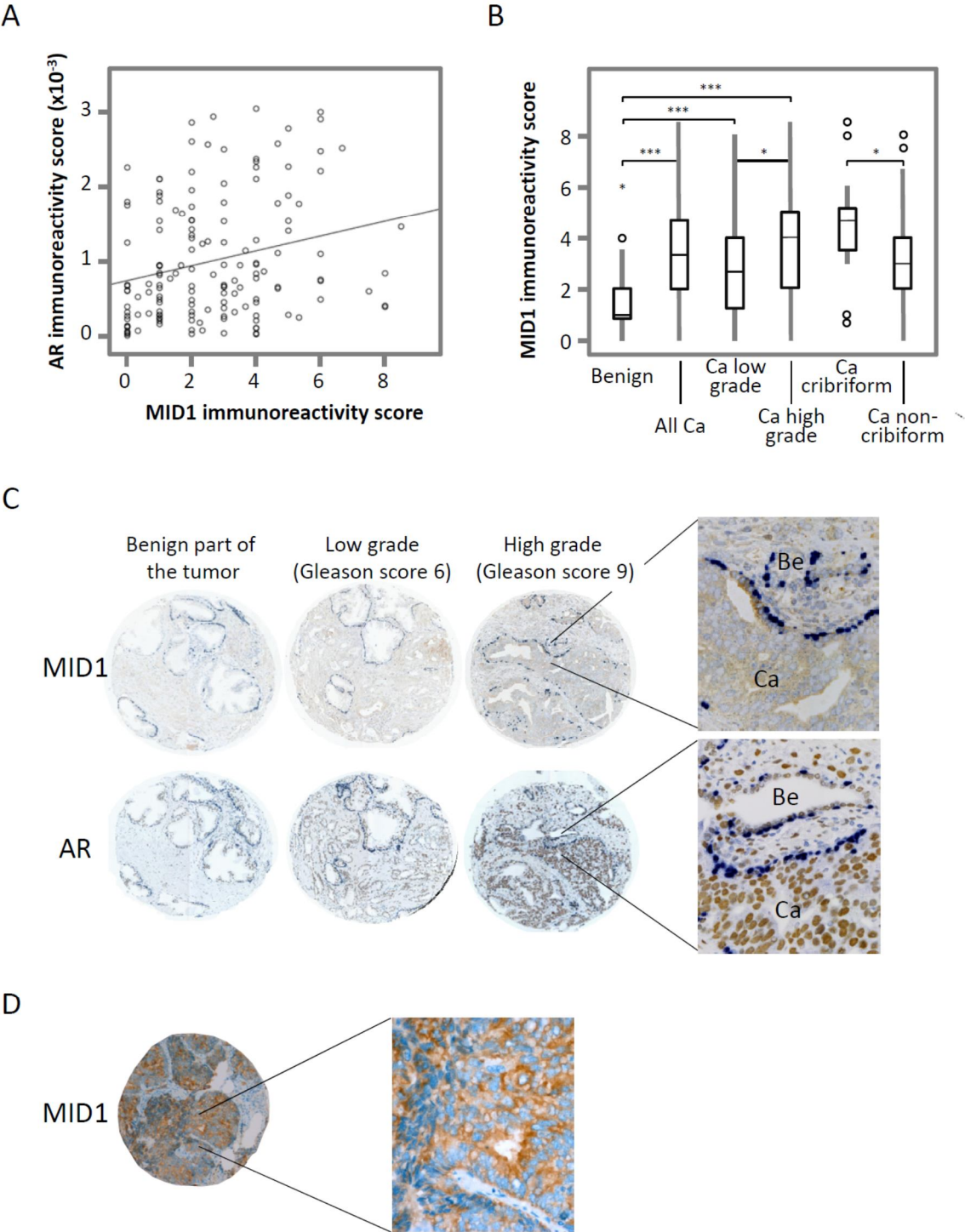
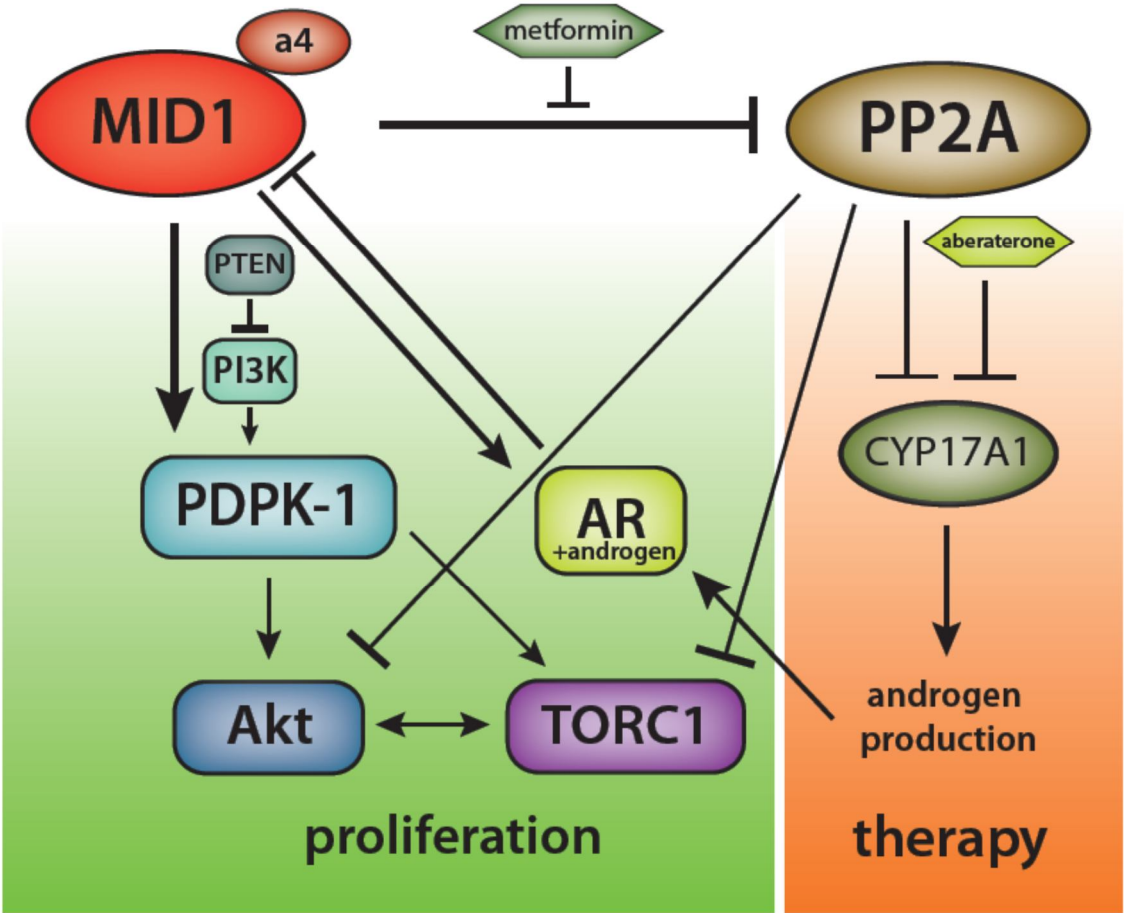
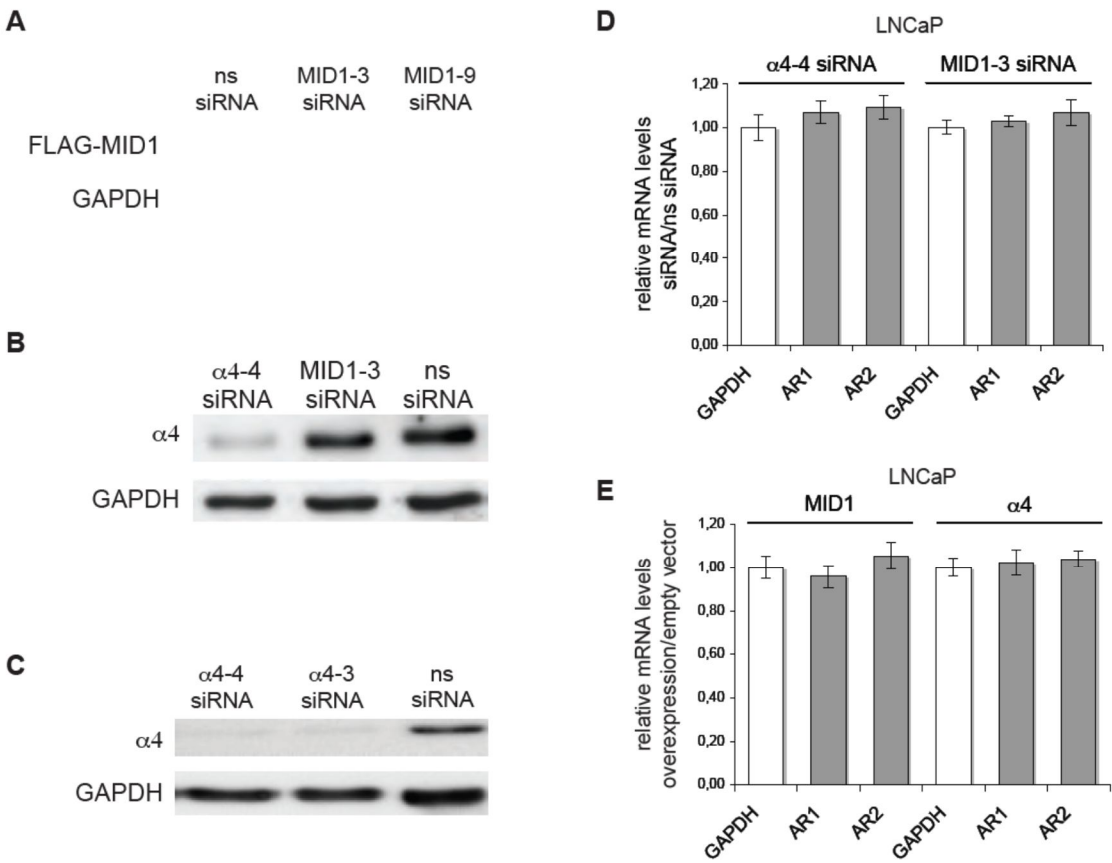


Figure 8.



Supplementary Figure 1.



5.2. Paper 2

Demir U, Köhler A, Schweiger S, Schneider R, Klocker H. **Metformin anti-tumor effect by disruption of the MID1 translational regulator complex and AR downregulation in prostate cancer cells.** Submitted to BMC Cancer, in revision.

Ummuhan Demir

Performed all in vitro experiments including RT-PCR, Westernblots, proliferation assays, CoIP analysis. Involved in planning of the project, data analysis, statistics and manuscript preparation and writing of the first version of the manuscript.

Andrea Köhler, Rainer Schneider and Susann Schweiger

Providing materials and expert opinions. Proofreading of the paper.

Helmut Klocker

Corresponding author. Involved in preparation, organization, collaboration and planning of project, proofreading of the paper.

Metformin anti-tumour effect by disruption of the MID1 translational regulator complex and AR downregulation in prostate cancer cells

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Running title: Metformin inhibition of prostate cancer cells

Key words: Metformin, androgen receptor, MID1- α 4/PP2A protein complex, translational regulation, CoIP.

Abstract

Background:

Metformin is a safe drug prescribed for diabetes. Its role as an anti-cancer agent has drawn significant attention because of its minimal side effects and low cost. However, its mechanism of anti-tumour action has not been fully clarified yet.

Methods:

The effect on cell growth was assessed by cell counting. Western blot was used for analysis of protein levels, Boyden chamber assays for analyses of cell migration and co-immunoprecipitation (CoIP) followed by western blot, PCR or qPCR for analysis of protein-protein and protein-mRNA interactions.

Results:

Metformin showed an anti-proliferative effect on a wide range of prostate cancer cells. It disrupted the AR translational MID1 regulator complex leading to release of the associated AR mRNA and subsequently to downregulation of AR protein in AR positive cell lines. Inhibition of AR positive and negative prostate cancer cells by metformin suggests involvement of additional targets. The activity of metformin was independent of AMPK activation and was mimicked by disruption of the MID1- $\alpha 4$ /PP2A protein complex by siRNA knockdown of MID1 or $\alpha 4$.

Conclusions:

The herein reported findings uncover a mechanism for the anti-tumor activity of metformin in prostate cancer, which is independent of its anti-diabetic effects. These data provide a

rationale for the use of metformin in the treatment of hormone naïve and castration-resistant prostate cancer and suggest AR as an indirect prime target of metformin.

Keywords

Metformin, androgen receptor, MID1- α 4/PP2A protein complex, AMPK, translational regulation, CoIP.

Background

Metformin is a commonly prescribed anti-diabetic drug. Epidemiological studies revealed a link between the use of metformin and a lower risk of several cancers, such as those of the breast, lung, colon and prostate [1, 2]. On the other hand, a recent meta-analysis denied an influence of metformin on prostate cancer risk [3]. Despite these ambiguous data metformin inhibits many tumour cells in-vitro, among them prostate cancer cells [4] and a number of clinical studies have been initiated to test the therapeutic efficacy of metformin in different cancer entities. Metformin targets several tumor-associated pathways [5, 6], however, the mechanism of its anti-cancer activity is not fully understood yet.

In diabetic patients, metformin reduces hepatic glucose production by inhibiting gluconeogenesis. This effect is mainly achieved by the inhibition of the mitochondrial respiratory chain I complex. This reduces the ATP/AMP ratio, which in turn activates AMPK to inhibit gene expression of gluconeogenesis enzymes and fructose-1,6-biphosphatase activity to terminate gluconeogenesis. In addition, activation of AMPK also shifts cells from an anabolic to a catabolic state by inhibiting protein, glucose and lipid synthesis, and inducing glucose uptake through the glucose transporters GLUT1 and GLUT4 [7].

The role of AMPK activation by metformin for its anti-cancer effects is discussed controversial. AMPK for example inhibits mTOR a key player in the tumour-promoting PI3K-Akt-mTOR survival pathway [8] and up-regulates the p53-p21 tumour suppressor axis [9]. Studies in prostate cancer models provided contradictory results. On one hand inhibition of AMPK was reported to accelerate cell proliferation and promote malignant behaviour of tumour cells suggesting a tumour-suppressive activity [10]. On the other hand, increased

AMPK activation through overexpression of its activator calmodulin kinase kinase was found in prostate cancer tumours and stimulated growth and malignant properties of tumour cells [11, 12].

Recently Kickstein et al. studied the action of metformin on tau phosphorylation in Alzheimer's disease [13]. The authors showed that metformin disturbs the assembly of the proteins midline-1 (MID1) and the regulatory ($\alpha 4$) and the catalytic subunits of protein phosphatase 2A (PP2A), which, together form a microtubule-associated ribonuclear protein complex [14]. Through the ubiquitin ligase activity of MID1 this complex acts as a negative regulator of protein phosphatase 2A (PP2A) by mediating its degradation [15]. Disruption of the MID1- $\alpha 4$ /PP2A complex by metformin thus leads to increased PP2A activity. Due to the tumour-suppressive function of PP2A acting as an antagonist of protein kinases this may be relevant for the anti-tumour effects of metformin [16, 17]. Loss of MID1 function through mutations and subsequent overactivation of PP2A is found in Opitz G/BBB syndrome (OS) that is characterized by defects of midline organ development, e.g. heart, lip, palate, anus, and male urethra [15, 18].

In addition to regulation of the PP2A phosphatase, the MID1- $\alpha 4$ /PP2A complex also acts as a translational enhancer of mRNAs associated with it [19, 20]. Disruption of the complex by metformin should affect translation of associated mRNAs, which bind through specific G-rich motives and are transported to different cellular locations [14, 19]. As an example, huntingtin mRNA harbouring an extended CAG repeat is associated with and translationally-regulated by the MID1 complex [20].

The anti-tumour functions of PP2A and associated mRNAs anticipate a regulatory role of the MID1 complex in cancer as well. In colorectal cancer a comparative study identified MID1

as one member of a 5-gene signature associated with lymph node involvement and overall survival [21]. With relevance to prostate cancer our previous investigations revealed association of AR mRNA with the MID1 ribonuclear complex through its trinucleotide repeat motives and upregulation of AR protein level through this complex (Koehler et al, submitted). Furthermore, we found overexpression of MID1 in prostate tumours, particularly in tumours with a more aggressive phenotype.

Observations that metformin has beneficial effects in prostate cancer together with these findings and the data showing that metformin targets the MID1- α 4/PP2A complex made us hypothesize that metformin might interfere with AR protein synthesis via this complex. Our results show that indeed metformin disrupts the MID1 protein complex and reduces AR protein levels in prostate cancer cells concomitant with inhibition of proliferation and migration. This provides a mechanism involved in anti-prostate tumour activity of metformin and strongly supports the usage of metformin in prostate cancer therapy.

Results

Metformin inhibits growth and reduces AR protein levels of prostate cancer cell lines.

The anti-proliferative effect of metformin has been reported for LNCaP, C4-2, PC-3, and Du-145 prostate cancer cell lines. In our experimental setting, a wide range of prostate cell lines including AR-positive (LNCaP, VCaP, DuCaP, LNCaP-abl), AR-negative (PC-3 and Du-145), and benign epithelial cell lines (RWPE-1 and EP-156T) were used to assess the effect of metformin (Figure 1A-C). The numbers of cells counted after 96 h of treatment with increasing concentrations of metformin up to 5 mM decreased significantly. While metformin affected proliferation of all cell lines tested the benign prostate epithelial cells were the least and the androgen receptor positive cell lines DuCaP and LNCaP the most sensitive ones. In the AR positive cell lines AR protein levels decreased upon metformin treatment in a dose-dependent manner (Figure 1D,E). DuCaP cells, which showed the strongest anti-proliferative effect upon metformin treatment, also responded with the most significant AR downregulation. Of note, AR protein was also efficiently downregulated in LNCaP-abl cells, which represent a castration-resistant prostate cancer phenotype.

Metformin inhibits migration of prostate cancer cell lines

In a next experimental setting to determine whether metformin affects tumourigenic properties of cancer cells other than proliferation we analyzed cell migration. Metformin inhibited the migration of LNCaP as well as PC-3 prostate cancer cells (Figure 2). The effect was again much more pronounced in the AR positive LNCaP than in the AR negative PC-3 cells.

The effect of metformin on prostate cancer cell proliferation is independent of AMPK

Metformin activates AMPK and it is often anticipated that it also exerts its anti-proliferative effect through this protein kinase. To test this hypothesis we used another direct activator of AMPK, i.e. the AMP mimetic AICAR. As expected, AMPK was activated as indicated by increased levels of the phosphorylated form (P-AMPK) (Figure 3A). In contrast to metformin, however, AICAR had no anti-proliferative effect on prostate cancer cell lines and AR protein levels remained constant during treatment (Figure 3A). These data confirm that the activation of AMPK is not the mechanism responsible for metformin's anti-proliferative effect and its effect on AR protein level.

To further complete the picture rescue of metformin effects on cell proliferation and AR protein synthesis upon AMPK inhibition was tested. The specific AMPK inhibitor compound-C alone showed a similar rather than an opposite effect than metformin on cell proliferation and AR protein level, although less pronounced (Figure 3B,C). At a concentration of 10 μ M, which prevented AMPK phosphorylation almost completely (Figure 3D), compound-C led to a decrease of AR protein level of about 30% and of cell numbers of around 50%. Metformin added to compound-C still further inhibited cell growth and reduced AR protein level despite very low AMPK phosphorylation (Figure 3E,F). Collectively these data indicate that AMPK activation is not required for metformin inhibition of prostate cancer cells.

Disruption of the MID1- α 4/PP2A protein complex inhibits prostate cancer cell growth and decreases AR protein levels.

Metformin targets the MID1- α 4/PP2A translational regulator complex and after exclusion of AMPK as the responsible target, we hypothesized that interference with this protein complex leads to the metformin effects observed in prostate cancer cells. To further elucidate this we

used $\alpha 4$ antibody pull-down in LNCaP cells overexpressing flag-tagged MID1 to confirm the physical association of MID1, $\alpha 4$ and PP2A in these cells (Fig. 4A). In a next step disruption of the MID1 protein complex by siRNA knockdown of either MID1 or $\alpha 4$ was carried out. MID1 significantly reduced AR protein levels in LNCaP and LNCaP-abl cells (Figure 3B). The same effect was achieved with $\alpha 4$ knockdown as shown for LNCaP cells (Figure 3C). Disruption of the complex by siRNA knockdown resulted in a decrease of cell proliferation of the AR positive cell lines similarly to what we had observed with metformin (Figure 3B). Interestingly, MID1 knockdown also exerted a negative effect on AR negative PC-3 whereas overexpression increased cell numbers (Fig. 3D) indicating that AR protein synthesis is not the single and only target.

Metformin disrupts the association of AR mRNA with the MID1 complex

The MID1- $\alpha 4$ /PP2A complex binds mRNA containing purine-rich sequences including so called MIDAS motives and trinucleotide repeats [19, 20]. AR mRNA is one of the bound mRNAs. We therefore proposed that metformin may cause disassociation of the AR mRNA from the complex. To test this notion we immunoprecipitated the complex from control or metformin treated cells using an $\alpha 4$ antibody. AR mRNA was detected in control samples (Figure 5A, lane 1; Figure 5B, column 1), but was absent or strongly reduced in samples treated with 5 mM metformin (Figure 5A lane 3; Figure 5B, column 2) as shown by PCR amplification of a cDNA fragment containing the AR CAG region (Figure 5A) or by qPCR of an AR cDNA fragment of the hormone binding domain (Figure 5B). These data confirm the MID1- $\alpha 4$ /PP2A complex with associated mRNAs as a target for metformin and provides a mechanism for AR protein downregulation.

Discussion

The anti-tumour effect of metformin has been observed in different types of cancers but a clear mechanism of action remained elusive. Several clinical trials are currently being performed to assess the effect of metformin alone or in combination with different drugs in various types of cancer including prostate cancer [22]; (<http://www.clinicaltrials.gov>, <https://www.clinicaltrialsregister.eu>). A better knowledge of the cellular target(s) and the molecular mechanism can support selection of patients and the set up of treatment in order to achieve best therapeutic efficacy.

Metformin has an effect on the translation of mRNAs. It is no global inhibition of translation such as that expected when cells attempt to spare energy; rather, it is restricted to a specific pool of mRNAs [23]. In our previous investigations we established the post-transcriptional regulation of AR protein levels by the MID1- α 4/PP2A ribonuclear protein complex (Koehler et al, submitted). The results presented herein establish a link between the effect of metformin and AR through this translational regulator complex. Metformin disrupts this complex as first shown by Kickstein et al. [13]. In conformity with this mechanism of action our data show release of AR mRNA associated with the complex by metformin treatment and downregulation of AR protein levels and subsequent growth inhibition of prostate cancer cells. Accordingly, disruption of the complex by silencing either MID1 or α 4 yielded the same outcome as treatment with metformin. AR positive cell lines were the most sensitive prostate cancer cells for the inhibitory effects of metformin supporting a mediation of these effects through reduction of AR protein levels. In agreement with our findings Colquhoun et al. reported inhibition of colony formation in AR positive LNCaP cells at much lower metformin concentrations than in AR negative PC-3 and Du-145 cells and enhancement of

the antiproliferative effects of the antiandrogen bicalutamide [24]. In agreement with Ben Sahra et al. we also observed the least sensitivity in benign cell lines [4]. However, also AR negative cells were inhibited, suggesting additional targets beyond AR. A likely candidate is the PTEN-Akt pathway, a pathway supporting proliferation, survival and migration of prostate cancer cells, which is often overactivated in prostate cancer through loss or inactivation of the tumour suppressor PTEN [25, 26]. Disruption of the MID1- α 4/PP2A complex targets the PTEN-Akt pathway through interference with the translation of the Akt-kinase PDPK-1 and enhancement of the activity of the protein kinase antagonist PP2A [19]. Importantly in terms of prostate cancer treatment LNCaP-abl cells, a model for castration resistant prostate cancer with gain of AR function [27], was also highly sensitive to metformin treatment. This suggests efficacy of metformin in castration resistant prostate cancer recommending in particular a combination of metformin with other drugs in late stage disease. In line with a mode of action of metformin through AR protein level modulation metformin was found to reduce serum androgen levels and endometrial androgen receptor levels in polycystic ovarian syndrome (PCOS), a disease characterized by elevated action of androgen and/or androgen receptor [7, 28].

A concern expressed about the use of metformin in cancer patients is its unclear effect on glucose levels in non-diabetic patients. It has been suggested that metformin reduces blood glucose levels only in diabetics, but not so in non-diabetics [5]. This is consistent with the preliminary results of clinical trials, which show that the side effects do not include hypoglycemia [29]. Our data suggest that metformin's anti-proliferative effect on prostate cancer cells is independent of the activation of AMPK, which, as a metabolic sensor, is the main effector molecule of metformin on metabolism and inhibition of gluconeogenesis. Metformin-independent AMPK activation showed no obvious effect whereas inhibition was

inhibitory and exerted a synergistic inhibitory effect together with metformin. This agrees with recent investigations that identified AMPK as over-activated in prostate tumours and as a driver of tumour progression and facilitator of castration resistance that stimulates growth and survival [11, 12]. Taken together these data suggest best therapeutic efficacy by a combination of metformin and AMPK inhibition.

The migration potential of cancer cells is essential for the development of metastases. Metformin inhibited the migration of AR-positive as well as AR-negative prostate cancer cells. Again the effect was stronger in the AR-positive cells. It was recently reported that activation of PP2A via inhibition of MID1 reduced the migration of neural crest cells [30]. Metformin might mediate a similar effect in prostate cancer cells. Furthermore, mesenchymal to epithelial transition (EMT) stimulated by TGF- β and its interplay with AR signalling is important for prostate cancer cell migration [31, 32]. Metformin was found to inhibit EMT through interference with TGF- β regulation in renal and in breast cancer cells [33, 34] and it affects translation of AR as shown herein and other EMT effectors such as MMP14 [19].

Conclusions

In conclusion the results of our study support the use of metformin for treatment of all stages of prostate cancer. The standard treatment for advanced prostate cancer is androgen deprivation therapy. It is initially effective in the majority of tumours but its long-term use is associated with side effects such as cardiovascular problems, metabolic disease and diabetes mellitus, and development of therapy resistance [35]. A combination of metformin with ADT might be a promising combination to improve efficacy and relieve side effects. Upregulation of AR through enhanced activity of the MID1 translational regulator complex could be abrogated by metformin and improve androgen deprivation therapy. Our data confirm the

MID1- α 4/PP2A ribonuclear protein complex as a target for anti-tumourigenic effects of metformin. Furthermore we establish AR as an indirect metformin target. A better understanding of the mechanism of action will support the setup and interpretation of clinical studies and help to optimize treatment efficacy and minimize side effects.

Methods

Reagents, chemicals and media

Compound-C Sigma-Aldrich (St. Louis, MO, USA) was dissolved in DMSO, metformin and AICAR (both Sigma-Aldrich) in water to prepare stock solutions. Cell culture media and supplements were obtained from PAA (Vienna, Austria), Pansorbin cells from Calbiochem (Billerica, MA, USA).

Cell culture and cell counting

LNCaP, Du-145, VCaP and PC-3 cells were purchased from ATCC. DuCaP cells were gifted by Dr. Schalken, Nijmegen. The LNCaP-abl cell line, a model for castration-resistant prostate cancer, was established in our laboratory after long-term culturing in steroid-free medium [27]. The immortalized primary epithelial cell lines RWPE1 were gifted by Dr. Watson, Dublin, EP156 cells were established by hTERT immortalization of primary epithelial prostate cells [36]. Media and culture conditions for cell lines are provided as supplementary data. Cell numbers were determined using a cell counting system (Schaerf System, Reutlingen, Germany).

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris.HCl, pH=8.0, 150 mM NaCl, 0.5 % Na-deoxycholate, 1% NP-40) supplemented with 1% phosphatase and 1% protease inhibitor cocktails, 5 mM NaF and 1 mM PMSF (all Sigma-Aldrich). Gel electrophoresis was performed according to standard protocols [37]. Antibodies and working dilutions for western blot: AR (1:100, Genetex, Irvine, CA, USA), GAPDH (1:100,000, Millipore, Billerica, MA, USA), AMPK and p-AMPK-Thr¹⁷² (1:1000, Cell signalling, Danvers, MA, USA), MID1

(1:400, Sigma-Aldrich), $\alpha 4$ (1:500, Abcam, Cambridge, UK), N-flag (1:1000, Sigma-Aldrich), PP2A (1:1000, Millipore). Immunoblot bands were scanned and quantified using a scanning densitometer (Odyssey; Li-Cor Biosciences, Lincoln, NE, USA). The housekeeping protein GAPDH served as loading control.

Cell transfections

Nanofectin (PAA) was used for transfection of pCMV vectors containing full-length or Flag-tagged MID1 cDNA or empty vector following the recommendations of the supplier. For siRNA transfections, $\alpha 4$ -siRNAs were purchased from Dharmacon (Thermo-Fisher, Waltham, MA, USA), MID1-siRNAs as reported previously [19] from GenXpress (Vienna, Austria). Nanofectin siRNA reagent (PAA) was used for siRNA transfections.

Migration assay

After metformin treatment for 72 h, cells were seeded in 24-well BD cell culture inserts and metformin treatment was continued a further 48 h. 20% FBS for LNCaP and 10% FBS for PC-3 cells were used as chemo-attractants in the lower chambers. After 48 h, cells on the upper side of the membrane were removed by scraping with cotton swabs while cells on the lower side were fixed with methanol and stained with the nuclear stain DAPI. Cells migrated through the membrane were viewed with an immunofluorescence microscope (Carl Zeiss GMBH, Oberkochen, Germany) and quantified with TissueFAXs software (TissueGnostics, Vienna; Austria).

Co-immunoprecipitations and analysis of associated proteins and mRNA.

Cells were lysed in 100 mM NaCl, 20 mM Tris-HCl, 0.5 mM DTT, 10% glycerol and 0.1% NP-40 and precleared with normal rabbit-serum-saturated pansorbin cells. After incubation

with $\alpha 4$ antibody or rabbit control IgG (Santa Cruz, Dallas, TX, USA) overnight, the antigen-antibody complexes were immunoprecipitated with pansorbin cells. The pellets were washed with RIPA buffer 4 times. After boiling in SDS buffer, western blotting was performed with specific antibodies to visualize proteins interacting with $\alpha 4$. For RNA isolation from immunoprecipitates, poly(A) competitor RNA was added to pansorbin cells before pull-down and also to the last wash buffer. The pelleted pansorbin cells were washed 4 times with RIPA buffer supplemented with RNase inhibitor, and with metformin for the treated samples. Pellets were resuspended in RIPA buffer and trizol reagent, incubated at 65°C for 15 min and shaking and total RNA was isolated from following the protocol of the Directzol RNA extraction kit (Zymo Research, Irvine, CA, USA). RNA was reverse-transcribed to cDNA using the iScript select cDNA synthesis kit (Biorad, Hercules, CA, USA). An AR cDNA fragment containing the GAG repeat region was amplified using conventional PCR (GoTaq, Promega, Fitchburg, WI, USA), or AR mRNA was quantified by qPCR (ABI 7500 PCR System, Foster City, CA, USA). Primer and probe sequences are provided as supplementary data.

Statistics

All numerical data are presented as mean $\pm$ SEM from at least three independent experiments. Values were related to controls, which were set to 100%. Student's t-test was used to compare groups. Statistically significant differences are denoted * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Abbreviations

AID, androgen deprivation therapy; CoIP, co-immunoprecipitation; EMT, epithelial to mesenchymal transition; FBS, fetal bovine serum

Competing Interests

The authors declare that they have no competing interests.

Authors' contributions

UD designed and carried out the experiments with the help of AK, analyzed the data, performed statistical analysis and drafted the manuscript. RS, SS and HK conceived and designed the study and participated in the drafting of the manuscript. HK coordinated the study and finalized the manuscript. All authors read and approved the final manuscript.

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Legends to figures

Figure 1

The anti-diabetic drug metformin inhibits prostate cancer cell growth and reduces AR protein levels. Prostate cancer and immortalized benign prostate epithelial cells were treated with increasing concentrations of metformin. After 96 h cell numbers were counted and AR levels determined by western blot. (A), AR-positive cell lines, (B), AR negative cell lines, and (C), immortalised benign prostate epithelial cell lines. (D, E), AR protein levels determined by quantisation of western blot bands in AR positive cell lines. Each experiment was repeated at least 3 times. Representative western blot flourosan images are shown in (E).

Figure 2

Metformin inhibits cell migration. After 72 hours of treatment with metformin, LNCaP and PC3 cells were seeded in Boyden chambers and the number of migrated cells was quantified 48 hours later. Each experiment was repeated at least 3 times.

Figure 3

AMPK activation is not involved in metformin inhibition of prostate cancer cells. Prostate cancer cells were treated with increasing concentrations of an AMPK activator or inhibitor, respectively. (A), LNCaP cells were treated with the AMPK activator AICAR. After 96 h AR levels were quantified by western blot and normalized to GAPDH. Activation of AICAR was

controlled by detection of phosphorylated AMPK by western blot. (B, C), prostate cancer cells were treated with increasing concentrations of the AMPK inhibitor compound-C (0-10 μ M) and cell numbers (B) and AR protein levels (C) in LNCaP and DuCaP cells were determined after 96 h. Representative fluoroscan images of LNCaP and DuCaP cell western blots are shown in (D). Inhibition of AMPK was controlled by detection of phosphorylated AMPK. (E,F), addition of 1 mM of metformin to the AMPK inhibitor compound-C resulted in an additive inhibition of cell proliferation in LNCaP and PC3 cells (E) and reduction of AR protein levels in LNCaP cells (F). AR levels were quantified by western blot. The histograms show the data of at least three independent experiments, the fluoroscan image shows a representative western blot of LNCaP cells.

Figure 4

Regulation of AR protein level and cell growth through the MID1- α 4/PP2A transcriptional regulator complex. MID1, α 4 and PP2A form the core of a ribonuclear protein complex that enhances translation of associated mRNAs. (A), physical interaction was confirmed in LNCaP cells overexpressing a flag-tagged MID1. MID1 and PP2A were coprecipitated in an α 4 pulldown (α 4); normal rabbit IgG was used as negative control (IgG). A representative western blot of two independent experiments is shown in (A). (B-D), disruption of the complex by MID1 or α 4 protein knockdown mimics the effect of metformin. Knockdown of MID1 in LNCaP and LNCaP-abl cells (B) or α 4 (C) in LNCaP cells resulted in the inhibition of cell growth and a reduction of AR protein levels. In the AR negative PC3 cells MID1 overexpression lead to enhancement, knockdown to the inhibition of cell growth (D). Luci siRNA was used as negative control for siRNA transfections, empty vector for

overexpression control. Cell numbers were determined after 10 days. Western blot inserts show flourosan images of representative experiments.

Figure 5

Metformin disrupts the MID1- α 4/PP2A complex and releases associated AR mRNA. DuCaP prostate cancer cells were treated with 5 mM of metformin or vehicle control for 24 h. Afterwards the MID1- α 4/PP2A complex was immunoprecipitated using an α 4 specific antibody. Normal rabbit IgG was used as negative control. Complex-associated RNA was isolated and transcribed to cDNA. Using PCR and real-time PCR amplification an AR cDNA fragments containing the CAG-repeat region (A) or a fragment of the AR hormone-binding region (B), respectively, were amplified. (A), representative agarose gel image of 3 independent experiments, the histogram (B) shows the relative real-time PCR AR fragment levels standardized to the input amount and normalized to the control for 3 independent experiments. NeCo, negative control; Inpt, input. PCR primer and fragment information is provided in supplement data.

Supplementary Information

Metformin anti-tumour effect by disruption of the MID1 translational regulator complex and AR downregulation in prostate cancer cells

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Cell culture media used for the different prostate cancer cell lines

Prostate tumor and immortalized benign epithelial prostate cells were routinely cultured at 37°C in a humidified atmosphere of 5 % CO₂ in air using the following media:

Cell line	PC-3	Du-145	LNCaP	LNCaP -abl	DuCaP	VCaP	RWPE1	EP156T
Basal Medium	RPMI	RPMI	DMEM	RPMI	RPMI	DMEM	K-SFM	MCDB153
FBS	10%	10%	10%	10%*	10%	10%		1%
100x glutamax solution	1%	1%	1%	1%	1%	2%	1%	1%
100x penicillin/streptomycin solution	1%	1%	1%	1%	1%	1%	1%	1%
Na-pyruvate			1 mM	1 mM				
D-Glucose			2.3 g/L	2.3 g/L		2.3 g/L		
HEPES pH=7.2			10 mM					
BPE							0.05mg/ml	50ng/ml
EGF							5 ng/ml	5 mg/ml
R1881								10 nM

* Char-coal stripped, steroid-free FCS

Androgen receptor PCR primers and Probes:

For amplification of a cDNA fragment of 295 bp in length containing the CAG triplet repeat (AR mRNA transcript variant 1, bases 1175 – 1470) the forward primer: 5'-AGGAGCTTTCCAGAATCTGTT-3' and the reverse primer 5'-GCTGTGAAGGTTGCTGTTCTC-3' were used. PCR amplification was performed with initial denaturation for 2 min followed by 5 cycles with and 27 cycles with denaturation for 30s at 95°C, annealing for 1min at 55°C and synthesis for 40s at 72°C followed by a

final synthesis step of 5min. The PCR product was visualized in a 2% agarose gel using sybrgreen staining.

AR mRNA quantification by qPCR followed the taqman technique. Primers 5`AGGATGCTCTACTTCGCCCC-3` (forward) and 5`-ACTGGCTGT ACATCCGGGAC-3` (reverse) and the taqman probe FAM-5`-TGGTTTCAATGAGTACCGCATGCACA-3`-TAMRA were used to amplify an 72 bp AR cDNA fragment of the ligand-binding domain (AR mRNA transcript variant 1 bases 3396-4368) applying the standard protocol of the ABI 7500 real-time PCR system.

Figure 1.

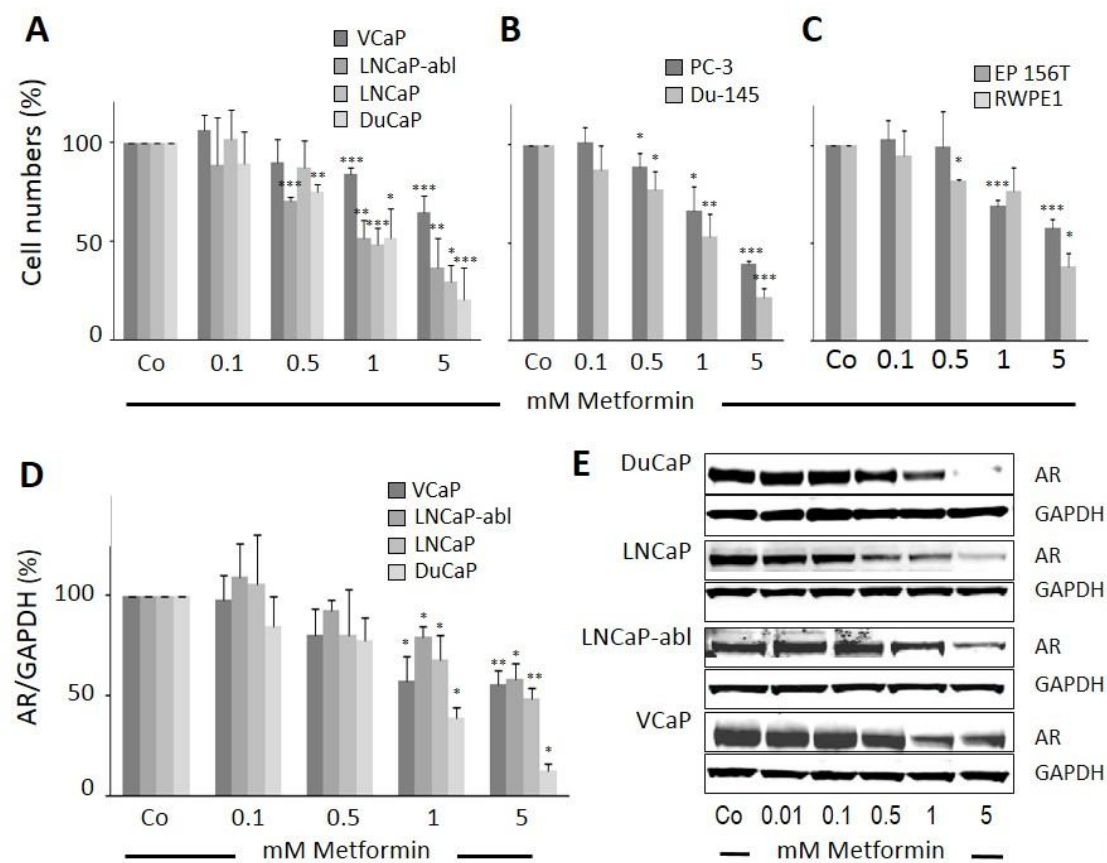


Fig.1

Figure 2.

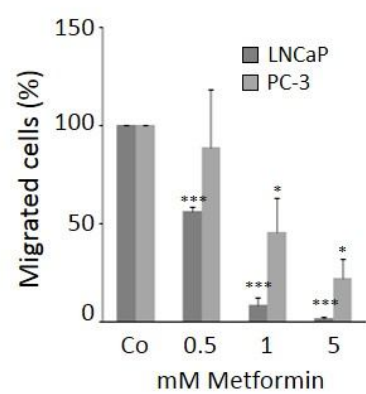


Fig.2

Figure 3.

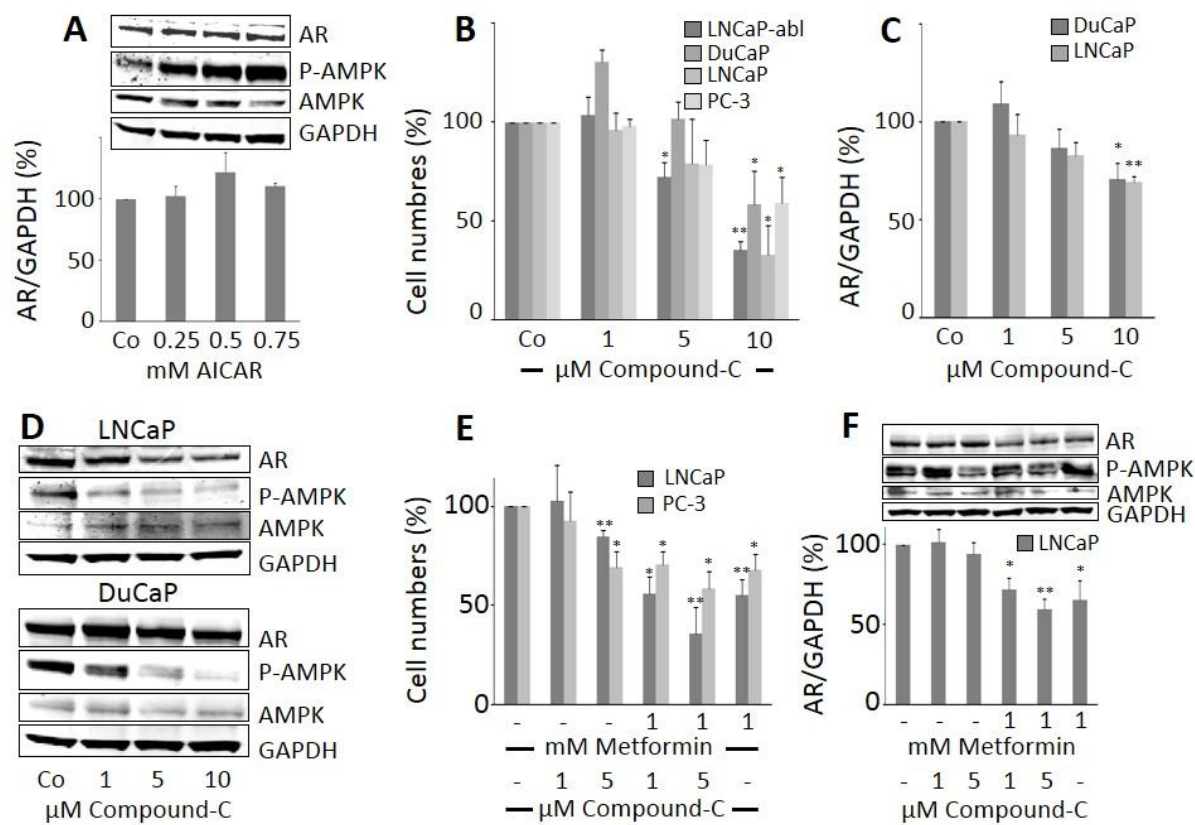


Fig. 3

Figure 4.

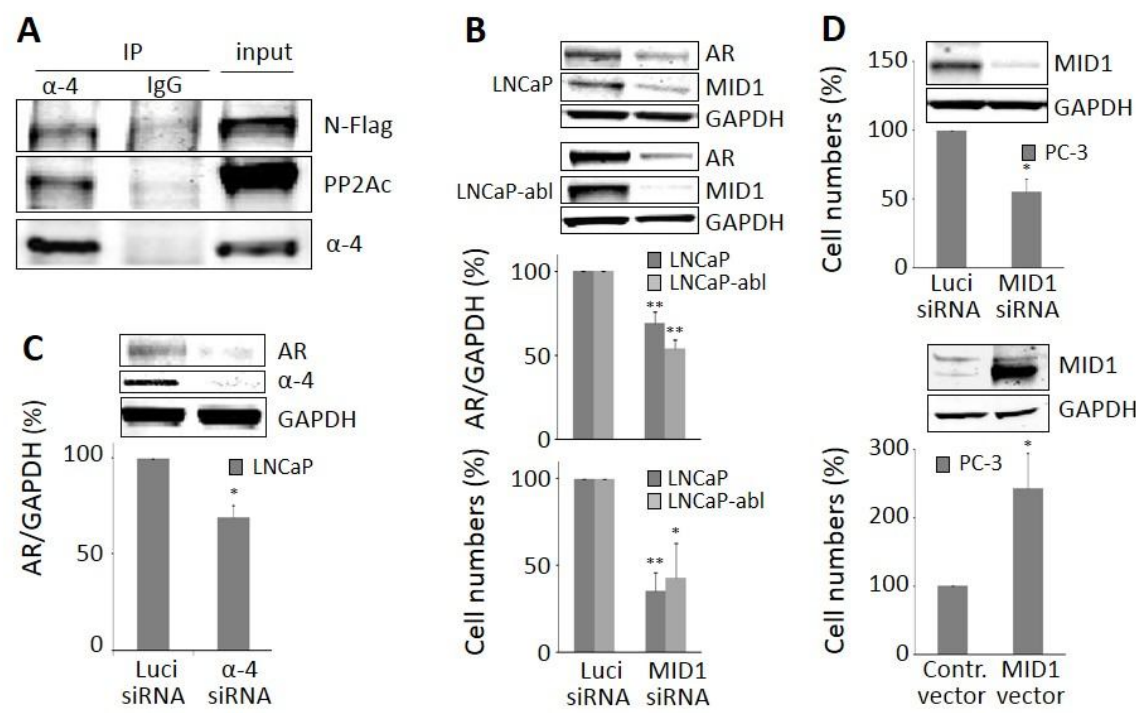


Fig.4

Figure 5.

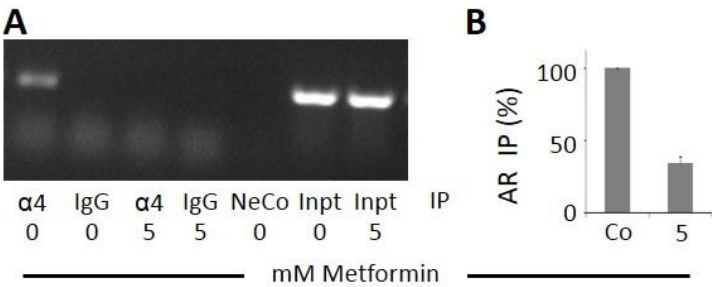


Fig. 5

6. General Discussion

The MID1 gene is mutated in Opitz G/BBB syndrome and the studies in Opitz patients shedded some light on the function of the MID1 protein. It forms a ribonuclear protein complex including PP2A, $\alpha 4$ and several other proteins and mRNAs carrying G-quartet motifs. MID1 acts as a translational enhancer for the mRNAs associated with the complex. Our collaborator group found out that AR mRNA is one of these mRNAs. Because of the well-established role of AR in prostate cancer, it is important to elucidate how it is regulated and how this regulation mechanism is disrupted in prostate cancer. Therefore, the aim of our study was to understand the role of MID1/PP2A/ $\alpha 4$ complex in the progression of prostate cancer. We established a reciprocal feedback regulation between AR and MID1. MID1 regulates AR posttranscriptionally and AR decreases MID1 transcription in an androgen dependent manner. In tumor tissue MID1 expression is increased with increasing tumor grade, most probably because of aberration of the regulation mechanisms between AR and MID1. Figure 7 summarizes our findings and the proposed negative feedback regulation model.

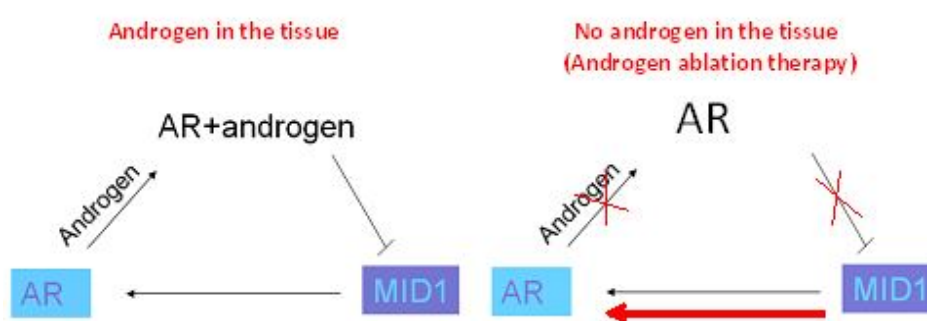


Figure 7: Proposed model for a negative feedback regulation between MID1 and AR.

6.1. MID1 Family Proteins Play Crucial Roles in Different Cancers

MID1 is an ubiquitin ligase carrying a RING finger motif. RING finger E3 ligases are the most abundant class of E3 ubiquitin ligases that mediate protein degradation. Their function is crucial

for the cell to regulate protein homeostasis. They can have tumor promoting or suppressing effects. Some examples are: 1. The E3 ligase MDM2 targets the tumor suppressor p53. It is overexpressed, amplified or over-activated in several types of cancer leading to inactivation of p53. 2. The tumor suppressor Von Hippel-Lindau (VHL) targets the hypoxia inducible factor α (HIF α) for proteosomal degradation. Inactivation of VHL in sporadic or familial kidney cancer leads to accumulation of HIF α . HIF α acts as a transcription factor to increase the expression of angiogenic and growth genes. 3. The CBL family E3 ligases target receptor tyrosine kinases (RTKs) for degradation thus controlling the activity of growth factor and survival pathways. CBL proteins inactivating mutations have been detected in myeloid neoplasms. This inactivation leads to hyperactivation of RTKs and survival pathways [112].

MID1 is an E3 ligase targeting PP2A for proteosomal degradation. PP2A is an essential phosphatase in the cell and known for its tumor suppressive activities. Therefore, MID1 may be declared as a tumor promoter. There have been some preclinical studies to target E3 ligases with small molecule inhibitors, for example with MDM2 inhibitors. This rationale may also be applied to MID1 for therapy of cancer, in particular for therapy of prostate cancer.

MID1 is also a member of the tripartite motif (TRIM) proteins and is therefore also termed TRIM18. This protein family contains two zinc-binding motifs and B-boxes in addition to a RING finger motif. TRIM proteins also have E3 ubiquitin ligase activity. There are 70 members of this protein family and some of them were reported to have tumor promoter activities. Beyond that, TRIM proteins play additional roles in tumors. Several of them are involved in translocations in various types of cancer. TRIM 19 (PML) fuses with the retinoic acid receptor- α (RAR α) gene in acute promyelocytic leukemia (APL). TRIM24 is involved in rearrangements with FGFR1 in myeloproliferative syndrome. In addition, some of the TRIM proteins are involved in the regulation of the activity of nuclear receptors. Interestingly, TRIM 24 interacts with AR and increases its transcriptional activity. TRIM 68, which was found upregulated in prostate cancer, may also function as a cofactor for induction of AR-mediated transcription [113].

In breast cancer a similar role as proposed here for MID1 (TRIM18) in prostate cancer was described for TRIM 25. Transcription of the TRIM25 gene is regulated by estrogens and TRIM25 enhances ER α mediated transcription [113]. In this way there is a feed forward mechanism between TRIM25 and ER α as opposed to a negative feedback regulation we propose for MID1 and AR.

6.2. Negative Regulation by Androgens and AR

Although the positive regulation of gene transcription by AR is studied extensively, negative regulation is not so clear. In our study, we showed that the MID1 gene is negatively regulated by AR; however we couldn't see any negative regulation in reporter gene assays when testing two AR binding sites in the MID1 gene. One mechanism proposed in the literature that could apply here is involvement of several ARBSs together [114]. This could mean that only one ARBS is not enough to achieve negative regulation. Another idea is the interaction with other transcription factors and binding sites to achieve the negative regulation. One of the most likely interacting transcription factors is SP1. It is involved in negative regulation of cyclin D1, TGF β and c-MET by AR [32]. In breast cancer cells it was proposed that both SP1 and AR bound to their respective response elements interact in the chromatin context to induce DNA looping and recruitment of HDAC1 and DAX-1 to form a repression complex [115]. MID1 crosstalks with the Sonic Hedgehog (SHH) and the TGF β pathway, and with cyclin D1. GLI1 a downstream transcription factor of the SHH is known as a negative modulator of AR [116]. Therefore, it is a good candidate for interaction with AR to achieve negative transcriptional regulation. Cyclin D1, in addition to its well known cell cycle regulator function, acts as a transcription factor. It also has been reported as a negative regulator of AR transcriptional activity. Recent reports revealed different variants of Cyclin D1 playing various roles in prostate cancer [117]. Therefore, cyclin D1 may be involved in negative regulation of MID1 by AR as well. SMADs are the downstream transcription factors of the TGF β pathway. Although it is not clear if SMADS are involved in regulation of AR transcriptional activity, involvement of the TGF β pathway in prostate cancer

progression is well known [118]. Therefore, it is important to consider also SMADs in a negative regulation model.

6.3. MID1 and Androgen Deprivation Therapy

Androgen deprivation therapy (ADT) is the preferred therapy for advanced prostate cancer. However, sooner or later tumors become therapy resistant a tumor stages called CRPC. Several mechanisms are proposed for progression to CRPC as explained in the Introduction section. It is very important to note that AR has a different transcriptional program in CRPC [52].

We propose a role of MID1 in the progression to CRPC. MID1 is negatively regulated by androgens and during ADT MID1 expression is induced. Because MID1 is a positive translational inducer of the AR an increase of MID1 expression would further enhance AR translation and thus contribute to the upregulation of AR protein levels. As outlined above AR protein upregulation is often observed in prostate cancer and considered an important androgen ablation therapy escape mechanism.

In the same manner as MID1, two other important regulatory pathways in the prostate and prostate cancer are negatively regulated by androgens, the SHH and TGF β pathways [114, 119]. Therefore, during ADT, all these pathways are upregulated. It is interesting to note that these pathways all crosstalk with each other and are important for prostate development. Their increased activity during CRPC development suggests activation of developmental programs, which may lead to more aggressive and less differentiated tumor phenotypes.

In our study, we showed that MID1 expression increased with increasing Gleason score. However, we used primary cancer tissues and small number of metastasis as the latter are hardly available. Based on our findings, it would be most suitable to compare hormone deprivation therapy sensitive and corresponding CRPC tissue samples.

6.4. Metformin in Prostate Cancer

Metformin has been drawing substantial attention recently because of its anti-cancer effect in a wide range of cancer types. However, its mechanism of action is not clear. In our study, we proposed that metformin targets the MID1/PP2A/ α 4 complex in prostate cancer based on the study of Kickstein et. al. In their study, they showed that metformin caused dissociation of this complex. The dissociation of this complex eventuates in the ineffective translation of the mRNAs associated with the complex. We proved this phenomenon in the case of AR. However, there are other mRNAs associated with the complex. It is not clear if this translational/posttranscriptional regulation of mRNAs associated with the complex is the same in every cell or tissue type. Otherwise, inefficient translation of several mRNAs in tissue or cell type specific manner can explain different effectiveness of metformin against cancer in different organs.

It was also proposed for metformin to interfere with translation of mRNAs because of its inhibitory effect on mTORC1 signaling. However, the idea was that this inhibitory effect is indirectly through AMPK and AMPK inhibits mTORC1 by phosphorylating TSC2 [95]. Larsson et. al provided first direct evidence that metformin interferes with translation of specific mRNAs. Interestingly, the subset of mRNAs, of which metformin inhibits translation overlaps with the subset of mRNAs inhibited by mTORC1 inhibitors. However, they are not exactly the same subset [98]. This means that metformin has another target other than mTORC1 for its translation inhibition effect. In our study, we proposed that this target is the MID1/PP2A/ α 4 complex. This complex is associated with specific mRNAs containing MIDAS motifs and enhances their translation. AR mRNA was confirmed to be one of this mRNAs. We showed that addition of metformin caused dissociation of AR mRNA from the complex by isolation of mRNAs associated with the complex in the presence or absence of metformin. However, it is still an open question to globally analyze of which other mRNAs the translation is effected by metformin treatment in prostate cancer cells.

The efficiency of metformin in cancer therapy was proved in many in vitro and in vivo studies. There are several clinical trials going on in many cancer types including prostate cancer. These clinical trials evaluate the efficacy of metformin in castrate resistant prostate cancer cases as well as a first line therapy for advanced prostate cancer (<http://www.clinicaltrials.gov>).

A retrospective study performed on progression to CRPC in prostate cancer patients provided valuable information and promising results. Metformin use gave an advantage to the prostate cancer patients in terms of several parameters such as overall survival, distant metastasis free survival. Interestingly, only 4% of metformin users progressed to CRPC compared to 43% diabetic non-metformin users and 26% of nondiabetic prostate cancer patients [113]. This clinical study supports our findings. Our results suggest that the MID1/PP2A/ α 4 complex has a role in the progression to CRPC because of the negative feedback regulation between AR and MID1 and metformin may prevent this by inhibiting the complex.

6.5. AR and AMPK in Prostate Cancer Cells

As the central regulator in prostate epithelial and cancer cells the AR was also shown to be important for the regulation of metabolism. Several bottleneck and rate-limiting enzymes in metabolism and biosynthetic pathways are target genes of AR transcriptional activity such as fatty acid synthase (FASN), acetyl-CoA carboxylase α (ACACA), mTOR and CAMKK2. The latter, phosphorylates and activates AMPK. Therefore, AMPK is an indirect target of AR. The AR regulated CAMKK2 protein is overexpressed in patients' tumor samples; in cell lines, androgen-sensitive as well as androgen-deprivation-resistant ones [111].

Our results revealed that action of metformin is independent of AMPK. Actually, inhibition of AMPK in combination with metformin resulted in an enhanced inhibitory effect. Although AMPK is considered as a tumor suppressor in the literature it behaves like a tumor promoter in prostate cancer cells. This can be explained by its indirect regulation by AR through CAMKK2 [111].

7. Conclusions and Outlook

AR is a very important player in prostate cancer progression. This is also evident by the fact that AR pathway targeted therapies have been proven to be useful for prostate cancer and new therapies targeting the AR pathway are under development [45-47]. However, tumor cells overcome AR targeted therapies by overactivating AR by several ways [53-60]. Therefore, it is important to block the pathway also upstream of AR. There are many studies going on to understand the AR target genes and their functions in prostate cancer and AR transcriptional program in normal and cancerous prostate. However, knowledge about the upstream regulators of AR pathway is limited. In our study, we defined a novel regulator of AR translation, MID1/PP2A/ α 4 complex. In addition to this novel finding, we found out that this complex plays a role in progression to the castrate resistance because of a negative feedback regulation between AR and MID1. All these findings make us think that this complex is a suitable drug target for prostate cancer. Fortunately, metformin was described recently as the disruptor of this complex. The clinical trials about metformin are going on in prostate cancer patients. Our results provide clues about selecting proper patient populations and evaluation criteria for drug performance for clinical trials and further clinical use of metformin.

Our study is the first study introducing MID1 as an important player in the prostate cancer field. However, further studies are urgently needed to confirm our findings and bring more insights about the role of MID1/PP2A/ α 4 complex in prostate cancer. We found out that expression of MID1 in prostate cancer tissue is increasing with increasing Gleason score. It is important to find out what causes the increase in MID1 expression. Although we identified MID1/PP2A/ α 4 complex as a translational regulator of AR, it is not clear which role each member of the complex plays in prostate cancer and what is the exact function of each member.

MID1 plays a role in the development of midline structures. Another important question is whether MID1 has a role also in the development of the prostate. This information can also

provide clues about the progression of prostate cancer and how developmental programs are turned on again in prostate cancer.

We found out that MID1 is negatively regulated by androgens. Although there are few models for positive regulations by androgens, the knowledge about how AR suppresses gene transcription is limited. MID1 is a good model for further studies because AREs are dispersed both in promoter site and inside the MID1 gene.

8. References

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9. Appendix

Curriculum Vitae- Ummuhan Demir

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Work

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EDUCATION

2009- 2013 PhD student in Doctoral program Molecular Cell Biology and Oncology, Section of Experimental Urology, Dept. of Urology, Innsbruck Medical School, Austria

2006- 2008 Master of Science, Eskisehir Osman Gazi University, Turkey

Medical Genetics GPA: 3.86/4.00

1999-2004 Bachelor of Science, Bogazici University, Turkey

Major: Molecular Biology and Genetics GPA: 2.99/4.0

RESEARCH EXPERIENCE

2009- 2013 PhD project in Dept. of Urology Section of experimental Urology

(Supervisor: Helmut Klocker: Helmut.Klocker@uki.at)

PhD thesis title: Interaction of AR with MID1/PP2A complex and implications on prostate cancer progression and therapy

2006-2008 Master Thesis in Dept. Of Medical Genetics, Eskisehir Osman Gazi University, Turkey

(Supervisor: M. Hamza Muslumanoglu)

Master thesis title: Analyzing genomic sequences of miR-125a and miR-34a microRNAs in breast cancer samples with sequencing.

2003 July-Sept Scientific practice in Section of Experimental Urology, Dept. of Urology, Innsbruck Medical School, Austria

COMPUTER SKILLS

Microsoft Office: Word, Excel, Power Point

Bioinformatic databases: NCBI, Ensembl.

LANGUAGE SKILLS

English and Turkish: Fluent, excellent reading and writing skills

English TOEFL score: 267

German: Elementary level

AWARDS AND HONORS

Boğaziçi University, Undergraduate School Fellowship, 1999

MCBO Doctoral Program, PhD Fellowship, 2008

Best poster Award, 27th Annual EAU Congress, Paris, France, 2012.

PUBLICATIONS

PAPERS;

Demir, U., Koehler, A., Kickstein, E., Krauss, S., Aigner, J., Aranda-Orgilles, B., Karagiannidis, A.I., Achmueller, C., Bu, H., Wunderlich, A., Schweiger M.R., Schaefer, G., Schweiger, S., Klocker, H. and Schneider, R. A hormone-dependent feedback-loop controls androgen receptor levels by limiting MID1, a novel translation enhancer and promoter of oncogenic signaling. (Manuscript submitted to NEOPLASIA)

Demir, U., Koehler, A., Schneider, R., Schweiger, S. and Klocker, H. Metformin anti-tumor effect by disruption of the MID1 translational regulator complex and AR downregulation in prostate cancer cells. (Manuscript in revision in BMC Cancer.)

ABSTRACTS;

Cilingiroglu, O., Ozdemir, M., Muslumanoglu, M.H., Tepeli, E., Bademci, G., Korkmaz, C., Kutlay, O., **Demir, U.,** Aslan, H., Durak, B., and Artan, S. MEFV mutations in Turkish patients suffering from Familial Mediterranean Fever. European Journal of Human Genetics 16 (2): 248, May 2008, poster

Demir, U., Schaefer, G., Koehler, A., Schneider, R., Schweiger, S. and Klocker, H. Androgen receptor deregulation in prostate cancer: analysis of androgen receptor regulation through the midline 1 ribonuclear protein complex. Annual Meeting of the Austrian Association of Molecular Life Sciences and Biotechnology, Vienna (A), 2010, poster.

Demir, U., Schaefer, G., Koehler, A., Schneider, R., Schweiger, S. and Klocker, H. Androgen receptor deregulation in prostate cancer: analysis of androgen receptor regulation through the midline 1 ribonuclear protein complex. First International Symposium of the Journal Hormone Molecular Biology and Clinical Investigation, Seefeld (A), 2010, poster.

Demir, U., Schaefer, G., Koehler, A., Schneider, R., Schweiger, S. and Klocker, H. Metformin decreases prostate cancer cell proliferation by androgen receptor down-regulation through MID1/PP2A/alpha-4 complex. 9th World Congress in Urological Research, Innsbruck (A), 2011, poster.

Demir, U., Schaefer, G., Koehler, A., Wunderlich, A., Schneider, R., Schweiger M.R., Schweiger, S. and Klocker, H. A novel androgen receptor regulatory loop: Posttranscriptional androgen receptor regulation through Midline 1 ribonuclear protein complex. 27th Annual EAU Congress Paris (FR), 2012, poster.

Demir, U., Schaefer, G., Koehler, A., Wunderlich, A., Schneider, R., Schweiger M.R., Schweiger, S. and Klocker, H. A novel androgen receptor regulatory loop: Posttranscriptional

androgen receptor regulation through Midline 1 ribonuclear protein complex. AACR 103rd Annual meeting, Chicago (USA), 2012, poster.

Demir, U., Schaefer, G., Koehler, A., Schneider, R., Schweiger, S. and Klocker, H. Metformin decreases prostate cancer cell proliferation by androgen receptor down-regulation through MID1/PP2A/alpha-4 complex. 20th Meeting of the EAU Section of Urological Research (ESUR), Strasburg (FR), 2012, poster.