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Cellular and molecular mechanisms underlying the role played by the MEN1 gene in prostate cancer cells

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Cellular and molecular mechanisms underlying the role played by the *MEN1* gene in prostate cancer cells

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Abstract

MEN1 mutations predispose patients to multiple endocrine neoplasia type 1 (MEN1), a genetic syndrome associated with the predominant co-occurrence of endocrine tumors. Accumulating evidence suggests that menin, the *MEN1* gene product, could also be involved in the development of prostate cancer, a major hormone-related cancer. Intriguingly, our previous study found that menin plays dual role in prostate cancer (PCa) cells.

The androgen receptor (AR) acts as a vital transcription factor in development and progression of PCa. Our previous results unveiled that menin is involved in the transcriptional regulation of AR in AR-dependent PCa cells. Nevertheless, the role and mechanism of menin in prostate cancer still remain poorly understood.

The aim of my thesis project was to dissect the molecular mechanisms underlying the biological role of menin respectively in AR-dependent and AR-independent prostate cancer cells. In the first part of my works, our data demonstrated that menin exerts oncogenic effects by modulating *AR* transcription specifically in AR-dependent PCa cells. Furthermore, different analyses revealed that menin exerted a critical role on the MYC binding site on the *AR* promoter and was essential for the regulation of MYC-mediated *AR* transcription. Interestingly, my work also showed that menin was involved in the regulation of MYC-related lncRNA *PCAT1* in AR-dependent PCa cells, through both its positive regulation of *EZH2* transcription and its physical interaction with *EZH2*, as well as their co-binding on the regulatory sequence of *PCAT1* in AR-dependent PCa cells.

In the second part, my work was to determine its role in AR-independent PCa cells. Our results indicated that *MEN1* silencing in AR-independent cells resulted in an increase in cell anchorage independence and cell migration, accompanied by sustained MYC expression and the nuclear translocation of both JunD and β -catenin. Moreover, the expression of several molecular markers of EMT and stemness was

altered after *MEN1* silencing in AR-independent cells. In addition, the analyses using cultured cell and PC3-GFP xenografts in the mouse demonstrated that both JunD and β -catenin are necessary for the altered tumorigenic potential triggered by *MEN1* inactivation in AR-independent PCa cells.

Thus, firstly, my thesis works uncovered an essential role of menin in regulating *MYC* transcription, MYC-mediated AR transcription and the MYC pathway, involving *PCAT1* regulation via EZH2, specifically in AR-dependent PCa cells. Furthermore, the work highlighted an unrecognized oncosuppressive role of menin specifically in AR-independent PCa cells, via regulating the JunD and β -catenin pathways.

Résumé

Les mutations du gène *MEN1* prédisposent les patients à la néoplasie endocrinienne multiple de type 1 (MEN1), un syndrome génétique qui peut entraîner la survenue de tumeurs endocrines. De plus en plus de preuves ont suggéré que la ménine, la protéine codée par le gène *MEN1*, pourrait également être impliquée dans le développement de cancers de la prostate, un cancer hormono-dépendant majeur. De manière intéressante, une étude que nous avons précédemment menée a révélé que la ménine joue un double rôle dans les cellules du cancer de la prostate (PCa).

Le récepteur aux androgènes (AR) agit comme un facteur de transcription essentiel dans le développement et la progression du PCa. L'étude précédemment que nous avons menée a démontré que la ménine est impliquée dans la régulation transcriptionnelle d'AR dans les cellules PCa AR-dépendantes. Néanmoins, le rôle et le mécanisme de la ménine dans le PCa restent encore mal compris.

L'objectif de mon projet de thèse était de disséquer les mécanismes moléculaires sous-jacents du rôle biologique de la ménine dans les cellules cancéreuses de la prostate AR-dépendantes et AR-indépendantes. La première partie mes travaux a démontré que la ménine exerce des effets oncogènes en modulant la transcription d'AR, spécifiquement dans les cellules PCa AR-dépendantes. De plus, différentes analyses ont révélé que la ménine a un rôle critique sur le site de liaison de MYC sur le promoteur d'AR, et qu'elle est essentielle pour la régulation de la transcription de AR médiée par MYC. De manière intéressante, mes travaux ont également démontré que la ménine est impliquée dans la régulation du ARNlnc *PCAT1* lié à MYC dans les cellules PCa AR-dépendantes, à la fois par sa régulation positive de la transcription d'EZH2 et par son interaction physique avec EZH2, ainsi que par leur co-liaison sur la séquence régulatrice de *PCAT1* dans les cellules PCa AR-dépendantes.

La deuxième partie de mes travaux vise à déterminer le rôle de la ménine dans les cellules PCa AR-indépendantes. Mes résultats ont indiqué que l'inactivation de *MEN1*

dans les cellules AR-indépendantes entraîne une augmentation de la croissance cellulaire sans adhésion et de la migration cellulaire, accompagnée d'une surexpression de MYC, et de la translocation nucléaire à la fois de JunD et de la β -caténine. De plus, l'expression de plusieurs marqueurs moléculaires de la TEM et des cellules souches a été modifiée dans les cellules AR-indépendantes suite à l'inhibition de l'expression de *MEN1*. En outre, les analyses dans des lignées cellulaires en culture et dans un modèle murin xéno greffé PC3-GFP ont démontré que JunD et la β -caténine sont nécessaires à l'altération du potentiel tumorigène induite par l'inactivation de *MEN1* dans les cellules PCa AR-indépendantes.

Ainsi, mes travaux de thèse ont permis de mettre en évidence un rôle essentiel de la ménine dans la régulation de la transcription de *MYC*, de la transcription d'*AR* médiée par MYC, et de la voie MYC, impliquant la régulation *PCAT1* via EZH2, spécifiquement dans les cellules PCa AR-dépendantes. De plus, mes travaux ont révélé un rôle oncosuppresseur jusqu'ici inconnu de la ménine spécifiquement dans les cellules PCa AR-indépendantes, via la régulation des voies JunD et la β -caténine.

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List of abbreviations

ADT: Androgen deprivation therapies
AP-1: Activator protein 1
AR: Androgen receptor
AREs: Androgen response elements
ASK: Activator of S-phase kinase
BAX: BCL2 Associated X
BC: Breast cancer
BRCA1 and 2: Breast cancers 1 and 2
CD44: Cluster of Differentiation 44
CDK: Cyclin-dependent kinase
COMPASS: Complex of proteins associated with Set 1
CRPC: Castration-resistant prostate cancer
CSCs: Cancer stem cells
DBD: DNA-binding domain
DHT: Dihydrotestosterone
DNMTs: DNA methyltransferases
EIT-6: Estrogen Induced Tag-6
EGF: Epidermal growth factor
ER: Estrogen receptor
ERE: Estrogen response element
EZH2: Enhancer of zeste homolog 2
FOX: Forkhead box
ECM: Extracellular Matrix
EMT: Epithelial Mesenchymal Transition
FKBP5: FK506 binding protein

H3K4me3: Tri-methylation at the 4th lysine residue of the histone H3 protein

HAT: Histone Acetyltransferase

HCC: Hepatocellular carcinoma

HDAC: Histone deacetylase

HER2: Human epidermal growth factor receptor 2

HGPIN: High-Grade Prostatic Intraepithelial Neoplasia

HMT: Histone Methyltransferase

HOX: Homeobox

Hsp: Heat-shock proteins

IGF-1: insulin-like growth factor-1

IGFBP-3: Insulin-like growth factor-binding protein 3

KMTs: histone lysine methyltransferases

LAR: Luminal-androgen receptor

LBD: Ligand-binding domain

LEDGF: Lens epithelium-derived growth factor

LGPIN: Low-grade prostatic intraepithelial neoplasia

LOH: Loss of heterozygosity

mCRPC: Metastatic CRPC

MEF: Mouse embryonic fibroblast

MEN1: Multiple Endocrine Neoplasia type 1

MI: Molecule inhibitor of menin-MLL interaction

MIN: Mammary intraepithelial neoplasia

MLL1/2: mixed lineage leukemia 1/2 (KMT2A and 2B)

MTA1: metastasis-associated protein 1

mTOR: Mammalian target of rapamycin

NEPC: Neuroendocrine prostate cancer

NF- κ B: nuclear factor- κ B

NKX3.1: NK3 Homeobox 1
NLS: Nuclear localization sequence
NTD: N-terminal Domain
PCa: Prostate cancer
PCSCs: Prostate cancer stem cells
PR: Progesterone receptor
PRMT5: Protein arginine N-methyltransferase 5
PRC2: Polycomb repressive complex 2
PSA: Prostate-specific antigen
PTEN: Phosphatase and TENsin homolog
ROS: Reactive oxygen species
SCC: Small cell carcinomas
SERDs: Selective estrogen receptor downregulators
SERMs: Selective estrogen receptor modulators
TGF- β : Transforming growth factor beta
Th2: T- helper type 2
TIT-5: Tamoxifen Induced Tag-5
TMPRSS2: Transmembrane protease serine 2
TNBC: Triple negative breast cancer
TNF α : Tumor necrosis factor alpha
TSS: Transcription start site

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Chapter I Introduction

1. The prostate

1.1 Anatomy

The prostate is the male sexual accessory gland. It is located on the floor of the pelvis and surrounds the neck of the bladder and urethra (Fig. 1.1). In men, the urethra serves two purposes; urination and ejaculation. It runs from the bladder through the prostate and to the tip of the penis. The section of the urethra running through the prostate is known as the prostatic urethra. After being produced in the testicles, sperm moves into a coiled mass, known as the epididymis for maturation. It then goes into two muscular tubes known as the vas deferens, which coil around the bladder and seminal vesicles. The seminal vesicle can house the sperm for several days until ejaculation. During ejaculation, the prostate muscles contract and the sperm into the prostatic urethra towards the tip of the penis (Blandy *et al.*, 1986; Khan *et al.*, 2011).

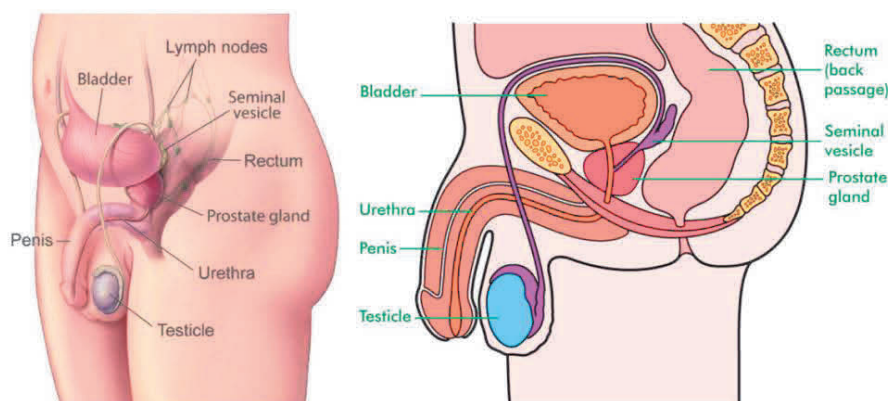


Fig. 1.1 The anatomical relations of the prostate gland. *Obtained from:*

<https://www.cancer.gov/types/prostate/understanding-prostate-changes>

The average weight of a healthy prostate is approximately 11 grams, ranging between 7 and 16 grams (Leissner *et al.*, 1979). It is encapsulated by a fibroelastic tissue layer, leading to septa extending inwards and dividing the prostate into different lobes. The lobes accommodate nearly 50 irregularly branched saccular glands,

excretory ducts, stroma (connective tissue cells), blood vessels and nerves. This transitional epithelium or urothelium has the ability to contract and expand according to the volume of fluid within (Cohen *et al.*, 2008).

It has been described four histologically distinct zones within the prostate (Fig. 1.2). The peripheral zone is found postero-laterally and forms 70% of the prostate. Approximately 70-80% of prostate cancers arise in the peripheral zone. The central zone forms 25% of the prostate and is positioned anterior to the peripheral zone. Fewer than 5% of prostate cancers arise in the central zone.

The transitional zone surrounds the prostatic urethra and is the exclusive site of origin of BPH. It forms only 5% of the prostate but approximately 10% of prostate cancers arise here. Finally, the anterior fibro-muscular zone is devoid of glandular components, and forms the anterior surface of the prostate (Cohen *et al.*, 2008).

The main male hormone is testosterone and is produced in the testicles. The prostate is regulated by dihydrotestosterone, which is synthesized from testosterone in the peripheral tissue (Blandy *et al.*, 1986).

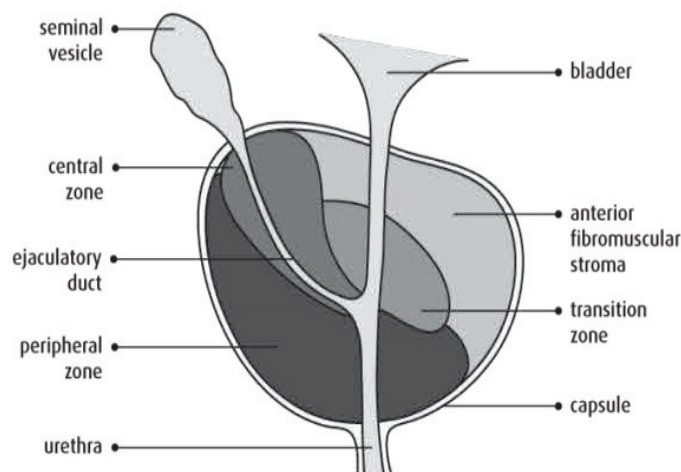


Fig. 1.2 Zonal anatomy of the prostate (Patek, 2018).

1.2 Prostate Histology

Within the prostatic epithelium, there are at least three distinct cell types that can be distinguished by their morphological characteristics, functional significance and

relevance for carcinogenesis (Fig. 1.3). The glandular lumen is lined by secretory luminal epithelial cells which express the androgen receptor (AR). These tall, columnar epithelial cells secrete prostatic acid phosphatase (PAP), PSA and human kallikrein-2 into the lumen of the gland to form seminal fluid. Basal epithelial cells separate luminal epithelial cells from the basement membrane and are thought to secrete components of the basement membrane. These low, cuboidal epithelial cells have low expression of AR. Amongst the basal cell population, it is proposed that progenitor stem cells produce prostatic epithelial cells via intermediate cell stages (van Leenders *et al.*, 2000). Neuroendocrine cells, the third epithelial cell type found within the prostate, are irregularly distributed throughout the glands. They do not express AR and their role is not fully understood.

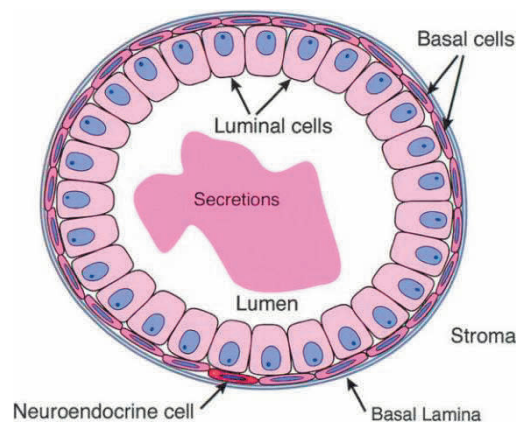


Fig. 1.3 Schematic depiction of the cell types within a human prostatic duct.

Note that the rare neuroendocrine cells are morphologically indistinguishable from basal cells. **Obtained from:** Abate-Shen and Shen, 2000.

1.3 Prostate function

The prostate gland is located in front of the rectum, just below the urinary bladder, and surrounds the urethra. The main function of the prostate is to synthesize and secrete proteins and fluids that, together with contributions from the seminal vesicles, form most of the ejaculate. Although the prostate is involved in fertility, it is not required for reproduction. The major protein produced by the prostate is a protease, prostate specific antigen (PSA) that helps to liquefy the semen so that the sperms way to the egg is facilitated (Balk *et al.*, 2003). Normally PSA is secreted into the prostate

lumen, transported to the urethra and removed during ejaculation. During conditions such as prostate cancer, benign prostate hyperplasia (BPH) and inflammation, the basal epithelial layer and basal membrane are disrupted and PSA leaks into the surrounding stroma and vasculature. Thereby PSA can be elevated in the blood and used as a diagnostic marker for prostate diseases (Brawer *et al.*, 1989).

2. Prostate cancer

2.1 General background

Prostate cancer, with its near 1,414,259 new cases and more than 375,304 deaths reported in 2020 in the world (Sung *et al.*, 2021), represents continuously a major cause of cancer-related mortality and morbidity in men worldwide. Prostate cancer is mainly a disease of the elderly and most of the men are diagnosed with prostate cancer between ages of 70-74 years, while the majority of prostate cancer deaths occur in men over 79 years. The single most significant risk factor for developing prostate cancer is advanced age. The incidence and mortality for prostate cancer varies in different regions around the world and the environment and diet/lifestyle could be important factors that may explain those differences. In addition, the genetic background may also affect the disease risk (Lichtenstein *et al.*, 2000; Sun *et al.*, 2007; Rebbeck, 2017; Pernar *et al.*, 2018).

2.2 Clinical and histopathological aspects of prostate hyperplasia, neoplasia and cancer

2.2.1 Benign Prostatic Hyperplasia (BHP)

The prostate is the only internal organ in men that continues to grow throughout adulthood. In BPH, there is benign proliferation of both stromal and epithelial components of the prostate, occurring exclusively in the transitional zone (Figure 1.4).

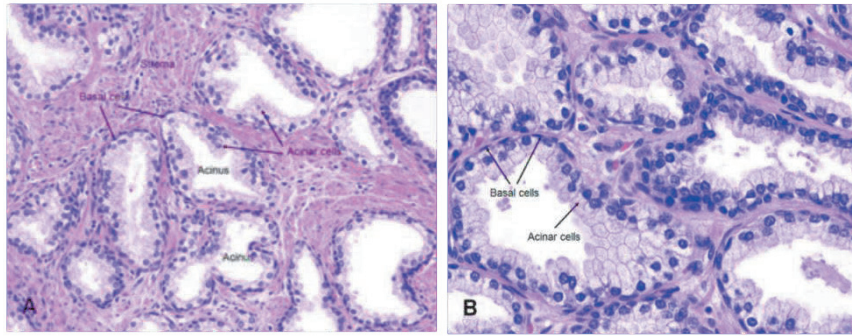


Fig. 1.4 Histopathology of normal prostate tissue and BPH. (A) Normal prostate tissue and (B) BPH characterised by epithelial and stromal. Obtained from Association AU. Pathology for Urologists:

<http://www.auanet.org/education/auauniversity/education-products-and-resources/pathologyfor-urologi>

As the transitional zone enlarges, there is compression of the peripheral zone. Androgens are known to play a role in BPH; castrated pre-pubescent males do not develop BPH in later life. BPH is so common in men, that it is viewed by many as a normal part of the ageing process. It is estimated that 20% of men in their 40s will have BPH, rising to 80-90% of men in their 70s and 80s (Roehrborn *et al.*, 2005). The clinical manifestation of BPH is with bothersome lower urinary tract symptoms (LUTS) comprised of urinary frequency, urinary urgency, hesitancy and incomplete bladder emptying. Complications of BPH include recurrent urinary tract infection (UTI), bladder calculi and acute urinary retention. There is no association between BPH and the later development of prostate cancer (Schenk *et al.*, 2011). Treatment for BPH includes conservative management with lifestyle advice initially where appropriate, before progressing to medical treatment and/or surgery if required. Medical management is with 5-alpha reductase inhibitors, which inhibits the conversion of testosterone to the more potent dihydrotestosterone (DHT), and alpha-blockers, which cause prostatic smooth muscle relaxation. The mainstay of surgical management in BPH is transurethral resection of the prostate (TURP), however this is becoming less common with the success of medical management.

2.2.2 High-grade Prostatic Intraepithelial Neoplasia (HGPIN)

Known as prostatic intraepithelial neoplasia (PIN) (McNeal and Bostwick 1986), this lesion can be classified into four common architectural types: tufting, micropapillary,

cribiform, and flat (Bostwick and Brawer 1987; Nagle *et al.* 1991; Bostwick, 1999). PIN is recognized as a continuum between low-grade and high-grade forms, with high-grade PIN thought to represent the immediate precursor of early invasive carcinoma.

High-grade prostatic intraepithelial neoplasia (HGPIN) is an asymptomatic, pathological entity that is a premalignant lesion of prostate adenocarcinoma. There is increased proliferation of luminal epithelial cells, with complete or partial preservation of the basal cell layer but no invasion into the surrounding stroma. In addition, it is characterised by the presence of atypical epithelial cells with prominent nucleoli, increased nuclear-to-cytoplasmic ratio and increased nuclear size. (Figure 1.5). As with prostate cancer, HGPIN is most commonly identified in the peripheral zone and can be multifocal. HGPIN is more common with advancing age (Bostwick *et al.*, 2004). There is no evidence that HGPIN causes elevation of serum PSA. HGPIN has been found to be independently associated with increased risk of developing prostate cancer, particularly if multifocal (Merrimen *et al.*, 2009). As such, patients with HGPIN in ≥ 3 biopsy sites on an otherwise negative prostate biopsy are recommended to have repeat biopsy (Mottet *et al.*, 2017).

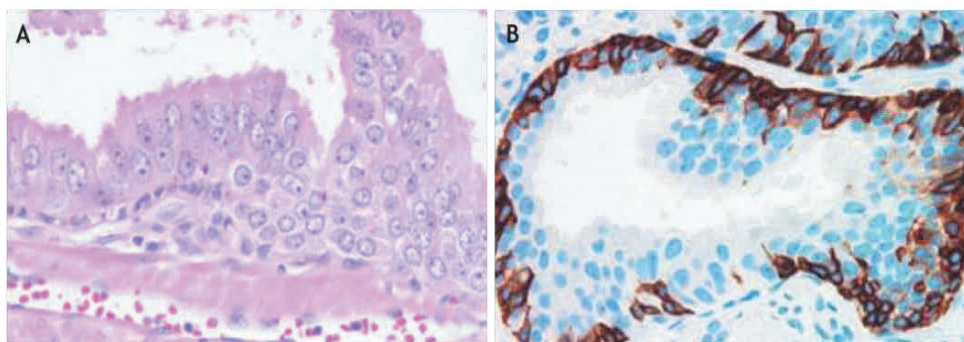


Fig. 1.5 Histopathology of high-grade prostatic intraepithelial neoplasia. (A) Haematoxylin and eosin (H&E) stained section of HGPIN with luminal epithelial cell proliferation and prominent nuclei and nucleoli (B) Prostate biopsy section stained for high molecular weight keratin, a basal cell marker, showing HGPIN with disruption of the basal cell layer. *Obtained from* Bostwick and, Qian, 2004.

2.2.3 Primary PCa

The majority of primary PCa, approaching to 99%, is prostatic adenocarcinoma. Its histological diagnosis is based on the assessment for the three major criteria: glandular architecture, loss of basal cells and nuclear features of the glandular lining cells (Table 1.1) (Mostofi *et al.* 1993; Humphrey *et al.* 2012).

For primary PCa, histologically, basal cells are lost and epithelial cells become more proliferative and less differentiated. The emerging atypical cells develop aberrant nuclei and nucleoli, and are less able to form glandular structures, instead clumping into sheets of malignant cells. There is also a change in protein and receptor expression, leading to altered communication with surrounding cells. Indeed, stromal paracrine signals can promote malignant proliferation and control anti-survival signals in cancer cells (Picard *et al.*, 1986; Miller *et al.*, 1989).

Table 1.1 Criteria for diagnosis of prostatic adenocarcinoma.

Adapt from: Humphrey, 2017

Diagnosis of prostatic adenocarcinoma
<i>Major criteria</i>
Architectural: Infiltrative small glands or cribriform glands too large or irregular to represent high-grade prostatic intraepithelial neoplasia (PIN)
Single-cell layer (absence of basal cells)
Nuclear atypia: nuclear and nucleolar enlargement
<i>Minor criteria</i>
Intraluminal wispy blue mucin (blue-tinged or basophilic mucinous secretions)
Pink amorphous secretions
Mitotic figures
Intraluminal crystalloids
Adjacent high-grade PIN
Amphophilic cytoplasm

2.2.4 PCa invasion and metastasis

Prostate cancer invasion is the spread of the cancer cells out of the prostate glands into nearby tissues, and to further distant sites such as lymph nodes, lung and bone.

The process begins with biological changes within the cancer cells, which enhance the capacity of, or excite the potential for, cancer cell movement and lead to

local invasion. This is followed by intravasation into blood and lymphatic vessels, transit through the invaded circulatory system, and extravasation from the vessel into the parenchyma of distinct tissue. In the secondary site, small nodules of cancer cells form and grow from micro-metastases into large macro-metastatic lesions (Hanahan and Weinberg, 2011; Leach *et al.*, 2015; Leach and Buchanan, 2017).

Within the environs of the prostate, invasion and metastasis is limited by cancer cell movement, which can be achieved by collective-cell or single-cell mechanisms. Collective cell movement involves a multitude of cells moving as large coordinated mass, or as small cohorts of cells (Friedl and Wolf, 2003). Singular cell movement occurs without stress fibre formation or protease activity, squeezing through extracellular matrix (ECM) components (Sabeh *et al.*, 2009). Singular cell movement is unequivocally the most noted form of movement in prostate cancer, which may be facilitated by epithelial mesenchymal transition (EMT) of cancer cells. As a result of EMT, cancer epithelial cells exhibit changes in cadherin expression (E-/N-cadherin), acquire mesenchymal properties and detach from surrounding cells and the ECM. Cells that have undergone EMT secrete proteases that degrade the ECM, facilitating their movement out of the prostate (Nauseef and Henry, 2011; Leach *et al.*, 2015).

2.2.5 CRPC and metastasis CRPC

2.2.5.1 CRPC

In general, PCa is strongly driven by androgen receptor (AR) regulated transcription (Sehgal *et al.*, 2019). However, PCa can become “castration resistant” (CRPC) after escaping conventional androgen deprivation treatment (ADT, with luteinizing hormone releasing hormone (LHRH) agonists and/or the AR antagonist enzalutamide (ENZ)). CRPC status is defined as the presence of castrate serum testosterone levels (<50 ng/dL or 1.7 nmol/L) plus either biochemical progression (three consecutive rises in prostate-specific antigen (PSA) 1 week apart, resulting in two 50% increases over the nadir, and PSA >2 ng/mL) or radiologic progression (Cornford *et al.*, 2017).

As prolonged and intense targeting AR in CRPC patients results in a survival benefit, “castration resistance” often remains AR driven (Luo *et al.*, 2018). However,

histological and clinical resistance cancer types can emerge after AR-inhibition, in which the tumors become androgen independent prostate cancer (AIPC), neuroendocrine (NEPC) and other rare histological variants (Graham and Schweizer, 2016) (Table 1.2).

Table 1.2 Clinical, histological and molecular characteristics of most common CRPC variants. Adapt from: Vlachostergios *et al.*, 2017

CRPC variant	Survival	Histology	Molecular aberrations	References
AIPC	To be defined	Adenocarcinoma, AR (low/absent)	AR signaling low, RB1 loss, TP53 loss, high EZH2	Beltran <i>et al.</i> , 2016; Mu <i>et al.</i> , 2017; Ku <i>et al.</i> , 2017
Neuroendocrine (NEPC)	1-2 years	chromogranin (+/-), synaptophysin (+/-), AR low/absent	low AR signaling, RB1 loss, TP53 loss, REST, high MYCN, AURKA, EZH2, PEG10, SRRM4, DLL3, epigenetic	Wang <i>et al.</i> , 2008; Beltran <i>et al.</i> , 2011; Marcus <i>et al.</i> , 2012; Deorah <i>et al.</i> , 2012; Wang <i>et al.</i> , 2014; Tsai <i>et al.</i> , 2015; Lee <i>et al.</i> , 2016
Small cell carcinoma	7 months	Small blue/oat cells		
Aggressive variant	9-17 months	Heterogeneous (Adenocarcinoma or NEPC)	RB1, TP53, PTEN loss	Tzelepi <i>et al.</i> , 2012; Aparicio <i>et al.</i> , 2013; Aparicio <i>et al.</i> , 2016
Ductal	84 months	High Gleason, endometrioid	MMR gene alterations/hypermethylation	Tu <i>et al.</i> , 2009; Schweizer <i>et al.</i> , 2016
Intermediate atypical	To be defined	To be defined	Mixed/overlapping molecular features with adenocarcinoma and NEPC	Small <i>et al.</i> , 2015; Small <i>et al.</i> , 2016

2.2.5.2 mCRPC

In mCRPC patients, prolonged AR pathway inhibition can alter the typical course of the disease, manifest by histological differentiation to a small cell/high-grade neuroendocrine phenotype in 11-17% of cases (Aggarwal *et al.*, 2018; Abida *et al.*, 2019).

Despite recent advances in the treatment of patients progressing with mCRPC, average survival remains approximately three years (Roviello *et al.*, 2016). The heterogeneous appearance of these changes underlies the multifocal nature of PCa. It is thought that AR insensitive clones will survive the therapeutic selection pressure by AR targeting treatments. Clinical evaluation for adverse prognostic indicators in mCRPC involves either histopathologic/immunohistochemical assessment of biopsy specimens or molecular analysis of biopsy material or circulating tumor cells. Some adverse prognostic indicators include small cell/high-grade neuroendocrine transformation on histopathologic/IHC evaluation, alterations such as AR splice

variant 7, those involving TP53, RB1, DNA damage response genes, and AR/PI3K pathways on molecular profiling (Antonarakis *et al.*, 2014; Conteduca *et al.*, 2017; Annala *et al.*, 2018).

2.2.6 NEPC

The diagnosis of neuroendocrine prostate cancer (NEPC) is histologically defined, encompassing morphologies that overlap with neuroendocrine tumors arising from other primary sites (eg., lung, bladder), and may occur with mixed or overlapping features with prostate adenocarcinoma (Epstein *et al.*, 2016; Vlachostergios *et al.*, 2017). Immunohistochemical detection of neuroendocrine markers such as synaptophysin, chromogranin, and CD56 may help support the diagnosis of NEPC but are not required in the presence of morphologic characteristics (Watson *et al.*, 2015). Pure small-cell neuroendocrine prostate carcinoma is a subtype of NEPC that has the most aggressive biologic behavior and poor outcomes. The clinical significance of other NEPC subtypes, especially mixed or hybrid phenotypes, is not well defined (Watson *et al.*, 2015; Epstein *et al.*, 2016; Vlachostergios *et al.*, 2017).

Although NEPC rarely arises *de novo* and accounts for less than 2% of patients at the time of diagnosis of prostate cancer (Parimi *et al.*, 2014), NEPC is enriched in the advanced disease setting and reported as high as 10-20% in CRPC (Nadal *et al.*, 2014). Clinically, NEPC is often suspected in patients that progress in the setting of low or moderately rising PSA levels (suggesting less AR driven disease) with a predominance of visceral and/or lytic bone metastases. Morphologically, NEPC shares features with other high grade neuroendocrine cancers, including presence of small cells with ‘salt and pepper’ chromatin, high mitotic count and nuclear molding (Wang *et al.*, 2008). In the majority of cases, at least one neuroendocrine immunohistochemical (IHC) marker, such as neuron-specific enolase, synaptophysin, chromogranin, or CD56 stains positive. NEPC is often negative for classical luminal markers of prostatic glandular differentiation (eg., prostate-specific antigen (PSA) and prostatic acid phosphatase). Uncommonly, polypeptide hormones may be present

(including ACTH, antidiuretic hormone (ADH), and corticotropin-releasing factor) (Wang *et al.*, 2008; Vlachostergios *et al.*, 2017).

2.3 Prostate cancer staging and grading

2.3.1 Staging: TNM classification system

Once a patient has been diagnosed with a prostate tumor, the latter must be staged to determine if it has spread beyond the prostate. Staging also provides a better insight into the risk of the disease spreading further, so the correct treatment option is selected. The TNM stage was developed by the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) (Wallace *et al.*, 1975). It is used to evaluate the extent of the primary tumor (T), the affected regional lymph nodes (N) and if it has spread or metastasized (M). There are four stages: in stage I only a small part of the prostate is cancerous, most of the cells are normal and the gland feels normal. In stage II, a lump can be felt in the prostate to the examining finger and a larger part of the prostate is affected. In stage III, the tumor has spread beyond the prostate but very locally, and in stage IV, it has spread to lymph nodes or nearby organs. A more detailed view can be found in [Table 1.3](#).

2.3.2 Grading: Gleason Grading System

Ultrasound guided needle biopsies are taken from the prostate in patients with elevated PSA levels, and if a biopsy contains cancer, it should be examined by a pathologist using microscopic analysis and scored according to the Gleason system, which is the strongest prognostic tool available today for prostate cancer (Gleason and Mellinger, 1974). The differentiation pattern of the tumor is scored on a scale ranging from 1 to 5, where 5 represents the less differentiated and most aggressive tumor pattern. The most common areas of differentiation are summarized into the Gleason score (GS) (Coffey and Isaacs, 1981; Westin *et al.*, 1995). GS is a good predictor of outcome in patients with low GS < 6 or high GS 8-10, but the majority of patients

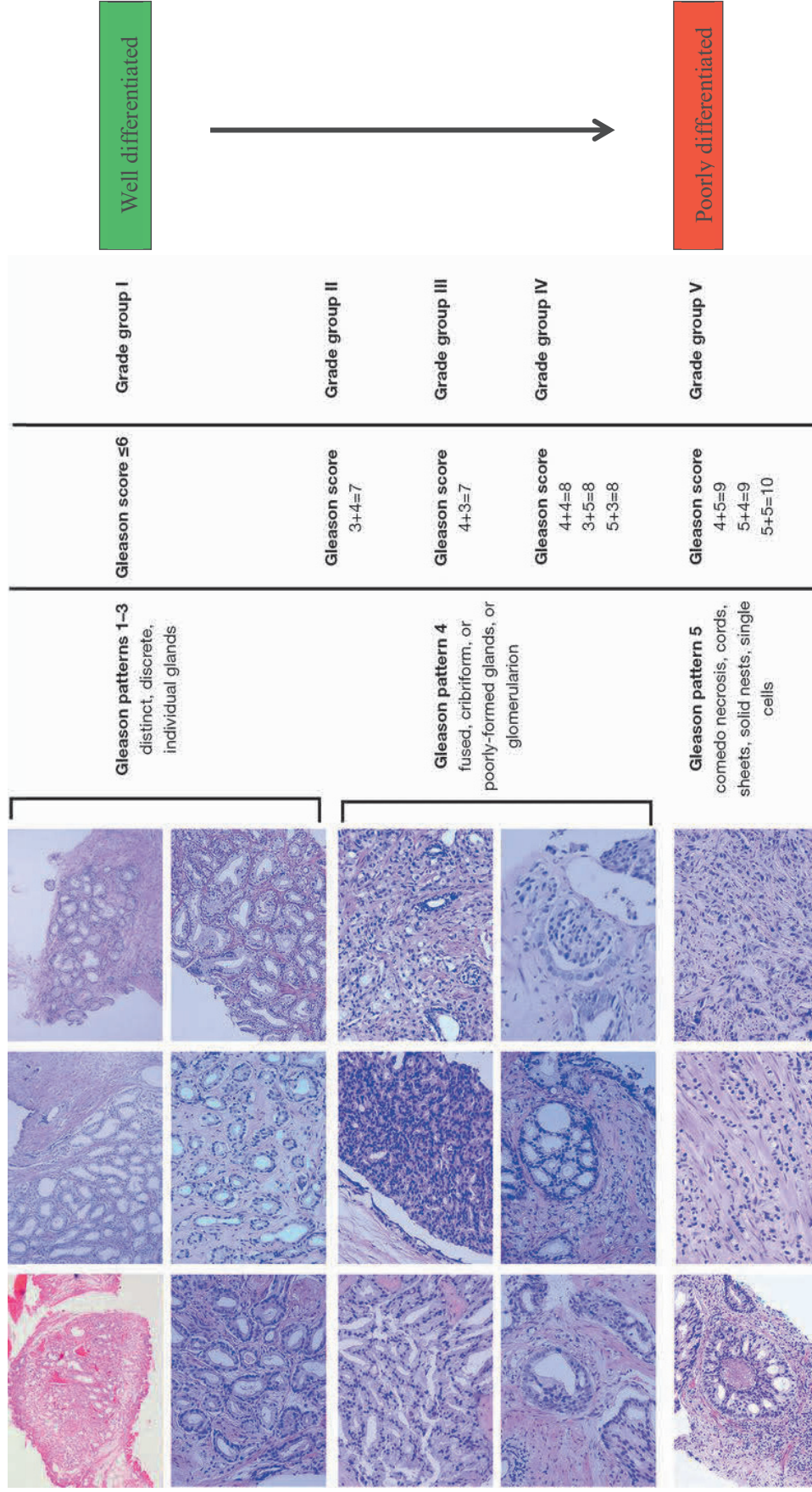
have GS 6-7 where the outcome is very variable and today largely unpredictable (Andren *et al.*, 2006). The Gleason patterns are detailed in Fig. 1.6.

Table 1.3 The TNM classification system in Prostate Cancer.

Obtained from: NICE. Prostate Cancer: Diagnosis and Treatment. Clinical Guideline. 2014. Available from: <https://www.nice.org.uk/guidance/cg175>

Primary Tumour (T)	<p>TX Primary tumour cannot be assessed</p> <p>T0 No evidence of primary tumour</p> <p>Ta Non invasive papillary carcinoma</p> <p>Tis Carcinoma in situ: "flat tumour"</p> <p>T1 Tumour invades sub epithelial cells</p> <p>T2 Tumour invades muscle</p> <p>T2a Tumour invades superficial muscle (inner half)</p> <p>T2b Tumour invades deep muscle (outer half)</p> <p>T3 Tumour invades perivesical tissue</p> <p>T3a Microscopically</p> <p>T3b Macroscopically</p> <p>T4 Tumour invades prostate</p> <p>T4a Tumour invades prostate</p> <p>T4b Tumour invades pelvic wall or abdominal wall</p>
Regional Lymph Nodes (N)	<p>NX Regional lymph nodes cannot be assessed</p> <p>N0 No regional lymph node metastasis</p> <p>N1 Metastasis in a single lymph node 2cm or less in greatest dimension</p> <p>N2 Metastasis in a single lymph node 2cm but no more than 5cm in greatest dimension</p> <p>N3 Multiple lymph nodes, none more than 5cm in greatest dimension</p> <p>Metastasis in a lymph node no more than 5cm in greatest dimension</p>
Distant Metastasis (M)	<p>MX Distant metastatic cannot be assessed</p> <p>M0 No distant metastatic</p> <p>M1 Distant metastatic</p>

Fig. 1.6 Gleason patterns and the Group Grade system. Adapt from: Chen and Zhou Q, 2016.



2.4 Diagnosis and prognosis of prostate cancer

2.4.1 DRE

Digital rectal examination (DRE) is an important part of the assessment of a patient suspected of having prostate cancer. Since most prostate cancers arise in the peripheral zone (McNeal *et al.*, 1988) (i.e. the posterior part of the prostate), larger or advanced lesions can be palpable on DRE. An abnormal DRE may be defined by asymmetry, a palpable nodule or fixed craggy mass. An abnormal DRE initial assessment is highly predictive for high grade prostate cancer (odds ratio 6:1) and estimating prostate size also improves diagnostic accuracy when combined with PSA (Roobol *et al.*, 2012).

Previous work by Epstein *et al* (Epstein *et al.*, 1994) had already highlighted the importance of assessing the estimated prostate volume in conjunction with the patient's PSA reading, finding that a PSA density of less than 0.1 ng/ml per gram was predictive of no adverse pathological findings on needle biopsy and a PSA density of 0.1- 0.15 ng/ml per gram indicative of low or intermediate grade cancer smaller than 3 mm in one needle core biopsy specimen. Thus, DRE still plays an important role in the diagnostic evaluation of men suspected of having prostate cancer, although the positive predictive value of DRE in primary care remains extremely variable (Hoogendam *et al.*, 1999).

2.4.2 PSA

The prostate-specific antigen (PSA) is a glycoprotein enzyme responsible for liquefaction of semen thus enabling fertilisation. It is produced by columnar acinar and ductal prostatic epithelial cells, being present in both benign and malignant cells. PSA is mainly secreted into the semen with small quantities found in the urine and blood. Normally there are significant tissue barriers (basal cell layer, basement membrane, stromal layer and the capillary wall itself), between prostatic cells and

capillaries. In prostate cancer these barriers are compromised and thus PSA leaks into circulating capillaries and hence serum PSA values rise.

Stamey *et al* first published results of 699 patients who underwent both PSA testing and prostatic acid phosphatase (PAP) testing (this was the only serum marker for prostate cancer prior to PSA), concluding that PSA was more sensitive than PAP in detecting prostate cancer (Stamey *et al.*, 1987). The original work on PSA thresholds conducted by Catalona *et al* in the early 1990s (Catalona *et al.*, 1994) suggested a PSA cut-off value of 4ng/ml when guiding whether to perform prostate biopsy in absence of any positive examination findings. They found that prostate cancer was detected in 26% of men with a PSA in the range 4-10 ng/ml and in 53% of men with PSA > 10 ng/ml. Oesterling *et al* (Oesterling *et al.*, 1993) conducted work which concluded that age-specific PSA thresholds were more useful. They suggested the following cut-off points to determine a raised PSA based on age (Table 1.4):

Table 1.4 Age specific PSA values.

Age (years)	PSA (ng/ml)
40-50	2.5
50-60	3.5
60-70	4.5
70-80	6.5

Furthermore, PSA has been shown to predict pathological stage in prostate cancer, even allowing for the increased diagnosis of low-risk, localised prostate cancers (Freedland *et al.*, 2008). PSA is currently used in the NICE guidelines for risk stratification for men with localised prostate cancer (Chun *et al.*, 2007) (Table 1.5). PSA has also been used in assessing response to treatment and disease progression.

Table 1.5 Risk stratification of men with localised prostate cancer. NICE. Prostate Cancer: Diagnosis and Treatment. Clinical Guideline. 2014.

Risk category	Serum PSA		Gleason score		Clinical stage
Low	<10 ng/ml	and	≤6	and	T1-T2a
Intermediate	10-20 ng/ml	or	7	or	T2b
High	>20 ng/ml	or	8-10	or	≥T2c

3. Androgen receptor (AR)

The androgen receptor (AR) is a transcriptional factor, belonging to the steroid receptor superfamily and activated by testosterone and dihydrotestosterone, and required for prostate development and normal prostate function (Cunha *et al.*, 2004). AR and the modulators of AR activity remain important in prostate cancer, even in castration-resistant prostate cancer (CRPC) (Knudsen and Kelly, 2011). Approximately 80-90% of prostate cancers are dependent on androgen at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR (Denis and Griffiths, 2000). Studies of AR in CRPC revealed that AR was still active in CRPC, and it remains as a potential target to treat CRPC.

3.1 Structure of the AR gene

The AR gene is located on chromosome X (Xq11-12) and consists of 8 exons coding a protein about 110 kDa. AR has four regions: from the N-terminal, an NH2 terminal transactivation domain (NTD) encoded by exon 1, a DNA-binding domain (DBD) encoded by exons 2–3, a hinge region encoded by exon 4, and a ligand binding domain (LBD) encoded by exons 5-8 (Fig. 1.7).

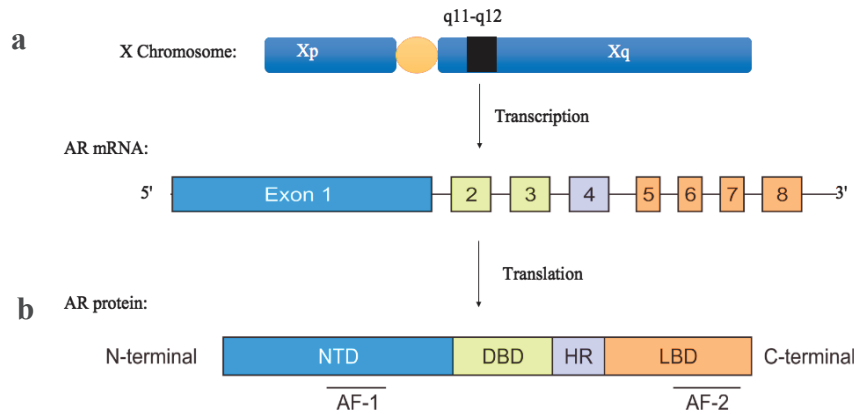


Fig.1.7 Structure of androgen receptor (AR). **a.** Full-length AR is composed of 8 exons. **b.** NTD: N-terminal domain, DBD: DNA-binding domain, HR: hinge region, LBD: ligand-binding domain, AF-1: activation function-1, AF-2: activation function-2, CE: cryptic exon. *Adapt from:* Fujita and Nonomura, 2019.

The NTD has glutamine repeats (CAG repeats) varying in size (most men have 19–25 repeats), approximately 919 amino acids, which results in the variation of all amino acids in AR (Gottlieb *et al.*, 2012). Shorter glutamine repeats are associated with high transcriptional activity of AR (Beilin *et al.*, 2000). It has been reported that men with shorter glutamine repeats have a higher risk of prostate cancer (Giovannucci *et al.*, 1997). In contrast, patients with Kennedy disease have long CAG repeats (>40), which results in low AR-transcriptional activity with gynecomastia, erectile dysfunction, testicular atrophy, and muscular atrophy (Finsterer *et al.*, 2009). The NTD includes the transcriptional regulatory region, activation function-1 (AF-1), and the LBD includes activation function-2 (AF-2) (Jenster *et al.*, 1991). Upon DNA binding, the AR dimer forms a complex with coactivator and coregulatory proteins at the AF-1 and AF-2 regions (Jenster *et al.*, 1991; Antonarakis *et al.*, 2016; Fujita and Nonomura, 2019), including SRC1, SRC2, SRC3, p300/CBP, and AEA54, among many others.

The hinge region between the DBD and LBD is involved in nuclear localization and degradation. The N-terminus has a unique LxxLL-like motif, which binds to a hydrophobic cleft of the C-terminus generated by ligand binding to the receptor. The initial N-C interaction occurs in the cytoplasm, which stabilizes the ligand binding caused by physical interaction between the N-terminal and C-terminal of the receptor

(Jin *et al.*, 2019). In the absence of androgens, heat shock protein (HSP) binds to AR, therefore, AR remains inactive in the cytoplasm. Binding of androgens with AR induces a conformational change, resulting in the dissociation of HSP from AR. Then, the AR dimer translocates into the nucleus where it binds to androgen responsive elements of genomic DNA and regulate its target genes involved in growth and proliferation (Chen *et al.*, 2004; Mills, 2014) (Fig. 1.8).

AR regulates the gene expressions with diverse functions located downstream of the androgen-response element, including secreted proteins (KLK3, KLK2), fusion genes (TMPRSS2-ERG), growth stimulators (IGF1R, APP), PI3K modulation (FKBP5), transcription factors (NKX3.1, FOXP1), metabolic enzyme (CAMKK2), cell cycle regulators (UBE2C, TACC2), and glucuronidation (UGT1A1) (Takayama and Inoue, 2013).

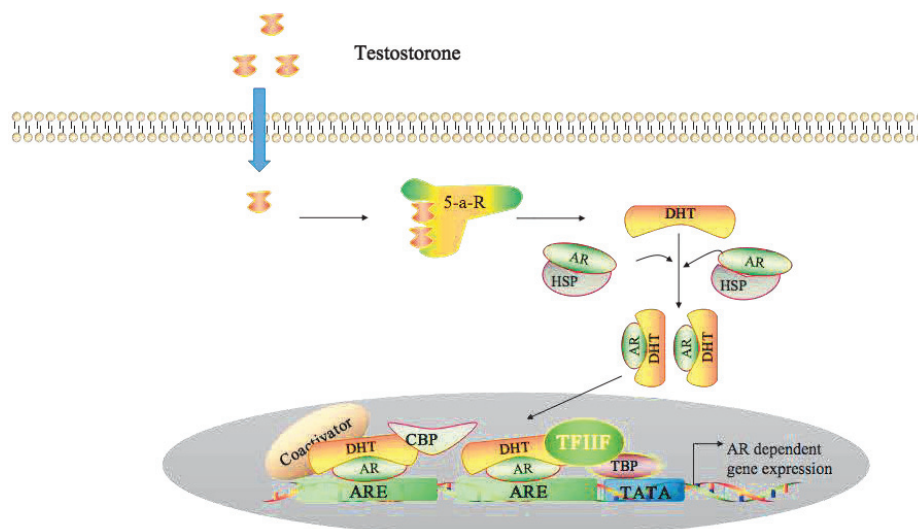


Fig. 1.8 The androgen-AR signaling pathway (Ligand-dependent). Testosterone diffuses into the cells and gets converted into dihydrotestosterone (DHT) via the action 5- α -reductase (5-a-R). DHT binds to the ligand binding pocket of AR and promotes its dissociation from the heat shock protein (HSP). Free AR then translocates into the nucleus and binds to androgen receptor element (ARE) present in the promoter region of AR responsive genes. At the promoter, AR recruits components of basal transcriptional machinery such as TATA binding protein (TBP), transcription factor IIF (TFIIF), and cAMP responsive element binding protein (CRBP) which ensure the transcription of AR responsive genes. *Adapt from:* Heinlein and Chang, 2014.

3.2 AR variants

It has been known for more than two decades that AR has splicing variants (Bryce *et al.*, 2016) (Table 1.6). More than 20 AR variants (AR-Vs) have been reported, and most are missing some C-terminal domain including LBDs, thus functionally active without androgens (Hu *et al.*, 2009; van der Steen *et al.*, 2013) (Fig. 1.9).

Most AR-Vs contain an intact DNA-binding domain (DBD), whereas AR-V3 (aka AR6) lacks the second zinc finger of the DBD. Nevertheless, it contains the first zinc finger of DBD, can constitutively activate AR-responsive promoters in prostate cancer cells (Dehm *et al.* 2008).

Table 1.6 AR variants/isoforms expressed in tissues and cell lines. *Adapt from: Pelekanou et al., 2013.*

AR variants						
	AR45	AR8	ARv567es	ARV1	ARV7	AR23
Tissues, cells and cell lines expressed	Tissues: Prostate, Testis, Uterus, Breast, Heart, Lung, Trachea, Liver, Kidney and Muscle. Cells and cell lines: LNCaP, Cardiomyocytes, HepG2, hepatocellular carcinoma cells.	Tissues: Benign and malignant. Cells and cell lines: prostate cells, LNCaP, c4-2 and C4-2B cells.	Tissues: Normal and malignant. Cells and cell lines: prostate epithelial cells, LuCap xenograft.	Cells and cell lines: prostate cancer cells from CRCP patients; Primary tumors and bone metastases	Tissues: Normal (low levels) and malignant (high levels), prostate epithelial cells, prostate cancer cells from CRPC models and CRPC patients. Cells and cell lines: VCaP and Myc-CaP, LuCap xenografts	Metastatic prostate cancer after anti-androgen treatment.
Function	Inhibits AR function by the formation of AR-AR45 heterodimers.	Primarily localized at the plasma membrane its overexpression promotes association of Src and AR with EGF receptor.	Increasing the expression of AR.	Constitutive and ligand independent activation; Confer castration resistant growth.	Constitutive and ligand independent activation; Confer castration resistant growth.	No nuclear localization and activation of androgen responsive receptors; Increasing AR activation.
References	Ahrens-Fath <i>et al.</i> , 2005; Wu <i>et al.</i> , 2008; Tsuei <i>et al.</i> , 2011;	Yang <i>et al.</i> , 2011	Sun <i>et al.</i> , 2010; Hu <i>et al.</i> , 2012	Hu <i>et al.</i> , 2009; Hörnberg <i>et al.</i> , 2011; Zhao <i>et al.</i> , 2012	Watson <i>et al.</i> , 2010; Hu <i>et al.</i> , 2012	Jagla <i>et al.</i> , 2007; Steinkamp <i>et al.</i> , 2009

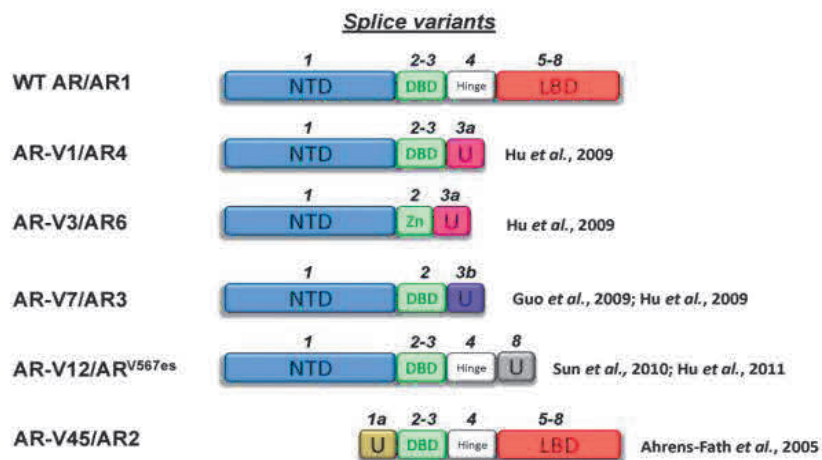


Fig. 1.9 Common splice variants of the AR. *Adapt from: Lallous et al., 2013.*

Some of these variants are constitutively active, such as AR-V7 and ARv567es (aka AR-V12), whereas some others are conditionally active, depending on the cellular context, such as AR-V1 (Hu *et al.* 2011). The latter is truncated at the end of exon 3 and contains 19 amino acids from cryptic exon 1. AR-V1 and AR-V7 were the most abundant variants, with 20-fold higher expression in CRPC compared with hormone-naïve prostate cancer (Hu *et al.*, 2009).

AR-V567es has exons 5–7 spliced out and only contains a small portion of the LBD (Shafi *et al.*, 2013). ARv567es was found to be enriched in prostate epithelium of patients with prostate cancer and in CRPC bone metastasis where they were associated with increased nuclear AR, aberrant cell cycle regulation, and reduced overall survival (Sun *et al.*, 2010; Hornberg *et al.*, 2011). Expression of AR-V567es and/or AR-V7 was associated with poor survival (Sharp *et al.*, 2019).

AR-V7 is truncated at the end of exon 3 and contains 16 amino acids from cryptic exon 3. AR-V7 is located in the nucleus under androgen-depleted conditions and is constitutively active in androgen-responsive genes. More recently, ARv7 expression was detected in circulating tumor cells of patients treated with antiandrogen enzalutamide (12 of 31) or Cyp17A1 inhibitor abiraterone (6 of 31), and its expression was correlated with therapy-resistance (Antonarakis *et al.*, 2014). These studies suggested the potential of using AR-V7 as a predictive marker of response to enzalutamide and abiraterone.

AR45 is truncated in the N-terminal domain, while all the other variants contain an intact N-terminal domain but lack portions of the ligand-binding domain (LBD). As the N-terminal domain harbors the two trans-activating regions (Tau1 and Tau5/AF5)(Need *et al.*, 2009), AR45 loses its transactivating ability and acts as a dominant-negative variant to inhibit the function of the full-length AR (AR-FL) by forming a heterodimer with AR-FL. Moreover, overexpression of AR45 in LNCaP cells inhibited proliferation (Ahrens-Fath *et al.*, 2005). Together, these data indicate that AR45 is a negative regulator of AR signaling.

3.3 AR mutations

Somatic AR mutations may occur selectively in response to androgen deprivation (Hoang *et al.*, 2017). A review of 27 clinical studies found that AR mutations in androgen-dependent tumors ranged from 2-25%, while the incidence in CRPC tumors was slightly higher at 10-40% (Koochekpour, 2010; Hoang *et al.*, 2017). Additional work has identified the AR LBD as a mutational hotspot, placing the incidence of AR LBD point mutations in CRPC at ~15-20% (Grasso *et al.*, 2012; Robinson *et al.*, 2015). Two well-known examples are AR-T877A and AR-W741C, originally described in LNCaP cells, which convert the antiandrogens flutamide and bicalutamide to partial agonists, respectively (Otsuka *et al.*, 2011). Recently, emergence of AR-T878A and AR-L702H was observed in 13% of CRPC tumors progressing on abiraterone treatment, while total AR copy number remained unchanged pre- and post-treatment with abiraterone (Jernberg *et al.*, 2017). Mechanistically, these AR LBD mutations were demonstrated to sensitize AR to activation by progesterone (T878A) (Chen *et al.*, 2015) and glucocorticoids (L702H) (Attard *et al.*, 2012), circumventing the effects of CYP17A1 inhibition on AR signaling.

3.4 AR dependent mechanisms driving prostate cancer progression

3.4.1 AR amplification, mutations and variants

AR amplification/overexpression is the most common genomic aberration in CRPC patients; up to 80% of these patients show AR overexpression (Edwards *et al.*, 2003). This type of adaptation is more common in patients who progressed during or after the treatment with new generation hormonal therapy than in treatment-naïve patients, so it has been considered as a potential resistance mechanism (Crona and Whang, 2017).

AR mutations can be found in up to 30% of CRPC patients treated with ADT. Their incidence may increase during treatment with abiraterone and enzalutamide

because, when AR signalling is more effectively suppressed, clonal selection of tumour cells can enhance AR somatic mutations and the consequent aberrant transcription. Most AR point mutations are clustered in the LBD, altering the steroid-binding pocket and enabling its activation by alternative ligands including progesterone, hydrocortisone, oestradiol, and some AR antagonists (Taplin *et al.*, 1995).

Constitutively active AR splice variants (AR-Vs), lacking the LBD, play a critical role in the development and progression of CRPC, even without androgen binding (Dehm and Tindall, 2011). ARV expression is significantly increased during ADT and is related to PC progression. The most common AR splicing variant, AR-V7, is associated with resistance to both abiraterone and enzalutamide and with short survival (Antonarakis *et al.*, 2014).

3.4.2 Treatment of castration-resistant prostate cancer

Given that AR signaling remains active in CRPC patients, it is recommended that ADT should be continued by adding an AR antagonist such as bicalutamide to the castration therapy (combined androgen blockade). The first-line treatment for patients who relapse after ADT are chemotherapy in form of docetaxel (Tannock *et al.*, 2004; Kellokumpu-Lehtinen *et al.*, 2013). The second line approved treatment options are cabazitaxel (de Bono *et al.*, 2010) (chemotherapy), abiraterone acetate (CYP17 inhibitor) (de Bono *et al.*, 2011), enzalutamide (Scher *et al.*, 2012) (AR antagonist/AR inhibitor), and alfaradin (Radium 223 radioisotope) (Parker *et al.*, 2013) (Fig. 1.10).

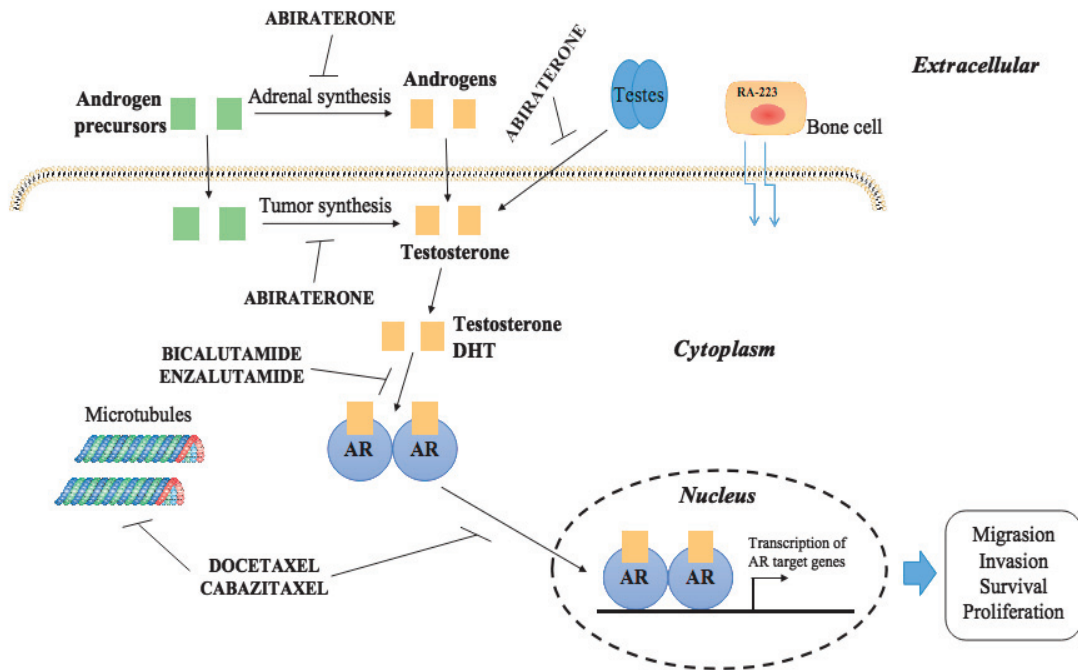


Fig.1.10 Schematic illustration of the mechanisms of action of drugs used in castration-resistant prostate cancer. Abiraterone is a CYP17 inhibitor that blocks androgen synthesis not only in the testis but also in the adrenal gland and in tumor tissue. Bicalutamide and enzalutamide are AR antagonists that bind the AR ligand site, thus preventing ligands to bind to the AR. Cabazitaxel and docetaxel are taxanes that stabilize microtubule which results in blocking of cell division, thereby inducing cell death, taxanes also inhibit nuclear translocation of the AR. Alpharadin (Radium 223 radioisotope) targets new bone growth in and around bone metastases and induces double-strand DNA breaks through alpha radiation over a short distance, thereby inducing cell death. Adapted from Emma Jernberg, 2013.

3.5 AR gene regulation

3.5.1 Regulation of the *AR* gene by AR itself

Grad with colleagues found that the *AR* gene itself is regulated by AR in osteoblast-like cells. Mechanistically, a 350-bp fragment consisting of two androgen responsive elements (ARE) have been identified in exons 4 and 5 of the *AR* gene. These elements selectively control the auto-activation of the *AR* promoter, being responsible for androgen-mediated upregulation of *AR* mRNA in U2OS cells (Grad *et al.*, 2001). Furthermore, it was reported that the expression of the *AR* gene in PCa was directly suppressed by itself through recruitment of lysine-specific demethylase 1 to

the enhancer in the second intron of *AR* and demethylation of H3K4me1 and H3K4me2 (Cai *et al.*, 2011).

3.5.2 AR gene regulation by other factors

3.5.2.1 MYC

a. *MYC* gene and MYC protein

The *MYC* oncogene family, consisting of MYC, MYCN, and MYCL, (Blackwood *et al.*, 1991) encodes transcription factors that contain a basic region/helix-loop-helix/leucine zipper (bHLHZip) type of DNA-binding and protein interaction domain (Dang *et al.*, 2012). All MYC proteins heterodimerize with the obligatory bHLHZip protein MAX, which enables the MYC: MAX heterodimer to bind so-called E-box DNA sequences (CACGTG and similar sequences) situated in regulatory regions of target genes (Eilers *et al.*, 2008; Meyer *et al.*, 2008; Larsson *et al.*, 2010; Hydbring *et al.*, 2017).

The *MYC* gene is present on human chromosome 8q24 and encodes MYC protein which is a transcription factor that plays a key role in regulating a number of cellular processes including cell cycle progression, metabolism, ribosome biogenesis, protein synthesis, mitochondrial function, and stem cell self renewal (Knoepfler *et al.*, 2008; Eilers *et al.*, 2008; Dang *et al.*, 2012). Mechanistically, MYC has the ability to regulate target genes by recruiting different cofactors participating in chromatin modification and remodeling and/or in the initiation and elongation of RNA Pol I, II, and III-mediated transcription. Recently, two components of the multisubunit COMPASS/mixed lineage leukemia (MLL) histone H3 methylase complexes, ASH2L (Ullius *et al.*, 2014) and WRD5 (Thomas *et al.*, 2015), were found to interact with MYC. The WRD5 seems to stabilize MYC's interaction with chromatin (Thomas *et al.*, 2015), while the interaction between the ASH2L and MYC was shown to promote histone H3 lysine 27 (H3K27) demethylation and subsequent H3K27 acetylation (Ullius *et al.*, 2014). H3K27 acetylation is, like histone H3 lysine 4 (H3K4)

methylation, a mark for actively transcribed genes (Shilatifard *et al.*, 2012). However, accumulating results showed that MYC mediated also repression of transcription through interacting with MIZ1 (Staller *et al.*, 2001; Seoane *et al.*, 2001), SP1 (Gartel *et al.*, 2001; Parisi *et al.*, 2007) and SMADs (Seoane *et al.*, 2001).

MYC protein is very short-lived, having a half-life of around 30 min. A number of E3 ubiquitin ligases have been implicated in ubiquitylation of MYC, including SKP2 (Carrano *et al.*, 1999), FBW7 (Popov *et al.*, 2010), HUWE1/HECTH9 (Zhao *et al.*, 2008), FBX29 (Koch *et al.*, 2007), TRUSS (Yada *et al.*, 2004) and FBXL14 (Thomas & Tansey, 2011; Farrell & Sears, 2014). In addition, MYC protein levels are regulated at the level of mRNA translation through the 5' cap mRNA-binding eIF4F complex but also through internal ribosomal entry site elements that are bound by eIF4A-containing complexes, both of which are controlled by mammalian target of rapamycin (mTOR) signaling (Wolfe *et al.*, 2014; Bhat *et al.*, 2015; Castell & Larsson, 2015; Wiegering *et al.*, 2015).

b. MYC in prostate cancer

The *MYC* oncogene is key player in cancer initiation and progression, being critical for maintaining the tumorigenic state in numerous cancer types, including prostate cancer. The overexpression of MYC in PCa has been a well-recognized phenomenon since 1986, when Fleming *et al.* showed a significantly higher level of its expression in adenocarcinoma of the prostate than in benign prostate hyperplasia by Northern blotting (Fleming *et al.*, 1986). Furthermore, Sato *et al* reported that MYC amplification is strongly associated with higher histopathological grades and Gleason scores, as well as with earlier disease progression and cancer-associated death (Sato *et al.*, 2006). Gene amplification of MYC is a key event at the precancer stage (i.e. PIN) of the PCa development (Ellwood-Yen *et al.*, 2003; Gurel *et al.*, 2008; Koh *et al.*, 2010;).

Since MYC is so commonly overexpressed at the protein level in PCa, the current findings raise the possibility that MYC might be an excellent therapeutic target in this disease. New approaches to target MYC in MYC-related tumors are

being developed (Goga *et al.*, 2007), and if these, or similar approaches, reach the clinic trial stage, it will be important to test their therapeutic efficacy in prostate cancer patients.

c. AR regulation by MYC

The MYC has a well-documented role in driving AR oncogenic functions in PCa cells (Grad *et al.*, 1999), and was recently exposed as a key activator in the regulation of *AR* transcription (Nadiminty *et al.*, 2012). They identified miR-let-7c as a key regulator of expression of AR in human prostate cancer cells by targeting its transcription via MYC (Nadiminty *et al.*, 2012).

More recently, Bai *et al* reported a role of MYC in accounting for coordinated AR-full length (AR-FL) and AR- variants (AR-Vs) expression (Bai *et al.*, 2019). Using shRNA knockdown, they confirmed MYC regulation of expression and activity of AR-FL and AR-Vs in cell lines (LNCaP, 22Rv1 and VCaP) and a patient-derived xenograft model. Mechanistically, MYC promotes *AR* transcription and enhances the stability of AR-FL and AR-Vs proteins without altering *AR* RNA splicing. In addition, analysis of gene-expression data from 159 metastatic CRPC samples and 2142 primary prostate tumors showed that the level of MYC is positively correlated with that of individual AR isoforms. More importantly, inhibiting MYC sensitizes enzalutamide-resistant cells to growth inhibition by enzalutamide (Bai *et al.*, 2019). Taken together, these results highlight a critical role of MYC in regulating the coordinated expression of AR-FL and AR-Vs that is commonly observed in CRPC, suggesting the utility of targeting MYC as an adjuvant to AR-directed therapy. However, the mechanism of AR regulation by MYC is still not clear.

3.5.2.2 EZH2

a. The function of EZH2

Enhancer of Zeste Homolog 2 (EZH2), a key component of the Polycomb repressive complex 2 (PRC2) that helps to maintain cell identity during development (Czermin *et al.*, 2002; Müller *et al.*, 2002; Simon & Kingston, 2009), has been found to

contribute to gene repression via the histone H3 lysine 27 trimethylation (H3K27me3) (Cao and Zhang, 2004) catalyzed by its SET domain. EZH2 regulates several cellular processes, including cell fate determination, cell cycle regulation, senescence, cell differentiation and carcinogenesis (Sauvageau & Sauvageau, 2010). Furthermore, EZH2 has been reported to be overexpressed and to function as an oncogene in various cancers by mediating the expression of target genes (Sauvageau & Sauvageau, 2010; Chase & Cross, 2011), including prostate cancer (Varambally *et al.*, 2002), breast cancer (Chang *et al.*, 2011; Yoo & Hennighausen, 2012), hepatocellular carcinoma (Feng *et al.*, 2015), colorectal cancer (Fornaro *et al.*, 2012), gastric cancer (Bai *et al.*, 2014), ovarian cancer (Wang *et al.*, 2015), melanoma (Tiffen *et al.*, 2015) and cervical cancer (Ding *et al.*, 2015). Additionally, EZH2 has been found to act as an epigenetic modifier during the TGF- β -induced epithelial-mesenchymal transition (EMT) and to control the p38 mitogen-activated protein kinase (MAPK) signaling pathway during breast cancer cell migration, invasion and metastasis (Moore *et al.*, 2013).

b. AR regulation by EZH2

EZH2 has recently been shown to coordinate with AR on transcriptional repression. Through ChIP-seq analysis of the key transcription factors and histone marks, they identified an integrative transcriptional network involving AR, EZH2, and ETS-related gene-1 (ERG1) in PCa cells (Yu *et al.*, 2010). ERG belongs to the ETS family of transcription factors and is frequently fused with the 5' untranslated region of the Tmprss2 gene in PCa (Tomlins *et al.*, 2005). They showed that ERG cooccupies with AR on many ARE-containing genomic loci to inhibit gene expression, which may be mediated, at least partially, by direct induction of EZH2 expression and recruitment to the target sites for epigenetic silencing. Further, integrating genome-wide AR localization data with androgen-induced gene expression data, they have identified a large number of genes whose expression is directly inhibited by AR but can be rescued through EZH2 inhibition (Zhao *et al.*,

2012; Fong *et al.*, 2017) Collectively, their data demonstrated that EZH2 facilitates AR-transcriptional repression through catalyzing H3K27me3.

More interestingly, it has been reported that EZH2 activates *AR* transcription through direct occupancy at its promoter (Kim *et al.*, 2018). Importantly, this activating role of EZH2 is independent of PRC2 and its methyltransferase activities. The authors also demonstrated an enhanced efficacy of enzymatic EZH2 inhibitors (GSK126 or EPZ) when used in combination with AR antagonists (enzalutamide) in blocking the dual roles of EZH2 and suppressing prostate cancer progression in cell models (LNCaP, LAPC4, C4-2B and 22Rv1) and C4-2B xenograft mouse model. More recently, Liu *et al* reported that EZH2 physically interacts with AR in PCa cells. Furthermore, they also demonstrated that EZH2 regulates AR protein expression and AR downstream targets, such as, PSA, TMPRSS2 and FKBP5, in AR positive PCa cell lines (Liu *et al.*, 2019). Taken together, the above data showed EZH2 as a transcriptional activator, a key target of which is AR, suggesting a drug-combinatory approach to treat advanced prostate cancer.

3.5.2.3 Pioneering factors: FOXA1, GATA2 and HOXB13

By analyzing enriched motifs around the histone modifications in AR binding sites (ARBSs), AR-associated transcription partners such as Forkhead box protein A1 (FOXA1) (Gao *et al.*, 2003), GATA2 (He *et al.*, 2014), HOXB13 (Huang *et al.*, 2007) have been mapped to the prostate cancer genome and these studies suggested that these factors activate AR-driven transcriptional program. Among them, a chromatin-opening transcription factor, FOXA1, is able to directly bind to the chromatin to open up the local nucleosomal domain (Cirillo *et al.*, 2002). In prostate cells, FOXA1 protein has been shown physically interact with AR and plays critical roles in regulating the transcription of prostate genes such as PSA (Gao *et al.*, 2003). Besides FOXA1, several other transcription factors such as GATA2 and HOXB13 may have similar pioneering cofactor effects on AR-chromatin binding and transcriptional regulation. Similar as FOXA1, GATA family transcription factors have

also been shown to open compact chromatin through their conserved zinc finger domains (Boyes *et al.*, 1998). In addition, GATA2 expression is also essential for AR-mediated transcription of prostate genes such as PSA and TMPRSS2 (Perez-Stable *et al.*, 2000; Wang *et al.*, 2007). Likewise, HOXB13 is a member of the homeodomain family of sequence-specific transcription factors. Mouse studies have shown that HOXB13 plays an essential role in prostate development (Huang *et al.*, 2007). Through analysis of several candidate genes, HOXB13 has been shown as a regulator of AR-chromatin interaction, similar as FOXA1 (Norris *et al.*, 2009). Specifically, HOXB13 was found to inhibit transcription of genes regulated by AR binding to ARE, but enhance AR binding to cis-regulatory regions containing HOX elements juxtaposed to AREs, thereby inducing corresponding gene expression. How GATA2 and HOXB13 regulate genome-wide AR binding profile, however, are yet to be carefully examined.

3.5.2.4 PCAT1

Prostate Cancer Associated Transcript 1 (*PCAT1*) is a long non-coding RNA with a length of ~1900 nt, made of 2 exons. Exon 1 contains a retroviral long terminal repeat (LTR) sequence and exon 2 contains sequences from the HSMAR1 mariner family transposase bisected by an AluY repeat element (Prensner *et al.*, 2011). The *PCAT1* gene locates at chromosome 8q24 and 725 kb upstream of the *MYC* gene. Chr8q24 locus is an area commonly studied in prostate cancer due to frequent amplification and the presence of prostate cancer susceptibility SNP loci (Gudmundsson *et al.*, 2007; Al *et al.*, 2009).

A recent study demonstrated that *PCAT1* was regulated by an SNP located in its enhancer region (Guo *et al.*, 2016). Specifically, the prostate cancer risk-associated SNP rs7463708 was associated with increased expression of *PCAT1* through modulating ONECUT2 and AR binding at a distal enhancer. In addition, *PCAT1* interacted with AR and lysine-specific demethylase 1 (LSD1) and was required for their recruitment to the enhancers of the glycine N-methyltransferase (*GNMT*) and

24-dehydrocholesterol reductase (*DHCR24*) genes (two androgen late-response genes implicated in prostate cancer development and progression) to activate their transcription (Guo *et al.*, 2016) (Fig. 1.11).

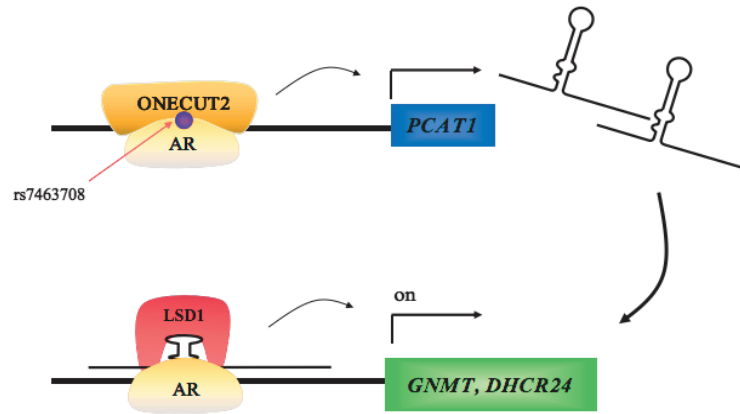


Fig. 1.11 Schematic model of regulation of *PCAT1* at SNP rs7463708. The prostate cancer risk-associated SNP rs7463708 is associated with increased expression of *PCAT1* expression via modulating ONECUT2 and AR binding at adistal enhancer. *PCAT1* recruits AR and LSD1 to the *GNMT* and *DHCR24* enhancers to activate transcription of these genes. Abbreviations: *PCAT1*, prostate cancer-associated transcript 1; SNP, singlenucleotide polymorphism; ONECUT2, one cut homeobox 2; AR, androgen receptor; LSD1, lysine-specific demethylase 1; *GNMT*, glycine N-methyl-transferase; *DHCR24*, 24-dehydrocholesterolreductase. *Adapt from:* Guo *et al.*, 2016.

PCAT1 promotes cell proliferation and is upregulated in major types of cancers (Yan *et al.*, 2015; Zhao *et al.*, 2015; Qiao *et al.*, 2017; Cui *et al.*, 2017), including prostate cancer (Fagerberg *et al.*, 2014), particularly mCRPC (Salinas *et al.*, 2014). In addition, *PCAT1* overexpression was closely associated with clinicopathological characteristics, including tumor size, lymphatic metastasis, distant metastasis, tumor-node-metastasis stage and poor prognosis (Salinas *et al.*, 2014). These results suggested that *PCAT1* may function as a potential prognostic biomarker and therapeutic target in prostate cancer.

3.5.2.5 Other factors: ONECUT2 and Sp1

a. ONECUT2

According to recent reports by Rotinen *et al* and Guo *et al*, ONECUT2 (one cut homeobox 2) plays a critical role, as a master transcriptional regulator, in poorly differentiated neuroendocrine prostate tumors (Rotinen *et al.*, 2018; Guo *et al.*, 2019). As a survival factor in mCRPC models, ONECUT2 depresses AR transcription-related program and activates NE differentiation genes, accelerating tumor progression to lethal disease (Rotinen *et al.*, 2018). Besides, overexpression of ONECUT2 in prostate adenocarcinoma under hypoxia condition is able to inhibit AR signaling and induce NE phenotype (Guo *et al.*, 2019). Given the crucial role of hypoxia in angiogenesis, the authors postulate that ONECUT2 may also contribute to the angiogenic phenotype of NEPC. Indeed, one study in ovarian cancer demonstrated that silencing *ONECUT2* reduced VEGF expression and vascularization in xenografted tumors (Lu *et al.*, 2018).

b. Sp1

Sp1, the first mammalian transcription factor identified and initially named according to the purification procedure adopted (Sephacryl and phosphocellulose columns), is now more commonly named Specificity protein 1 (Kadonaga *et al.*, 1987; Berg, 1992). It is a ubiquitously expressed transcription factor and belongs to a zinc finger family (Lania *et al.*, 1997; Philipsen *et al.*, 1999; O'Connor *et al.*, 2016). It is reported that Sp1 regulates gene transcription by binding to a GC rich element (consensus sequence: GGGGCGGGG), as well as GT/CACC (GGTGTGGGG) boxes in the promoter of target genes (Hagen *et al.*, 1992; Kingsley & Winoto, 1992; Matsumoto *et al.*, 1998). Sp1 not only regulates the expression of AR but also acts as its coregulator. Yuan *et al.* showed that Sp1 can enhance transcriptional activity of the *AR* promoter and of androgen upregulated gene promoters (PSA and hK2)(Yuan *et al.*, 2005). Additionally, Hay *et al.* also demonstrated that Sp1 binds to GC-rich motifs of the *AR* promoter and activates AR transcription, whereas the associated antagonistic transcription factor pur- α can bind to the same region and inhibit AR transcription in PCa cells (Hay *et al.*, 2015).

3.5.3 AR gene regulation by pathways

3.5.3.1 Wnt/ β -catenin pathway

WNT signaling is an evolutionary highly conserved signaling system throughout the eukaryotic kingdom (Nelson and Nusse, 2004). During embryonic and postnatal development, WNT signaling controls many cellular processes, including proliferation, survival and differentiation (Grigoryan *et al.*, 2008; Klaus and Birchmeier, 2008; Lien and Fuchs, 2014). Deregulation in WNT signaling leads to an imbalance of such processes, often resulting in aberrant development or disease (Clevers *et al.*, 2006; Polakis, 2007). In particular, deregulated WNT signaling is common in human cancers, including malignancies of the intestine (Taketo *et al.*, 2004; White *et al.*, 2012), liver (Sato *et al.*, 2000), skin (Chan *et al.*, 1999), breast (Shulewitz *et al.*, 2006) and prostate (Hu *et al.*, 2016).

The WNT signaling transduction can be mediated by three different, essentially separating but interacting pathways, the canonical Wnt/ β -catenin, non-canonical planar cell polarity and the non-canonical Wnt/Calcium pathways (Fig. 1.12) (Polakis, 2012). The best-studied Wnt signaling pathway is the canonical Wnt/ β -catenin pathway in which Wnt ligands form a complex with Frizzled (FZD) receptor and coreceptors such as the low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6. Upon receptor activation, the “destruction complex” which includes adenomatous polyposis coli (APC) protein and Axin is inhibited, thereby blocking the phosphorylation of β -catenin by both casein kinase I α and glycogen synthase kinase-3 β (GSK-3 β), which is prerequisites for β -catenin degradation (Gómez-Orte *et al.*, 2013; Sprowl *et al.*, 2013).

A hallmark of canonical Wnt signaling is the stabilization and nuclear localization of β -catenin. Phosphorylation of β -catenin normally targets β -catenin for degradation. Its inhibition results in cytoplasmic β -catenin stabilization and accumulation which facilitates its translocation into the nucleus. Nuclear β -catenin acts as a transcriptional coactivator and interacts with transcription factors such as T-cell factor (TCF) and lymphoid enhancer factor (LEF) and leads to increased

transcription of target genes, such as *MMP7*, *MYC*, *c-Jun*, *Fra* and other members of the c-Fos family. In addition, Wnt signaling can also regulate other nonconventional pathways such as the JNK pathway independent of β -catenin leading to changes in cell polarity, movement and survival (Polakis, 2012; Gómez-Orte *et al.*, 2013; Sprowl *et al.*, 2013).

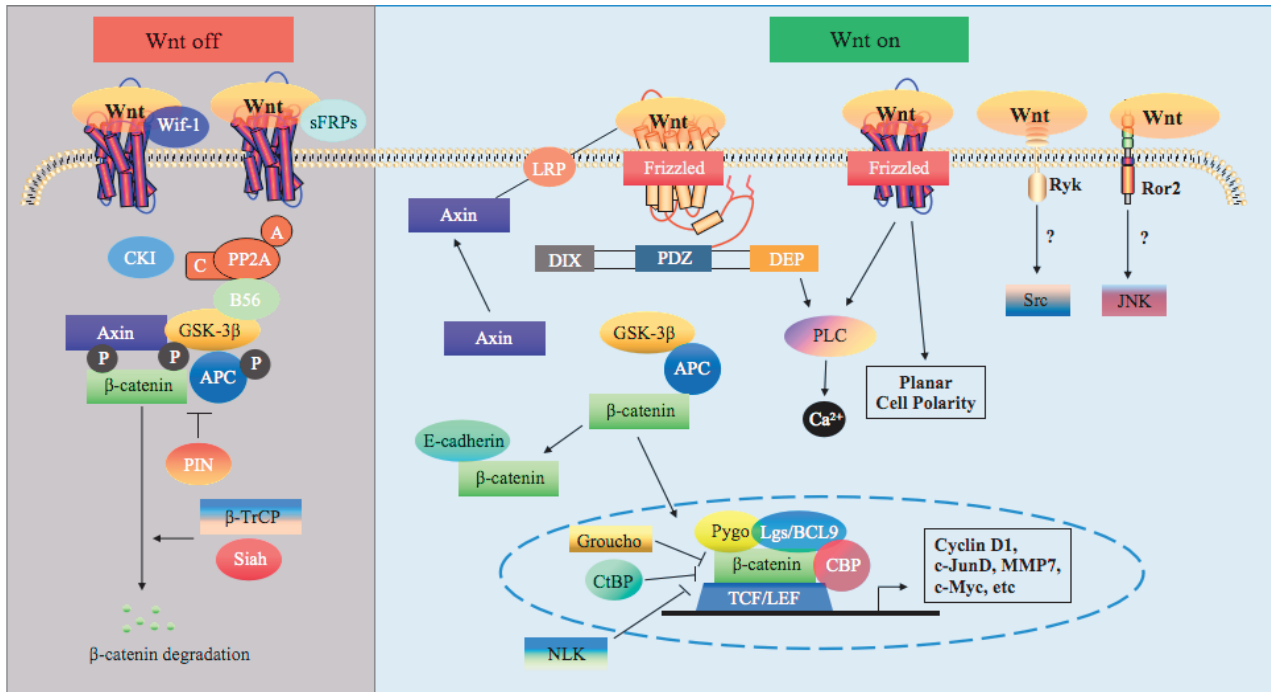


Fig. 1.12 Overview of the Wnt signaling pathway. In the “Wnt-Off” state, Wnt/receptor interactions are interrupted by secreted Wnt antagonists, including sFRPs, DKKs and WIF1; and β -catenin is degraded by the formation of the “destruction complex” consisting of APC protein, Axin and others. In the “Wnt-On” state, Wnts are lipid modified by the acyl transferase porcupine in the endoplasmic reticulum, and act in an autocrine and paracrine fashion. The Wnts form a complex with Frizzled receptor and coreceptors LRP. Upon receptor activation, the “destruction complex” which includes APC, Axin and others is inhibited thereby blocking β -catenin phosphorylation for degradation. This inhibition results in cytoplasmic β -catenin stabilization and accumulation which facilitates its translocation into the nucleus. Nuclear β -catenin acts as a transcriptional co-activator for LEF1/TCF, leading gene transcription of Wnt target genes, such as *JUN*, *Cyclin-D1*, and *MMP7*. In addition, Wnts bind to tyrosine-protein kinase transmembrane receptors ROR2 and RYK to activate other non-canonical planar cell polarity, the Wnt/JNK and the Wnt/Calcium pathways. *Adapt from: Yokoyama et al., 2014.*

Accumulating evidence supports that the WNT/ β -catenin pathway plays an important role in CRPC, by interacting with AR signaling (Cheshire *et al.*, 2000; Wang *et al.*, 2008; Schweizer *et al.*, 2008; Yokoyama *et al.*, 2014). Findings of a

protein-protein interaction between AR and β -catenin suggested the biological significance of β -catenin in PCa cells. In 2000, Truica *et al.* showed that β -catenin could directly bind to AR to enhance its transcriptional activity stimulated by androgen, androstenedione, or estradiol in LNCaP cells (Truica *et al.*, 2000). In 2002, Yang *et al.* demonstrated that β -catenin preferentially and directly bound to the LBD of AR in the presence of DHT over several other steroid hormone receptors (Yang *et al.*, 2002). Further studies revealed that β -catenin bound to the AF-2 region of the AR LBD, and modulated the transcriptional effects of the AR NTD as well as the p160 coactivator transcriptional intermediary factor 2 (TIF2); importantly, a single AR lysine residue (K720) has been shown to be necessary for the AR/ β -catenin and TIF2/ β -catenin interactions (Song *et al.*, 2003; Masiello *et al.*, 2004). In β -catenin, early mapping experiments suggested that the NH2 terminus and the first six armadillo repeats of β -catenin were involved in its interaction with AR. In particular, deletion of repeat 6 fully abolished the physical interaction between AR and β -catenin, suggesting a key role of this repeat in the interaction (Yang *et al.*, 2002). Phenotypically, transient over-expression of β -catenin in AR-positive PCa cell lines CWR22-Rv1 and LAPC-4 enhanced AR-mediated transcription of its target genes, in an androgen-dependent manner (Chesire *et al.*, 2004). Hence, β -catenin (wild-type or mutated) is considered as a ligand-dependent co-activator of the AR-driven transcription. Binding of β -catenin to ligand-engaged AR also facilitates the movement of β -catenin into the nucleus (Mulholland *et al.*, 2005). Furthermore, it was shown that WNT/ β -catenin signaling could increase *AR* transcription via the TCF/LEF-1 binding sites in the *AR* promoter (Li *et al.*, 2009). Thus, in hormone-naïve PCa, WNT/ β -catenin signaling serves as a positive regulator of AR signaling in an androgen-dependent manner.

β -catenin is also one of the three AR coactivators (other two AR specific coactivators are ARA70 and ARA55) that can enhance *AR* transcriptional activity in LNCaP cells when treated with 17 β -estradiol (Yang *et al.*, 2002; Song *et al.*, 2003; Masiello *et al.*, 2004). In addition, β -catenin can function as a coactivator with altered

AR with mutations W741C and T877A in prostate cancer cell lines (Song *et al.*, 2003). These AR mutations were detected in CRPC patients that have been treated with bicalutamide leading to the W741C mutation and also in CRPC patients with lymph node metastatic lesions containing the T877A mutation (Song *et al.*, 2003). In rodent studies, Chesire *et al.* reported that castrated mice receiving androgen treatment exhibited nuclear co-localization of AR and β -catenin in normal prostatic epithelium (Chesire and Isaacs, 2003). Nuclear β -catenin localization was found to occur concomitantly with androgen-induced regrowth of normal rat prostate from androgen deprivation induced regression. Furthermore, Wang *et al.* observed increased expression and nuclear colocalization of AR and β -catenin as well as the interaction between endogenous AR and β -catenin in CRPC from castrated mice (Wang *et al.*, 2008). However, they found no interaction or colocalization of AR and β -catenin in xenografts from noncastrated mice. Mutations of β -catenin are uncommon in prostate cancer (< 5%) (Voeller *et al.*, 1998; Chesire *et al.*, 2000).

3.5.3.2 PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR signaling pathway has a diverse array of functions, including the regulation of cellular survival, differentiation and stem cell-like properties, growth, proliferation, metabolism, migration, and angiogenesis (Guba *et al.*, 2002; Dubrovskaja *et al.*, 2009; Liu *et al.*, 2009; Courtney *et al.*, 2010; Furic *et al.*, 2010; Hsieh *et al.*, 2012). There are three classes of PI3K that are differentiated by their structural characteristics and substrate specificities (Courtney *et al.*, 2010). Class I PI3Ks are activated by receptor tyrosine kinases (RTKs), G-protein-coupled receptors, and some oncogenes, such as rat sarcoma oncogene (RAS), and can be further subdivided into class IA and IB, of which class IA PI3Ks are most frequently implicated in cancer. Class IA PI3Ks consist of two subunits: a regulatory subunit, p85, and a catalytic subunit, p110. There are three isoforms of p85 (p85 α , p85 β , and p85 γ) encoded by the genes PIK3R1, PIK3R2, and PIK3R3 respectively and three isoforms of class IA p110 (α , β , and δ) encoded by the genes PIK3CA, PIK3CB,

and PIK3CD respectively (Courtney *et al.*, 2010). Activation of PI3K leads to the phosphorylation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] to phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃] and subsequent recruitment of AKT to the plasma membrane where Akt is activated (Liu *et al.*, 2009, Courtney *et al.*, 2010). AKT activation is mediated through phosphorylation at two residues: T308 by phosphoinositide-dependent kinase-1 and S473 by the mTOR complex 2 (mTORC2). Both phosphorylation events are required for full activation of AKT (Sarbassov *et al.*, 2005). Upon activation, AKT phosphorylates a host of other proteins including glycogen synthase kinase 3 (GSK3), FOXO transcription factors, and tuberous sclerosis complex (TSC) and thereby regulates a range of cellular processes, including protein synthesis, cell survival, proliferation, and metabolism (Liu *et al.*, 2009; Courtney *et al.*, 2010). In prostate cancer, PI3K signaling has been shown to repress *AR* transcriptional activity, illustrating the lineage-specific complexity of this pathway (Carver *et al.* 2011).

mTOR is a serine threonine kinase and participates in the formation of the catalytic subunit of the functionally distinct mTORC1 and mTORC2. Depending on available nutrients and the cellular environment, mTORC1 controls the growth of the cell through phosphorylation of S6K and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Sparks and Guertin, 2010). mTORC2 does not bind to and is generally insensitive to rapamycin and has been shown to signal independently of mTORC1. mTORC2 is believed to mediate cell proliferation and cell survival through phosphorylation of its substrates, which include AKT, glucocorticoid-induced protein kinase (SGK), and protein kinase C (Sparks & Guertin 2010).

PI3K/AKT/mTOR signaling is negatively regulated by the tumor suppressor phosphatases and tensin homolog (PTEN) and inositol polyphosphate-4-phosphatase, type II (INPP4B), which convert PI(3,4,5)P₃ to PI(4,5)P₂ and PI(3,4)P₂ to PI(3)P respectively. mTORC1-activated S6K has also been shown to negatively regulate PI3K/AKT/mTOR by phosphorylating mTORC2, resulting in a reduction in mTORC2-dependent S473 phosphorylation of Akt (Dibble *et al.*, 2009). In addition,

S6K negatively regulates RTK signaling and hence PI3K/AKT/mTOR and RAS/RAF/MEK signaling by phosphorylating and causing the degradation of insulin receptor substrate 1 (IRS-1) (O'Reilly *et al.*, 2006; Carracedo *et al.*, 2008; Rodrik-Outmezguine *et al.*, 2011).

The PI3K/AKT/mTOR and AR signaling pathways have recently been shown to regulate each other through complex reciprocal feedback mechanisms (Wen *et al.*, 2000; Lin *et al.*, 2001; Li *et al.*, 2008; Sarker *et al.*, 2009; Carver *et al.*, 2011; Mulholland *et al.*, 2011). PI3K/AKT/mTOR signaling inhibits AR signaling via feedback inhibition of human epidermal growth factor 2/3 (HER2/3) kinases, which have been shown to promote AR stability and transcriptional activity (Carver *et al.*, 2011). In addition, PTEN increases the transcriptional activity of AR by negatively regulating the expression of the transcription factors Egr1 and c-JUN, which inhibit AR-targeted gene expression, such as FKBP5 (Mulholland *et al.*, 2011). Meanwhile, AR signaling downregulates PI3K/AKT/mTOR signaling through FK506-binding protein-5 (FKBP-5)-mediated stabilization of the Akt phosphatase PHLPP (Carver *et al.*, 2011). Activation of the PI3K/AKT/mTOR pathway as a result of treatments targeting AR signaling or due to *PTEN* loss may, therefore, enable prostate cancer cells to survive and proliferate in androgen-reduced conditions. Conversely, PTEN controls the transcription of *NKX3.1*, which negatively regulates the *AR* promoter and reduces AR pathway signaling. When *PTEN* is lost, this brake on AR signaling is also lost (Lei *et al.*, 2006). Therefore, inhibition of the PI3K/AKT/mTOR pathway may lead to a compensatory increase in AR activity and may promote prostate cancer progression and development of resistance to single-agent PI3K pathway inhibitor therapy. This concept of reciprocal inhibition is further supported by the recent finding that use of antiandrogens for chemoprevention actually accelerates progression to invasive prostate cancer in a *Pten*-null mouse model (Jia *et al.*, 2013). More importantly, PI3K-AKT-mTOR pathway inhibition is associated with augmented AR signaling that can contribute to drug resistance and promote prostate cancer progression (Carver *et al.*, 2011; Thomas *et al.*, 2013; Qi *et al.*, 2015;

Audet-Walsh *et al.*, 2017). Carver and colleagues showed that PI3K/mTOR inhibition activates AR signaling in human xenograft and transgenic mouse models of prostate cancer, and that co-treatment with the PI3K/mTOR inhibitor BEZ235 and the antiandrogen MDV3100 (enzalutamide) significantly reduced tumor burden relative to BEZ235 monotherapy (Carver *et al.*, 2011; Shorning *et al.*, 2020). In corroboration, resistance to the AKT inhibitor capivasertib (AZD5363) in LNCaP prostate cancer xenografts is also associated with elevated AR signaling, and combining AZD5363 treatment with the antiandrogen bicalutamide prolonged disease stabilization (Thomas *et al.*, 2013). Furthermore, mTOR and EGFR co-inhibition with everolimus and gefitinib has shown limited sensitivity in patients owing to enhanced AR activity and PSA levels (Audet-Walsh *et al.*, 2017), providing further rationale for combining AR and PI3K-AKT-mTOR blockade to treat prostate cancer (Shorning *et al.*, 2020).

Several distinct molecular mechanisms have been identified that underpin AR reactivation upon AKT inhibition. Notably, AKT inhibition can prevent FOXOs from AKT-mediated nuclear exclusion, which can lead to augmented transcription of FOXO-target genes such as RTKs (e.g., ERBB2/3 encoding HER2/3) (Figure 3B) (Yao *et al.*, 2009; Carver *et al.*, 2011; Chandarlapaty *et al.*, 2011). HER2/3 activity has been shown to promote AR signaling by protecting AR from ubiquitination and proteasomal degradation, and by enhancing AR binding to ARE target sequences and stimulating *AR* transcriptional activity (Yeh *et al.*, 1999; Mellinghoff *et al.*, 2004). Nonetheless, the role of FOXO-dependent signaling in PI3K/AKT/mTOR and AR pathway crosstalk is complex. Although FOXO transcription factors upregulate the expression of RTKs (Chandarlapaty *et al.*, 2011) causing a subsequent increase in AR signaling (Mellinghoff *et al.*, 2004), the ectopic expression of FOXO1 conversely dampens *AR* activity (Liu *et al.*, 2008; Shorning *et al.*, 2020).

3.5.3.3 RAS/RAF/MEK pathway

The RAS/RAF/MEK pathway is also involved in extensive cross talk with the PI3K/AKT/mTOR pathway (Fig. 1.13), and activation of this pathway has been

associated with decreased sensitivity to PI3K/AKT/mTOR pathway inhibitors (Ihle *et al.*, 2009). Recently, a study of prostate cancer tissue microarrays found that the RAS/RAF/MEK pathway was significantly elevated in both primary and metastatic lesions (Mulholland *et al.*, 2012). In murine models, *PTEN* deletion results in the development of prostate cancer (Wang *et al.*, 2003); while the combination of *PTEN* deletion and RAS activation significantly accelerated prostate cancer progression caused by *PTEN* loss, and this was accompanied by EMT and macrometastasis (Mulholland *et al.*, 2012). Furthermore, inhibition of the RAS/RAF/MEK pathway with a MEK inhibitor (PD325901) significantly reduced metastatic progression initiated by *PTEN*-deficient and *KRAS*-activated stem/progenitor cells (Mulholland *et al.*, 2012). Thus, cross talk between the PI3K/AKT/mTOR pathway and the RAS/RAF/MEK pathway in prostate cancer is likely clinically important in promoting metastasis.

Receptor tyrosine kinases (RTKs), which activate both the PI3K/AKT/mTOR and the RAS/RAF/MEK pathways, are in turn negatively regulated by mTORC1 activity; inhibition of mTORC1 leads to a relief of an inhibitory signal from S6K to IRS-1, a receptor substrate for multiple cell surface receptors, such as insulin and IGF, HER2/3 and epidermal growth factor receptor (EGFR), and others (O'Reilly *et al.*, 2006; Carver *et al.*, 2011, Rodrik-Outmezguine *et al.*, 2011). Relief of this feedback inhibition with single-agent rapamycin analogs may lead to rapid compensatory PI3K and Akt re-activation and limit the pharmacodynamics and clinical impact of these agents on tumor cell survival and invasion/metastasis (Rodrik-Outmezguine *et al.*, 2011). Combined inhibition of cell surface receptors with mTORC1 inhibitors has demonstrated an ability to overcome this feedback loop and is an area of ongoing investigation. Whether combination therapy with PI3K, AR, cell surface receptor, and/or RAS/RAF/MEK pathway inhibition is needed for optimal therapy in preclinical or clinical prostate cancer is not known yet.

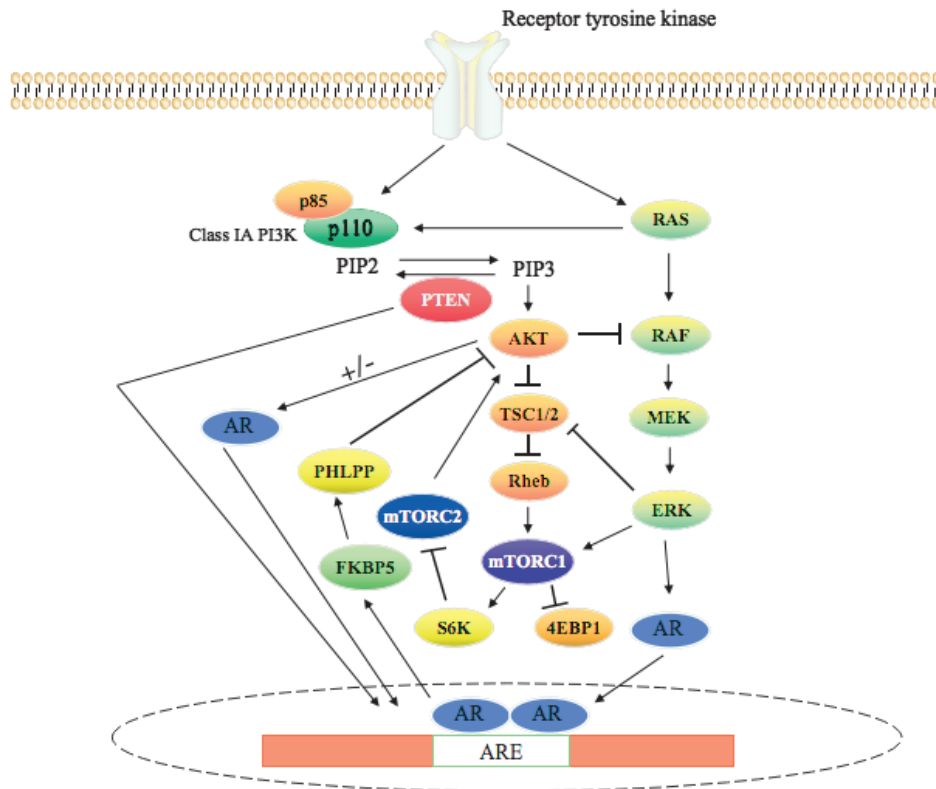


Fig. 1.13 The PI3K/AKT/mTOR pathway and cross talk with the AR signaling and RAS/RAF/MEK pathways. AR, androgen receptor; ARE, androgen-responsive element; DHT, dihydrotestosterone; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; ERK, extracellular signal-related kinase; FKBP5, FK506-binding protein 5; MEK, mitogen-activated protein/ERK kinase; PHLPP, PH and leucine-rich repeat phosphatase; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; RAS, rat sarcoma oncogene; Rheb, RAS homolog enriched in the brain; S6K, S6 kinase; TSC, tuberous sclerosis protein; TORC, target of rapamycin complex; +/-, Akt activity can both enhance and suppress AR signaling. Possible mechanisms of AKT-mediated regulation of AR activity include direct phosphorylation of AR (Wen *et al.*, 2000; Lin *et al.*, 2001).

3.6 The function of AR

3.6.1 AR in normal tissues

In humans, AR is expressed in all organs with the exception of the spleen and bone marrow. It is essential for the development of male reproductive organs, puberty, male fertility and male sexual function. In the prostate, androgens are essential for normal development and function. AR is expressed in both epithelial cells and stromal cells. In epithelial cells, AR has a role in cellular differentiation, survival and the

expression of secretory proteins (Cunha *et al.*, 2004; Olsen *et al.*, 2016). Stromal AR has a role in embryonic prostate development, epithelial differentiation and determining secretory protein expression (Wen *et al.*, 2015).

3.6.2. Cell cycle regulator

Several papers have already shown that AR is a critical regulator of G1-S transition in AR-dependent cell cycle progression in prostate cancer cells. The p21^{cip} has been validated as a direct AR target, and the induction of p21^{cip} upon androgen stimulation may assist in assembling active CDK4/cyclin D1 complexes (Lu *et al.*, 1999; Alt *et al.*, 2002; Sherr & Roberts, 2004). This supposition is consistent with the findings revealing that p21^{cip} expression was enhanced in tumors, and correlated with a higher proliferative index and Gleason grade (Aaltomaa *et al.*, 1999; Baretton *et al.*, 1999). Furthermore, Knudsen *et al.* showed that androgen depletion induces p27^{Kip1}, which likely contributes to the observed reductions in CDK2 activity (Knudsen *et al.*, 1998). And, p27^{Kip1} loss in the context of a *PTEN* mutation promotes a tumorigenic phenotype in the prostate (Di Cristofano *et al.*, 2001; Gao *et al.*, 2004).

3.6.3 Apoptosis regulator

Kerr *et al* first suggested the term “apoptosis” and confirmed it as a general mechanism of controlled cell deletion, which is complementary to mitosis in the regulation of animal cell populations (Kerr *et al.*, 1972). Rokhlin *et al* found androgen/AR signaling could directly regulate p53 to suppress apoptosis. Mechanistically, androgen suppresses TNF- α /Fas-induced apoptosis through inhibition of p53 expression and caspase-2 activation in LNCaP and C4-2 cells (Rokhlin *et al.*, 2005). Other studies also showed that androgen could block apoptosis induced by Fas activation and TNF- α (Kimura *et al.*, 2001). Besides, p21 has been proven to be able to protect against p53-mediated apoptosis (Gorospe *et al.*, 1997). Asada found that overexpression of p21 may induce cell cycle arrest and led to an apoptosis-resistant phenotype (Asada *et al.*, 1999). Furthermore, AR stimulated

endogenous *p21* mRNA and protein levels and functioned as an apoptosis inhibitor to promote PCa LNCaP cell growth (Lu *et al.*, 1999). Interestingly, Frezza *et al* found that co-treatment with Bortezomib and the AR antagonist Casodex caused significant decrease in AR expression and led to caspase-3 activity increase in LNCaP and PC3-AR cells, suggesting that AR might suppress caspase-3 expression (Frezza *et al.*, 2011). Liao *et al* also showed that knockdown of *AR* via siRNA led to apoptotic death in PCa cells (Liao *et al.*, 2005).

3.6.4 AR as a transcriptional repressor

Zhao *et al.* have shown that AR can act as a transcriptional repressor to directly inhibit gene expression. This repression is mediated by AR binding to AREs and facilitated by EZH2-mediated repressive chromatin remodeling (Zhao *et al.*, 2012). EZH2 thus cooperates with AR in transcriptional repression of target genes. Furthermore, Wu *et al.* have nominated a number of robust AR-repressed genes (*CCN3/NOV* gene family), through meta-analysis of microarray datasets profiling androgen-treated PCa cells. Interestingly, they demonstrated that AR activation recruits the polycomb group protein EZH2, which subsequently catalyzes histone H3K27me3 around the *NOV* promoter, thus leading to repressive chromatin remodeling and epigenetic silencing. Concordantly, AR and EZH2 inhibition synergistically restored *NOV* expression in LNCaP cells. More importantly, they analyzed *NOV* expression in a microarray data set that profiled gene expression in a panel of benign prostate, clinically localized and metastatic prostate cancer tissues. They found that *NOV* mRNA was remarkably downregulated in metastatic tumors. Furthermore, the level of *NOV* was negatively correlated with the level of androgen signaling represented by PSA expression. Finally, they concluded that *CCN3/NOV* genes may have important cellular functions and their repression by androgen may be critical for prostate physiology and disease (Wu *et al.*, 2014).

3.6.5 Histone modifiers: acetyltransferases and methyltransferases

Acetyltransferases

Some of these histone modifications are associated with transcriptional activation (e.g., acetylation), and others are indicative of active or repressed genes (e.g. methylation) (Mellor, 2006; Li *et al.*, 2007). Dynamic changes in the modification state of covalent histones are related to androgen-stimulated transcription. These include modifications that activate histone 3 such as acetylation of lysine 9 and lysine 14, dimethylation of arginine 17, phosphorylation of serine 10, and dimethylation as well as trimethylation of lysine 4 (Kang *et al.*, 2004). Fu *et al.* demonstrated a direct interaction between p300, CBP, P/CAF and AR. The potentiation of ligand-induced AR transactivation by these three coactivators relies on the presence of a functional histone acetylase domain. Furthermore, p300 and P/CAF acetylate AR at three lysine residues in its DBD-hinge region. They conclude that CBP and p300 function as a direct bridge between DNA-bound AR and the basal transcriptional machinery (Fu *et al.*, 2000).

Methyltransferases

AR-dependent transcription relies on methyltransferase activities. Androgen stimulation leads to recruitment of CARM-1 (coactivator-associated arginine methyltransferase-1) to androgen-responsive enhancers (Majumder *et al.*, 2006). Moreover, the presence of CARM-1 enhances AR transactivation by p160 family members. Loss of CARM-1 reduces transcription of androgen-responsive genes (Chen *et al.*, 1999; Majumder *et al.*, 2006). CARM-1 also recruited to the AR transcriptional complex through p44, a component of the methylosome. p44, found on the promoters of AR target genes upon androgen stimulation, interacts directly with AR and CARM-1 and stimulates the transcription of some AR-target genes such as, *NKX3.1*. Lee *et al.* showed that G9a, a histone H3 Lys-9 methyltransferase localized in euchromatin, functions as a coactivator for AR, although weakly. G9a cooperates synergistically with CARM-1 and p300 in activating transcription by the AR. This synergy is strongly dependent on the activity of CARM-1 arginine-specific protein

methyltransferase, which indicates a link between histone arginine and lysine methylation during AR-mediated transcription (Lee *et al.*, 2006).

4. Other mechanisms underlying prostate cancer initiation and progression

4.1 Genes involved in the carcinogenesis of prostate cells

4.1.1 NKX3.1

NKX3.1 is a prostate-specific tumor suppressor located on chromosome 8p, whose loss or reduction represents a key initiating event in prostate cancer (Cancer Genome Atlas Research, 2015; Baca *et al.*, 2013). Inactivation of the *NKX3.1* homeobox gene, expressed in normal prostate luminal cells, represents a frequent and critical event in prostate cancer initiation, likely involving multiple mechanisms (Abate-Shen *et al.*, 2008). Previous study showed that NKX3.1 expression is completely lost in advanced cancers (Bowen *et al.*, 2000), more recent analyses using a highly sensitive antibody indicate that low levels of NKX3.1 expression can be demonstrated in nearly all prostate cancers and metastases examined (Gurel *et al.*, 2010)

Interestingly, several studies reported *NKX3.1* as a critical regulator of prostate epithelial differentiation and stem cell function in mouse models. During development, *NKX3.1* is expressed in all epithelial cells of the nascent prostate buds from the urogenital sinus, and represents the earliest known marker for the prostate epithelium (Bhatia-Gaur *et al.* 1999). In the absence of *NKX3.1*, there is a significant decrease in prostatic ductal branching, as well as in production of secretory proteins (Bhatia-Gaur *et al.* 1999; Schneider *et al.* 2000). Notably, young adult *Nkx3.1* heterozygous and homozygous mutants frequently display prostate epithelial hyperplasia and dysplasia, and often develop Prostatic Intraepithelial Neoplasia (PIN) by 1 year of age (Bhatia-Gaur *et al.* 1999; Schneider *et al.* 2000). In particular, *Nkx3.1* inactivation in mice results in a defective response to oxidative damage, while its expression in human prostate cancer cell lines protects against DNA damage and is

regulated by inflammation (Ouyang *et al.* 2005; Markowski *et al.* 2008; Bowen and Gelmann 2010). These findings are consistent with the tumor suppressor activity of NKX3.1 observed in cell culture and xenograft assays (Kim *et al.* 2002; Lei *et al.* 2006).

4.1.2 TMPRSS2-ERG translocations

One of the earliest genetic alterations in prostate cancer is overexpression of the *ERG* (*ETS-related gene*) oncogene, which occurs in over 50% of prostate cancers. In the majority of tumors, this is driven by fusion of the *ERG* gene with *transmembrane protease, serine 2* (*TMPRSS2*), a prostate-specific and androgen-regulated gene that maps very close to *ERG* on the same chromosome (Tomlins *et al.*, 2005; Perner *et al.*, 2006; Wang *et al.*, 2006; Clark *et al.*, 2007). This generates an androgen-responsive fusion oncoprotein, leading to overexpression of the respective ETS family member. *ERG* is a member of the large family of ETS transcription factors (Hollenhorst *et al.* 2011). This gene fusion has never been found in normal prostate but is present in tumor adjacent to PIN. The frequency of these *TMPRSS2-ERG* fusions is \approx 15% in HGPIN lesions and \approx 50% in localized prostate cancer (Clark *et al.*, 2008; Mosquera *et al.*, 2008; Albadine *et al.*, 2009; Taylor *et al.* 2010), suggesting that this rearrangement either occurs after cancer initiation, or alternatively corresponds to an early event that predisposes to clinical progression.

By comparison of global gene expression data for clinical prostate cancer samples with and without *ERG* overexpression, pathways associated with *ERG* overexpression have been identified. Data obtained by Iljin *et al.* (2006) indicated a role of the WNT pathway in *ERG*-associated prostate cancer and showed high expression of HDAC1 in *ERG*-overexpressing tumor samples. Also, activation of the transforming growth factor β (TGF β) pathway has been associated with *ERG* overexpression (Brase *et al.* 2011). Although the data reported in different studies are variable, a consistent association with *ERG* overexpression of more than ten genes, including *CACNA1D*, *TDRD1*, *PLA2G7*, and *NCALD*, has been found (Iljin *et al.*

2006, Jhavar *et al.* 2008, Taylor *et al.* 2010, Brase *et al.* 2011, Boormans *et al.* 2013). This does not mean that these genes are direct ERG target genes. They might be indirectly regulated by ERG or they might represent a common prostate cell type in which TMPRSS2-ERG fusion occurred. Interestingly, a recent study revealed that TMPRSS2-ERG was also involved in PCa bone metastasis via its regulation of the expression of osteoblastic markers (Delliaux *et al.*, 2018). The mechanism of expression of other ERG-associated genes remains to be investigated.

4.1.3 EZH2

The *EZH2* gene, situated on 7q35-36, encodes EZH2 protein, and EZH2 protein levels are significantly increased in prostate cancer compared with BPH tissue (Varambally *et al.*, 2002). EZH2 increases histone deacetylation, which is a repressor of transcription in prostate cancer. Elevated levels of EZH2 mRNA and protein in clinically localised prostate cancer predicts poor prognosis. In addition, repression of EZH2 by small interfering RNA (RNAi) decreases the rate of cell proliferation in PCa cell lines (Varambally *et al.*, 2002). This gene represents an interesting development both as a prognostic marker and also as a therapeutic target.

4.1.4 PTEN

Germline disruption of *Pten* in heterozygous mutants or conditional ablation in the prostate epithelium in the mouse results in PIN and/or adenocarcinoma development (Di Cristofano *et al.* 1998a; Podsypanina *et al.* 1999; Trotman *et al.* 2003; Wang *et al.* 2003). Fluorescence in situ hybridization (FISH) has been used to identify genomic *PTEN* loss, which is found in 9–23 % of HGPIN lesions (Schmitz *et al.*, 2007; Sircar *et al.*, 2009) and 10–70 % of prostate cancers (Suzuki *et al.*, 1998; Yoshimoto *et al.*, 2007), and is correlated with an overall poor prognosis (Yoshimoto *et al.*, 2007; Taylor *et al.*, 2010).

Notably, PTEN reduction or loss in prostate cancer is correlated to the emergence of castration-resistant prostate cancer (Mulholland *et al.* 2006; Shen and

Abate-Shen 2007). In particular, perturbation of PTEN expression in human prostate cancer cell lines or targeted *Pten* disruption in the mouse is sufficient for the development of castration resistance (Lin *et al.* 2004; Bertram *et al.* 2006; Gao *et al.* 2006; Wu *et al.* 2006). *PTEN* loss is more common in hormone refractory and metastatic prostate cancer than in hormone-dependent primary tumors, with homozygous *PTEN* loss in 10 and 50 % of hormone-dependent and metastatic/hormone refractory cases, respectively (Verhagen *et al.*, 2006; Yoshimoto *et al.*, 2006; Mulholland *et al.*, 2006; Schmitz *et al.*, 2007; Sircar *et al.*, 2009; Han *et al.*, 2009).

Moreover, loss of *PTEN* and *AR* expression has been correlated clinically with increased mortality in CRPC patients (Sircar *et al.*, 2009). Microarray-based Comparative Genomic Hybridization (aCGH) analysis on metastatic prostate cancer samples has also demonstrated frequent amplification of AR (73%), coinciding with aberrant deletion of PTEN (87%) (Friedlander *et al.*, 2012). Therefore, PTEN could serve as a prognostic marker for hormone refractory and metastatic disease.

4.1.5 The AKT/mTOR signaling pathways

As noted above 3.5.3.2, considerable evidence indicates that *Pten* loss of function results in up-regulation of the AKT/mTOR signaling pathway in prostate cancer, primarily through activation of *AKT1* (Thomas *et al.* 2004; ML Chen *et al.* 2006; Mulholland *et al.* 2006; Shen and Abate-Shen 2007). Upregulation of this pathway in prostate cancer can take place through activating mutations of *AKT1* (Boormans *et al.* 2008), or through activation of the p110 β isoform of PI3K (Hill *et al.* 2010; Lee *et al.* 2010). The consequences of AKT/mTOR pathway activation are particularly relevant for castration-resistant prostate cancer, as has been shown in genetically engineered mouse models, in gain-of-function studies with orthotopic grafting or tissue recombination models, as well as in human cell lines (Majumder *et al.* 2003; Uzgare and Isaacs 2004; Gao *et al.* 2006a; Xin *et al.* 2006). In addition, the consequences of Akt activation are mediated in part by activation of NF- κ B signaling via stimulation

of IKK (Dan *et al.* 2008). Conversely, functional studies in mouse models and correlative studies in human prostate cancer have implicated deregulated NF- κ B signaling in mediating androgen responsiveness, metastasis, and disease outcome (Fradet *et al.* 2004; Ismail *et al.* 2004; Lessard *et al.* 2006; Luo *et al.* 2007; Zhang *et al.* 2009).

4.1.6 TGF β

Several reports showed that AR crosstalks with transforming growth factor β (TGF β) in prostate cancer (Culig *et al.*, 2002; Yoon *et al.*, 2006; Zhu and Kyprianou, 2008). TGF β cooperates with AR signaling to promote prostate cancer cell growth, in part through the regulation of the Runx2 transcription factor (van der Deen *et al.*, 2010). Runx2 enhances PCa cell growth in response to both androgen and TGF β signaling. TGF β typically is growth stimulatory in stromal cells, but inhibitory in epithelial cells *in vitro*. However, there are a number of mechanisms by which PCa cells overcome this response including loss of TGF β receptor expression (Guo *et al.*, 1997; Shafi *et al.*, 2013), DHT mediated repression of receptor or Smad3 expression (Song *et al.*, 2010), over-expression of COUP-TFII resulting in inhibition of TGF β signaling through SMAD4 (Qin *et al.*, 2013) or induction of cytokines such as IL-6 (Park *et al.*, 2003).

TGF- β induces epithelial–mesenchymal transition (EMT) to facilitate tumor progression and metastasis (Jones *et al.*, 2009). In prostate cancer, TGF- β 1 induces EMT in prostate tumor epithelial cells and in a mouse model of tumorigenesis with a targeted SMAD3 disruption, supporting a contributing role of TGF- β signaling in EMT and prostatic cancer metastasis (Ao *et al.*, 2006; Roberts *et al.*, 2006). TGF- β induces EMT in prostate tumor through constitutively active Akt that inhibits translocation of SMAD3 and p21 to the nucleus (Ao *et al.*, 2006). In PC-3 prostate cancer cells, blockade of NF- κ B signaling leads to decreased vimentin expression and inhibition of their invasive capability, indicating functional involvement of NF- κ B in mediating TGF- β -induced EMT (Zhang *et al.*, 2009).

4.2 AR independent mechanisms driving prostate cancer progression

Progression to CRPC results from escape of ADT, which targets the AR signaling axis and inhibits AR-driven proliferation and survival pathways. The exact mechanisms underlying the transition from androgen-dependent PC to CRPC remain incompletely understood. At the same time, the presence of AR-negative cell populations in CRPC has also been identified. While AR signaling has been proposed as the primary driver of CRPC, AR-independent signaling pathways may represent additional mechanisms underlying CRPC progression.

4.2.1 Alternative steroid receptors: GR and PR

Steroid receptors, including AR, progesterone receptor (PR) and glucocorticoid receptor (GR) are high homology, particularly in the DBD binding region (Laudet *et al.*, 1992; Karmakar *et al.*, 2013). Almost all PCa were discovered to express AR, whereas GR is only present in 30% of PCa cases; however, GR expression increases in patients following ADT (Szmulewitz *et al.*, 2012). Recent studies indicated that AR and GR possess the same chromatin binding sites and regulate the expression of AR-specific genes, such as *FKBP5* (Sahu *et al.*, 2013). ADT can increase GR expression and the potential for GR signaling (Arora *et al.*, 2013; Xia *et al.*, 2015). GR can also act as a substitute for AR signaling. Montgomery *et al.* reported a series of clinical trials to assess the contributions of glucocorticoids and GR in patients with CRPC (Montgomery *et al.*, 2014; Montgomery *et al.*, 2015). In these trials, analysis of patients in clinical cohorts who received glucocorticoids showed poor prognostic features, compared with patients who did not receive such treatment; therefore, stimulation of GR signaling may pose negative effects to patients who have received androgen-targeted therapies (Song *et al.*, 2014; Montgomery *et al.*, 2015). Mechanistically, endogenous GR was strongly expressed in DU145, weakly in PC3 but not in LNCaP cells. AR was strongly expressed in LNCaP but not in DU145 cells. DHT treatment enhanced STAT5 phosphorylation (pSTAT5), GR translocating into the nucleus and promoted proliferation of all CRPC cell lines, including LNCaP,

DU145 and PC3 cells. Mifepristone treatment, that is a remarkably active antiprogestosterone and antiglucocorticosteroid agent, abolished DHT-induced cell proliferation and *STAT5* activation in both DU145 and PC3 cells but not in LNCaP cells. Similarly, GR-specific siRNA completely suppressed *STAT5* activation. They also confirmed direct complex formation between the GR and pSTAT5 by immunoprecipitation (Song *et al.*, 2014). In such conditions, the presence of increased glucocorticoids in serum and absence of androgen possibly enables the selection of ‘promiscuous’ AR variants with mutations in the LBD, allowing their activation by glucocorticoids (Lorente *et al.*, 2014); therefore, GR can bypass AR pathways and promote the development of CRPC.

On the other hand, PR is also a member of the steroid hormone nuclear receptor family, and it is structurally associated with AR (Levin and Hammes, 2016). As with GR, PR may have the ability to transcriptionally regulate a subset of AR target genes in PCa and thereby bypass AR (Chen *et al.*, 2017). A large retrospective analysis carried out recently demonstrated the association of high PR staining in primary PCa with its clinical recurrence (Grindstad *et al.*, 2015). A research on a cohort of > 500 patients also revealed the association of high PR expression in cancer cells with reduced clinical failure-free survival (Grindstad *et al.*, 2015). This evidence indicates that PR antagonists may possess therapeutic effects, suggesting that PR signaling can be an important carcinogenic target in the treatment of PCa. However, the mechanism still needs to be investigated.

4.2.2 Histologic transformation

4.2.2.1 Neuroendocrine transformation

Recent genomic profiling studies have shown a significant overexpression and gene amplification of Aurora Kinase A (AUR-KA) and N-MYC in 40% of NEPC and 5% of advanced PC, and a loss of AR target gene expression (Beltran *et al.*, 2016). AUR-KA is best known for its role in mitosis, but its interactions with the oncogene

N-myc are not completely understood. In neuroblastoma, a positive feedback loop has been described in which AUR-KA induces and stabilizes N-MYC (Beltran *et al.*, 2011). A phase II trial was recently conducted to evaluate if AUR-KA could be a new potential target in the treatment of NEPC. 60 patients were treated with alisertib, an oral AUR-KA inhibitor. Although the study did not meet its primary endpoint of 6-month radiographic progression free survival, a subset of patients with molecular features supporting AUR-KA and N-MYC activation achieved a significant clinical benefit. In particular, two patients achieved a remarkable response, with complete regression of liver lesions (Beltran *et al.*, 2019). Alisertib was also studied in combination with abiraterone in patients with mCRPC progressing during abiraterone treatment (Lin *et al.*, 2016).

4.2.2.2 Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) process is crucial for physiological embryonic development and differentiation of the urinary genital system, but it is also activated in pathological conditions, such as fibrosis or cancer progression (Lamouille *et al.*, 2014). While the epithelial phenotype is characterized by apical–basal cell polarity and strong intercellular adhesions, mesenchymal cells are totally different, being neither adherent nor polarized (Larue *et al.*, 2005). EMT can be induced by ADT in metastatic PC and it confers invasive potential to tumour epithelial cells. Snail and Twist are transcription factors able to reduce the expression of E-cadherin, a key event in EMT (Montanari *et al.*, 2017). The TGF- β superfamily members induce Snail transcriptional factor. Aberrant TGF- β pathway and the protein kinase C (PKC)/Twist1 signalling activation are putative mechanisms for the occurrence of enzalutamide resistance. There are currently no direct Twist inhibitors available, but Shiota *et al* investigated a PKC inhibitor (Ro31-8220) to switch back resistance in PC cell lines with promising results (Shiota *et al.*, 2014). Therapeutic targeting of EMT by a proteasome inhibitor suppressing Snail seems to be promising in mCRPC (Baritaki *et al.*, 2009).

4.3 AR-negative prostate cancer

4.3.1 Incidence of AR-negative prostate cancer

In a recent paper, Bluemn with colleagues showed that AR-directed therapies are resulting in the emergence of prostate metastases devoid of AR signaling and discover an effective pathway to target in these AR-null cancers (Bluemn *et al.*, 2017). They characterized metastatic CRPCs from 84 patients for AR status and neuroendocrine status (NE). Neuroendocrine tumors express markers of neuroendocrine differentiation and are a subtype of prostate carcinoma separate from the more common epithelial type. The CRPCs collected from patients between 1997-2011 were 85% AR-positive, 10% NE-positive, and 5% negative for both. However, in samples collected between 2012-2016, when AR-targeting therapies became available, 69% were AR-positive, 10% were NE-positive, and 21% were double-negative, demonstrating that double-negative metastases were four times as prevalent in recent cases (Bluemn *et al.*, 2017).

4.3.2 AR-negative cell populations: stem-like cells

Small CSC populations have also been detected in PCa (van Leenders *et al.*, 2001). Although the main hypothesis is that prostate cancer arises from terminally differentiated luminal cells, there is growing evidence that PCa arises from more undifferentiated cells with a basal phenotype (Nagle *et al.*, 1987; De Marzo *et al.*, 1998; Stoyanova *et al.*, 2013). Studies demonstrated that androgen-independent tumours arise from AR-negative cells at a frequency of 1:10⁵–10⁶ AR-positive cells (Craft *et al.*, 1999). Furthermore, several putative populations of prostate cancer stem-like cells (PCSCs) have been reported in the literature, most commonly identified through fluorescence-activated cell sorting (FACS) of bulk tumor cell lines, xenografted tumors or clinical samples for stem-like cell surface markers. Reported marker profiles include CD44⁺/α2β1⁺/CD133⁺ (Collins *et al.*, 2005), ALDH (van

den Hoogen *et al.*, 2010), TRA-1-60/CD151/CD166 (Rajasekhar *et al.*, 2011), and PSA⁻/lo (Qin *et al.*, 2012), among others.

A number of reports have suggested that PCSCs are AR-negative or express very low levels of AR, predicting lack of responsiveness to antiandrogens and other therapies targeting the AR signaling axis. Patrawala and colleagues demonstrated that CD44⁺ PCSCs were negative for AR expression. Moreover, putative CD44⁺/α2β1⁺/CD133⁺ PCSCs isolated from clinical PCs following prostatectomy were found to be AR-negative and exhibited a prostate basal cell phenotype (Collins *et al.*, 2005). More recent work supports enrichment of a basal cell gene expression profile in metastatic CRPC, establishing a link between expansion of a stem-like cell population and castrate resistance (Zhang *et al.*, 2016).

Importantly, at least one study demonstrated that tumours containing luminal, basal and neuroendocrine cells could be reconstituted in mice by using AR⁻ and p63⁻ negative cells expressing the stem cell markers CD44, PROM1, nestin (NES) and KIT (Gu *et al.*, 2007).

4.3.3 Preliminary study for AR-negative PCa therapy

Furthermore, Bluemn *et al* confirmed that gene sets reflecting the activity of FGF signaling, MAPK signaling, and MEK/ERK signaling are significantly enriched in AR-negative CRPC tumors by RNA sequencing. Across all of the CRPC metastases, AR activity was inversely associated with FGF8/9 expression and FGFR activity. They also found that the downstream mediator of FGF/MAPK signaling ID1 (inhibitor of differentiation 1) was upregulated in androgen independent LNCaP cells in comparison to wild-type LNCaP cells. Stimulation of wild-type LNCaP cells with FGF8 in androgen-deprived conditions led to an increase in ID1 mRNA and protein levels, indicating that FGF8 activation is sufficient to trigger ID1 expression. While ID1 knockdown did not impair wild type LNCaP cell growth, it significantly impaired androgen independent LNCaP cell growth, ID1 transcripts were diminished in mouse xenograft models after treatment with an FGFR inhibitor. Together, these data support

the hypothesis that ID1 mediates the FGFR dependent survival of AR-negative CRPCs. They suggested conducting a clinical trial of FGFR or MAPK antagonists in patients selected for AR activity status to conclusively determine if targeting these pathways will help patients with AR-negative prostate cancer (Bluemn *et al.*, 2017).

5. Multiple endocrine neoplasia type 1 (MEN1): from gene to disease

5.1 MEN1 disease

Multiple endocrine neoplasia type 1 (MEN1) (MIM#131100) is a hereditary syndrome transmitted as an autosomal dominant trait. It is characterized by multiple occurrences in a same patient or in several members of the same family of tumors in endocrine organs. The major endocrine organs affected in MEN1 comprise the parathyroids, the endocrine pancreas (and the duodenum) and the anterior pituitary.

In MEN1 patients, hyperplastic and tumor lesions of the parathyroids affect virtually all patients (90%), pancreatico-duodenal endocrine tumors occur in 40% of patients, followed by anterior pituitary lesions (functional prolactinomas in 20% of patients, GH secreting, GH/PRL secreting, ACTH secreting, non-functional in 17% of patients) (Falchetti *et al.*, 2009; Thakker, 2010). Non endocrine cutaneous tumors are frequent in MEN1 patients with prevalence at 40 years old of age 85% for facial angiofibromas and 70% for collagenomas (Table 1.7).

Table 1.7 MEN1 pathology(tumors and hyperplasia)

Tumor localisation	Tumor type	% of MEN1 patients	% of GEP tumors in MEN1
Endocrine	Multiglandular hyperplasia, adenomas(hyperparathyroidism due to PTH secretion)	90 - 100	
Gastro Entero Pancreatic (GEP)		20 - 100	
Duodenal	-Gastrinomas	40	21 - 61
Pancreatic			
	-Non-functional macroscopic PETs	20	0 - 13
	-Insulinomas	10	7-31
	-Glucagonomas		1 - 6
	-VIPomas		1 - 12
	-GRFomas		< 1
	-Somatostain secreting tumors		0 - 1
	-pancreatic gastrinomas	rare	
Stomach	-Type II gastric NET (non-functional) associated with ZES		
Anterior pituitary		10 - 60	
	-PRLomas	20	
	-GH	5	
	-GH/PRL	5	
	-ACTH	2	
	-TSH	rare	
	-Non-functional	17	
Adrenal(adrenocortical)		20 - 40	
	-Non-functional	20 - 40	
	-cortisol secreting	rare	
	-aldosterone secreting	rare	
	-pheochromocytomas	< 1	
Foregut carcinoids	-Thymic carcinoid	2 - 8	
	-Lung (bronchial) carcinoid	2 - 8	
Thyroid	Thyroid adenomas		
Non-endocrine			
Cutaneous	-Skin angiofibromas	85 - 88	
	-Collagenomas	70 - 72	
	-Lipomas	30 - 34	
Central Nervous System	-Meningiomas	5 - 8	
	-Ependydimas	1	
	-Schwannoma	ND	
Smooth muscle	-leiomyomas -leiomyosarcomas	10	

5.2 The *MEN1* gene

The *MEN1* gene was mapped to chromosome 11q13 in 1988 (Larsson *et al.*, 1988) and positionally cloned in 1997 (Chandrasekharappa *et al.*, 1997; Lemmens *et al.*, 1997). It is located at chromosome 11q13, spans 9.8 kb with 10 exons, the exon1 and distal part of exon10 are non-coding sequence. The human *MEN1* coding sequence consists of 1830bp. Two *MEN1* transcripts were detected by northern-blotting in human tissues, a main 2.8kb transcript expressed in most organs, and a 4.2 kb transcript expressed in thymus and pancreas (Lemmens *et al.*, 1997). In the mouse, the *Men1* gene is localized in the pericentromeric region of the chromosome 19, in a region sythenic to human 11q13 locus, with at least two reported transcripts, a 2.8kb and a 3.2 kb transcripts that result from differential alternative splicing of the noncoding exon1, containing a coding sequence of 1833bp, and are expressed in most tissues excepted skeletal muscle (Stewart *et al.*, 1998; Bassett *et al.*, 1999).

The *MEN1* gene encodes a 610 amino acid protein referred to as menin, with no homology to any know protein (Guru *et al.*, 1998). *MEN1* mRNA is the expressed in the majority of the tissues and cell lines examined, although its expression levels are variable among different tissues. Menin orthologues have been identified in vertebrates and also invertebrates, including fruits fly and snail, but not in the yeast *Saccharomyces crevisiae* or nematode *Caenorhabditis elegans*. Menin is mainly located in nuclei. Half-life of protein menin is about 6-8 h in hEK293T cells (Yaguchi *et al.* 2004), and 10 h in COS cells (Ikeo, *et al.*, 1999).

5.3 *MEN1* mutations

An extensive landscape of *MEN1* mutations has been described to date, with more than 1000 germinal mutations and more than 200 somatic mutations (occurring in sporadic tumors) having been reported. About 41% mutations are frameshift deletions or insertions, 23% are nonsense, 20% are missense, 9% affect splice-site and 6% are in-frame deletions or insertions. Interestingly, some mutations in *MEN1* patients are not localized in the coding sequence, but rather in the promoter or other untranslated

regions. Germline deletions of *MEN1* exons have also been reported. Most mutations are inactivating mutations, consistent with the tumor suppressing functions of the *MEN1* gene. No phenotype-genotype correlations have been reported so far, except the work reported by the French GTE showing that mutations affecting the interaction domain of the protein with JunD had a higher risk of death (Thevenon *et al.*, 2013).

In MEN1 endocrine tumors (90%), the loss of heterozygosity (LOH) of the MEN1 wild-type allele, leading to complete *MEN1* inactivation, can be systematically detected. The LOH seems, therefore, to be an essential event to initiate tumorigenesis, even if a report suggests that heterozygous *Men1*-disruption in the mouse may lead to a haploinsufficiency (Lejonklou *et al.*, 2012).

5.4 Menin is a scaffold protein that controls gene expression and cell signaling

During the past two decade, it has also been reported that menin plays a role in suppressing hyperplasia or tumors in several other organs, such as the lung, prostate, and breast, and it exacerbates diabetes in mouse models. Menin also influences the function of other organs such as bone and live. Detailed mechanisms for how menin impacts these organs are less clear, but accumulating evidence showed that menin is a key scaffold protein that functionally crosstalks with various partners to regulate gene transcription and interplay with multiple signaling pathways (Table 1.8). Here we review the progress in understanding the molecular mechanisms by which menin functions.

Table 1.8 Summaries for different partners of menin.

Overview of different partners of menin		
Protein	Function	References
<i>Transcriptional regulation</i>		
JunD	Transcriptional factor	-Repression of JunD transcriptional activity. -Inhibition of JunD activation by JNK by binding competition. -Repression of JunD mediated Gastrin gene expression in AGS cell lines. Agarwal et al., 1999; Gobl et al. 1999; Kim et al., 2003; Gallo et al., 2002; Huang et al., 2012; Nafio et al., 2005; Mensah-Osman et al., 2011
NF- κ B	Transcriptional factor	-Repression of NF- κ B mediated transcriptional activation by an unknown mechanism. Heppner et al., 2001
Runx2	Transcriptional factor	-Activation of mesenchymal stem cells differentiation into preosteoblasts. Sowa et al., 2003
β -catenin	Transcriptional factor	-Menin is necessary for the transcription activation of Wnt pathway target genes, such as, <i>Axin2</i> , by the trimethylation of H3K4 on its promoter. Chen et al., 2008
c-Myb	Transcriptional factor	-Recruitment of MLL complex to c-Myb DNA targets, such as <i>HOX9</i> gene locus in hematopoietic and leukemic cells. -Complex formation with GATA3 to recruit MLL to <i>IL-13</i> locus and subsequent transcriptional activation. Jin et al., 2010; Nakata et al., 2010; Kozuka et al., 2010
c-Myc	Transcriptional factor	-Promotes HIV-1 Tat transactivation. -Menin interacts with the TAD domain of MYC and co-localizes with MYC to E-Box to enhance the transcription of MYC target genes in a P-TEFb-dependent manner in liver cancer cells. Bres et al., 2009; Wu et al., 2017
mSin3a	Transcriptional factor	-Recruitment of HDCA to inhibit JunD-mediated transactivation. Gobl et al., 1999; Kim et al., 2003
Smad1/5	BMP(transcription co-regulator)	-Activation of mesenchymal stem cells differentiation into preosteoblasts induced by BMP2- mediated transcriptional activation of Runx2. -Positively regulates transcriptional activity of Smad1 in Leyding cells. Sowa et al., 2003; Sowa et al., 2004; Hussein et al., 2008
Smad3	TGF- β (transcription co-regulator)	-Mediates TGF- β -induced growth inhibition in anterior pituitary cell lines. -Repression of osteoblast maturation by positively regulating TGF- β induced repression of differentiation. -Potentially mediates Activin-induced repression of proliferation and Prolactin gene expression in pituitary cell lines. -Potential positive role in TGF- β mediated repression of PTH expression and repression of proliferation in primary cells from human parathyroid tumor(physical interaction was not studied). Kaji et al., 2001; Sowa et al., 2003; Lacerte et al., 2004; Aziz et al., 2009; Sowa et al., 2004
Rpb2 Ser5P	RNA polymerase II subunit	-Coupling of H3K4me3 and transcription. Hughes et al., 2004
PRMT5	H4R3 symmetric dimethyl transferase	-Inhibition of Hedgehog signaling by recruiting PRMT5 to promotes and subsequent repression of Gas2 expression. Gurung et al., 2013
SUV39H1	H3K9 methyl transferase	-Potential recruitment of SUV39H1 to target promotes (such as, <i>Gbx2</i>) and subsequent transcriptional repression by repressive histone mark H3K9me3 in MEF cells. Yang et al., 2013
Ledgf	Chromatin-associated protein, cofactor	- Complex formed by menin/Ledgf/MLL1 is crucial for MLL1 and mll1 fusion proteins activity. Yokoyama and Cleary, 2008; Huang et al., 2013

Continue above

ER α	Estrogen nuclear receptor	- Ligand-induced ER α transcriptional activity (recruitment of methyltransferase activity to ER α target genes). - Upregulation of BRC1 and Rad51 expression in melanoma cell lines through a MLL1-dependent mechanism.	Dreijerink et al., 2006; Imachi et al., 2010; Fang et al., 2013
AR	Androgen receptor	-Menin bound to the proximal promoter of AR and regulated AR transcription via H3K4me3 histone mark in AR-dependent prostate cancer cells.	Teinturier et al., 2021
VitaminD nuclear receptor	VitaminD nuclear receptor	-Menin acts as a co-activator, ligand-independent, of VDR-mediated transcription in parathyroid cells (with a potential role of menin-mediated HMT activity at VDR target genes)	Dreijerink et al., 2009
PPAR γ	Free fatty acid, prostaglandin nuclear receptor	Essential for PPAR mediated differentiation of 3TAL1 and mouse embryonic fibroblast into adipocytes (by recruiting HMT activity to PPAR target genes).	Dreijerink et al., 2009
Pem	Transcriptional factor	Possible role in mouse spermatogenesis (no Pem homolog in human)	Lemmens et al., 2001
Hlx9	Homeobox transcription factor (repressor)	Indirect interaction in MN6 cells but weak in MEF cells. Possible role in the regulation of Hlx9 protein expression or regulation of Hlx9 target genes.	Shi et al., 2013
Cell signaling			
β -catenin	cell adhesion, transcription factor	-Nuclear exclusion of β -catenin -Coactivation of β -catenin target gene Axin 2 through H3K4me3 in α and β cell lines -Positive regulation of β -catenin transcription, effect in mediating mesenchymal cells differentiation into osteoblast.	Cao et al., 2009; Chen et al., 2008; Inoue et al., 2011.
TCF3	Transcriptional factor (effector of β -catenin transcription activity)	Coactivation of β -catenin target gene Axin2 through H3K4me3 in α and β cell lines.	Chen et al., 2008
IQGAP1	GTPase-activating protein	Intercellular adhesion by stabilizing E-cadherin and β -catenin at cytoplasmic membrane.	Yan et al., 2009
AKT	Serine/threonine protein kinase	Represses of ASK induced proliferation.	Schnepp. et al., 2004
Nm23	Nucleoside diphosphate kinase, protein kinase	Activation of menin GTPase activity.	Ohkura et al., 2011; Yaguchi et al., 2002
Ches1 (FoxN3)	Transcription factor; Interacts with SKIP	Important for G1-S arrest in response to DNA damage induced by ionizing radiation in Drosophila and MEF cells.	Busygina et al., 2006
DNA repair			
FANCD2	DNA repair	Possible role in FANCD2-mediated DNA repair pathway.	Jin et al., 2003
RPA2	DNA repair	Roles in DNA replication, recombination and repair and possibly apoptosis and transcription.	Sukhodolets et al., 2003
Cytoskeleton			
Vimentin	Type III intermediate filament proteins	Possible sequestration of menin out of the nucleus in glioma cell lines during S and G2 phase of cell cycle.	Lopez-Egido et al., 2002
NMMHC II-A	Cell structure	Possible role in cytokinesis.	Obungu et al., 2003

5.4.1 Regulation of histone modifying enzymes by menin

Menin-interacting chromatin modifying proteins include histone methyltransferase MLL (H3K4me3), protein arginine methyltransferase 5 (PRMT5) (H4R3me2s), SUV39H1(H3K9me3), HDAC/SIRT and EZH2, and menin affects the binding of these histone modifiers to the promoter of its target genes and influences their function.

5.4.1.1 MLL and H3K4me3

Chromosomal rearrangements of the *MLL* (mixed lineage leukemia) gene located at chromosome band 11q23 are found in patients with de novo acute myeloid (AML) and acute lymphoblastic (ALL) leukemias (Pui *et al.*, 2002; Liu *et al.*, 2009; Zeisig *et al.*, 2014; Milne *et al.*, 2015), and in therapy related leukemia or myelodysplastic syndrome (MDS) (Rowley *et al.*, 2002). The MLL proteins of the lysine methyltransferase (KMT) family, MLL1 (KMT2A) and MLL2 (KMT2B) are part of a protein complex that catalyzes a specific histone modification for gene activation, histone H3 lysine-4 trimethylation mark (H3K4me3) (Shilatifard 2012). MLL1 translocation with several different genes that encode transcription factors leads to the formation of MLL1-fusions that activate the expression of genes including the *HOX* genes (Ayton *et al.*, 2003; Yokoyama 2017). The role of *HOXA* genes in leukemic transformation has been observed both in vitro (Argiropoulos and Humphries, 2007) and in vivo (Rice and Licht, 2007) models, demonstrating that MLL fusion protein mediated upregulation of *HOXA9* and *MEIS1* genes resulted in enhanced proliferation and blockage of hematopoietic differentiation, ultimately leading to acute leukemia (Armstrong *et al.*, 2002; Ayton *et al.*, 2003). Menin was identified as an oncogenic co-factor of MLL fusion proteins in leukemia (Yokoyama *et al.*, 2005; Chen *et al.*, 2006). Loss of menin has been shown to coincide with the loss of H3K4me3 and the gain of H3K27me3, that is an epigenetic mark of gene repression, at specific genes (Agarwal and Jothi 2012; Lin, *et al.* 2015; Scacheri, *et al.* 2006). The interaction of menin in the Trithorax-like MLL protein complex has been shown to be essential for the oncogenic activity of MLL1-fusion proteins to cause leukemia (Hughes, *et al.*

2004; Yokoyama, *et al.* 2005). Evidence from studies conducted by using bone marrow of mouse models has confirmed this pro-oncogenic action of menin (Chen, *et al.* 2006).

Therefore, menin-MLL interaction inhibitors have been developed, facilitated by the deciphering the 3D structure of menin with the MLL1 menin-binding motif (MLL1 MBM) (Thiel *et al.*, 2012; Grembecka, *et al.* 2012). These inhibitors named MI and further improved versions of these inhibitors (MI-463, MI-503, MI-538 and compound 27) have been shown to block the proliferation of MLL1-fusion leukemia cells from patients in vitro and in mouse xenografts in vivo (Borkin, *et al.* 2016). Such menin-MLL interaction inhibitors hold promise to conduct future clinical trials as a potential treatment for patients with MLL1-fusion leukemia. Menin-MLL interaction inhibitors have also been studied in mice to block the growth of specific types of prostate cancer, Ewing sarcoma, and childhood gliomas, respectively (Funato, *et al.* 2014; Malik, *et al.* 2015; Svoboda, *et al.* 2017).

On the other hand, in endocrine cells or mouse embryonic fibroblasts (MEFs), menin is required for MLL1 binding to the cyclin-dependent kinase (CDK) inhibitors, p27Kip1 and p18Ink4c loci to increase H3K4me3 and induce their expression (Milne *et al.* 2005). Moreover, menin forms a complex with MLL and several other cofactors, including WDR5 (Song and Kingston 2008) and ASH2L (Hughes *et al.* 2004), leading to H3K4me3 at the promoter of the target genes. Interestingly, menin is also required for recruiting MLL1 to the *GATA3* locus to regulate *GATA3* expression and Th2 cytokine production in T helper type 2 (Th2) cells (Onodera *et al.* 2010). These studies all strongly suggested that the target genes regulated by menin-MLL methyltransferase complex vary in different cells and tissues.

Besides directly binding to the N-terminus of MLL1 via the central pocket (Borkin, *et al.* 2016), menin also directly binds chromatin-associating protein LEDGF (lens epithelium-derived growth factor) (Yokoyama & Cleary 2008, van Nuland *et al.* 2013). LEDGF is crucial for co-localization of menin and wild-type MLL1 or MLL fusions to the loci of menin/MLL target genes such as *HOX* and *CDKIs* (Yokoyama & Cleary 2008). Importantly, crystallographic studies indicate that menin, the

N-terminal part of MLL, and LEDGF form a ternary complex in which a helical structure from LEDGF sits on a 'V' shape structure formed from a MLL helix and menin. This structure clearly shows that menin acts as a scaffold to recruit both MLL and LEDGF (Huang *et al.* 2012). Moreover, although menin has no DNA-binding domains, it was reported that menin could associate with chromatin (Jin *et al.* 2003), and directly bound to genomic DNA via the positively charged amino acid residues in the nuclear localization signals (NLSs) of menin (La *et al.* 2006). These findings indicate that menin acts as a scaffold by recruiting the MLL complex to its target genes partly via its binding to genomic DNA.

5.4.1.2 PRMT5

Menin also regulates PRMT5 function (Gurung *et al.* 2013b), a member of PRMT family (Bedford & Clarke 2009; Stopa *et al.* 2015). PRMT5, a type II enzyme, is found in both the cytoplasm and the nucleus of cells (Pasternack *et al.*, 2007; Banasavadi-Siddegowda *et al.*, 2017), and involved in many physiological processes, including adipogenesis (LeBlanc *et al.*, 2012), hematopoiesis (Liu *et al.*, 2016), and spermatogenesis (Wang *et al.*, 2015; Wang *et al.*, 2018). The overexpression of PRMT5 in various tumors and in leukemia has also been reported (Yoshimatsu *et al.*, 2011; Karkhanis *et al.*, 2011; Wang *et al.*, 2018). PRMT5 works together with its co-factor MEP50 to mediate the methylation of histones H2A and H4 at arginine 3 and histone H3 at arginine 8 (Karkhanis *et al.* 2011). In addition, PRMT5 can repress globin gene expression through recruitment of DNA methyltransferase 3A (DNMT3A)(Zhao *et al.*, 2009), indicating a potential crosstalk between histone arginine methylation and DNA methylation (Girardot *et al.* 2014). Posttranslational histone modifications catalyzed by PRMT5 significantly affect gene expression and regulate cell growth and proliferation (Scoumanne *et al.* 2009). Menin regulates PRMT5's function by several modes. First, menin can suppress glucagon-like peptide-1 (GLP1)-induced and PKA-mediated phosphorylation of both FOXO1 and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), likely through PRMT5 or PRMT5-like enzyme-mediated methylation of FOXO1 and

CREB (Muhammad *et al.* 2017). Second, menin recruits PRMT5 to the promoter of the *Gas1* gene, increases repressive H4R3me2s and suppresses *Gas1* expression. Third, menin can also recruit PRMT5 to the promoter of *Gli1* and subsequently repress *Gli1* expression and thus Hedgehog signaling (Gurung *et al.* 2013a). However, further molecular details remain to be investigated.

5.4.1.3 H3K9me3 and Daxx

Several reports have revealed that menin silences the transcription of target genes by interacting with the suppressor of variegation 3-9 homologous protein 1 (SUV39H1), a histone 3 lysine 9 (H3K9) methyltransferase (Rea *et al.* 2000), and increasing histone 3 lysine 9 trimethylation (H3K9me3) at the promoter of the target genes (Yang *et al.* 2013, Song *et al.* 2014, Feng *et al.* 2017). Menin interacts with SUV39H1 through 360–445 amino acid region of menin and recruits SUV39H1 to the promoters of *GBX2* (Yang *et al.* 2013) and *IL6* (Song *et al.* 2014) and represses their expression via enhancing H3K9me3.

Interestingly, previous works reported that menin directly binds to Daxx/ATRAX complex and further recruits SUV39H1 to the promoter of membrane metallo-endopeptidase (MME, or CD10) and represses MME expression by enhancing H3K9me3 at the *MME* promoter (Feng *et al.* 2017). Daxx is a H3.3-specific histone molecular chaperone. It deposits H3.3 in specific chromatin regions together with ATRAX (Drane *et al.*, 2010; Lewis *et al.*, 2010), and is often mutated in neuroendocrine tumors (Jiao *et al.*, 2011). Menin and Daxx are required for each other to recruit SUV39H1 to the *MME* promoter. Daxx also directly binds to ATRAX, which may also bind SUV39H1-binding domain (Tang *et al.* 2004, Yang *et al.* 2013, Feng *et al.* 2017). These findings suggest that menin and Daxx/ATRAX firstly form a complex and recruit SUV39H1 to the *MME* promoter, leading to the suppression of MME expression.

5.4.1.4 Histone deacetylases: HDAC/SIRT

The silencing by menin of JunD-mediated transcription has been attributed to

repressive chromatin modifications. Gobl and coworkers showed that repression of JunD-dependent transcription could be reversed using the histone deacetylase (HDAC) inhibitor trichostatin A (Gobl *et al.* 1999). Kim and coworkers found an association of menin with SIN3A-HDAC complexes (Kim *et al.* 2003), which could not be confirmed by quantitative proteomic analysis of HeLa cells (van Nuland *et al.* 2013). In a more recent study, an interaction between menin and another histone deacetylase sirtuin 1 (SIRT1) was reported. Menin was shown to be required for the recruitment of *SIRT* to the *CD36* gene promoter in mouse hepatocytes (Cao *et al.* 2013).

5.4.1.5 Menin regulates EZH2

Unlike the SET domain in MLL that methylates H3K4 (Yokoyama *et al.*,2004), the EZH2 SET domain specifically methylates H3K27, and the methylated H3K27 can be recognized by other specific binding proteins to compress chromatin structure, leading to repression of gene transcription (Cao *et al.*, 2002; Sparmann and van Lohuizen, 2006). Gao *et al.* reported that menin bound to the promoter of *Pleiotrophin* (PTN) and recruited the Polycomb group (PcG) complex to the locus, resulting in H3K27 trimethylation, PTN suppression, and inhibition of proliferation of lung cancer cells(Gao *et al.*, 2009). Moreover, they also reported that menin and EZH2 co-occupy a large number of promoters from ChIP-on-ChIP analysis in HCC specimens(Gao *et al.*, 2014). On the other hand, in endocrine tissues, the important genetic interaction between MEN1, EZH2 and *Inhbb* (activinB) in β -cell proliferation has been revealed by Gherardi *et al* (Gherardi *et al* 2017). They showed that activin B expression is mediated through a direct modulation of H3K27me3 marks on the *Inhbb* locus in *Men1*-KO cell lines. More importantly, they demonstrated that menin bound to the *Inhbb* promoter where it favours the recruitment of EZH2 likely via an indirect mechanism involving AKT phosphorylation (Gherardi *et al* 2017).

5.4.2 Menin interacts with numerous transcriptional factors

By investigating the possible molecular links between different transcription factors

and menin, we speculated that we might gain further insight into the possible role played by menin in MEN1 tumors and other cancers (Fig. 1.14).

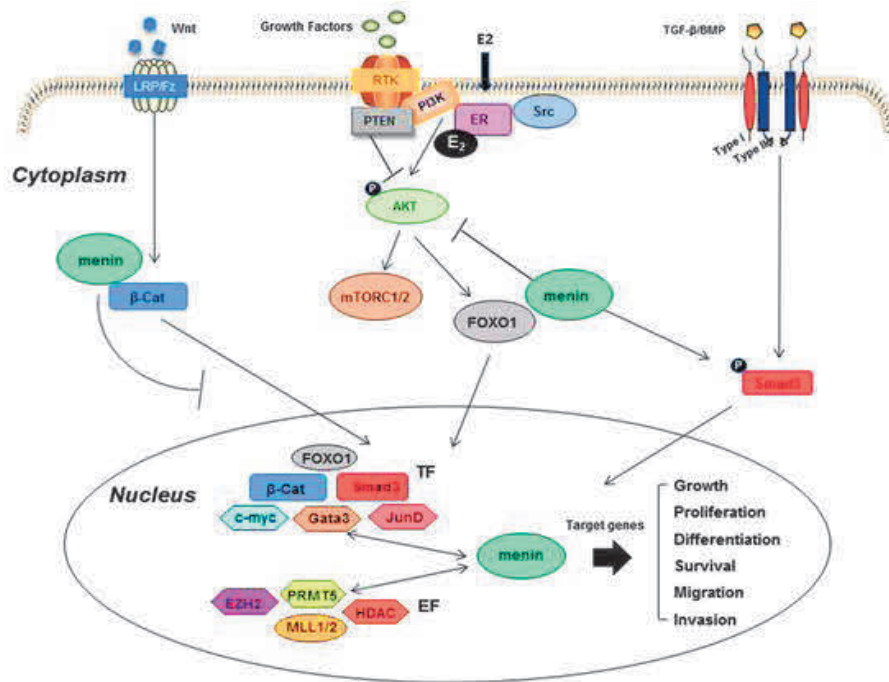


Fig. 1.14 Mechanistic clues underlying the involvement of menin in tumorigenesis. Menin interacts with numerous menin-interacting factors, consequently participating in the regulation of many target genes and interfering with different signaling pathways strongly implicated in breast and prostate cancers. EF: epigenetic factors; TF: transcriptional factors.

5.4.2.1 JunD

JunD, a basic leucine-zipper (bZIP) transcription factor, is a member of Jun family (c-Jun, which is the cellular counterpart of the v-Jun oncogene, JunB and JunD), forming the dimeric transcription factor complex AP-1 with Fos members (c-Fos, FosB, Fra1 and Fra2). These bZIP transcription factor family members dimerize at their C-terminal leucine zipper domains in synergy with their adjacent basic domain binding to DNA. They regulated transcription on promoters by binding to a consensus TRE (12-O-teradecanoylphorbol-13-acetate [TPA] responsive element) sites. The hallmark of the AP-1 protein complex c-Fos/c-Jun is its role in regulation of proliferation responses (Jochum *et al.*, 2001). Additional study has shown that JunD is involved in negative control of cell proliferation in human intestinal epithelial cells or rat tumor cell lines (Xiao *et al.*, 2007; Fries *et al.*, 2007;

Hernandez *et al.* 2008).

The transcription factor JunD was the first to be identified as a direct-interacting partner of menin by using yeast two-hybrid screening (Agarwal *et al.*, 1999; Gobl *et al.*, 1999). Menin interacts with JunD and represses the JunD activity (Agarwal *et al.* 1999, Huang *et al.* 2012). The crystal structural study showed that menin binds JunD via its central pocket, which can also bind MLL1 (Huang *et al.* 2012). In other words, menin uses the same pocket to bind either MLL1 or JunD, but having much more affinity with the former. A conserved sequence (FPXXP) is found in the menin-binding domain (MBD) of both JunD and MLL (Huang *et al.* 2012). The region of JunD that binds menin spans 27-47 amino acid residues (Agarwal *et al.* 1999, Huang *et al.* 2012). Earlier biochemical studies have suggested that menin binds to JunD to repress JunD-induced transcription through interaction with co-repressors mSin3A and HDAC 1 or 2 in cultured cells (Agarwal *et al.* 1999, Gobl *et al.* 1999, Kim *et al.* 2003). Furthermore, a new mechanism was reported whereby menin binds JunD and thus block c-Jun N-terminal kinase (JNK)-mediated phosphorylation of JunD at residues S90, S100 and T117, an event crucial for activating JunD function in transcriptional regulation (Huang *et al.* 2012).

Coincidentally, both the basic residues and the leucine residues in JunD are bound by JNK to phosphorylate JunD, and these residues are also bound by menin. As such, menin binding to JunD effectively blocks JNK's interaction with JunD and thus inhibits JNK-mediated phosphorylation and activation of JunD. This finding well explains why JunD activates proliferation of MEFs in the absence of menin, but suppresses proliferation in the presence of menin (Agarwal *et al.* 2003). In addition, JunD has two isoforms: full-length and truncated isoforms, and menin only can bind to full-length isoform of JunD (Yazgan and Pfarr, 2001).

5.4.2.2 MYC

MYC (c-Myc) is a transcription factor that binds to E-boxes in the DNA to activate transcription. Increased MYC expression and activity is correlated with cell proliferation rates, and this is observed in many cancer types. Both activating and

repressive effects of menin on MYC action have been reported. Menin has been suggested to interact with MYC and the SKI-interacting protein (SKIP) coactivator at the HIV-1 promoter (Bres *et al.* 2009). In HEK293 embryonic kidney cells, menin and the mixed-lineage leukemia (MLL) complex were found to interact with the transcription factor FUSE-binding protein 1 (FBP1/FUSBP1) to stimulate MYC expression (Zaman *et al.* 2014). More in line with its tumor suppressive function, loss of menin was shown to result in the upregulation of the *MYC* mRNA in mouse-derived pancreatic cells, possibly via increased Hedgehog signaling pathway activity (Gurung *et al.* 2013).

A recent report shows that menin can directly interact with the transactivation domain (TAD) of MYC and then bind to E-boxes to enhance transcription of Myc target genes. This enhanced transcription of MYC target genes depends on P-TEFb, a key factor to facilitate transcription regulation by MYC (Wu *et al.* 2017). The above findings suggest that, by transcriptionally promoting the expression of MYC target genes, menin can stimulate cell proliferation, cellular metabolism and cancer progression in certain types of cancers cells.

5.4.2.3 Nuclear receptors: ER α and AR

Several works showed that menin is a generic co-regulator for nuclear receptor-mediated transcription, as it interacts with many other NR family members including the vitamin D receptor (VDR) (Dreijerink *et al.*, 2009a), the retinoid X receptor (RXR) (Cheng *et al.*, 2011), the peroxisome proliferator-activated receptors (PPAR) alpha (Dreijerink *et al.*, 2009b), the liver X receptor alpha (LXR α) (Cheng *et al.*, 2015) and the androgen receptor (AR) (Malik *et al.* 2015; Teinturier *et al.*, 2021).

ER α

Menin is able to interact with the estrogen receptor alpha (ER α) ligand-binding domain in an estradiol (E2)-dependent fashion. Menin interacts with hormone activated estrogen receptor alpha (ER α) and links ER α to MLL1 and MLL2 methyltransferase complexes. This interaction is important for transcription of ER α target genes through recruitment of H3K4me3 methyltransferase activity, such as the

estrogen regulated *TFF1* gene (Dreijerink *et al.* 2006). Menin also regulates transcription of the *ESR1* gene via H3K4me3 histone marker in MCF-7 cells (Dreijerink *et al.* 2017). Interestingly, they revealed that menin-dependent *ESR1* gene promoters display looping to distal enhancers that are bound by menin, FOXA1 and GATA3 in MCF-7 cells.

More importantly, studies hypothesized that the expression of menin in breast cancer cells might be associated with resistance to antiestrogen therapy. The analysis of ER positive breast cancer samples revealed that patients with menin-positive tumors had a worse outcome than those with menin-negative tumors (Imachi *et al.*, 2010; Imachi *et al.*, 2011). Therefore, inhibition of the menin interaction with MLL methyltransferases may impair co-activation of the estrogen receptor and may offer a therapeutic benefit for patients with breast cancers dependent on ER signaling.

AR

Almost a decade after discovering the interaction between menin and ER α , menin was identified as an important co-factor for AR signaling, by its physical interaction with AR-NTD and the recruitment of the MLL histone methyltransferase complex to AR target genes. Inhibition of menin-MLL interaction with a small-molecule inhibitor (MI) impaired AR signaling and inhibited the growth of castration-resistant tumors in xenograft experiments in mice (Malik *et al.*, 2015). Hence, these results suggest that menin can facilitate tumor growth through AR signaling in prostate cancer.

More recently, our work suggested that menin could play a tumor suppressor role during the tumorigenesis of mouse prostate cells. We found that prostate-specific *Men1*-deficient mice developed accelerated mPIN and later displayed microinvasion adenocarcinoma. More importantly, *Men1*-KO led to markedly downregulation of AR in early-stage lesions. Interestingly, we also found the expression of AR was decreased in *MEN1* silencing LNCaP, 22Rv1 and VCaP cells. Moreover, we demonstrated menin bound to the proximal *AR* promoter and positively regulated *AR* transcription via the H3K4me3 histone mark in PCa cells. These findings highlight the regulation of the AR promoter by menin and the crosstalk between menin and the AR pathway (Teinturier *et al.*, 2021).

5.4.2.4 Forkhead box proteins and GATA3

Forkhead box proteins are gene-specific transcription factors that bind to DNA sequences and are downstream effectors of several signaling pathways. Menin has been reported to interact with several FOX family members including FOXN1/checkpoint suppressor 1 (CHES1) and FOXO1 (Busygina *et al.* 2006, Wuescher *et al.* 2011). Menin has also been recently shown to interact with GATA3 and FOXA1 (Dreijerink *et al.* 2017) in breast cancer to regulate the *ESR1* promoter as we detailed before, GATA3 and FOXA1 being markers of luminal breast cancer especially luminal A (Perou *et al.*, 2000; van de Vijver *et al.*, 2002; Oh *et al.*, 2006) and highly associated with ER α and required for the proper function of most of its target genes (Lacroix *et al.*, 2004; Carroll *et al.*, 2005; Carroll and Brown 2006). Menin is also known to interact with GATA3 and c-Myb to activate the Th2 cell maturation in primary human peripheral blood T cells (Nakata *et al.*, 2010). In mouse embryonic stem cell (mESC)-derived embryoid bodies, lower *Foxa1* and *Foxa2* mRNA levels were observed in the absence of *Men1* gene expression (Zhang *et al.* 2011). In addition, menin was found to be able to interact with FOXA2 and to regulate FOXA2 expression in mouse pancreatic cells and derived cell lines, indicating a potential role in *MEN1*-associated pNET tumorigenesis (Bonnavion *et al.* 2017). It is worth mentioning that FOXA1 plays a crucial role in the AR signaling, and possibly in CRPC occurrence (Obinata *et al.*, 2017).

5.4.2.5 TGF- β and Smad3

Transforming growth factor β (TGF- β) and bone morphogenic protein (BMP) signaling is transduced to gene transcription via SMA and MAD (SMAD) gene specific transcription factors. Genomic SMAD-binding sites can be both at promoter-proximal and enhancer elements. Menin regulates TGF- β signaling and TGF β -induced gene transcription by interacting with SMAD3, a TGF- β downstream effector (Kaji *et al.* 2001). Menin directly interacts with SMAD3, and antisense menin suppresses TGF- β -induced and Smad3-induced transcriptional activity by inhibiting

Smad3/4-DNA binding at specific transcriptional regulatory sites (Kaji *et al.* 2001). When the *Men1* gene is deleted in Leydig cells, the effect of TGF- β -induced inhibition of proliferation was reduced (Hussein *et al.* 2008). Consistently, Canaff and coworkers showed that menin mutation compromises menin's function in promoting TGF- β -induced SMAD3 transcriptional activity (Canaff *et al.* 2012). Thus, menin mutation deprives its function in promoting TGF- β signaling-induced repression of cell proliferation, likely contributing to the development of MEN1. On the other hand, it is also reported that TGF- β upregulates menin expression in MLL-AF9-transformed mouse bone marrow cells (Zhang *et al.* 2011). Moreover, menin expression was downregulated in MLL-AF9-transformed mouse bone marrow cells when T β RII, a vital component in TGF- β signaling pathway, was deleted. Menin expression was also decreased in liver samples from the conditional T β RIIknockout mice after T β RII excision (Zhang *et al.* 2011).

5.4.2.6 Runx2 and Smad1/5

Another recognized function of menin is its role in the commitment of multipotential mesenchymal stem cells into the osteoblast lineage and osteoblast differentiation. However, menin also inhibits the later differentiation of these cells (Hendy *et al.*, 2005). In the TGF superfamily, bone morphogenetic proteins (BMPs) and TGF- β are crucial regulators of bone formation. The interactions of menin with the BMP-2 signaling pathway and Runx2 might be different depending on the osteoblast differentiation stage (Hendy *et al.*, 2005).

Menin promotes the commitment of multipotential mesenchymal stem cells to the osteoblast lineage through interactions with Smad1 to Smad5 and Runx2, whereas the interaction of menin and Smad3 inhibits later osteoblast differentiation by negatively regulating the BMP-2–Runx2 cascade after the commitment to the osteoblast lineage (Sowa *et al.*, 2004).

5.4.3 Menin regulates various signaling pathways

5.4.3.1 Wnt/ β -catenin

The wingless-related integration site (Wnt)/ β -catenin signaling has important roles not only in development, but also in cancer biology. Menin was shown to be crucial for regulating canonical Wnt/ β -catenin signaling in cultured rodent islet tumor cells by interaction with β -catenin (Chen *et al.* 2008). In *Men1*-null MEFs and insulinomas from β -cell-specific *Men1*-knockout mice, β -catenin accumulates in the nucleus, but overexpression of menin reduces nuclear accumulation of β -catenin and suppresses its transcriptional activity (Cao *et al.* 2009). Wnt signaling stimulates pancreatic islet β cell proliferation, possibly by increasing expression of paired-like homeodomain 2 (*Pitx2*) (Rulifson *et al.* 2007). When menin and activated β -catenin are overexpressed in islet tumor cells, the Wnt/ β -catenin downstream target gene, *Axin2*, is significantly enhanced, correlating with increased H3K4me3 at the promoter of the *Axin2* gene (Chen *et al.* 2008). These opposite results for the role of menin on the Wnt signaling pathway and cell proliferation may depend on distinct context of cells. Inhibition of Wnt signaling resulted in reduced proliferation of *Men1*-deficient rodent β -cells. Accordingly, combining a β cell-specific mouse knockout model of the *Men1* gene with knockout of the *Ctnnb1* gene (encoding β -catenin) resulted in lower tumor numbers and sizes in mice defective in *Men1* and *Ctnnb1* vs *Men1* alone (Jiang *et al.* 2014), accompanied with the inhibition of the expression of pro-proliferative genes and improved hyperinsulinemia and hypoglycemia. Antagonizing β -catenin signaling by the small molecule inhibitor PKF115–584 in *Men1*-deficient mice also suppresses tumor cell proliferation *in vitro* and *in vivo* (Jiang *et al.* 2014). These results suggest that activated Wnt signaling after loss of *MEN1* gene expression could be a targetable mechanism in MEN1-related tumors.

5.4.3.2 PI3K/AKT/PTEN/mTOR signaling

More recently, Wong and colleagues generated mouse models with insulin-specific biallelic inactivation of *Men1* and *Pten* in β -cells, and showed that their concomitant loss accelerated islet cell tumorigenesis. Co-mutations of *MEN1* and *PTEN* were observed in a small percentage of human PanNETs (Jiao *et al.*, 2011;

Scarpa *et al.*, 2017), suggesting that menin and Pten may function synergistically to suppress tumorigenesis.

Several studies have focused on the relationship between the PAM (PI3K/AKT/mTOR) and resistance to endocrine therapy in pre-clinical BC models (Boulay *et al.*, 2005), in which the authors showed that Akt can activate the ER α pathway independently of estrogen availability and that the combination of mTOR inhibitors and endocrine therapy can overcome this resistance (Crowder *et al.*, 2009). In addition, the PAM pathway has also been implicated in trastuzumab resistance in HER2-overexpressing BCs (Nagata *et al.*, 2004). Interestingly, menin interacts with AKT1, downregulates its kinase activity and suppresses both AKT1 induced proliferation and anti-apoptosis in endocrine and non-endocrine cells, mainly by reducing the translocation of AKT1 from the cytoplasm to the plasma membrane during growth factor stimulation (Wang *et al.*, 2011). Another study showed that menin can interact with FOXO1, a downstream effector of AKT, in the hepatocytic cancer cell line HepG2 and in MEFs (Wuescher *et al.*, 2011). A recent study revealed that menin regulates milk protein synthesis through mTOR signaling in normal mammary epithelial cells (Li *et al.*, 2017). According to the authors, menin overexpression caused significant suppression of factors involved in the mTOR pathway, as well as milk protein κ -casein (CSNK). All of the abovementioned data suggest that menin may regulate the PI3K/AKT/mTOR pathway in different tissues and cells, including mammary cells.

5.4.3.3 Hedgehog signaling

In pancreatic ductal adenocarcinoma, menin expression was suppressed by DNA methyltransferase 1 (DNMT1) downstream of the Hedgehog signaling pathway, and menin overexpression strongly antagonized the positive effect of Hedgehog signaling on pancreatic cancer cell proliferation (Cheng *et al.* 2016). Gurung *et al.* study indicated that menin antagonizes Hedgehog signaling, partly via increasing PRMT5-mediated repressive H4R3me2s at the *Gas1* promoter in neuroendocrine cells (Gurung *et al.* 2013b). *Men1*-null cells supplemented with menin mutants cannot

inhibit *Gas1* mRNA levels, but wild type can do (Gurung *et al.* 2013b). Moreover, menin and PRMT5 also suppress Hedgehog signaling at a second step besides *Gas1*, by inhibiting the expression of Hedgehog downstream effector *Gli1*. These findings indicate that loss of menin-mediated repressive histone methylation of H4R3me2 sites at the *Gas1* and *Gli1* promoters and resulting upregulation of Hedgehog signaling play a role, at least in part, in the pathogenesis of MEN1 syndrome.

5.4.3.4 Menin inactivation triggered pancreatic islet α cell to β cell transdifferentiation

Menin is also a crucial factor maintaining the cell differentiation of pancreatic islet α -and β -cells. Mice with α -cell-specific knockout of the *Men1* gene developed insulin-secreting β -cell tumors or insulinomas 6 months following *Men1* disruption (Lu *et al.* 2010). Genetic cell lineage tracing analysis showed that insulinoma cells were directly derived from transdifferentiating glucagon-expressing cells (Lu *et al.* 2010), associated with altered expression of transcription factors critical for islet cell differentiation, including MAF-B and FOXA2. It remains to further investigate as to what pathway(s) is(are) perturbed or the epigenetic program altered in the *Men1*-deficient α cells, resulting in α -cell to β -cell trans-differentiation.

5.4.3.5 Menin and K-Ras signaling regulate each other

Menin suppresses both proliferation and migration of lung adenocarcinoma cells partly via inhibiting PTN and its receptor RPTP β/ζ signaling (Gao *et al.* 2009, Feng *et al.* 2010). However, menin also is repressed by K-Ras through DNMT1-dependent DNA demethylation of the promoter of the *MEN1* gene in lung adenocarcinoma cells and inversely menin reduces the level of active Ras-GTP at least partly by preventing GRB2 and SOS1 from binding to Ras (Wu *et al.* 2012); these studies suggest that there exists a potential negative feedback loop between menin and K-Ras, and that menin plays a crucial role in K-Ras-induced lung cancer development.

5.4.3.6 Cell cycle control & growth factor

Kaji *et al.* demonstrated that menin could suppress cell proliferation via the transforming growth factor- β (TGF- β) pathway in the rat pituitary cell line by interacting with SMAD3 (Kaji *et al.*, 2001). Agarwal *et al.* reported that menin is essential for JunD-mediated inhibition of cell proliferation (Agarwal *et al.*, 1999). Ratineau *et al.* showed that menin represses cell proliferation in rat intestinal epithelial cells (Ratineau *et al.*, 2004) by inhibiting the expression of Cyclin D1, Cyclin D3, and CDK4. Based on a transcriptomic study of differentially expression genes in mouse *Men1* insulinomas, our team demonstrated that *Men1* ablation in mouse islet cells greatly affected the expression of factors involved in cell cycle and cell growth control, such as Cyclin A2, B2, and D2 for the former; and IGF2, IGFBP3, and 6 for the latter (Fontanière *et al.*, 2006). Menin can also repress cell proliferation by interacting and inhibiting ASK (S-phase kinase) (Schnepp *et al.*, 2004). In addition, menin was reported to upregulate the expression of Cyclin-dependent kinase inhibitors p18^{ink4c} and p27^{kip1} with the help of the MLL compass-like complex (Fontanière *et al.*, 2006), which adds H3K4me3 marks on their promoters, thus activating gene expression (Karnik *et al.*, 2005; Pei *et al.*, 2009; Stephens *et al.*, 2012). Interestingly, p18 has recently been shown to be a downstream target of GATA3 in luminal BC and to suppress luminal progenitor cell proliferation and tumorigenesis (Milne *et al.*, 2005). P27 is ranked as one of the 18 most significantly mutated genes in luminal A BC, and loss of p27 was associated with poor outcome in BC patients (Schnepp *et al.*, 2006). Schnepp *et al.* revealed that the infection of cells using menin-expressing adenoviruses could trigger apoptosis in MEFs (Lindsten *et al.*, 2000; Schnepp *et al.*, 2003) by activating an apoptotic pathway that depends on Bax (Milne *et al.*, 2005). They also highlighted that *Men1* disruption increased resistance to TNF α -induced apoptosis in *Men1*^{+/-} mouse model, further supporting a vital role for menin in regulating apoptosis.

5.5 Regulation of menin expression

5.5.1 Regulation of menin expression by prolactin signaling

As menin plays an important and pleiotropic role in regulating multiple functions of islets, it is conceivable that its expression is controlled by numerous signals. During late stage of pregnancy of mice, prolactin is produced, and prolactin binds its cell surface receptor, and phosphorylates and activate STAT1 (Jabbour, *et al.* 1998; Karnik, *et al.* 2007; Tourkine, *et al.* 1995). The activated STAT1 translocates into the nuclear in β cells, and then binds to the promoter of the *Men1* gene to suppress *Men1* transcription (Karnik *et al.* 2007). Notably, controlled expression of the *Men1* transgene in the pancreatic islets attenuates the prolactin-induced repression of the *Men1* expression in the pancreatic islets, leading to gestational diabetes in the mice (Karnik *et al.* 2007). These findings indicate that menin is physiologically regulated to adapt the pancreatic islets to counteract the development of gestational diabetes. More work remains to investigate whether this molecular circuitry is also conserved in human pancreatic islets.

5.5.2 Regulation by FOXO1

Forkhead box O1 (FOXO1) is a member of Forkhead box containing transcription factor family (Anderson, *et al.* 1998). The key feature of this family of transcription factors is that many of them can be phosphorylated by receptor tyrosine kinases (RTKs) by activating the PI3K/AKT pathway (Brunet, *et al.* 1999). AKT can phosphorylate FOXO1 at three different sites Thr24, Ser256 and Ser319. For example, insulin induces phosphorylation of FOXO1 by activating PI3K and AKT axis, and phosphorylated FOXO1 binds to 14-3-3 protein and is isolated in the cytoplasm (Tzivion, *et al.* 2011). In addition, phosphorylated FOXO1 in the nucleus can also be exported to the cytoplasm. Thus, the net result of the phosphorylation of FOXO1 is to reduce its nuclear localization and therefore its activity in transcriptional activation (Vogt, *et al.* 2005). Further, it is reported that FOXO1 is crucial for regulating insulin signaling and also cell metabolism (Kitamura 2013).

Menin expression is activated by FOXO1 in β -cells. It has been reported that glucose induces activation of AKT and suppresses FOXO1 activity, leading to decreased menin expression and activated β -cell activity (Zhang *et al.*, 2012).

Importantly, the authors also demonstrated that Foxo1 bound to the proximal *Men1* promoter and regulated its activity in β -cells (Zhang *et al.*, 2012). Therefore, menin regulation is linked to an important signaling pathway regulating β cell, metabolism and diabetes.

5.5.3 Somatostatin increases menin expression

Somatostatin is a peptide hormone and inhibitor of gastrin expression and secretion (Karnik *et al.*, 1989). Menin also inhibits the expression of gastrin. Octreotide is a somatostatin analog that increases mRNA and menin protein levels in the duodenum of mice. While octreotide inhibited PKA enzyme activity, forskolin treatment, which increases cellular cAMP and PKA activity, suppressed menin protein level (Mensah-Osman, *et al.* 2008). However, whether and how the somatostatin pathway regulates PKA to induce menin expression remains to be investigated.

5.5.4 Regulation of menin by posttranslational modifications

5.5.4.1 Ubiquitination

Yaguchi *et al* found that missense mutant menin protein has much short half-life as compared to wild type menin protein in transfected 293 cells (Yaguchi *et al.*, 2004). Further studies revealed that the reduced levels of the mutant proteins were a result of rapid menin degradation through ubiquitin-mediated degradation. Several reports further revealed that unstable menin mutants, but not wild type menin, were associated with a heat shock protein Hsp70 and its co-chaperone CHIP, which was shown to function as a ubiquitin E3 ligase towards several substrates presented by Hsp70 and Hsp90 (Connell *et al.*, 2001; Meacham *et al.*, 2001). Recently, Wu *et al* reported that the menin inhibitors (MI), including MI463 and MI503, induced reduction of H3K4m3, and HOXA9 expression was rescued with a proteasome inhibitor that blocks MI-induced menin protein degradation in human leukemia cell lines. Mechanistically, MIs promote the interaction of menin with Hsp70-associated ubiquitin ligase CHIP, resulting in increased menin ubiquitination, leading to

increased menin degradation (Wu *et al.*, 2019).

5.5.4.2 Phosphorylation

The first phosphorylation modification of menin was found in 2006 by Matthew *et al* (MacConaill, *et al.* 2006). They showed that menin is phosphorylated on two serine residues, Ser543 and Ser583. However, the functional study of the phosphorylation by mutating both serine residues had no impact on menin's ability to recruit trithorax family complex proteins Ash2L, Rbbp5, and MLL2, nor on cell proliferation. While these two phosphorylation sites are situated between the two NLSs located at the C-terminal part of menin, mutations at both of the serine residues do not affect menin localization into the nucleus (MacConaill *et al.* 2006). Further study showed that Ser394 and Ser487 were also identified by mass spectrometry analysis. Phosphorylation of menin at Ser394 was induced in response to irradiation (IR) or UV treatment, while Ser487 was phosphorylated under normal cell culture conditions. Nonetheless, HMT activity assay showed both Ser394 and Ser487 mutated menin mutant was still able to immunoprecipitate methylated histone 3 (Francis *et al.* 2011).

5.6 Menin and miRNAs

5.6.1 Let-7a

Abnormal microRNA (miRNA) regulation has been attributed to all phases of cancer and affects several of the cancer hallmarks (Peng *et al.*, 2016; Romano *et al.*, 2017). Discovered in *C. elegans*, let-7 (lethal-7) miRNA family functions as an important regulator of differentiation (Reinhart *et al.*, 2000; Copley *et al.*, 2013). It is categorized as a tumor suppressor because it reduces cancer aggressiveness, chemoresistance, and radioresistance.

Menin does not affect levels of primary-let-7a (pri-let-7a), but increases the levels of mature let-7a (Gurung *et al.* 2014). Let-7a targets, including insulin receptor (INSR) and insulin receptor substrate 2 (IRS2), pro-proliferative genes that are crucial for insulin-mediated signaling, are up-regulated in *Men1*-excised cells (Gurung *et al.* 2014). Inhibition of let-7a using anti-miRNA in wild type cells is sufficient to enhance

the expression of IRS2. Depletion of menin inhibits conversion of pri-miRNA to pre-miRNA. These findings unravel a mechanism whereby menin suppresses cell proliferation, at least partly by promoting the biogenesis and processing of certain miRNAs, including *let-7a*, to insulin signaling and likely endocrine cell proliferation (Gurung *et al.* 2014).

5.6.2 MiR-24-1

The human miR-24 is located at chromosome 19 of the human genome and transcribed as a part of miR-23a-27a-24-2 cluster (Chhabra *et al.*, 2010). Dysregulation of miR-24 has been reported in various human cancers, such as non small cell lung cancer (Franchina *et al.*, 2014), hepatocellular carcinoma (Chen *et al.*, 2016), breast cancer (Lu *et al.*, 2015), ec. The miR-24 had different functions in different cancer types (Wang *et al.*, 2018; Zhang *et al.*, 2019). Interestingly, even in the same type of cancer, miR-24 can play opposite function (Duan *et al.*, 2014; Zhang *et al.*, 2016). Therefore, further studies are needed to confirm its functions and adverse effects.

MiR-24-1 directly binds to the highly conserved 3'UTR region of *MEN1* mRNA, and represses menin expression, and the negative feedback loop between miR-24-1 and menin protein is essential for MEN1 tumorigenesis (Luzi, *et al.* 2012). Vijayaraghavan *et al.* also found that miR-24 directly decreases menin expression and impacts downstream cell cycle inhibitors in pancreatic cancer (Vijayaraghavan, *et al.* 2014). Moreover, miR-24 inhibition increases menin expression and decreases cholangiocarcinoma cell proliferation (Ehrlich, *et al.* 2017).

5.7 The involvement of the *MEN1* gene in hormone-related cancers

5.7.1 Hormone-related cancers observed in *Men1* mouse models

5.7.1.1 Mammary gland lesions in mouse *Men1* models

Our team has been focused on the studies on the role of *MEN1* using *Men1* mutant mouse models. Our previous works showed that aged heterozygous *Men1* mutant

mice, in addition to endocrine tumors, developed mammary gland carcinomas in female and prostate cancers in male mutant mice at low frequencies (Bertolino *et al.*, 2003). To further confirm and understand the role of menin in the development of mammary lesions, a conditional mammary-specific *Men1* knock-out mouse model was generated by crossing the mice carrying floxed *Men1* alleles (*Men1^{F/F}*) with WapCre transgenic mice expressing *Cre* recombinase under the control of the whey acidic protein (*Wap*) promoter, which is known to be expressed in luminal mammary epithelial cells. Female *Men1^{F/F}*-WapCre mice developed substantially higher amounts of early mammary intraepithelial neoplasia (MIN), which are precursor lesions, in comparison with control *Men1^{+/+}*-WapCre mice. Interestingly, ER α expression and the number of ER α -positive cells were clearly reduced in MIN lesions of mutant mice compared with normal mammary glands. In addition, cell membrane expression of β -Catenin and E-Cadherin was almost absent in the mammary lesions of *Men1^{F/F}*-WapCre mice compared with control mice; neither β -Catenin nor E-cadherin were detected in the TS1 cell line derived from a mouse *Men1* BC (Seigne *et al.*, 2013).

5.7.1.2 Prostate lesions in mouse *Men1* models

By following a cohort of 47 male heterozygous *Men1* mutant mice (*Men1^{+/-}*) and 23 male wild-type (*Men1^{+/+}*) age-matched littermate mice from 18 to 26 months of age, Seigne *et al.* found that six *Men1^{+/-}* mice (6/47, 12.8%) developed prostate cancer, including two adenocarcinomas and four in situ carcinomas, while none of the control mice developed cancerous lesions. No prostate carcinoma was found in age-matched *Men1^{+/+}* littermates (0/23). In addition, these carcinomas exhibited loss of the non-target *Men1* allele (LOH), therefore supporting a tumor suppressor role for the *Men1* gene in prostate glands. Moreover, the AR and p27 expression was decreased in tumor lesions, likely facilitating prostate cell tumorigenesis due to *Men1* inactivation (Seigne *et al.*, 2010).

More recently, our team generated a conditional *Men1*-KO mouse model to decipher the role played by *Men1* in the initiation of prostate cell tumorigenesis.

Men1^{F/F} mice (Bertolino *et al.*, 2003) were crossed with *Nkx3.1Cre*^{ERT2} mice, in which the activity of Cre recombinase controlled by the endogenous *Nkx3.1* promoter can be induced in prostate luminal cells upon tamoxifen injection. *Men1*^{F/F}–*Nkx3.1Cre*^{ERT2}–/+ mice thus generated were subjected to tamoxifen treatment (*Men1*NT) or not (*Men1*N) at 1.5 months of age, and prostate glands were subjected to pathological examination 1.5, 4.5, and 8.5 months after tamoxifen injection. Having shown that a majority of prostate cells within lesions in *Men1*NT mice lost menin expression, we observed that these mice progressively developed more advanced lesions with prominent pleomorphic changes and microinvasive carcinoma (MIC) at 6 (2/7, 28%) and 10 months (4/7, 57%) of age. As experimental controls, no age-matched *Men1*N mice developed MIC (n = 5), *Men1*^{F/F} mice either treated or not with tamoxifen developed no mPIN lesions within the 10-month experimental time course, and tamoxifen-treated *Nkx3.1Cre*^{ERT2}–/+ mice developed no MIC lesions at 5 and 10 months of age (n = 5). Our results indicated that prostate-specific *Men1* disruption in mice leads to the acceleration of tumorigenesis in *Nkx3.1*^{–/+} prostate cells (Teinturier *et al.*, 2021).

Taken together, all of the data obtained from mouse models suggest a tumor-suppressive role for menin during the initiation and development of murine breast and prostate cancers.

5.7.2 *MEN1* gene in human breast cancer

Over the last two decades, several case reports have described breast cancer cases related to *MEN1*. In 2004, a 44-year-old Japanese woman was diagnosed with *MEN1* syndrome, having hyperparathyroidism, primary aldosteronism, and also scirrhous breast carcinoma. The DNA taken from her parathyroid adenoma and breast cancer tissues showed germline *MEN1* mutation at codon 451 in exon 10, which resulted in alanine-to-tyrosine substitution (A541T), as well as LOH (Honda *et al.*, 2004). Another study by Jeong *et al.* reported a case of a patient with both *MEN1*-associated tumors and breast cancer. They found a germline *MEN1* mutation manifested as a 5-bp duplication in exon 3 (named:196_200dupAGCCC), which resulted in a

frameshift mutation. In addition, the tested exon 10 showed a polymorphism at codon 423 with substitution of a cytidine to a thymidine (C423T), causing a change of amino acid (Jeong *et al.*, 2014). More recently, a 41-year old patient with no familial history of breast cancer but with a mother with primary hyperparathyroidism (PHP) was found carrying a variant p.C421R/p.426R in the *MEN1* gene. The patient's histopathological study revealed hormone receptor negativity, as well as HER-2 and p53 negativity. A family study showed positive findings for MEN1 in a sister, two maternal nephews, and one of the patient's daughters, with no record of breast cancer development in any of these people (Herranz-Antolín *et al.*, 2018).

The most relevant evidence of the likely involvement of menin in BC arose from the observation that female MEN1 patients were at a higher risk of developing BC (Dreijerink *et al.*, 2014). In this study, Dreijerink *et al.* referred to the Dutch longitudinal MEN1 database to assess the incidence of BC in MEN1 patients, and found that out of 190 female patients, the relative risk of invasive BC was 2.83 ($p < 0.001$) and the mean (\pm SD) age at diagnosis of essentially luminal-type BC was 48 ± 8.8 years, compared with an age range of 60 to 65 years in the general population. This feature is often observed in the patients harboring a genetic predisposition. The authors validated their results using other independent MEN1 patient cohorts, especially that from France ($P = 0.03$), which provided similar values for relative risk as those obtained in the Dutch cohort, with an average age at diagnosis of 51 years. Furthermore, 8 out of 10 BC samples obtained from Dutch MEN1 patients displayed more than 50% reduction of menin expression in the nucleus, and subsequent analysis showed loss of heterozygosity at the *MEN1* locus in 3 of 9 tumors. Overall, these observations strongly suggest that *MEN1* mutations could be involved in human breast tumorigenesis as a tumor suppressor. More recently, a study in which the whole-genome sequences of 560 BCs were analyzed highlighted sporadic *MEN1* mutations, albeit at low frequency, as being among driver mutations (such as *BRCA1*, *TP53*, *PIK3CA*, *MYC*, *CCND1*, *PTEN*) in BC (Nik-Zainal *et al.*, 2019). In addition, several other case reports identified *MEN1* mutations among sporadic BC patients, independent or not of germline mutations in *BRCA1* and *BRCA2* genes that are

usually associated with hereditary BC (Jeong *et al.*, 2014; Papi *et al.*, 2009; Ghataorhe *et al.*, 2007).

However, in a clinical study conducted by Imachi *et al.* with 65 ER α -positive BC samples treated with tamoxifen for 2–5 years as adjuvant therapies, the authors observed that menin-positive tumors (20 patients) had a worse clinical outcome and were more resistant to tamoxifen than menin-negative tumors (46 patients) (Imachi *et al.*, 2007). They, therefore, proposed that menin could be a predictive factor of resistance to tamoxifen. Furthermore, they found that raloxifene could inhibit the binding of menin to the AF2 domain of ER α and proposed raloxifene as the therapeutic options for menin-positive and ER α -positive BC (Imachi *et al.*, 2011). Their works suggest an oncogenic role for menin, which raised the controversy as to its precise role in BC.

5.7.3 *MEN1* gene in human prostate cancer

Perakakis *et al.* reported two cases of PCa seen in a *MEN1* family with atypical tumor spectrum (Perakakis *et al.*, 2016). The DNA sequencing analysis revealed a novel mutation—Ser38 Cys (TCC > TGC) in exon 2, located in a region of menin that is responsible for interaction with the transcription factor JunD. The latter has recently been associated with prostate cancer.

Only limited sporadic *MEN1* mutations have so far been reported in human sporadic PCa (Manson-Bahr *et al.*, 2015). Manson-Bahr *et al.* found that missense mutations of the *MEN1* gene were detected in 2 of 8 formalin-fixed prostate needle biopsy materials (Imachi *et al.*, 2011). Interestingly, Grasso *et al.* analyzed 58 human CRPC samples by aCGH and found that 17.2% of all samples (10 of 58) harbored mutations in the MLL complex, including the *MEN1* gene (Grasso *et al.*, 2012). MLL functions as part of a multi-protein complex containing menin (Varier *et al.*, 2011). Many members of the complex have different levels of aberrations in CRPC (Grasso *et al.*, 2012). Noticeably, Chen *et al.* analyzed 150 cases for advanced and metastatic human PCa. They observed that the percentage of *PTEN* and *MEN1* co-loss was almost the same as the co-loss of *PTEN* and *PML* (Promyelocytic Leukemia), which

is around 11% in all cases (Chen *et al.*, 2018). Conversely, Paris *et al.* reported that the *MEN1* locus was amplified in some patients and was predictive of post-operative recurrence (Paris *et al.*, 2004). The similar observation was made Kerstin *et al.* (Heselmeyer-Haddad *et al.*, 2014).

More importantly, Malik *et al.* examined menin expression in a set of human prostate cancer tissue samples (benign: n=38; localized: n=118; metastatic: n=55). By using RNA sequencing (RNA-seq), they observed that *MEN1* mRNA expression was associated with disease progression, with significantly elevated levels seen in metastatic prostate cancer compared to those observed in hormone-naive prostate cancer and benign prostate. They also found that menin protein levels were elevated during prostate cancer progression, with notably higher protein levels in metastatic compared to localized disease. Furthermore, the *MEN1* mRNA overexpression was predictive of poor patient survival (Malik *et al.*, 2015). Taken together, these data establish that menin is upregulated at both the transcript and protein levels in localized and metastatic prostate cancer and its expression is associated with poor survival. In total, the current data obtained from human studies suggest that the *MEN1* gene could play a complex even opposite role in the development of human breast and prostate cancers.

Preface

I. My initial PhD research theme

I started my PhD research works with my participation in characterizing a mouse model where the *Men1* and *Nkx3.1* genes were conditionally disrupted in prostate luminal cells. The major findings achieved in this work are:

Men1 disruption accelerates tumorigenesis of prostate cells in *Nkx3.1*-deficient mice. *Men1/Nkx3.1*-deficient lesions display low AR and high CD44 expression, accompanied by reduced CK18 and E-cadherin expression. Mechanistically, we demonstrated that menin bound to the *AR* promoter and regulated *AR* transcription via H3K4me3 in human PCa cells. More interestingly, we also found menin could play an opposite role in AR-dependent versus AR-independent PCa cells.

My major contribution to this work was to dissecting the molecular mechanisms underlying the regulation of *AR* transcription by menin. In particular, the data I have obtained allowed us to uncover that the *MEN1* gene plays distinct roles in controlling cell proliferation in AR-dependent versus AR-independent PCa cells. Consequently, I've focused my major PhD research them to investigate the cellular and molecular mechanisms underlying the phenomenon.

Publication: Teinturier R#, Luo Y#, Decaussin-Petrucci M, Vlaeminck-Guillem V, Vacherot F, Firlej V, Bonnavion R, Abou Ziki R, Gherardi S, Goddard I, Gadot N, Bertolino P, Le Romancer M, Zhang CX. *Men1* disruption in *Nkx3.1*-deficient mice results in AR^{low}/CD44⁺ microinvasive carcinoma development with the dysregulated AR pathway. *Oncogene*. 2021;40(6):1118-1127. doi: 10.1038/s41388-020-01589-1. (#: co-first author) Please see the paper in Appendices part.

II. My major research themes

II-1. Crosstalk between menin, MYC and AR in AR-dependent prostate cancer cells

Our results demonstrated that menin exerted its oncogenic effects and was critical for *MYC* transcription and *MYC*-mediated *AR* transcription in AR-dependent cells. Interestingly, we also showed that menin was involved in the regulation of *MYC*-related LncRNA *PCAT1* in AR-dependent PCa cells, through its interaction with EZH2 and their co-binding on the regulatory sequence of *PCAT1*.

Prepared manuscript: Yakun Luo, Virginie Vlaeminck-Guillem, Romain Teinturier, Razan Abou Ziki, Philippe Bertolino, Muriel Le Romancer and Chang Xian Zhang. Menin is essential for activating the *MYC* locus and *MYC*-mediated androgen receptor transcription in AR-dependent prostate cancer cells. *Cancer Communication*. (Under review)

II-2. Activation of JunD and B-catenin triggered by MEN1 silencing in AR-independent PCa cells.

Our data provide evidence that menin plays a tumor suppressor role in AR-independent PCa cells. We found that *MEN1* inactivation promoted the tumorigenic potential of these cells via triggering nuclear translocation of JunD and β -catenin. Mechanistically, both JunD and β -catenin could replace menin to bind to the regulatory sequences of the *MYC* locus, ultimately resulting in maintaining *MYC* expression. Interestingly, we also found that *MEN1* inactivation altered the expression of several molecular markers of EMT and stemness, including E-cadherin, BMI1, Twist1 and HIF1 α . Finally, our analyses using cultured AR-independent PCa cells and PC3-GFP xenografts in the mouse demonstrated that both JunD and β -catenin are necessary for the altered tumorigenic potential triggered by *MEN1* inactivation.

Prepared manuscript: Yakun Luo, Virginie Vlaeminck-Guillem, Silvère Baron, Chang Xian Zhang and Muriel Le Romancer. *MEN1* silencing aggravates tumorigenic potential of AR-independent prostate cancer cells through nuclear translocation and activation of JunD and β -catenin. *Journal of Experimental & Clinical Cancer Research* (Under review)

ChapterII Results

Article 1. Menin is essential for activating the MYC locus and MYC-mediated androgen receptor transcription in AR-dependent prostate cancer cells

Short Report

Menin is essential for activating the MYC locus and MYC-mediated androgen receptor transcription in AR-dependent prostate cancer cells

Running title *Crosstalk between menin, AR and MYC in AR-dependent PCa cells*

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Conflict of interest statements

Nothing to disclose

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Abstract

The androgen receptor (AR) acts as a vital transcription factor in prostate cancer (PCa) development and progression and the proto-oncogene MYC is key for the regulation of *AR* transcription in PCa. We lately demonstrated that menin, encoded by the *MEN1* gene, exerts oncogenic effects by modulating AR transcription specifically in AR-dependent PCa cells and wondered herein whether this effect may occur through its interaction with other crucial factors, in particular, MYC. We found that *MEN1*-KD led to a decrease in MYC expression in AR-dependent (LNCaP and 22RV1), but not AR-independent (PC3 and DU145), PCa cells. Importantly, ChIP analyses demonstrated that menin bound to the *MYC* promoter and its two enhancers. Moreover, the binding of menin to the *MYC* promoter and enhancer 2 was increased upon stimulation with the androgen hormone. Furthermore, our data demonstrated that menin exerted a critical role on the MYC binding site on the *AR* promoter and was essential for the regulation of MYC-mediated *AR* transcription. Interestingly, we also demonstrated that menin was involved in the regulation of MYC-related LncRNA *PCAT1*, through both its positive regulation of *EZH2* transcription and its physical interaction with *EZH2* and their co-binding on the regulatory sequence of *PCAT1*. Collectively, we uncovered an essential role of menin in regulating MYC transcription and MYC-mediated AR transcription, involving *PCAT1* regulation via *EZH2*, specifically in AR-dependent PCa cells.

Key words (6)

Prostate cancer, the *MEN1* gene, AR regulation, MYC, *EZH2*, *PCAT1*

The androgen receptor (AR) acts as a vital transcription factor in prostate cancer (PCa) development and progression. Pathway aberrations are extensive in primary and metastatic castration-resistant PCa (mCRPC), where they affect the transactivation activity and transcription of AR [1], although the latter remains elusive. The proto-oncogene *MYC* has a well-documented role in driving AR oncogenic functions in PCa cells [2], and was recently exposed as a key activator in the regulation of *AR* transcription [3]. However, the exact mechanisms underlying *MYC*-mediated AR regulation are lacking, and may be crucial for better understanding PCa development and improving its management. We lately demonstrated that the tumor suppressor menin, encoded by the *MEN1* gene, plays an oncosuppressive role in the initiation of prostate cell tumorigenesis and the generation of AR^{low}/CD44⁺ lesions in the mouse, but exerts oncogenic effects specifically in AR-dependent human PCa cells, both likely through modulating *AR* transcription [4]. Since menin interacts with numerous transcription and epigenetic factors, we wondered herein whether this newly uncovered function of menin may occur through its interaction with other crucial factors, in particular, *MYC*.

We initially examined the expression of *MYC* upon *MEN1* knockdown (KD) in PCa cells. We found that *MEN1*-KD led to a decrease in *MYC* expression at the transcriptional (Fig. 1a, Fig. S1a) and protein (Fig. 1b-c, Fig. S1b-c) levels in AR-dependent (LNCaP and 22RV1), but not AR-independent (PC3 and DU145), PCa cells. We also detected the interaction between menin and *MYC* in AR-dependent PCa cells (Fig. S2a-b), previously reported in HeLa cells [5]. Recently, several distal regulatory sequences in the *MYC* locus have been identified and reported to play important role in *MYC* transcription, including in particular 2 *MYC* enhancers situated 67 kb upstream and 20 kb downstream of the transcription start site (TSS) of *MYC* [6,7]. Importantly, ChIP analyses demonstrated that menin bound not only to the *MYC* promoter, but also to these two enhancers (Fig. 1d, Fig. S2c). Moreover, menin was necessary for major components of the MLL complex (*viz* KMT2A, KMT2B, ASH2L), to which menin belongs [8], to bind to and activate the *MYC* promoter and

enhancer 2 likely via H3K4me3 marks (Fig. 1e-g, Fig. S2d-g). Consistently, *MEN1*-KD resulted in decreased expression of known *MYC* target genes, enhanced cell cycling alterations (Fig. 1h, Fig. S3a-c), and decreased expression of AR variants (Fig. S3d-e), the latter being known to be activated by *MYC* expression in PCa cells and to contribute to mCRPC development. Notably, the expression of menin and *MYC* and their interaction, as well as the binding of menin to the *MYC* promoter and enhancer 2, were augmented upon stimulation with the androgen hormone, dihydrotestosterone (DHT) (Fig. 1i-k, Fig. S4a-c). The above data revealed that menin is a crucial factor positively regulating *MYC* transcription specifically in AR-dependent PCa cells.

Next, we investigated whether menin was involved in *MYC*-mediated regulation of *AR* transcription known to play a critical role in activating *AR* transcription [3]. ChIP analysis and Luciferase-reporter assays revealed that menin and *MYC* bound to the same area on the *AR* promoter, at the location of an E-box site (Fig. 2a-b, Fig. S5a-g). Deletion of the E-box on the *AR* promoter significantly reduced the transactivation activity of *MYC* and that of menin, in Luciferase-reporter assays, whereas this latter activity remained unaffected on other parts of the *AR* promoter (Fig. S6a-b). Remarkably, using reChIP analysis, we found that the physical interaction between *MYC* and menin may be a prerequisite for the binding of menin to the E-box site on the *AR* promoter (Fig. 2c, Fig. S6c-d). Our data indicate, for the first time, that menin exerts a regulatory role on the *MYC* binding site on the *AR* promoter and is essential for the regulation of *MYC*-mediated *AR* transcription.

In parallel, we assessed whether menin is involved in the regulation of LncRNA *PCAT1*, situated 800 kb upstream of the *MYC* locus and known to play an oncogenic role through increasing the stability of *MYC* during PCa development [9]. Markedly, *MEN1*-KD in AR-dependent PCa cell lines led to reduced *PCAT1* expression (Fig. 2d, Fig. S7a). Since EZH2 has been reported to directly regulate *PCAT1* expression and that the interaction between EZH2 and menin has been described in different contexts [9, 10], we investigated whether menin controls *PCAT1* transcription through EZH2

in PCa cells. Our data demonstrated that menin was crucial for the expression of EZH2 (Fig. 2e, Fig. S7b) via its physical interaction with EZH2 (Fig. S7c-d) and its binding to the *EZH2* promoter (Fig. 2f-g, Fig. S7e-f). Finally, ChIP analysis showed that menin bound to the same binding sites as EZH2 on the regulatory sequences of *PCAT1* (Fig. 2h, Fig. S7g), the binding being weakened upon *EZH2*-KD (Fig. 2i, Fig. S7h). Taken together, the current work revealed that menin is critically involved in the regulation of MYC-related *PCAT1*, through its interaction with and regulation of EZH2. The function of menin in the activation of *PCAT1* expression should further strengthen the activity of the MYC pathway.

Finally, to explore the relevance of our findings for future therapeutic strategies, we analyzed large PCa data sets through data mining. Significant positive correlations were observed between *MEN1* and *MYC* mRNA expression in mCRPC, between *MEN1* and *EZH2* mRNA expression in both primary PCa and mCRPC, and a marginal positive correlation between *MEN1* mRNA and *PCAT1* expression in primary PCa (Fig. 2j). Lastly, the combined use of siMEN1 and of the MYC/Max dimerization inhibitor 10058-F4 had a synergistic effect on reducing the proliferation of LNCaP and 22Rv1 (Fig. 2k, Fig. S8a-b) cells.

Collectively, we uncovered a critical crosstalk between menin, AR and MYC, involving *PCAT1* regulation via EZH2, specifically in AR-dependent PCa cells (Fig. 2l), providing novel clues as to the mechanisms underlying the regulation of *AR* transcription. Considering AR is not only crucial for the development of PCa, but also for its treatment, we believe that the current finding may provide useful clues to new targets for PCa management.

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DECLARATIONS

Consent for publication

All of the authors approved the contents and the data in this manuscript and agreed for its submission for publication.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Competing of Interests

The authors declare that they have no competing interest.

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AUTHORS' CONTRIBUTIONS

Y.L conducted the experiments, analysed and interpreted the data, prepared figures and manuscript; V.V.G, T.R, and R.A.Z provided technical and material support and participated in data interpretation; P.B participated in study design and provided critical revision of the manuscript; M.L.R and C.X.Z conceived and supervised the study and manuscript preparation, and obtained funding.

ABBREVIATIONS

AR, Androgen Receptor; ChIP, Chromatin Immunoprecipitation; DHT, dihydrotestosterone; EZH2: Enhancer of zeste homolog 2; MEN1, Multiple endocrine neoplasia type 1; mCRPC, metastatic castration-resistant PCa; PCa, prostate cancer; PCAT1, Prostate Cancer Associated Transcript 1; siRNA: Small interfering RNA; TSS, Transcription Start Site.

Figure legends:

Fig. 1 Menin is essential for activating the *MYC* locus and *MYC* targets in AR-dependent PCa cells. *MYC* mRNA and protein levels analyzed by RT-qPCR (a), Western blot analysis (b) and immunofluorescent staining (IF) (c) in siCtrl or siMEN(1)+(3)-treated LNCaP and PC3 cells. d-k Shown are the experiments conducted exclusively in AR-dependent LNCaP cells. ChIP-qPCR assessing the binding of menin to (d) and H3K4me3 marks (e) on the *MYC* promoter and enhancers in siCtrl or siMEN(1)+(3)-treated cells. *Chr1* used as negative control. f *MYC* mRNA and protein levels analyzed by RT-qPCR (left panel) and Western blot analysis (right panel) in siCtrl or siASH2L-treated cells. g ChIP-qPCR assessing the binding of menin to the *MYC* promoter in siCtrl or siASH2L-treated cells. h mRNA (left panel) and protein (middle panel) expressions of *CDK2*, *CDK4*, *CCNA*, *CCND* and *CCNE* were analyzed by RT-qPCR and Western blotting in siMEN(1)- or siMEN1(3)-treated cells and cell cycle arrest (right panel) at the G1 phase in siMEN(1)+(3)-treated cells by flow cytometry. i Proximity ligation assay (PLA) showing menin and *MYC* interaction upon androgen hormone (DHT) treatment for 24 h with DAPI counterstained nuclei. Scale bar, 50 μ m. Quantification of PLA signals per cell is shown on the right. j Western blot analysis of AR, menin and *MYC* levels upon DHT treatment. GAPDH used as loading control. k ChIP-qPCR showing the binding of menin to the *MYC* promoter and enhancer 2 following treatment with DHT (10 nM) for 12 h and 24 h, respectively.

Fig. 2 Menin is crucial for *MYC*-mediated *AR* transcription and *EZH2*-regulated *PCAT* expression in AR-dependent PCa cells. a-i Shown are the experiments conducted exclusively in AR-dependent LNCaP cells. a ChIP-qPCR of the binding of menin to the *AR* promoter. b Relative luciferase activity after transfection of siCtrl or siMEN1(1)+(3)-treated cells with AR-Luc-2 or AR-Luc-2- Δ E. c ChIP-reChIP assay assessing the effect of siMEN1(1)+(3) on menin and *MYC* recruitment to the E-box

site of the *AR* promoter. **d** RT-qPCR analysis of *PCAT1* expression in siCtrl or siMEN1(1)+(3)-treated cells. **e** RT-qPCR showing *EZH2* expression in siCtrl or siMEN1(1)+(3)-treated cells. **f** ChIP evaluating the occupancy of menin on the *EZH2* promoter. **g** RT-qPCR analysis of *PCAT1* expression in siCtrl or siEZH2 (1)+(2)-treated cells. **h** ChIP showing the occupancy of menin on the *PCAT1* promoter. **i** ChIP showing the effect of siEZH2 (1)+(2) on the levels of menin recruitment to the *PCAT1* promoter. **j** Data mining analysis of the correlation between *MEN1* and *MYC* mRNA expression in human mCRPC (left), and between *MEN1* and *EZH2* (middle) or *MEN1* and *PCAT1* (right) mRNA expression in primary human PCa. **k** Combined use of MYC inhibitor (10058-F4) and siMEN1(1)+(3) showing effects on cell proliferation. **l** Schematic summary of uncovered functions of menin in AR-dependent PCa cells. For all RT-qPCR analyses, data normalized against *HPRT* and represented as fold change. For all the RT-qPCR and ChIP-PCR analyses, shown is the mean \pm s.d. (n=3). t-test, ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig.1 Menin is essential for activating the MYC locus and MYC targets

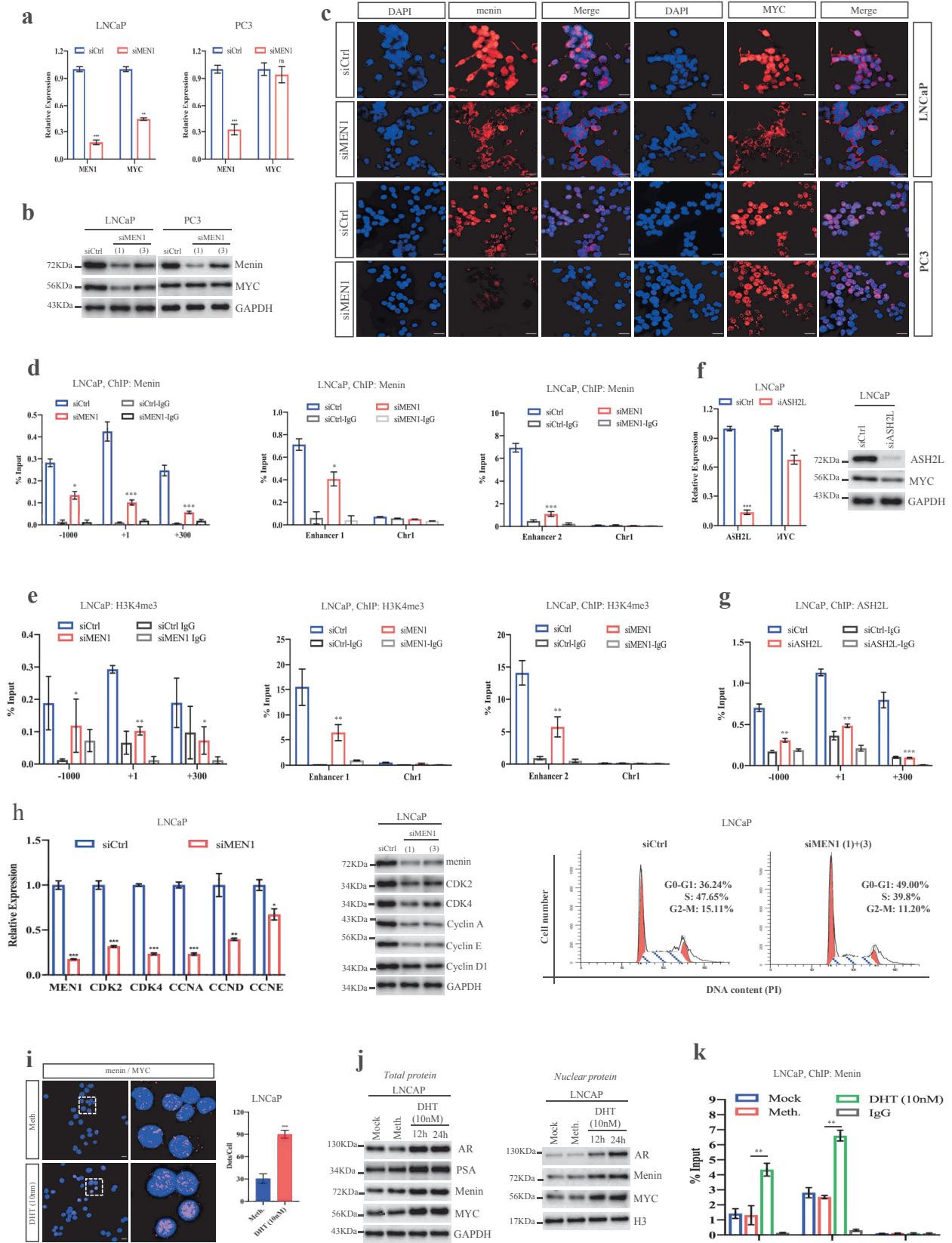
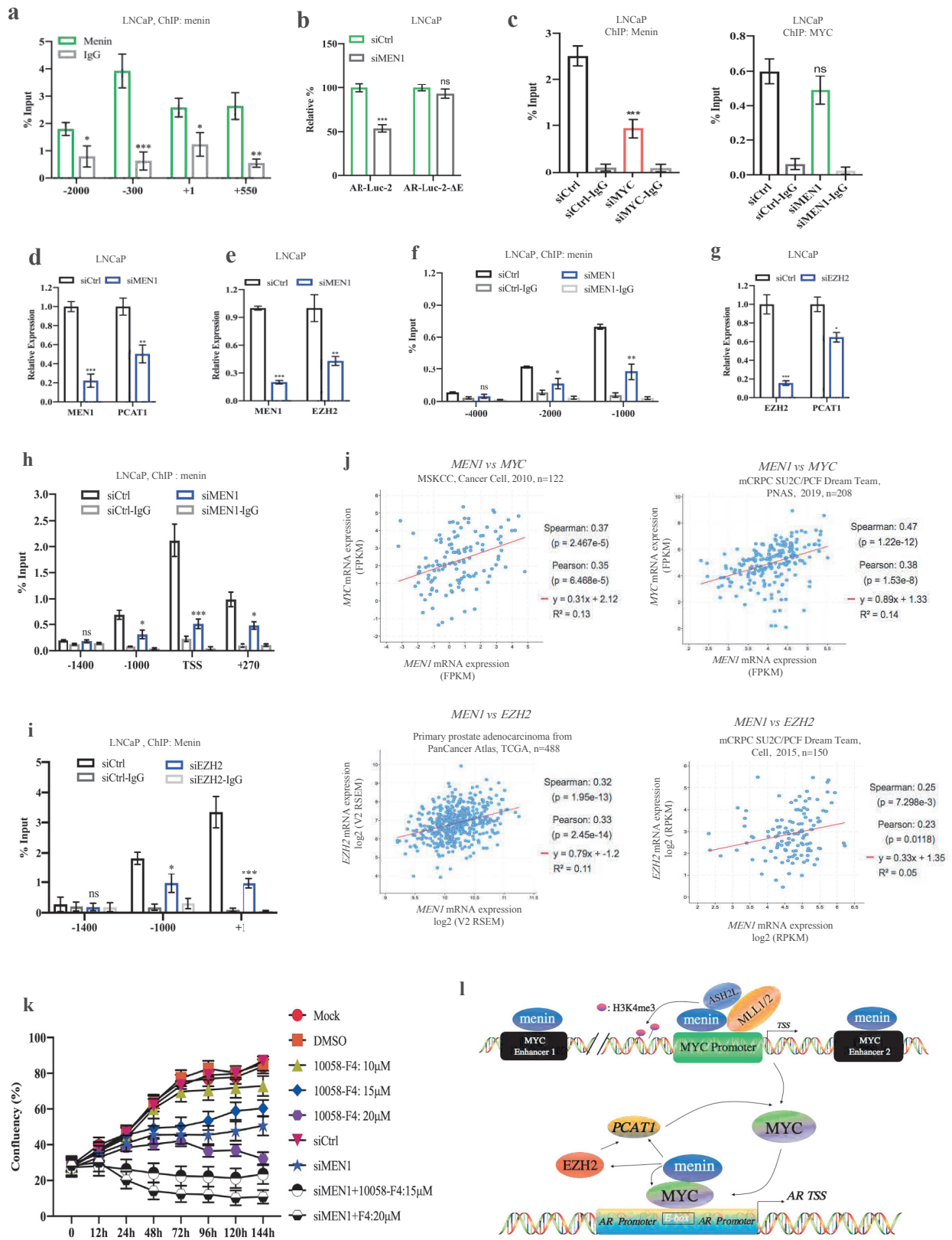


Fig.2 Menin is essential for MYC-mediated AR transcription in AR dependent PCa cells



Suppl. figure legends:

Fig. S1 Menin is crucial for maintaining MYC expression in AR-dependent PCa cells.

a RT-qPCR analysis for MYC transcription in *MEN1*-KD (with siMEN(1) + (3)) 22RV1 and DU145 PCa cells. Data were normalized against *HPRT* and represented as fold change. **b** Western blot showing MYC expression in *MEN1*-KD (with siMEN(1) or (3)) 22RV1 and DU145 PCa cells. **c** Representative images of immunofluorescent staining (IF) of MYC in siMEN1(1)+(3)-treated 22RV1 and DU145 cells (red). The nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S2 Menin recruits the MLL complex and activates MYC transcription in AR-dependent PCa cells.

a Representative images of in situ proximity ligation assay (PLA) showing the interaction between menin and MYC in LNCaP and 22RV1 PCa cells treated with siCtrl or siMEN1(1)+(3). The nuclei were counterstained with DAPI (blue), and quantification of PLA is shown on the right. Scale bar, 50 μ m. Enlarged view of the insets in the left panel is shown in the right panel. **b** Analysis of the interaction between menin and MYC by co-immunoprecipitation (IP) with endogenous menin and MYC proteins from LNCaP and 22RV1 cells, using anti-menin or IgG antibodies. **c** ChIP-qPCR showing menin recruitment on the *MYC* promoter and enhancers 1 and 2 in 22RV1 cells. The X-axis shows the central location of the PCR products relative to the *MYC* transcription start site (+1 site). **d** ChIP-qPCR evaluating the abundance of H3K4me3 histone marks on the *MYC* promoter and that of the *MYC* enhancer 1 and 2 in siMEN1(1)+(3)-treated 22RV1 cells, compared to cells treated with siCtrl. **e** RT-qPCR analysis of the transcriptional expression of *MYC* in *KMT2A+KMT2B*-KD

22Rv1 cells. **f** RT-qPCR and Western blot analyzing the transcriptional (left panels) and protein (right panel) expression of MYC in *ASH2L*-KD 22Rv1 cells as indicated. **g** ChIP-qPCR, using anti-ASH2L antibody, showing the occupancy of ASH2L on the *MYC* promoter in siMEN1(1)+(3)-treated 22Rv1 cells. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S3 *MEN1*-KD reduces the expression of downstream MYC target genes and AR splice variants in AR-dependent PCa cells.

a and **b** mRNA (**a**) and protein (**b**) expressions of CDK2, CDK4, CCNA, CCND and CCNE were analyzed by RT-qPCR and Western blotting in *MEN1*-KD 22Rv1 cells. **c** *MEN1*-KD induces cell cycle arrest at the G1 phase in 22Rv1 cells, compared to the cells transfected with control siRNA by flow cytometry. Percentages (%) of cell populations at different stages of cell cycles are listed within the panels. The experiment was repeated three times, and representative results are presented. **d** RT-qPCR analysis detecting the expression of *AR* variants, *ARV1*, *ARV3*, *ARV567es* and *ARV7*, in 22Rv1 cells transfected with siCtrl or siMEN1(1)+(3). Data were normalized against *HPRT* and represented as fold change. **e** Western blot analysis of the expression of *ARV7* in siCtrl or siMEN1(1)+(3)-treated PCa cells, using *ARV7*-specific antibody (#68492, Cell Signaling Tech.). ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S4 The regulation of MYC by menin is androgen-dependent in PCa cells.

a Western blot analysis showing AR, menin and MYC expression (in total or nuclear protein as indicated) in DHT-treated 22Rv1 cells. **b** In situ proximity ligation assay (PLA) for menin and MYC dimers was performed in DHT- or methanol-treated 22Rv1 cells with menin and MYC-specific antibodies. The detected dimers were represented by red dots. Enlarged view of the insets in the left panel is shown in the

right panel The nuclei were counterstained with DAPI (blue). Scale bar =10 μ m. The quantification of the number of signals per cell is shown in the right panel. **c** ChIP-qPCR using anti-menin antibodies showing the binding of menin to the MYC promoter in DHT-treated 22Rv1 cells for 24 h. The X-axis shows the central location of the PCR products relative to the MYC TSS. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S5 Menin and MYC co-regulate AR transcription in PCa cells.

a Schematic representation of the position of ChIP-qPCR primers along the AR promoter. **b** ChIP-qPCR showing the binding of menin to the AR promoter in 22Rv1 cells using anti-menin. **c** ChIP assay to assess the levels of MYC recruitment to the AR promoter in LNCaP and 22Rv1 cells. **d** Schematic representation of the design of Luciferase reporter along the AR promoter. **e** and **f** Luciferase activity was measured in extracts from siMEN1(1)+(3) (**e**) and siMYC(1)+(2)-treated (**f**) LNCaP or 22RV1 cells transfected with different luciferase reporter constructs, AR-Luc-2 containing the E-box site or AR-Luc-1 and 3. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S6 Menin is crucial for the MYC-mediated regulation of the AR promoter through the E-box site in AR-dependent PCa cells.

a left panel: Schematic representation of the AR promoter construct with E-box site-deletion (AR-Luc-2- Δ E). Relative luciferase activity analyses in MEN1-KD (**a**, right panel) and MYC-KD (**b**) PCa cells as indicated transfected with AR-Luc-2- Δ E. **c** ChIP was performed to assess the influence of MYC-KD on the menin occupancy on the AR promoter in siMYC(1)+(2)-treated LNCaP cells as indicated. **d** ChIP was performed to assess the influence of MEN1-KD on the MYC occupancy on the E-box

site of the *AR* promoter in siMEN1(1)+(3)-treated LNCaP and 22Rv1 cells, respectively. ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S7 Menin participates in the regulation of PCAT1 through EZH2.

a RT-qPCR analysis showing downregulated *PCAT1* expression in *MEN1*-KD 22Rv1 cells. **b** mRNA and protein expressions of *EZH2* were analyzed by RT-PCR (left and middle panels) and Western blotting (right panel) in *MEN1*-KD 22RV1 cells. The physical interaction between menin and *EZH2* in LNCaP or 22Rv1 cells detected by PLA (**c**) and co-IP (**d**). **e** ChIP-qPCR analysis assessing the effect of *MEN1*-KD on the level of menin recruitment to the *EZH2* promoter in siCtrl or siMEN1(1)+(3)-treated LNCaP or 22Rv1 cells. **f** ChIP-qPCR analyzing the alteration of H3K4me3 histone marks on the *EZH2* promoter in *MEN1*-KD LNCaP and 22Rv1 cells. **g** and **h** ChIP-qPCR was used to analyze the menin recruitment to the *PCAT1* promoter in siMEN1(1)+(3)-treated (**g**) and in siEZH2(1)+(2)-treated 22Rv1 cells, with reduced *EZH2* expression confirmed by Western blot analysis (**h**). For all the above ChIP-qPCR analyses, ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Fig. S8 Synergistic effect on reducing cell proliferation in PCa cells treated with the combined use of siMEN1 and of the MYC inhibitor.

a IncuCyte ZOOM analysis showing the effect on the cell proliferation of combined treatment using siMEN1(1)+(3) and MYC inhibitor 10058-F4 with different doses as indicated in 22Rv1 cells. **b** MYC expression was evaluated d by Western blot analysis in LNCaP and 22Rv1 cells treated by MYC inhibitor 10058-F4.

Fig. S1

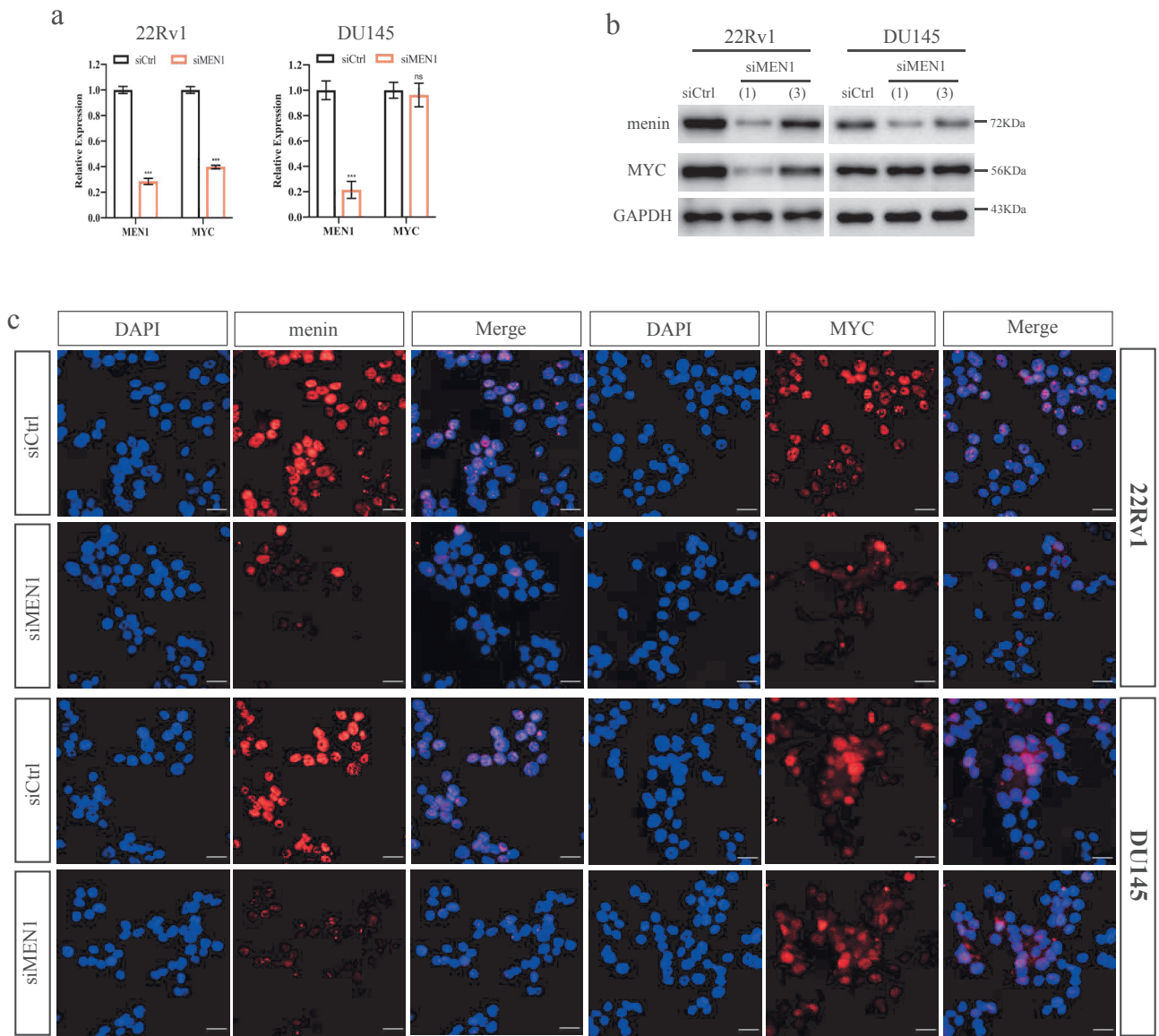


Fig. S2

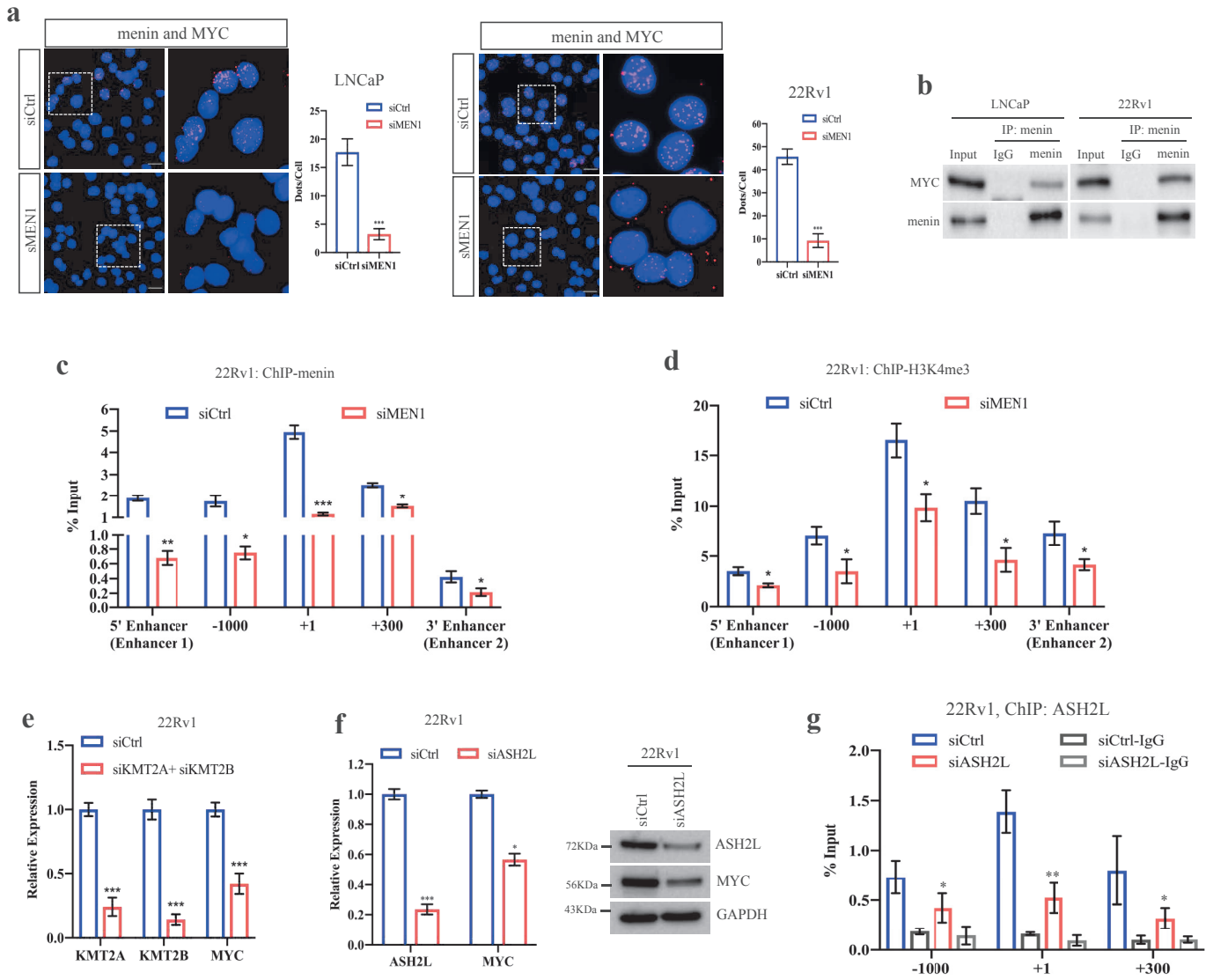


Fig. S3

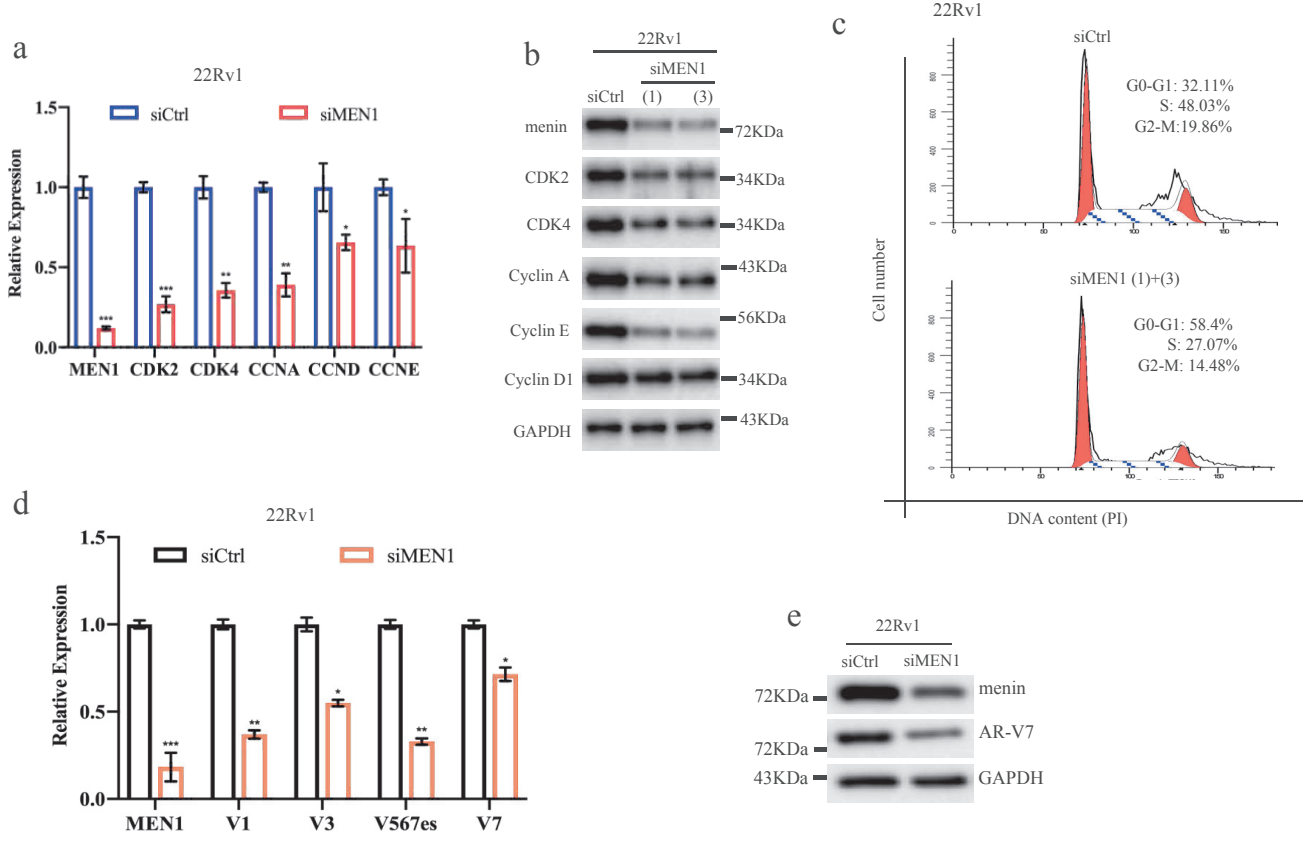


Fig. S4

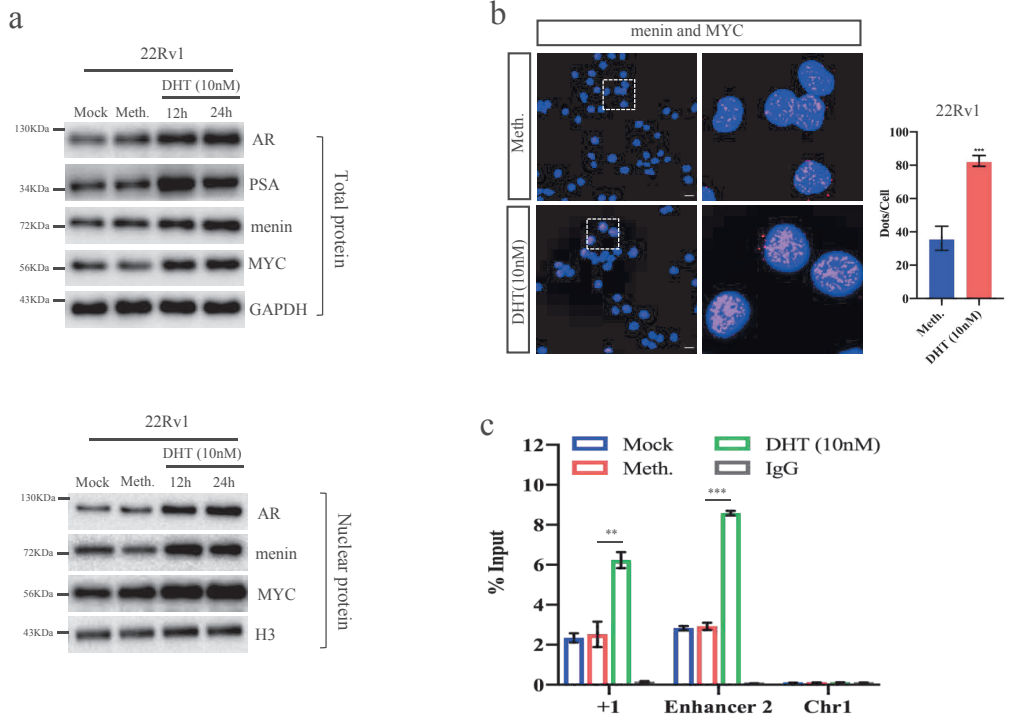


Fig. S5

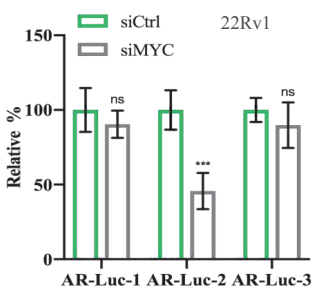
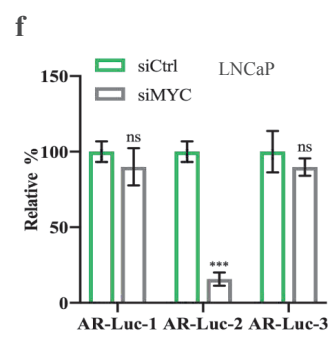
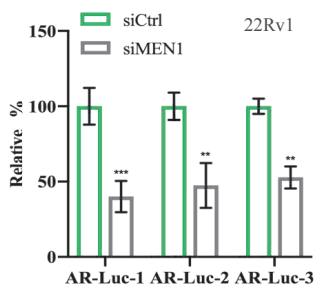
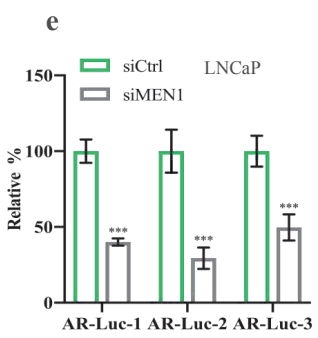
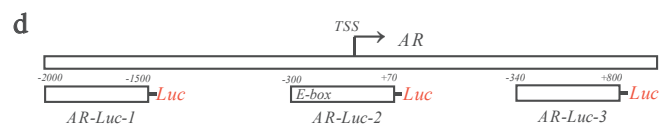
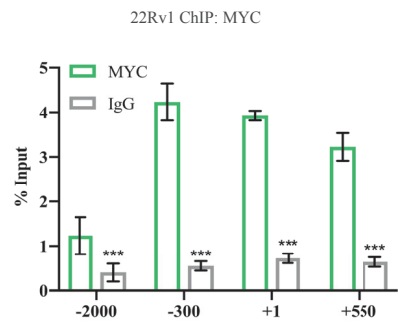
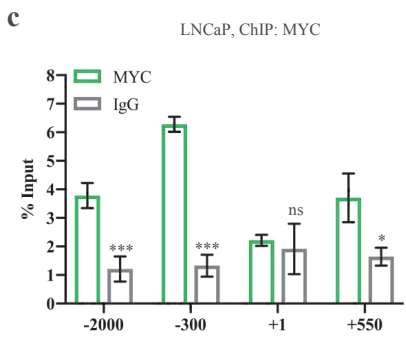
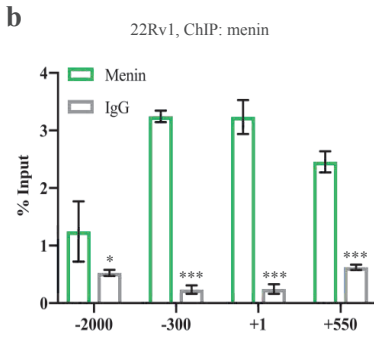
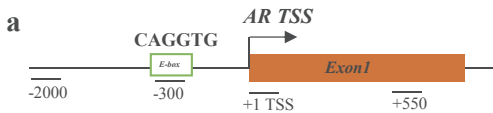


Fig. S6

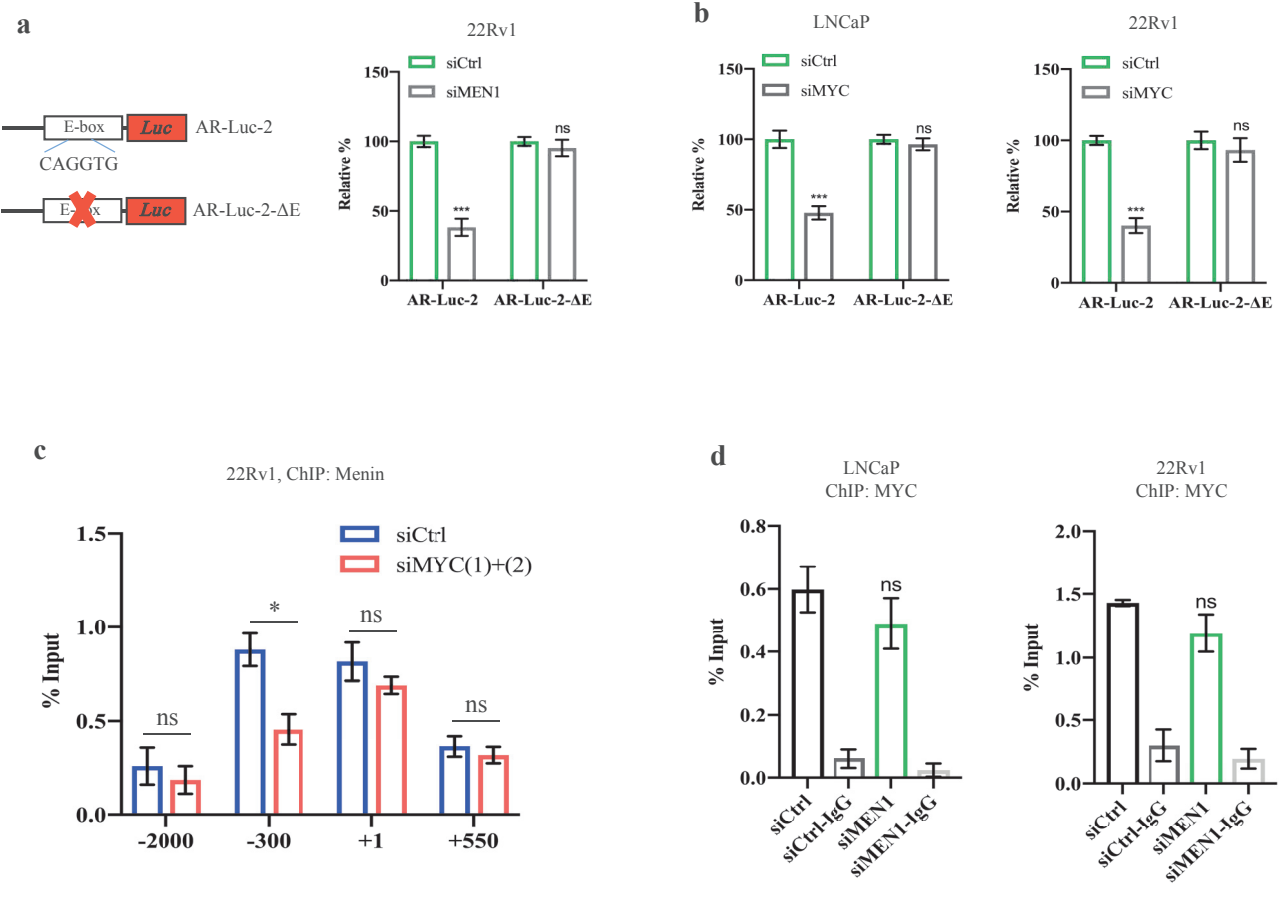
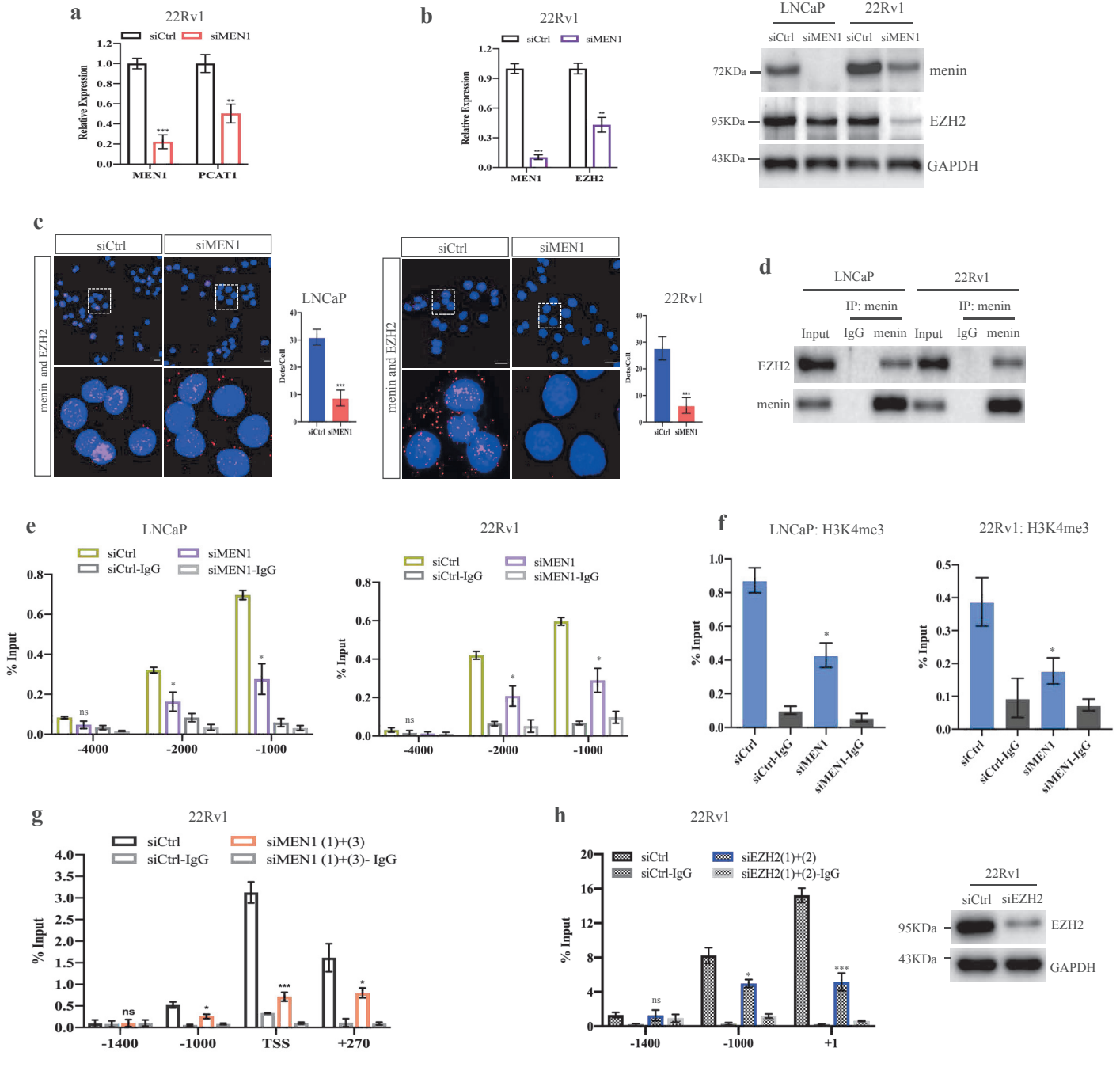


Fig. S7



Materials and Methods

Cell culture and treatment

Prostate cancer cell lines were purchased from ATCC. Their authentication was renewed recently and *Mycoplasma* testing was carried out regularly. LNCaP and 22Rv1 cells were maintained in RPMI-1640 Medium (Gibco), and DU145 cells in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), PC3 in DMEM/F-12 Medium and supplemented with 10% fetal bovine serum (FBS), 0.5% non-essential amino acids, 2 mM L-Glutamine, 100 unit/mL Penicillin, 100 µg/mL streptomycin, HEPES 10 mM, Sodium Pyruvate 1 mM. Cells were grown in a humidified atmosphere with 5% CO₂ at 37°C. Inhibition of MYC expression was achieved through the use of 10058-F4 [1] (F3680, Sigma), at the concentration of 10 µM, 15 µM, 20 µM, and androgen stimulation was performed with 10 nM Dihydrotestosterone (DHT) (Sigma, A-8380) for 12 and 24 h.

RNA interference and transfection

MEN1 siRNA sequences, that are siMEN1(1) (MEN1HSS106462) and siMEN1(3) (MEN1HSS181079), were designed by Invitrogen. *MYC* siRNA (siMCY(1), SASI_Hs01_00222677, siMCY(2), SASI_Hs01_00222676), *EZH2* siRNA (siEZH2(1), SASI_Hs01_00147882, siEZH2(2), SASI_Hs01_00022215), *ASH2L* siRNA (siASH2L, SASI_Hs01_00139134) were purchased from Sigma-MERCK. Non-targeting control pool (siCtrl) from Sigma-MERCK was used as negative control for each RNA interference experiment. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and incubated during 72 h.

Cell proliferation assay

Cell proliferation assays were performed as described previously [2]. Briefly, cells were seeded onto 96-well culture plates at 5×10^3 cells for 22Rv1, DU145 or PC3, and at 1×10^4 cells for LNCaP. 24 h later, cells were treated with 20 nM of siMEN1(1)+(3) or MYC inhibitor (10058-F4) at different concentrations (10 μ M, 15 μ M and 20 μ M), or with both 20 nM of siMEN(1)+(3) and 10058-F4 at different concentrations (15 μ M and 20 μ M). All experiments were performed in triplicate and at least three times.

Real time RT-PCR

RNA was isolated from cells using a NucleoSpin RNA Plus kit (ref. 740984, Macherey-Nagel) and mRNAs levels were assessed by quantitative real time RT-PCR as described previously [2]. Each experiment was repeated independently at least three times. Data were normalized against *HPRT* and represented as fold change. Primers used are listed in Supplementary information, Table S1.

Western blotting

Total protein extracts from cells were prepared and analyzed as described previously [3]. The cellular nuclear fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ref. 78833, Thermo Scientific). The following primary antibodies were used: Rabbit anti-menin (1/4000) (A300-105A, Bethyl), Rabbit anti-c-Myc (1/2000) (#9402, Cell Signaling Tech.), Rabbit anti-AR (1/2500) (A303-965A, Bethyl), Rabbit anti-ARV7 (1/2000) (#19672, Cell Signaling Tech.), Mouse anti-CDK2 (1/1000) (sc-6248, Santa Cruz), Mouse anti-CDK4 (1/1000) (sc-56277, Santa Cruz), Mouse anti-Cyclin A (1/500) (sc-271645, Santa Cruz), Mouse anti-Cyclin D1 (1/1000) (sc-20044, Santa Cruz), Mouse anti-Cyclin E (1/1000) (sc-377100, Santa Cruz), Rabbit anti-EZH2 (1/2000) (#5246, Cell Signaling Tech.), Rabbit anti-ASH2L (1/2000) (#5019, Cell Signaling Tech.). Secondary antibodies

included HRP Goat Anti-mouse (1/10000) (ref.111-035-003) and HRP Goat Anti-rabbit (1/10000) (ref. 111-035-003), and were purchased from Jackson immunoresearch. Mouse anti-GAPDH (1/1000) (sc-47724, Santa Cruz) and anti-Histone H3 (# 4499, Cell Signaling Tech.) antibodies were used as loading controls.

Flow cytometry analysis

Cells were treated with siCtrl or siMEN1(1)+(3) for 72 h, followed by harvesting and re-suspending in 100 μ l of Phosphate-Buffered Saline (PBS) and fixed with 70% ethanol at 4°C overnight, and stained with Propidium Iodide (P1304MP, Thermo Fisher) (50 μ g/ml) containing 100 μ g/ml RNase A (R6148, Sigma) for 15 min. Analysis of cell cycling was performed by flow cytometry on a FACScan instrument (Beckman Coulter, Brea, CA, USA).

Promoter and Luciferase reporter assays

Promoter constructs for the assays were generated by cloning the regions of the human *AR* promoter from -2000 to -1500, -300 to +70, +340 to 800, between the *XhoI* and *Hind III* restriction sites of the pGL3-Basic vector (Promega). After cloning and validation by sequencing, the constructs were named AR-Luc-1, AR-Luc-2 and AR-Luc-3. The AR-Luc-2- Δ E construct contains the deletion of the E-box site (CAGGTG), generated by overlapping PCR on the basis of AR-Luc-2. Primers used are listed in Supplementary information, Table S1.

Cells were plated in 6-well plates the day before transfections, then, co-transfected with 1 μ g of indicated plasmid and 10 ng Renilla luciferase plasmid pRL-TK (Promega) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were also transfected with 20 nM siRNA using the INTERFERin siRNA transfection reagent (#409-10, Polyplus transfection, France).

After 48 h transfection, cells were lysed and luciferase assays were performed with Promega Luciferase assay kit. The results obtained were normalized against Renilla luciferase activity and expressed relative to the activity of untreated cells transfected with the AR-Luc promoter plasmid. Promoter activity was reported as mean \pm SD value.

Proximity ligation assays (PLA), image acquisition and analysis

PLA assays were performed according to manufacturer's protocol as described previously [4]. Duolink In Situ PLA Probe Anti-Goat MINUS (#DUO92006, Sigma Aldrich) was used for menin (Goat anti-menin, A300-106A, Bethyl, TX, USA) and Duolink In Situ PLA Probe Anti-Rabbit PLUS (#DUO92002, Sigma Aldrich) for c-Myc or EZH2 (see above antibodies). Briefly, cells were grown on coverslips in 12-well plates and treated (as explained in figures and legends), then fixed with methanol for 5 min. After saturation in the blocking solution, cells were incubated with different pairs of primary antibodies at 37°C for 1 h. The PLA probes consisting of secondary antibodies conjugated with ligation were then added (30 min at 37°C), prior to the amplification phase, for 100 min at 37°C. Cells were finally counterstained with DAPI (DUO82040, Sigma-Aldrich) for 10 min. Images were acquired on an Eclipse-NiE NIKON microscope. For each sample, at least one hundred cells were counted. Analysis and quantification of these samples were performed using the ImageJ software (Version 1.52, NIH, MD, USA). PLA dots were quantified on 8-bit images using the 'Analyse Particles' command, whereas cells were counted using the cell counter plugin.

Immunofluorescence (IF) staining

For IF staining, cells were grown on glass coverslips, then fixed with methanol for 5 min at room temperature. Following fixation, cells were blocked with Dako buffer

(S0809, Agilent) for 1 h, and incubated with primary antibodies overnight at 4°C, then with appropriate secondary antibodies conjugated with Alexa 555 (red) or Alexa 488 (green) (Cell Signaling Technology). Cells were counterstained with DAPI (DUO82040, Sigma-Aldrich) for 10 min and visualized by fluorescence microscopy (Eclipse-NiE NIKON microscope). The following primary antibodies were used for immunostaining: Rabbit anti-menin (1/2000) (A300-105A, Bethyl), Rabbit anti-c-Myc (1/2000) (#9402, Cell Signaling Tech.)

Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) was performed with the Millipore ChIP Assay Kit (17-295). Cells were crosslinked with 1% formaldehyde for 10 min at 37°C. Chromatin was prepared according to the Millipore protocol and sonicated to an average size of 300-500 bp using a Diagenode Bioruptor. Chromatin fragments were immunoprecipitated at 4°C overnight with tested antibodies or normal rabbit IgG used as negative control, and immune complexes were collected on Protein A agarose beads (ChIP assay kit, Millipore). Chromatin complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃) and crosslinking was reversed at 65°C overnight [5]. DNA fragments were purified with the QIAquick PCR purification kit (Qiagen, 28104) and used for quantitative PCR reactions with SsoADV Univer SYBR Green Supermix (Bio-Rad). Each ChIP assay was repeated at least three times independently. Primers used for ChIP-qPCR are listed in Supplementary information, Table S1.

Raw data for mining analysis

The cBioPortal for cancer genomics (<http://www.cbioportal.org>), a recognized and widely used online portal providing analysis of large-scale cancer genomic datasets [6,7], was employed to analyze mRNA co-expression between *MEN1* and *MYC*, *MEN1*

and *EZH2*, *MEN1* and *PCAT1* in PCa. Data on the correlation between *MEN1* and *MYC* mRNA expression were download from SU2C/PCF Dream team (mCRPC) [8]. mRNA co-expression data for *MEN1* and *EZH2* were downloaded from Firehose Legacy team and PanCancer Atlas team (Primary prostate adenocarcinoma) [9]. Finally, mRNA co-expression data for *MEN1* and *PCAT1* were downloaded from the publication of SMMU team (Primary prostate adenocarcinoma) [10].

Statistical analysis

All experiments were repeated at least three times to ensure accuracy. The values are expressed as mean \pm standard deviation (S.D.) and analyzed by Student t-test. The level of significance: ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Table S1. Primers for this study

Application	Primers' name	Sequence	Reference
RT-qPCR	<i>MEN1</i>	F: 5'-GACCTGTCCCTCTATCCTCG-3'	1
		R: 5'-TGACCTCAGCTGTCTGCTCC-3'	
RT-qPCR	<i>MYC</i>	F: 5'-AACGATTCCTTCTAACAG-3'	2
		R: 5'-GGCTAAATCTTTC AGTCT-3'	
RT-qPCR	<i>AR</i>	F: 5'-CAGTGGATGGGCTGAAAAAT-3'	3
		R: 5'-GGAGCTTGGTGAGCTGGTAG-3'	
RT-qPCR	<i>ARV1</i>	F: 5'-GACCTTACGGGGACATGCG-3'	4
		R: 5'-GATTCTTTCAGAAACAACAACAGCTGCT-3'	
RT-qPCR	<i>ARV3</i>	F: 5'-GACCTTACGGGGACATGCG-3'	4
		R: 5'-AAATCCTTCAGCGGCTCTTTTG-3'	
RT-qPCR	<i>ARV567es</i>	F: CCT TGC TCT CTA GCC TCA ATG AA	5
		R: CTT GAT TAG CAG GTC AAA AGT GAA CT	
RT-qPCR	<i>ARV7</i>	F: 5'-TGTCGTCTTCGAAATGTTATGA-3'	4
		R: 5'-TCATTTTGAGATGCTTGCAATTG-3'	
RT-qPCR	<i>EZH2</i>	F: 5'-TGCAGTTGCTTCAGTACCCATAAT-3'	3
		R: 5'-ATCCCCGTGTACTTTCCCATCATAAT-3'	
RT-qPCR	<i>PCAT1</i>	F: 5'-TGAGAAGAGAAATCTATTGGAACC-3'	6
		R: 5'-GGTTTGTCTCCGCTGCTTTA-3'	
RT-qPCR	<i>HPRT</i>	F: 5'-TATGGCGACCCGACCCCT-3'	7
		R: 5'-CATCTCGAGCAAGACGTTTCA-3'	
RT-qPCR	<i>CDK2</i>	F: 5'-GCTAGCAGACTTTGGACTAGCCAG-3'	8
		R: 5'-AGCTCGGTACCA CAGGGTCA-3'	

RT-qPCR	<i>CDK4</i>	F: 5'-CTGGTGTTTGAGCATGTAGACC-3'	9
		R: 5'- GATCCTTGATCGTTT CGGCTG-3'	
RT-qPCR	<i>CCNA</i>	F: 5'-CTGCATTTGGCTGTGAACTAC-3'	10
		R: 5'-ACAAACTCTGCTACTTCTGGG-3'	
RT-qPCR	<i>CCND</i>	F: 5'-ACGAAGGTCTGCGCGTGT-3'	11
		R: 5'-CCGCTGGCCATGAACTACCT-3'	
RT-qPCR	<i>CCNE</i>	F: 5'-ATCAGCACTTCTTGAGCAACA-3'	11
		R: 5'-TTGTGCCAAGTAAAAGGTCTCC-3'	
RT-qPCR	<i>KMT2A</i>	F: 5'-AGCAGCTCTCATTTGCAGGT-3'	12
		F: 5'-ATGAGGAACACAACACTGCATCA-3'	
RT-qPCR	<i>KMT2B</i>	F: 5'-CCAGACCTGCTGCTTGAGT-3'	12
		F: 5'-CTCAGAGCTCGAAGCCTCAC-3'	

Continued above

Application	Primers' name	Sequence	Reference	
ChIP-PCR	ARpro: -2000	F: 5'-CCCACATCATCCTCTACGCC-3'	13	
		R: 5'-GTATGCAGCCGCTTGCTTTT-3'		
ChIP-PCR	ARpro: -300	F: 5'-GCGTGGTTGCTCCCGCAAG -3'		
		R: 5'-GGGTAGACCCTTCCCAGCCC-3'		
ChIP-qPCR	ARpro: +1(TSS)	F: 5'-CCGTCCAAGACCTACCGAGG-3'		
		R: 5'-CCGGGTTCTGGATCACTTCG-3'		
ChIP-qPCR	ARpro: +550	F: 5'-GCAACTCCTTCAGCAACAGC-3'		
		R: 5'-GACACCGACACTGCCTTACA-3'		
ChIP-qPCR	MYCpro: -1000	F: 5'-GAAGCGTAAATAAAATGTGAAT-3'		14
		R: 5'-GGCTGCCTTCCAGGCATTAA -3'		
ChIP-qPCR	MYCpro: +1(TSS)	F: 5'-CCAACAAATGCAATGGGAGT-3'		
		R: 5'-CCAGAGTCCCAGGGAGAGTG-3'		
ChIP-qPCR	MYCpro: +300	F: 5'-ACTCACAGGACAAGGATGCG-3'		15
		R: 5'-TGCTCCTCCGTAGCAGTACT-3'		
ChIP-qPCR	MYC enhancer-1(-67kb)	F: 5'-CATCCAATAAACCTTCTACCTGA-3'	16	
		R: 5'-TGGCAGGTGTCCTAGAGCAT-3'		
ChIP-qPCR	MYC enhancer-2(+20kb)	F: 5'-CCAGCGAATTATTCAGAA-3'	17	
		R: 5'-AATTACCATTGACTTCCTC-3'		
ChIP-qPCR	PCAT1pro- -1400	F: 5'-TGACACCAACTGGTTCCTATTA-3'	17	
		R: 5'-TAAATGCCAGAAGACTCACCTTG-3'		
ChIP-qPCR	PCAT1pro: -1000	F: 5'-TGCTAGTTAATTTCCAGCTCCTTC-3'		
		R: 5'-CAAGCGAGACTCTGTCTCAAAA-3'		

ChIP-qPCR	PCAT1pro: +1 (TSS)	F: 5'-GCACCTAGTCACTTCCCATATCA-3'	
		R: 5'- CCTCTTGGCTTCAGAAAAGTACA-3'	
ChIP-qPCR	PCAT1pro: +270	F: 5'-ACACTTAACTGGCACTTGTGGAA-3'	
		R: 5'-CACTGGTGTTCATGGCCTTATTA-3'	
ChIP-qPCR	EZH2pro: -4000	F: 5'-GCACATCAGCCACGCTTCT-3'	
		R: 5'-GGAGCTGAGGGAGCATTACTG-3'	
ChIP-qPCR	EZH2pro: -2000	F: 5'-CCAACATTGGAGTGATTCAG-3'	18
		R: 5'-TCATCAGATGATTAGCCCA-3'	
ChIP-qPCR	EZH2pro: -1000	F:5'-GACACGTGCTTAGAACTACGAACAG-3'	
		R: 5'-TTTGGCTGGCCGAGCTT-3'	
ChIP-qPCR	Chr1 Neg primers	F: 5'-CGGGGTCTTTTGGACCTT-3'	13
		R: 5'-GAAACACGGCTGCCAGAAAC-3'	

Continued above

Application	Primers' name	Sequence(5'-3')
Cloning	AR-Luc-1	F1: TA CTCGAGCCCACATCATCCTCTACGCC
		F2: ACT AAGCTTTTCACCGAAGAGGAAAGGGC
Cloning	AR-Luc-2	F1: TACTCGAGCAAGTATTAAGAGACAGACTG
		F2: ACT AAGCTTCGCTCTGGAACAGATTCTGGAAAG
Cloning	AR-Luc-3	F1:TACTCGAGGGTTGCGTCCCAGAGCCTGG
		F2: ACTAAGCTTCCGAAGCAGTCCCCTGGACT
Cloning	AR-Luc-2-ΔE	F1: GTAGCTGCAGCTAGCTGCCCGGGAAGCTCCAGAGAAGGAA
		F2: GGCAGCTAGCTGCAGCGACTACCGCATCATCACAGC

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Chapter II Results

Article 2. MEN1 silencing aggravates tumorigenic potential of AR-independent prostate cancer cells through nuclear translocation and activation of JunD and β -catenin

Research article

***MEN1* silencing aggravates tumorigenic potential of AR-independent prostate cancer cells through nuclear translocation and activation of JunD and β -catenin**

Running title *MEN1* exerts oncosuppressive functions in AR-independent PCa cells

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Abstract

Background: Recent studies highlighted the increased frequency of AR-low or -negative prostate cancers (PCas) and the importance of AR-independent mechanisms in driving metastatic castration-resistant PCa (mCRPC) development and progression. Several previous studies have highlighted the involvement of the *MEN1* gene in PCa. In the current study, we focused on its role specifically in AR-independent PCa cells.

Methods: Cell tumorigenic features were evaluated by proliferation assay, foci formation, colony formation in soft agar, wound healing assay and xenograft experiments in mice. Quantitative RT-PCR, Western blot and immunostaining were performed to determine the expression of different factors in human PCa lines. Different ChIP-qPCR-based assays were carried out to dissect the action of JunD and β -catenin.

Results: We found that *MEN1* silencing in AR-independent cell lines, DU145 and PC3, resulted in an increase in anchorage independence and cell migration, accompanied by sustained *MYC* expression. By searching for factors known to positively regulate *MYC* expression and play a relevant role in PCa development and progression, we uncovered that *MEN1*-KD triggered the nuclear translocation of JunD and β -catenin. ChIP and 3C analyses further demonstrated that *MEN1*-KD led to, on the one hand, augmented binding of JunD to the *MYC* 5' enhancer and increased formation of loop structure, and on the other hand, increased binding of β -catenin to the *MYC* promoter. Moreover, the expression of several molecular markers of EMT, including E-cadherin, BMI1, Twist1 and HIF1 α , was altered in *MEN1*-KD DU145 and PC3 cells. In addition, analyses using cultured cells and PC3-GFP xenografts in mice demonstrated that JunD and β -catenin are necessary for the altered tumorigenic potential triggered by *MEN1* inactivation in AR-independent PCa cells. Finally, we observed a significant negative clinical correlation between *MEN1* and *CTNNB1* mRNA expression in primary PCa and mCRPC datasets.

Conclusions: Our current work highlights an unrecognized oncosuppressive role for menin specifically in AR-independent PCa cells, through the activation of JunD and β -catenin pathways.

Keywords: (6)

Prostate cancer, AR-independent cells, *MEN1*, *MYC*, JunD, β -catenin

Background

With over 1.4 million new cases and more than 370,000 deaths reported worldwide in 2020 [1], prostate cancer (PCa) remains a major cause of cancer-related mortality and morbidity in men worldwide. The majority of PCas express the androgen receptor (AR), and dysregulation of the androgen pathway is key to the development and progression of PCa [2]. Androgen deprivation therapies (ADT) are, therefore, a highly effective frontline treatment for PCa [3]. However, ADT are characterized by the virtually unavoidable emergence of resistance, termed castration-resistant PCas (CRPCs), often metastatic (mCRPC) and with a high mortality rate [1, 4]. Genomic characterization of CRPCs has led to their subdivision into two subtypes: (1) AR-dependent CRPCs, containing alterations in the AR gene, such as amplification, point mutations, and generation of splice variants; and (2) AR-independent CRPCs, in which resistant cells lack AR expression or signaling [5]. Markedly, among these AR-independent CRPCs, some PCas express neither the AR nor markers of neuroendocrine (NE) differentiation (“AR null–NE null”, or double negative PCa, DNPC), and their incidence has risen over the past 2 decades from 5% in 1998-2011 to 23% in 2012-2016 [6]. Owing to the heterogeneous nature of the disease, addressing the mechanisms specifically underlying different subtypes of PCa is thus highly relevant.

Studies on DNPC and AR-independent mCRPC have considerably advanced our knowledge in this field over the last decade. By extensively characterizing cellular markers of the related lesions and cell models, cell dedifferentiation and/or altered cell plasticity were proposed to be critical for the development and evolution of these cancers [7]. Among these alterations, epithelial-to-mesenchymal transition (EMT), often in conjunction with stem cell-like changes, is considered to be crucial in the development of AR-negative PCas. The proto-oncogene *MYC*, a potent transcription factor that controls various biological processes [8-12], was reported to be one of the key drivers of CRPC and neuroendocrine prostate cancer (NEPC) [13-15]. Furthermore, several genetic factors and signaling pathways were shown to bypass

AR pathways and thus be involved in AR-independent PCa development, such as loss of P53 [16], RB1 [16], PTEN [17], glucocorticoid receptor (GR) [18], FGF [19] and Stat3/5, as well as the metabolic ACSL pathway [20, 21], whereas the underlying mechanisms remain elusive.

We have previously observed that male heterozygous *Men1* mutant mice developed PCa with a low but significant frequency [22]. However, Malik *et al.* reported that the physical interaction between menin and AR was essential for the growth of human PCa cell lines both in culture and in xenografts [23]. Recently, we demonstrated the decisive role played by the menin protein, encoded by the *MEN1* gene, in regulating *AR* transcription in AR-dependent PCa cell lines [24]. Our findings also highlighted disparities in the effects of this protein on the proliferation of PCa cells, since *MEN1* silencing had no impact (PC3 cells), or even increased (DU145 cells) the proliferation of AR-independent cells, whereas it led to a marked decrease in cell growth of AR-dependent lines (LNCaP, 22Rv1 and VCaP). The distinct role of the *MEN1* gene in these two PCa cell populations, albeit intriguing, is not surprising, considering the different cellular and molecular mechanisms involved in their tumorigenesis. Indeed, the *MEN1* gene is largely known as a tumor suppressor in several types of endocrine tissues, since its mutation predisposes patients to multiple endocrine neoplasia type 1 syndrome (MEN1 syndrome, OMIM 131100). However, it is now well established that menin displays oncogenic effects in certain types of leukemia containing fusion MLL caused by chromosome translocation [25]. Dreijerink *et al.* found that the gene expression profile obtained after *MEN1* silencing in normal mammary luminal progenitors was highly distinct from that found in ER-positive breast cancer MCF7 cells, suggesting that menin regulates different gene sets in normal mammary luminal cells versus ER-positive breast cancer cells [26]. Here, we focused on the mechanisms underlying the distinct effects observed upon *MEN1* silencing in AR-independent PCa cells. Through different analyses using cultured cells and *in vivo* experiments, our data unveiled the activation of the JunD and β -catenin pathways upon menin inactivation in AR-independent PCa cells,

underscoring menin as an oncosuppressive factor in these cells, in marked contrast to its role in AR-dependent PCa cells.

Materials and methods

Cell culture and treatment

Prostate cancer cell lines were purchased from ATCC. Their authentication was renewed recently and *Mycoplasma* testing was carried out regularly. LNCaP, 22Rv1 and DU145 were cultured in RPMI medium, and PC3 cells in F-12 medium (Gibco Invitrogen), at 37°C with 5% CO₂. Inhibition of menin-MLL interaction was achieved through the use of MI503 (Active Biochem).

Cell proliferation and foci formation assays

Cell proliferation assays were performed as described previously [24]. For foci formation assay, cells were seeded in 6-well culture plates at 5×10^2 cells for LNCaP, and at 2.5×10^2 cells for 22Rv1, DU145 or PC3. Cells were transfected with siRNA or treated with MI503, and cultured for 2 weeks. The ensuing colonies were stained with 0.05% crystal violet. The images of the plates were analyzed using Image J software. Each experiment was conducted in triplicate and statistical analyses were performed using the Prism software.

Scratch wound healing assay

Cell migration capacities were evaluated through wound healing assays. 5×10^3 cells were seeded onto 6-well plates. After 24 h in culture, cells were transfected with 20 nM of siRNA. 72 h after transfection, wounds were created, and wound closures were captured at 0, 6, and 12 h.

Colony formation in soft agar

Soft agar assays were performed as described previously [27]. After 4 weeks of incubation at 37°C, colonies were stained with 0.05% (w/v) crystal violet (Sigma) and colonies were counted using Image J software.

Xenograph tumor growth test

Xenograft has been performed using 8-weeks-old male NOD-Scidy mice by surgical implantation under the kidney capsule. 1×10^5 PC3-GFP cells have been encapsulated in a collagen matrix as previously described [28]. Five days after implantation, the mice were randomized onto two groups and treated three-times a week during 1 month. Mouse groups were treated with i.p. injections of menin inhibitor MI503 75 mg/kg (MedChemTronica) (n = 11) or Vehicle solution (DMSO/PEG300, Sigma-Aldrich) (n = 9). One month after, xenografted tissues were collected after necropsy and processed for further analyses. The quantification of the number of PC3-GFP cells in xenografted tissues by qPCR assay was performed as previously described [29]. All experiments were approved by Auvergne Ethics Committee (CEMEAA) and registered according the approval number 17296-2018102216428025 v3.

RNA interference and transfection

Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and incubated for 72 h.

RNA extraction, reverse transcription, and real-time PCR

Total RNA from cultured cells was extracted by using the RNeasy-Kits (Qiagen, Valencia, USA) as per manufacturer's instructions. cDNAs were amplified and

quantified in an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using the SYBR Green I dye (SsoAdvanced Universal SYBR Green, Bio-Rad). Data were normalized against the in-house control HPRT and represented as fold change. Primers used are listed in the Supplementary information.

Protein extraction and Western blotting

Subcellular fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ref. 78833, Thermo Scientific). Western blotting was carried out according to the method described previously [30].

Immunofluorescence (IF) and immunohistochemistry (IHC) staining

For IF staining, cells were grown on glass coverslips, then fixed with methanol for 5 min at room temperature. Following fixation, cells were blocked with Dako buffer (S0809, Agilent) for 1 h, and incubated with primary antibodies overnight at 4°C, then with appropriate secondary antibodies conjugated with Alexa 555 (red) or Alexa 488 (green) (Cell Signaling Technology). Cells were counterstained with DAPI (DUO82040, Sigma-Aldrich) for 10 min and visualized by fluorescence microscopy (Eclipse-NiE NIKON microscope).

Xenografts were collected and fixed in 4% PFA prior to paraffin embedding, sectioning, staining with hematoxylin and eosin, and with immunostaining conducted as described previously [24]. Images were acquired on a ZEISS Axioscope 5 microscope.

Chromatin immunoprecipitation (ChIP) and sequential ChIP (reChIP) assays

Chromatin immunoprecipitation (ChIP) was performed with the Millipore ChIP Assay Kit (17-295) as described previously [24]. For reChIP assays, the first immunoprecipitated chromatin complexes were washed and eluted with 10 mM dithiothreitol at 37°C for 30 min and diluted 50-fold with ChIP dilution buffer. The second immunoprecipitations were then performed [31]. Each ChIP or reChIP assay was repeated at least three times independently. Primers used for ChIP-qPCR are listed in the Supplementary information.

Chromosome Conformation Capture (3C) assay and ChIP-3C

3C was performed as described previously [32, 33] with minor modifications described in the Supplementary information. ChIP-3C assays were performed as formerly described [34]. Following 3C assays, we obtain cross-linking in 3C samples under ligation or non-ligation conditions. Then, by combining ChIP (as described above) and 3C assays we obtain ChIP-3C assays. Primers used for ChIP-3C qPCR are listed in the Supplementary information.

Raw data for mining analysis

The cBioPortal for cancer genomics (<http://www.cbioportal.org>) [35, 36] was employed to analyze mRNA co-expression between *MEN1* and *JunD*, *MEN1* and *CTNNB1* in PCa. Data on the correlation between *MEN1* and *JunD* mRNA expression were download from SU2C/PCF Dream team (mCRPC) [37] and Firehose Legacy team and PanCancer Atlas team (Primary prostate adenocarcinoma) [38].

Statistical analysis

All experiments were repeated at least three times to ensure accuracy. The values are expressed as mean \pm standard deviation (S.D.) and analyzed by *Student t-test*. The level of significance: ns, non-significant vs Ctrl, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, for all analyses.

Results

***MEN1* silencing promotes the tumorigenic potential and maintains MYC expression in AR-independent PCa cells.**

Our previous results, showing that *MEN1* silencing had distinct effects on cell proliferation of AR-dependent versus AR-independent PCa cells [24], prompted us to dissect cellular consequences of *MEN1* inactivation in AR-independent PCa cells. Firstly, we performed foci formation and soft agar assays to assess cell tumorigenic potential in *MEN1*-knockdown (KD) PCa cells. *MEN1*-KD resulted in a significant increase in colony formation in AR-independent PCa cells (DU145 and PC3) in both tests (Fig. 1a and 1b, S1a), whereas it had the opposite effect in AR-dependent PCa cells (LNCaP and 22Rv1) tested for foci formation (Fig. S1b). The efficiency of *MEN1* silencing was confirmed by Western blotting (Fig. S1a and S1c). In soft agar assays, *MEN1*-KD DU145 and PC3 cells gave rise not only to more foci (Fig. 1c), but also to larger ones, with colonies appearing more irregular in shape, compared with siCtrl-treated cells (Fig. 1b). Furthermore, to determine whether menin influenced cell migration, *MEN1*-KD DU145 and PC3 cells were subjected to scratch wound-healing assays, with wound closure being monitored at 6 h and 12 h. A significant increasing in DU145 and PC3 cell migration was observed at these time-points following *MEN1* silencing with siMEN1(1)+(3) (Fig. 1d-e), whereas this reduced 22Rv1 cell migration (Fig. S1d). Taken together, *MEN1*-KD in DU145 and PC3 cells promoted the cell growth in an anchorage-independent manner and increased cell migration, suggesting that menin plays a tumor suppressive role in AR-independent PCa cell lines, unlike its oncogenic role in AR-dependent PCa cell lines.

Intriguingly, Wu et al. demonstrated that menin physically interacts with MYC to enhance the transcription of MYC target genes in liver cancer cells [39]. We observed that, while *MEN1*-KD reduced the expression of MYC at the transcriptional (Fig. 1f, upper panels) and protein levels (Fig. 1g, upper panel) in LNCaP and 22Rv1 cells, its expression was maintained in the two *MEN1*-KD AR-independent PCa cells (Fig. 1f and 1g, lower panels), suggesting that the difference in cellular activity between AR-dependent and AR-independent PCa cells could be, at least partially, due to MYC expression. Having shown that *MEN1*-KD increased the tumorigenic potential of AR-independent PCa cells and that MYC expression was maintained, we wondered whether factors known to regulate MYC expression could contribute to the effects triggered by *MEN1* silencing.

Reduced menin expression triggers the nuclear translocation of JunD in AR-independent PCa cells.

One factor in particular, the proto-oncogene JunD, was recently reported to promote the proliferation of PCa cells through MYC signaling [8]. To better understand this mechanism, we investigated the distinct effects of *MEN1*-KD in AR-dependent and -independent PCa cells using MI503 as a means of comparison, since MI503 is known to inhibit not only the interaction between menin and MLL, but also that of menin and JunD [40]. Indeed, we previously demonstrated that MI503 treatment inhibited the proliferation of both types of PCa cell lines, unlike siMEN1 treatment [24]. Hence, we initially performed foci formation assays to confirm a significant decrease in colony formation in both AR-dependent and AR-independent PCa cells upon MI503 treatment (Fig. 2a-b). To understand the molecular differences triggered by siMEN1 and MI503, we examined the expression of MYC and JunD. The expression of *MEN1*, *MYC* and *JunD* was lower in all MI503-treated PCa cells at the mRNA (Fig. 2c and Fig. S2a) and protein levels (Fig. 2d). However, following *MEN1*-KD, only AR-dependent (LNCaP and 22Rv1) cells displayed a decrease in mRNA (Fig. 2e and

S2b, upper panel) and protein levels (Fig. 2f and S2c, left panel). Indeed, AR-independent PCa cells remained unaffected by this treatment (Fig. 2e-f and S2b-c, right panel), indicating that MI503 suppresses cell growth by inhibiting menin and JunD expression in PCa cells. Consequently, we confirmed the positive role of JunD on cell proliferation in *JunD*-KD DU145 and PC3 cells (Fig. S2d). We further evaluated JunD protein expression in *MEN1*-KD DU145 and PC3 cells, and found that, although *MEN1* silencing did not affect the JunD expression at the transcriptional (Fig. 2e and S2b, lower panel) and total protein levels (Fig. 2f and S2c, right panel), it triggered the nuclear translocation of JunD in these AR-independent PCa cells (Fig. 2g-h and S2e), indicative of JunD activation [41].

***MEN1* knockdown enhances the binding of JunD to the *MYC* locus in AR-independent PCa cells.**

Wang *et al.* identified four potential AP-1 binding sites in a 5' *MYC* enhancer, situated 67kb upstream of the transcription start site (TSS) of *MYC*, and demonstrated the binding of JunD to the enhancer to regulate *MYC* transcription in breast cancer cells [42]. We wondered whether the activation of JunD may allow maintaining *MYC* transcription in *MEN1*-KD AR-independent PCa cells. Through ChIP-qPCR analyses, we observed that menin bound to the *MYC* promoter and to its 5' enhancer in DU145 and PC3 cells (Fig. 3a), while JunD bound only to the *MYC* 5' enhancer (Fig. 3b). We then hypothesized that the increased JunD nuclear translocation triggered by *MEN1* silencing may augment its binding to the 5' *MYC* enhancer, which we confirmed by ChIP analysis upon *MEN1*-KD in DU145 and PC3 cells (Fig. 3c).

Next, we performed 3C assays to determine whether reduced menin expression modulates the “loop” structure between the distal enhancer region and the proximal promoter region [42]. For the 3C-PCR reaction, we amplified a 330bp DNA fragment (“Detection fragment”) to assess intramolecular ligation of the looping between the enhancer and promoter of the *MYC* locus, using the forward primer (DP1) that anneals

upstream of the distal enhancer and the reverse primer (DP2) that anneals downstream from the *Fat 1* site in the *MYC* promoter (Fig. 3d). As shown in Fig.3e and Fig. S3a, the detection fragment increased in *MEN1*-KD cells under the ligation condition, compared to siCtrl cells. As expected, the detection fragment failed to yield products in 3C assays under the non-ligation condition. This analysis clearly demonstrates that reduced menin expression triggers increased loop formation between the *MYC* enhancer and proximal promoter in DU145 and PC3 cells.

We then performed ChIP-3C [43] to further determine how the loop formed between the *MYC* enhancer and proximal promoter [44] might change in *MEN1*-KD AR-independent PCa cells. In the non-ligation condition, menin bound to the enhancer and promoter of the *MYC* locus (Fig. 3f and S3b), and JunD bound only to the 5' *MYC* enhancer in DU145 and PC3 cells (Fig. 3g and S3c), while, in the ligation condition, both menin and JunD could be detected on the 5' *MYC* enhancer and proximal promoter, as well as on the intramolecular ligation region (P3-P6), suggesting that they bound together to the looping structure. Interestingly, JunD binding increased at the intramolecular ligation region (P3-P6) of the *MYC* locus in *MEN1*-KD DU145 and PC3 cells under the ligation condition (Fig. 3g and S3c). These results provide first evidence that menin is present within the corresponding chromatin loop structure between the *MYC* enhancer and promoter, and that, importantly, its inactivation leads to increased binding of JunD to the 5' *MYC* enhancer and the loop structure. Taken together, our analyses strongly suggest that JunD, through its nuclear translocation, replaces menin on the *MYC* locus to maintain *MYC* transcription in *MEN1*-KD AR-independent PCa cells.

***MEN1* silencing triggers the nuclear translocation of β -catenin in AR-independent PCa cells.**

Our previous works highlighted the activation of β -catenin, a menin-interacting protein, upon *Men1* disruption in mouse insulinoma [45, 46]. Moreover, β -catenin is a

well-known oncogene in prostate cancer cells [47]. We, therefore, also analyzed the expression of β -catenin in *MEN1*-KD PCa cells, and found that *MEN1* silencing resulted in the accumulation of β -catenin in the nucleus of DU145 and PC3 cells, with its membrane and cytoplasmic expression being markedly reduced (Fig. 4a-b and Fig. S4a). Conversely, *MEN1*-KD significantly downregulated β -catenin total protein and cytoplasmic fraction levels in LNCaP cells and 22Rv1 cells, whereas its expression in nuclear and membrane subcellular fractions remained unaltered (Fig. S4b-d). Furthermore, we confirmed the positive role of β -catenin in cell proliferation in DU145 and PC3 cells knocked-down for *CTNNB1* coding for β -catenin (Fig. S4e). Collectively, these results indicate that, in *MEN1*-KD AR-independent PCa cells, the nuclear translocation of β -catenin is drastically increased, a hallmark of the activation of the WNT signaling pathway [48].

***MEN1* inactivation increases β -catenin binding to the *MYC* promoter in AR-independent PCa cells.**

It has previously been reported that *MYC* and β -catenin have a strong cooperative action in different cancers [49-51]. We thus investigated the eventual interplay between menin and β -catenin in regulating *MYC* expression in AR-independent PCa cells. Our ChIP analyses showed that β -catenin bound to the *MYC* promoter and its 3' enhancer, formerly described in colon cancer cells [50] (Fig. 4c and S5a), while menin bound to the promoter, but not to the *MYC* 3' enhancer in DU145 and PC3 cells (Fig. 4d and S5b). Furthermore, ChIP-reChIP and/ ChIP analyses showed that *MEN1*-KD elevated the β -catenin binding to the *MYC* promoter (Fig. 4e and S5c), but not its binding to the *MYC* 3' enhancer (Fig S5d) in DU145 and PC3 cells, whereas *CCTNBI*-KD did not affect the binding of menin to the *MYC* promoter in DU145 and PC3 cells (Fig. 4f and S5e). All these analyses indicate that nuclear translocation of β -catenin allows maintaining *MYC* transcription in *MEN1*-KD AR-independent PCa cells.

JunD and β -catenin are critical for the tumorigenic potential of AR-independent PCa cells and the expression of EMT markers.

To further investigate the role of JunD and β -catenin in AR-independent PCa cells, we initially performed soft agar assays to assess the anchorage independence in *JunD*-KD or *CTNNB1*-KD PCa cells. Both *JunD*-KD (Fig. 5a) and *CTNNB1*-KD (Fig. 5b) resulted in a significant decrease in colony formation in DU145 and PC3 cells. Concomitantly, *JunD*-KD (Fig. 5c-d) and *CTNNB1*-KD (Fig. 5e-f) upregulated mRNA and protein levels of the epithelial marker E-cadherin in DU145 and PC3 cells. Consistently, a marked decreased in mRNA and protein expression of Twist 1, a repressor of E-cadherin gene transcription and a known regulator of EMT [52], was observed. Importantly, the expression of HIF-1 α , a key mediator in EMT, inflammation and tumorigenesis under hypoxic conditions [53-55], was also reduced in *JunD*-KD and *CTNNB1*-KD DU145 and PC3 cells. These findings indicate that JunD and β -catenin are critical for the tumorigenic potential and the expression of EMT markers in AR-independent PCa cells.

The activation of JunD and β -catenin is needed to reverse the oncosuppressive role of menin in AR-independent PCa cells.

We then proceeded to further determine the specific roles of JunD and β -catenin in *MEN1*-KD AR-independent PCa cells. To this end, we used the foci formation assay to evaluate cell growth upon transfection with siMEN1, siJunD, siCTNNB1, siMEN1+siJunD, siMEN1+siCTNNB1 or siMEN1+siJunD+siCTNNB1 in DU145 (Fig. 6a) and PC3 (Fig. S6a) cells. Knockdown of *JunD* or *CTNNB1* significantly abolished the effect of *MEN1*-KD, whereas reduced menin expression significantly reversed the effects of *JunD*-KD or *CTNNB1*-KD in DU145 and PC3 cells (Fig. 6a and S6a). We obtained similar results on cell proliferation by Incucyte ZOOM analysis (Fig. S6b).

Having shown that both JunD and β -catenin activation are crucial for cell growth in *MEN1*-KD DU145 and PC3 cells, we hypothesized that *MEN1* silencing also alters the expression of genes promoting EMT in AR-independent PCa cell lines. As expected, *MEN1*-KD in DU145 and PC3 cells led to reduced E-cadherin expression at the mRNA and total protein levels (Fig. 6c-d). Moreover, this silencing led to an increase in the expression of HIF-1 α , Vimentin and BMI1 at the transcript and total protein levels in these cells, as well as the nuclear accumulation of HIF-1 α , Twist 1 and BMI1 (Fig. 6c-e). These results suggest that menin could be a key factor inhibiting the molecular program favoring EMT in AR-independent PCa cells.

Importantly, we performed cell line-derived xenografts under mouse kidney capsule using PC3-GFP cells to validate our data *in vivo* (Fig. S7a). Following the transplantation of PC3-GFP cells, mice were treated with either MI503 (n = 11) or vehicle (n = 9) for 1 month. We observed that *MEN1* inhibition with MI503 gave rise to significantly accelerated tumor growth, compared to the control treatment (Fig. 7a-b). Morphologically, MI503-treated PC3-GFP cells appeared more variable in size and more invasive towards surrounding mouse tissues, with disorientated alignment (Fig. 7a, c). In agreement with our *in vitro* observation, qPCR and immunostaining analyses of PC3-GFP xenografts confirmed reduced expression of menin and JunD in xenografted cells, but revealed increased *CTNNB1* transcription and overt nuclear expression of β -catenin in MI503-treated-PC3-GFP cells (Fig. 7c-d, S7b-d), suggesting that inhibition of both menin and JunD could still lead to aggravated tumorigenic potential of xenografted PC3-GFP cells, due to activated β -catenin.

In parallel, a significant negative correlation between *MEN1* and *CTNNB1* mRNA expression was seen in two different datasets from TCGA database [37] and mCRPC database [38] (Fig. 7e). These data further support the activated β -catenin due to MI503 treatment observed in PC3-GFP xenografts.

Overall, our data suggest that the activation of both JunD and β -catenin, although they individually play critical roles, is required to produce the effects of menin inactivation in AR-independent PCa cells.

Discussion

In the present study, we uncovered a previously unknown role for menin in preventing AR-independent PCa cells from evolving towards aggravated tumorigenic potential, contrasting drastically with its oncogenic role in AR-dependent PCa cells [24]. More importantly, we demonstrated that *MEN1* silencing in AR-independent PCa cells led to nuclear translocation and, therefore, increased binding of JunD and β -catenin to the regulatory sequences of the *MYC* gene, leading to maintained MYC expression and prominent cellular and molecular alterations.

Although well-known tumorigenic cell lines, *MEN1*-KD in DU145 and PC3 cells resulted in an even greater increase in their tumorigenic potential, including more marked loss of contact-inhibition, augmented cell anchorage independence and cell migration. Importantly, *in vivo* xenograft tests in mice further demonstrated that PC3-GFP cells treated with MI503 gave rise to significantly increased tumor growth. In parallel, we noticed that the changes in cellular behavior were accompanied by remarkable alterations in the expression of EMT makers, including BMI1, Twist1, HIN1a, E-Cadherin and Vimentin. Our data thus suggest that *MEN1* silencing in AR-independent PCa cells affects cell proliferation, cell differentiation and cell migration, resulting in altered cell plasticity and increased tumorigenicity. Both the changes in cell plasticity and abnormal expression of EMT markers are largely documented in PCa, in particular in clinical mCRPC samples [8]. However, we highlighted, for the first time, the involvement of menin in these procedures specifically in AR-independent PCa cells.

JunD was the first menin-interacting partner identified after the identification of the *MEN1* gene, with its possible oncogenic role upon menin inactivation being proposed from the beginning [56]. The hypothesis was further strengthened by menin 3D structure analysis, showing that JunD binds to the same menin protein pocket as KMT2A/B [16]. However, the role of JunD in MEN1 tumors has never been clarified. Interestingly, Wasylishen *et al.* recently reported that menin plays a tumor suppressive role in mouse Ras-related pancreatic cancer, likely through the activated JunD [57]. It is worth mentioning that our finding is in total agreement with the recent work reporting the role played by JunD in PCa through MYC regulation [8]. Importantly, our data provide new mechanisms showing that the prominent nuclear translocation of JunD triggered by *MEN1*-KD resulted not only in its increased binding to the regulatory sequence of the *MYC* locus, thus maintain MYC expression, but also in the altered expression of EMT markers, contributing to the aggravated tumorigenic potential seen in these *MEN1*-KD AR-independent PCa cells.

In PCa, the activation of β -catenin and the WNT signaling pathway is considered to be among the most commonly occurring molecular alterations involved in its development and progression [58, 59]. It has also been suggested that β -catenin could play a critical role in AR-independent CRPC [60]. We and our collaborators have previously demonstrated that menin physically interacts with β -catenin, and that *Men1* deficiency leads to nuclear translocation and activation of the latter in mouse *Men1* insulinoma [61, 62]. Interestingly, we uncovered in the current study that, in AR-independent PCa cells, the similar molecular switch can also happen, accompanied by decreased E-Cadherin and increased Vimentin expression, reminiscent of increased EMT. It is worth mentioning that similar changes were observed in mouse *Men1* insulinomas and mouse *Men1* mammary lesions [63, 64]. Furthermore, our ChIP analyses demonstrated an increase in the binding of β -catenin to the *MYC* promoter, contributing to maintaining MYC expression in these cells. Importantly, the fact that the nuclear expression of β -catenin increased in MI503-treated xenografted PC3-GFP cells further demonstrates that β -catenin

activation is critical for aggravated tumorigenicity triggered by menin inactivation observed in the current work.

Notably, we have depicted the increased nuclear expression of several factors related to cell dedifferentiation, including BMI1, Twist1 and HIF1 α . These factors are known to interact with both the JunD and β -catenin pathways and to play relevant roles in cancer progression [65-67]. Based on the published data, we could hypothesize that the activation of JunD and β -catenin may lead to, firstly, the activation of HIF1 α . Then, the latter could trigger the activation of other factors as previously described [68-71]. The interplay between menin inactivation and the altered expression of EMT makers revealed in the current study may explain the altered cellular activities observed, and it would be relevant to study whether similar situations could occur during PCa progression, especially in the DNPC subtype of mCRPC.

Our study may also provide insight into novel strategies for AR-independent PCa treatment, if these molecular perturbations could be further confirmed in clinical studies. Considering the data obtained from the current work, especially the above mentioned *in vivo* test, caution should be taken when using inhibitors of menin/MLL interaction, like MI503, in PCa therapeutic assays, especially in mCRPC cases.

Conclusion

The present work unveiled the activation of the JunD and β -catenin pathways upon menin inactivation specifically in AR-independent cells. Of note, the oncosuppressive role of menin in AR-independent PCa cells is closely associated with maintained MYC expression and altered expression of EMT markers (Fig 7f), which may pave the way for new strategies for PCa treatment.

Declarations

Ethics approval and consent to participate

All animal experiments were performed in accordance with the animal care guidelines of the European Union and were validated by the local Animal Ethics Evaluation Committee in agreement with the French Ministry of High School and Research. All experiments were approved by Auvergne Ethics Committee (CEMEAA) and registered according the approval number 17296-2018102216428025 v3.

Consent for publication

All of the authors approved the contents and the data in this manuscript and agreed for its submission for publication.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Competing of Interests

The authors declare that they have no competing interest.

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Authors' contributions

Y.L. conducted the experiments, analyzed and interpreted the data, prepared figures and manuscript; V.V. provided technical and material support and participated in the study design, data interpretation and manuscript preparation; S.B. and SD conducted mouse xenograft model tests, participated in analyses of xenografts and data

interpretation; M.L.R and C.X.Z conceived and supervised the study and manuscript preparation, and obtained funding.

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Figure legends:

Fig. 1 MEN1 silencing promotes anchorage independence and cell migration in AR-independent PCa cells with maintained MYC expression. **a Upper panel:** Representative images of foci formation assay with DU145 and PC3 cells treated with siMEN1(1)+(3) or siCtrl. **Lower panel:** Quantification of foci formation assay. **b** Representative images of soft agar colony formation assay in *MEN1*-KD DU145 or PC3 cells. Scale bar = 50 μm . **c** Bar charts showing colony formation 28 days post-transfection with siMEN1 or siCtrl, extrapolated from images using Image J software. **d** Representative images of scratch wound healing assays using siMEN1 or siCtrl-transfected DU145 and PC3 cells. Scale bar = 200 μm . **e** Graphs indicating cell migration displayed in terms of the % wound closure 6 h and 12 h post-wounding ($t = 0$, as control). **f and g** Quantitative RT-PCR (qRT-PCR) analysis of *MYC* transcripts (**f**) and Western blot analysis of MYC protein levels (**g**) in PCa cells treated with siCtrl or siMEN1(1)+(3) for 72 h.

Fig. 2 MEN1 silencing triggers the nuclear translocation of JunD in AR-independent PCa cells. Representative images of foci formation assays (**a**) **and** their quantification (**b**) using LNCaP, 22Rv1, DU145 and PC3 cells treated or not with MI503 (2.5 μM). qRT-PCR analysis of *JunD* and *MYC* mRNA expression (**c**) and Western blot analysis showing JunD and MYC expression (**d**) in 22Rv1 and DU145 cells treated or not with MI503 (2.5 μM). qRT-PCR analysis of *JunD* mRNA expression (**e**) and Western blot analysis of JunD protein expression (**f**) in *MEN1*-KD 22Rv1 and DU145 cells. **g** IF staining showing menin and JunD in DU145 cells treated with siCtrl or siMEN1(1)+(3). Scale bar = 25 μm . **h** Western blot analysis of JunD expression in different subcellular fractions in DU145 and PC3 cells treated with siCtrl or siMEN1(1)+(3).

Fig. 3 MEN1 knockdown leads to increased JunD binding and loop structure formation in the MYC locus in AR-independent PCa cells. **a** ChIP-qPCR analysis using anti-menin to evaluate the binding of menin to the *MYC* 5' enhancer (-67kb) and promoter in DU145 and PC3 cells treated with siCtrl or siMEN1(1)+(3). *Chr1* served as a negative control. **b** ChIP-qPCR analysis using anti-JunD to detect the binding of JunD to the *MYC* 5' enhancer and promoter in DU145 and PC3 cells treated with siCtrl or siJunD. **c** ChIP-qPCR analysis to assess the effect of *MEN1*-KD on the level of JunD recruitment to the *MYC* 5' enhancer in DU145 and PC3 cells. **d** Primer locations for qPCR of 3C and ChIP-3C analyses. **e** 3C detection results (DP1-DP2 fragment) and control (CP1-CP2) by PCR in siCtrl or siMEN1(1)+(3)-transfected DU145 cells upon ligation or non-ligation. ChIP-3C qPCR analysis detecting menin ChIP-qPCR analysis showing menin binding (**f**) and JunD binding (**g**) to the *MYC* 5' enhancer, *MYC* promoter and the looping fragment (F3-F6) in DU145 cells transfected with siCtrl or siMEN1(1)+(3) under ligation (right panel) or non-ligation (left panel) conditions, P1-P2 and P7-P8 were used as negative controls.

Fig. 4 MEN1 silencing results in nuclear translocation of β -catenin and an increase in its binding to the MYC promoter in AR-independent PCa cells. **a** Western blot analysis of β -catenin expression in different subcellular fractions in siCtrl or siMEN1(1)+(3)-treated DU145 and PC3 cells as indicated. **b** Double IF staining showing menin and β -catenin in siCtrl or siMEN1(1)+(3)-treated DU145 cells. Scale bar = 25 μ m. ChIP-qPCR analysis assessing β -catenin (**c**) or menin (**d**) binding to the *MYC* promoter and the *MYC* 3'enhancer in siCtrl or siMEN1(1)+(3)-treated DU145 cells. reChIP analysis evaluating the effect of *MEN1*-KD (**e**) and *CTNNB1*-KD (**f**) on menin (**left panel**) and β -catenin (**right panel**) co-occupancy on the *MYC* promoter in DU145 cells.

Fig. 5 JunD and β -catenin are involved in the regulation of EMT marker expression in AR-independent PCa cells. Representative images of soft agar colony formation assay

in *JunD*-KD (**a, left panel**) and *CTNNB1*-KD (**b, left panel**) DU145 or PC3 cells. Scale bar = 50 μ m. Graphs showing quantitative analysis of colony formation at 21 days post-transfection with siCtrl or siJunD(1)+(2) using Image J software (**right panel**). qRT-PCR (**c**) and Western blot (**d**) analyses of mRNA and protein expression of *HIF1A*, *E-cadherin* and *Twist 1* in *JunD*-KD DU145 and PC3 cells. qRT-PCR (**e**) and Western blot (**f**) analyses of mRNA and protein expression of *HIF1A*, *E-cadherin* and *Twist 1* in *CTNNB1*-KD DU145 and PC3 cells DU145 and PC3 cells.

Fig. 6 The expression of both JunD and β -catenin is critical for the tumorigenic potential of AR-independent PCa cells. a Representative images of foci formation of DU145 cells upon transfection with siMEN1, siJunD, siCTNNB1, siMEN1+siJunD, siMEN1+ siCTNNB1 or siMEN1+siJunD+siCTNNB1. The quantification of the data are shown in the right and lower panel. **b** qRT-PCR analysis showing mRNA expression of *HIF1A*, *E-cadherin*, *Vimentin*, *Twist 1* and *BMI1* in DU145 and PC3 cells transfected with siCtrl or siMEN1(1)+(3). **c** Western blot analysis showing total protein expression of menin, HIF-1 α , E-cadherin, Vimentin, Twist 1 and BMI1 in DU145 and PC3 cells transfected with siCtrl or siMEN1(1)+(3). **d** Western blot analysis showing menin, HIF-1 α , Vimentin, Twist 1 and BMI1 expression in nuclear and cytoplasmic subcellular fractions in siCtrl or siMEN1(1)+(3)-treated DU145 and PC3 cells.

Fig. 7 MI503-treated PC3-GFP xenografts display increased tumor growth with nuclear overexpression of β -catenin. a Representative images of HE stained xenografts treated with DMSO (upper) and MI503 (lower). Scale bar = 200 μ m. **b** qRT-PCR analysis evaluating the number of PC3-GFP cells transplanted in mouse kidney treated (n = 11) or not (n = 9) with MI503. Representative images of IHC staining (**c**, Scale bar = 100 μ m) for menin, JunD and β -catenin or IF staining (**d**, Scale bar = 50 μ m) for JunD and β -catenin in xenografts in the Ctrl group (DMSO

treatment, upper panels) or MI503 treatment group (lower panels) as indicated. **e** Data mining analyses investigating the clinical correlation between *MEN1* and *CTNNB1* mRNA expression in primary prostate cancer (left panel) and mCRPC (right panel) using existing prostate cancer datasets. **f** Schematic summary of oncosuppressive functions of menin in AR-independent PCa cells.

Fig. 1

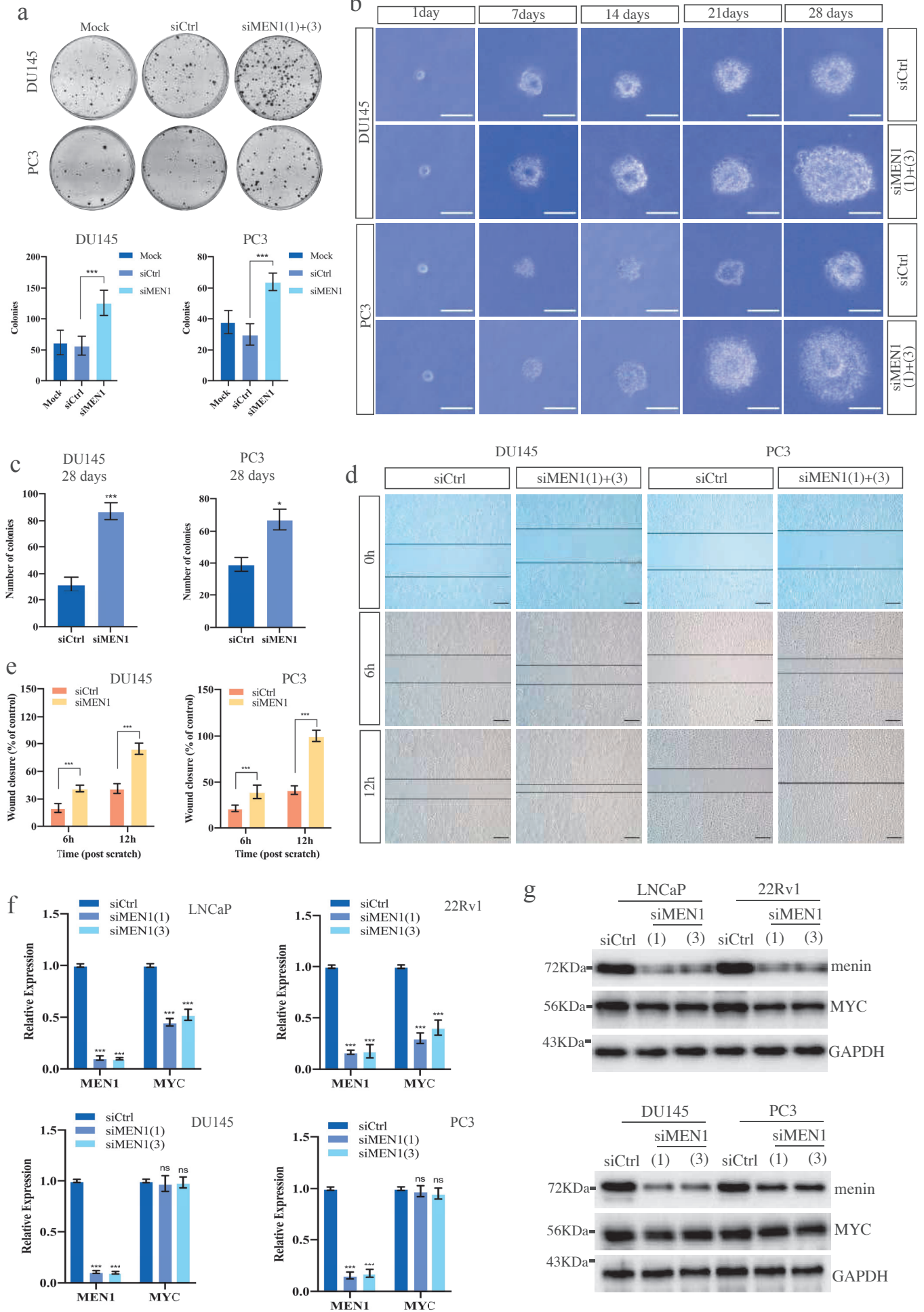


Fig. 2

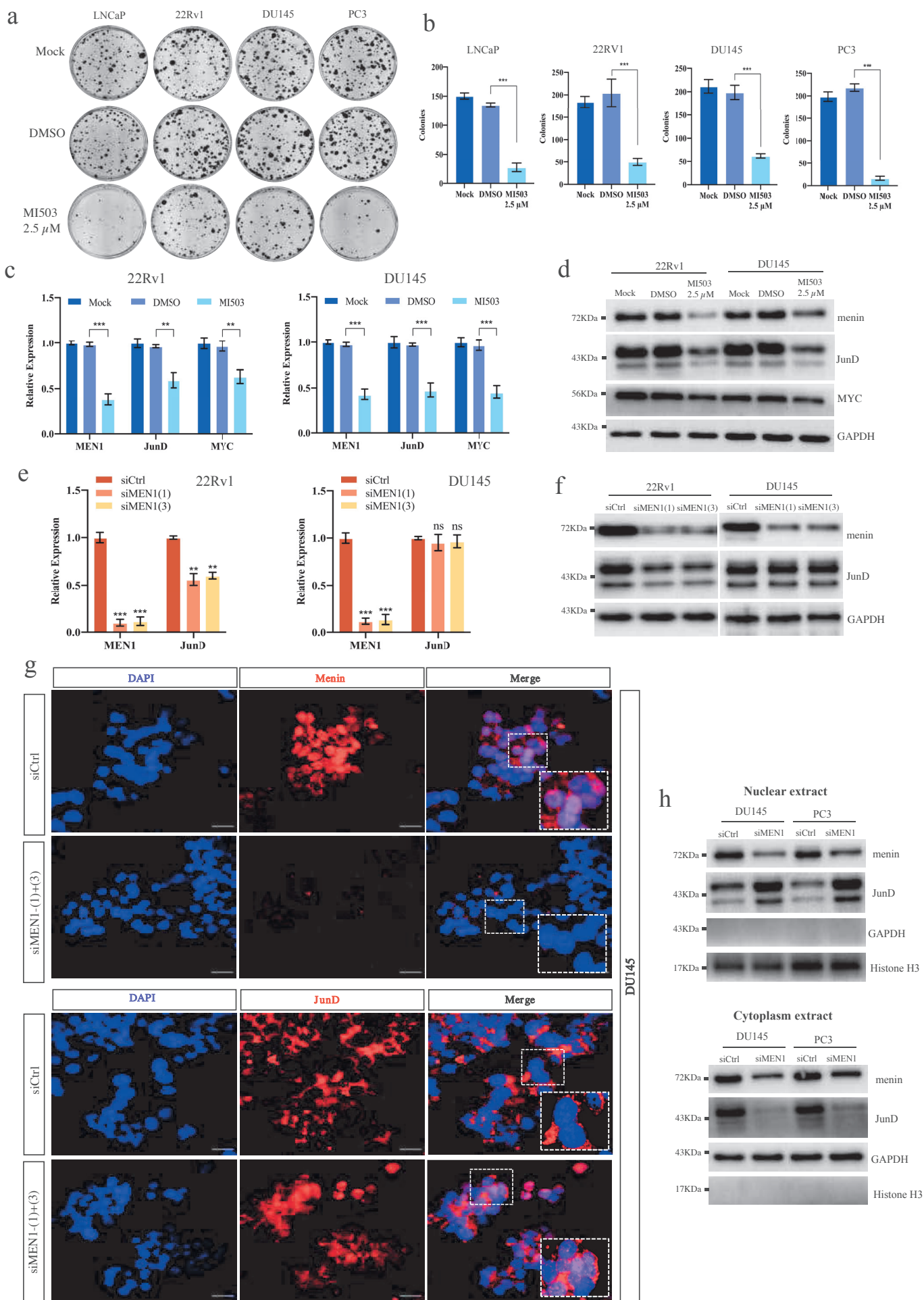


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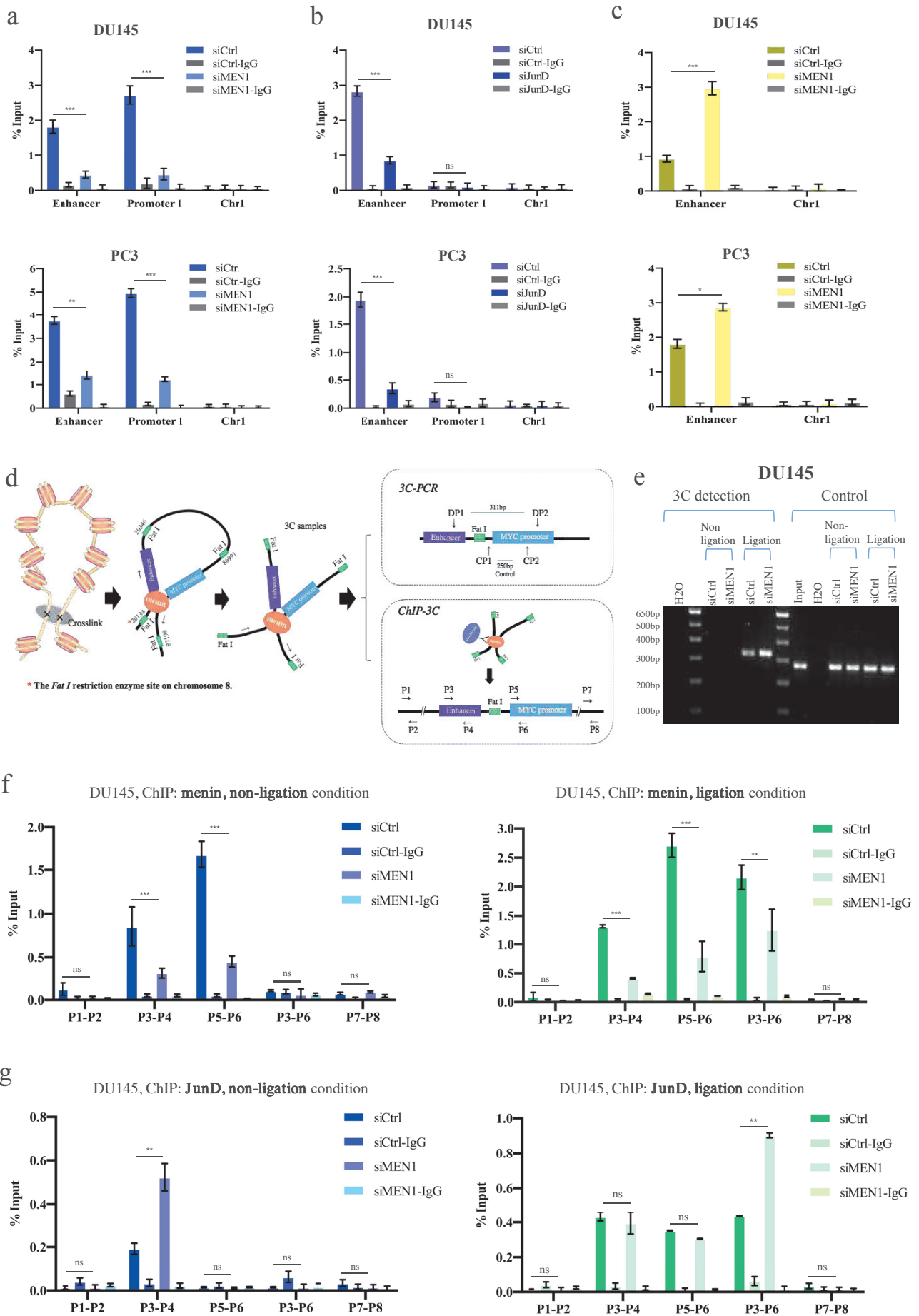


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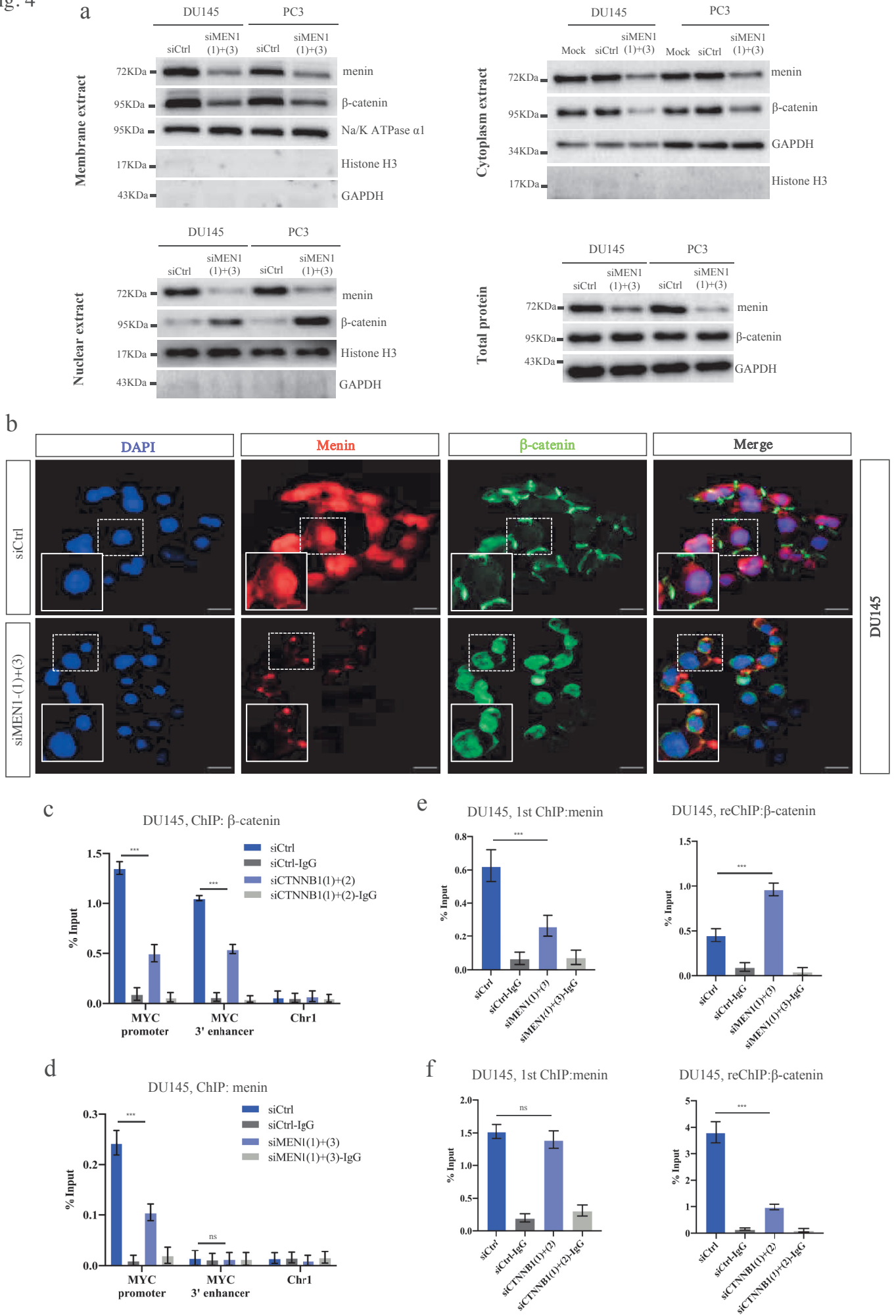


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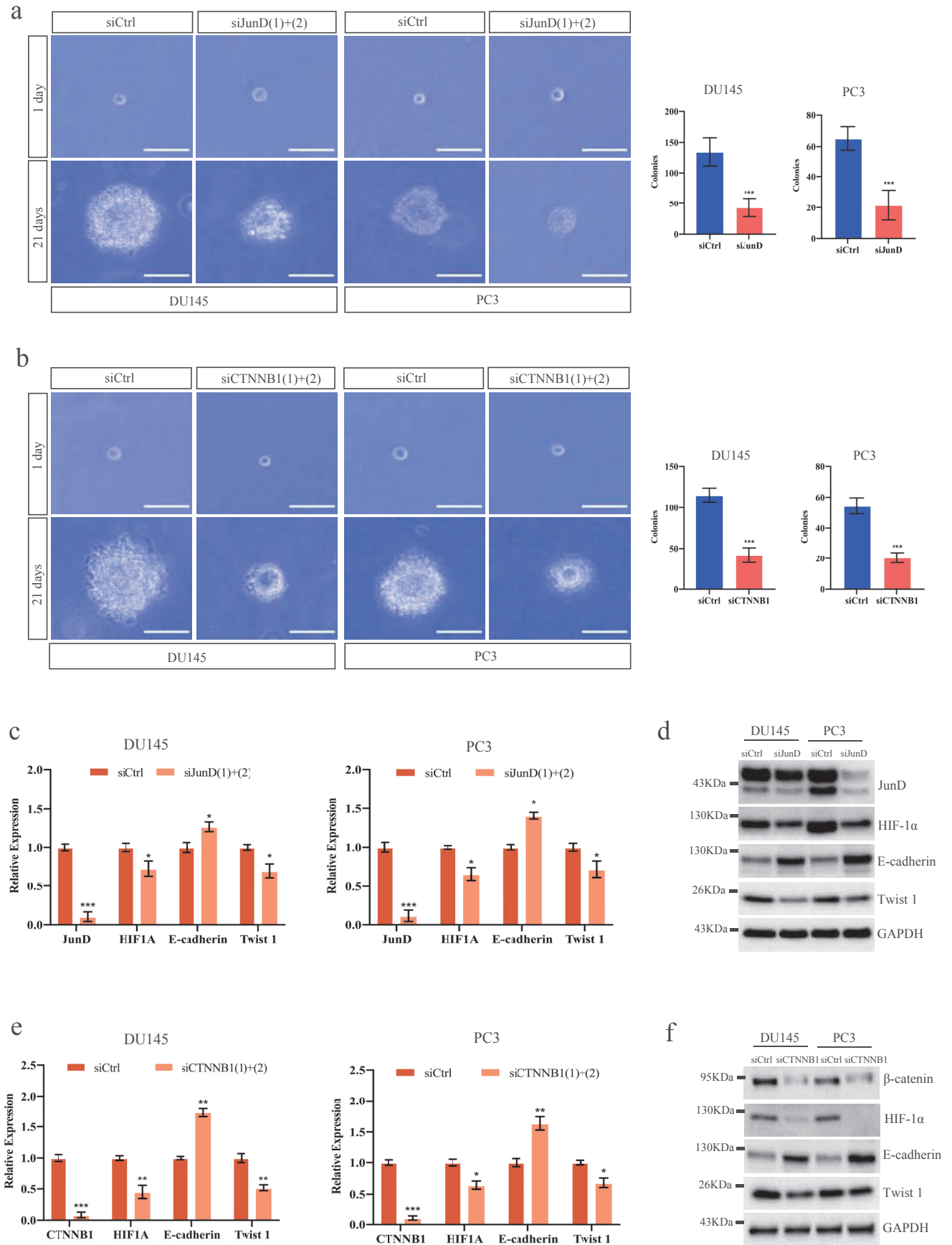


Fig. 6

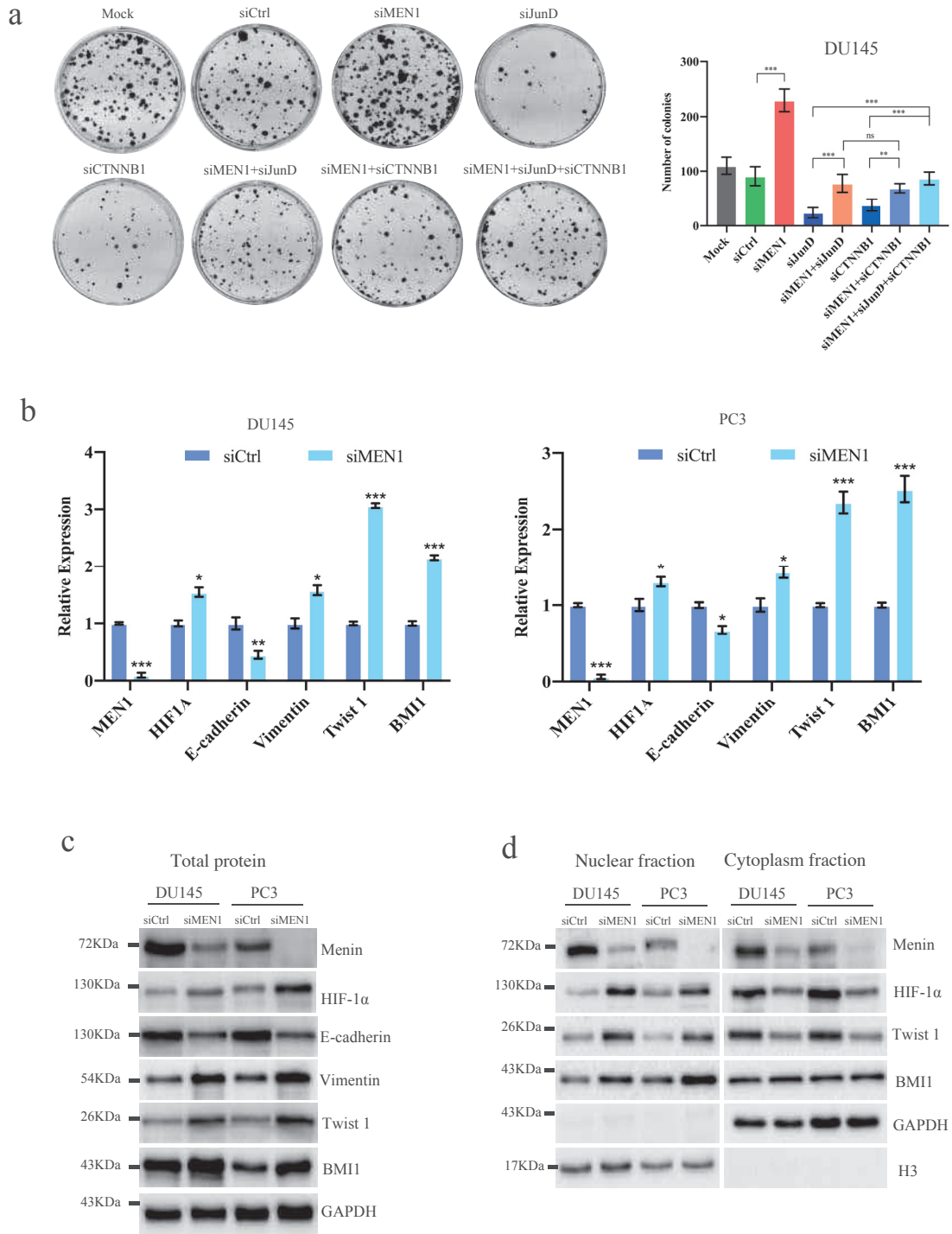
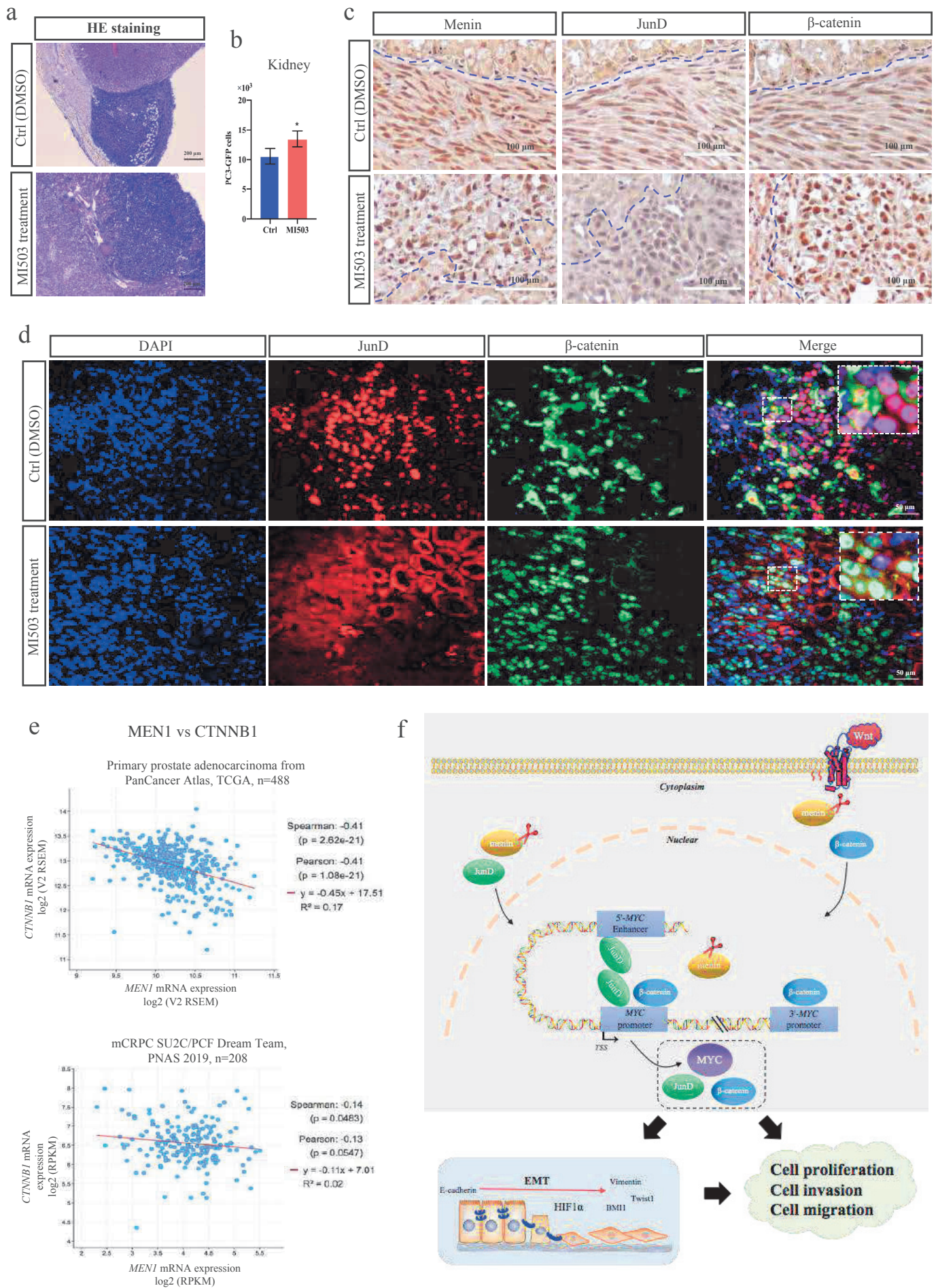


Fig. 7



Legends for supplementary figures

Fig. S1 *MEN1* silencing reduces cell growth and cell migration of AR-dependent PCa cells.

a Western blot analysis showing the efficacy of siMEN1(1)+(3) in DU145 and PC3 cells. **b** Representative images of foci formation assays and their quantification in *MEN1*-KD LNCaP and 22Rv1 cells. **c** Western blot analysis showing the efficacy of siMEN1(1)+(3) in LNCaP and 22Rv1 cells. **d** Representative images of wound healing assays and their quantification using siMEN1- or siCtrl-transfected 22Rv1 cells. Graphs showing cell migration displayed in terms of the % wound closure 6 h and 12 h post-wounding (t = 0, as control). Scale bar = 200 μ m.

Fig. S2 *MEN1* silencing triggers the nuclear translocation of JunD in AR-independent PCa cells, but not in AR-dependent cells.

a qRT-PCR analysis of *JunD* and *MYC* mRNA expression in LNCaP and PC3 cells treated with MI503 (2.5 μ M). **b** qRT-PCR analysis of *JunD* mRNA expression in *MEN1*-KD LNCaP and PC3 cells. **c** Western blot analysis of JunD in *MEN1*-KD LNCaP and PC3 cells. **d** Incucyte ZOOM analysis of DU145 and PC3 cell proliferation upon transfection with siCtrl, siJunD(1), siJunD(2) or siJunD(1)+(2). **e** Double IF staining for menin and JunD expression in siCtrl- or siMEN1(1)+(3)-transfected PC3 cells. Scale bar = 25 μ m.

Fig. S3 *MEN1* silencing elevates JunD binding to the MYC locus in AR-independent PCa cells.

a 3C detection results (DP1-DP2 fragment, right panel) and control (CP1-CP2, left panel) by PCR in siCtrl or siMEN1(1)+(3)-transfected PC3 cells upon ligation or non-ligation. ChIP-3C qPCR analysis detecting menin (**b**) and JunD (**c**) binding to the

MYC 5' enhancer (P3-P4), promoter (P5-P6) and looping fragment (F3-F6), in PC3 cells transfected with siCtrl or siMEN1(1)+(3) under ligation or non-ligation. P1-P2 and P7-P8 served as negative controls.

Fig. S4 *MEN1-KD induces the nuclear translocation of β -catenin in AR-independent, not AR-dependent, PCa cells.*

a Double IF staining showing menin and β -catenin in siCtrl- or siMEN1(1)+(3)-transfected PC3 cells. Scale bar = 25 μ m. **b** qRT-PCR analysis for mRNA expression of *CTNNB1* in *MEN1*-KD LNCaP and 22Rv1 cells. **c** Western blot detecting β -catenin protein levels in different subcellular fractions in siCtrl- or siMEN1(1)+(3)-transfected LNCaP cells as indicated. **d** Double IF staining for menin and β -catenin expression in siCtrl- or siMEN1(1)+(3)-transfected LNCaP cells. Scale bar = 25 μ m. **e** Incucyte ZOOM analysis showing proliferation of DU145 and PC3 cells upon transfection with siCtrl, siCTNNB1(1), siCTNNB1(2) or siCTNNB1(1)+(2).

Fig. S5 *β -catenin replace menin to bind to the MYC promoter in MEN1-KD AR-independent PCa cells.*

ChIP-qPCR analysis to assess β -catenin (**a**) and menin (**b**) binding to the *MYC* promoter and the *MYC* 3'enhancer in *CTNNB1*-KD PC3 cells. **c** ChIP-reChIP analysis evaluating the effect of *MEN1*-KD on menin (**left panel**) and β -catenin (**right panel**) co-occupancy on the *MYC* promoter in PC3 cells. **d** ChIP-qPCR analysis evaluating the effect of *MEN1*-KD on the binding of β -catenin to the *MYC* 3'enhancer in DU145 and PC3 cells. **e** ChIP-reChIP analysis assessing the effects of *CTNNB1*-KD on menin (**left panel**) and β -catenin (**right panel**) co-occupancy on the *MYC* promoter in PC3 cells as indicated.

Fig. S6 Both JunD and β -catenin are needed for increased tumorigenic potential triggered by menin inactivation in AR-independent PCa cells.

Foci formation (a) or Incucyte ZOOM (b) analysis of PC3 cell growth upon transfection with siMEN1, siJunD, siCTNNB1, siMEN1+siJunD, siMEN1+siCTNNB1 or siMEN1+siJunD+siCTNNB1.

Fig. S7 MI503-treated Xenografted PC3-GFP cells displayed accelerated tumor growth with increased nuclear β -catenin expression.

a Schematic diagram of the strategy used for evaluating the effect of MI503 on PC3-GFP cell growth by xenografts in mice. b qRT-PCR analysis detecting *MEN1*, *JunD* and *CTNNB1* mRNA expression in DMSO- or MI503-treated xenografts. Representative images of IHC staining for GFP, menin, JunD and β -catenin (c) and of IF staining for menin and GFP (d) in xenografts in the Ctrl group (DMSO treatment, upper panels) or MI503 treatment group (lower panels) as indicated.

Fig. S1

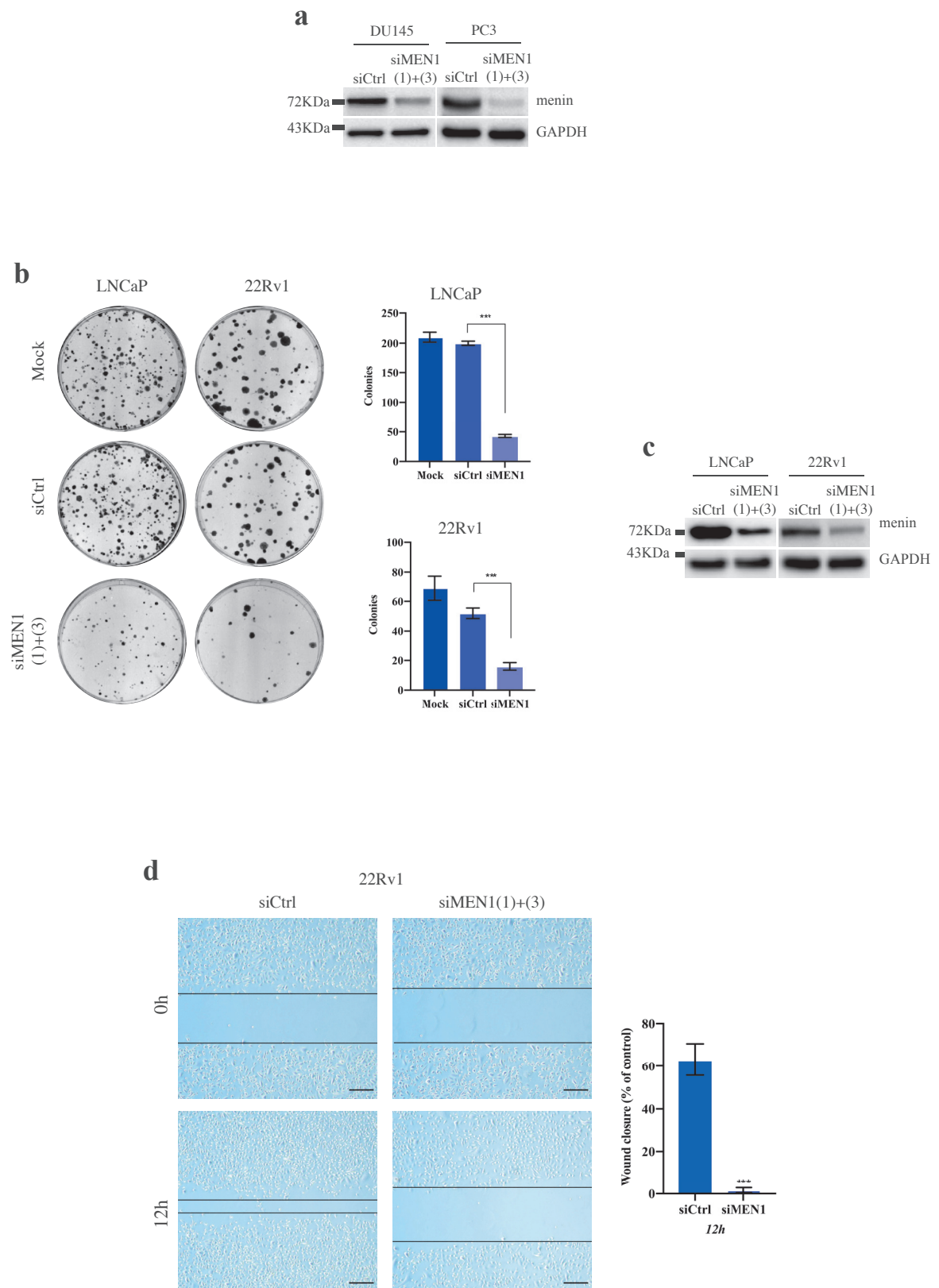


Fig. S2

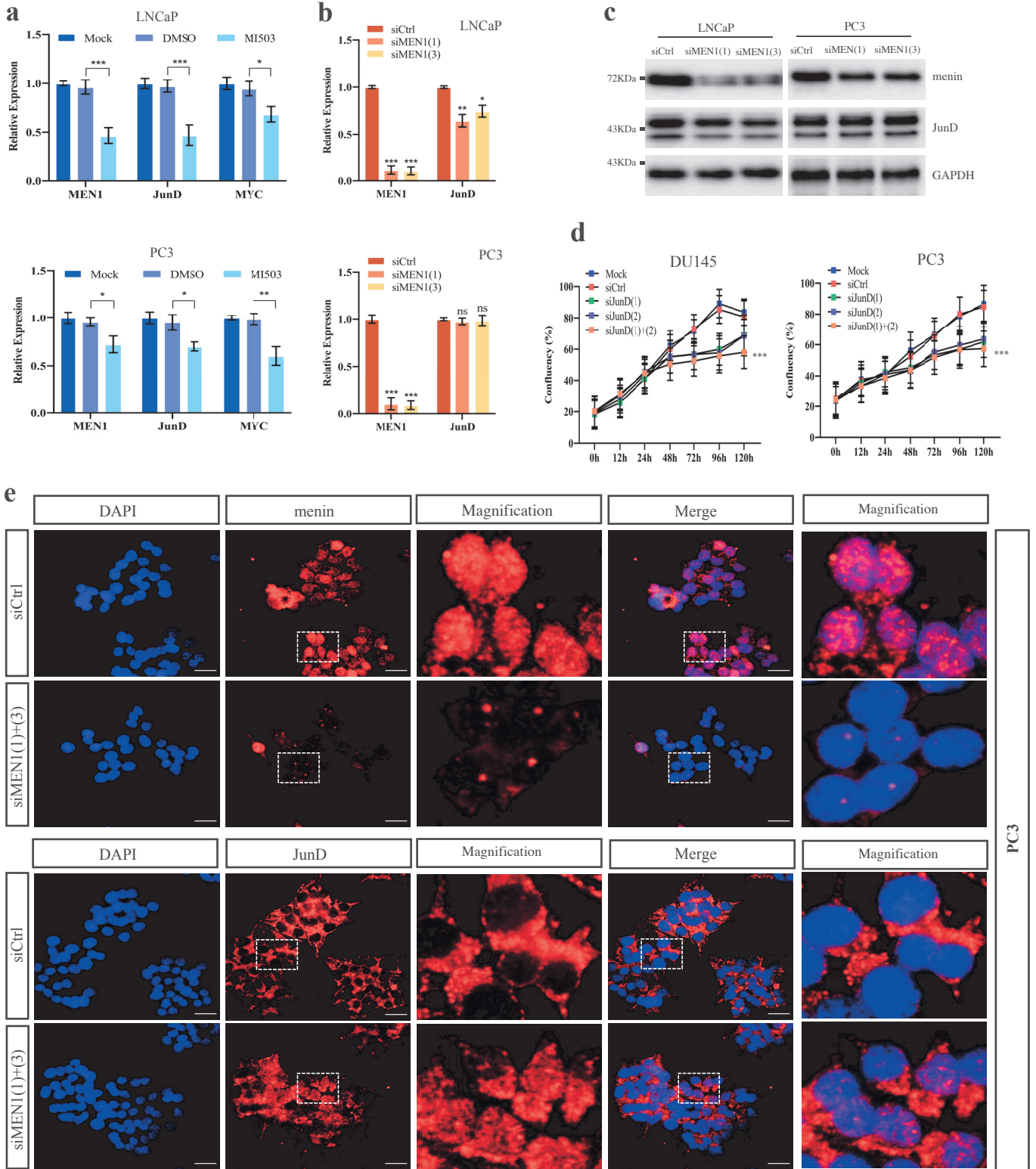


Fig. S3

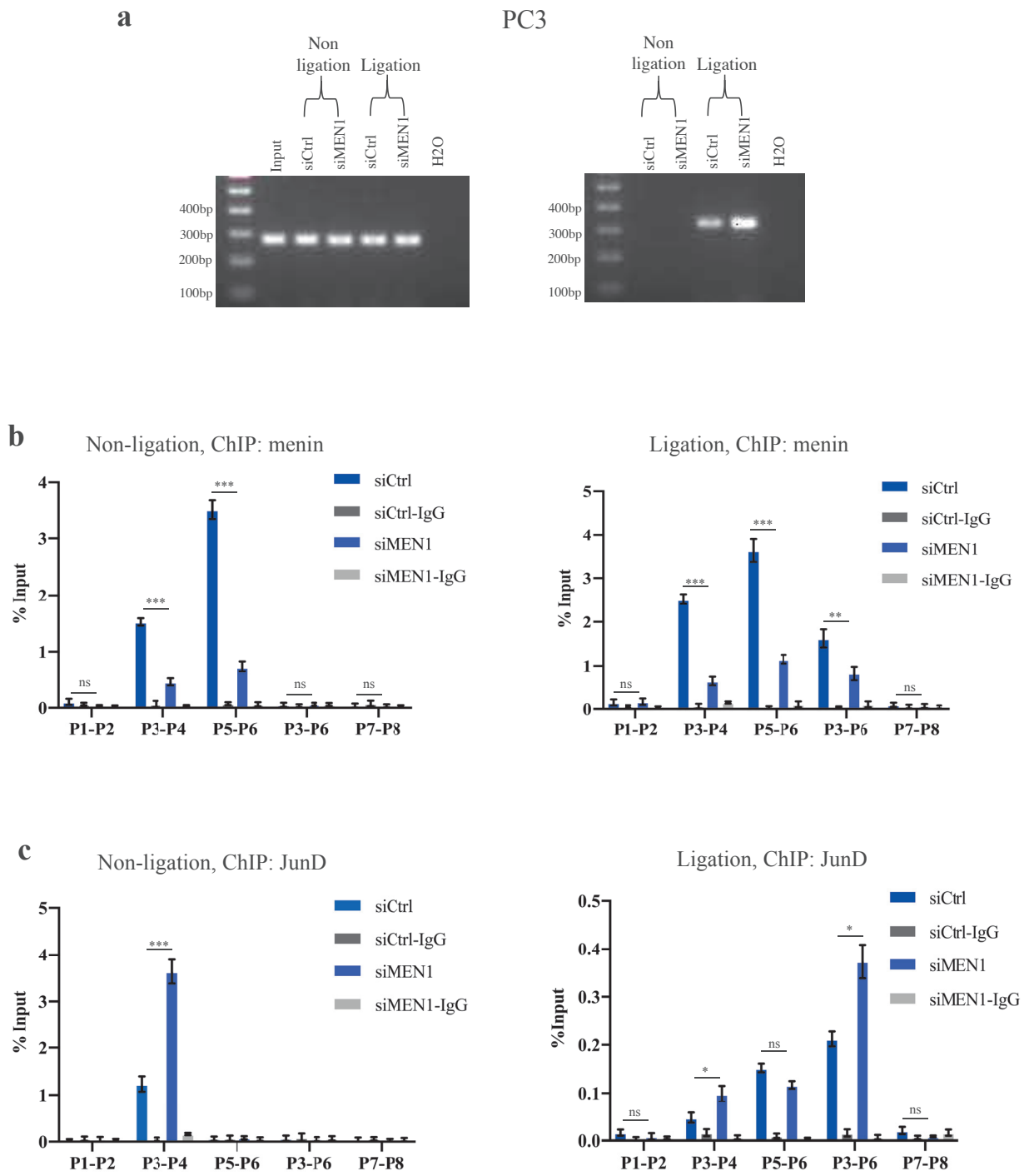


Fig. S4

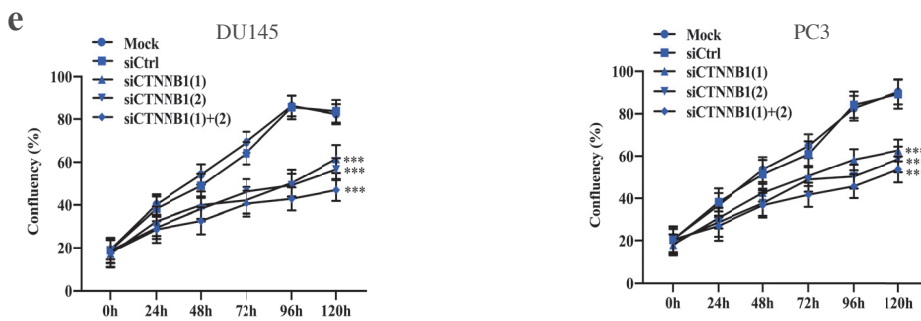
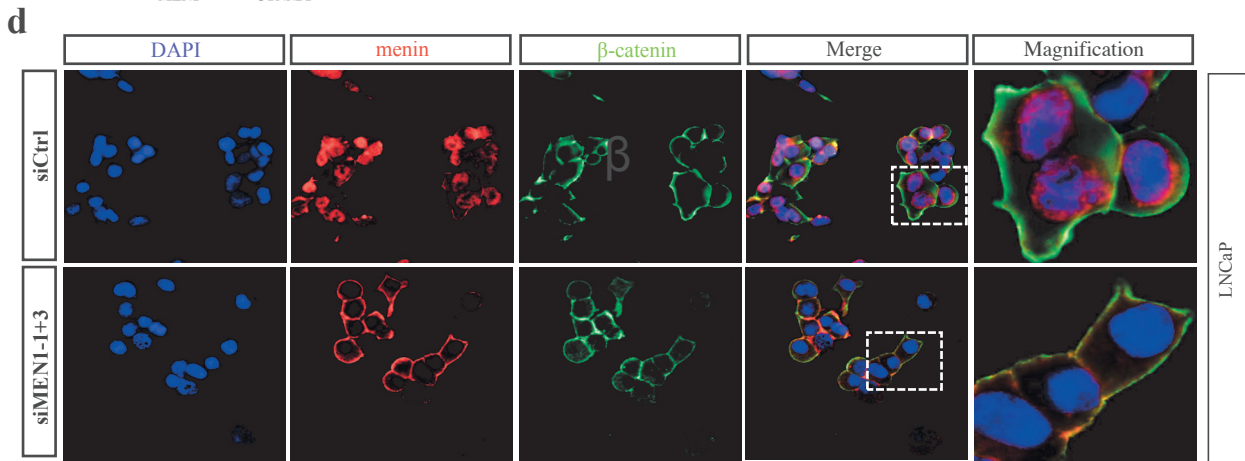
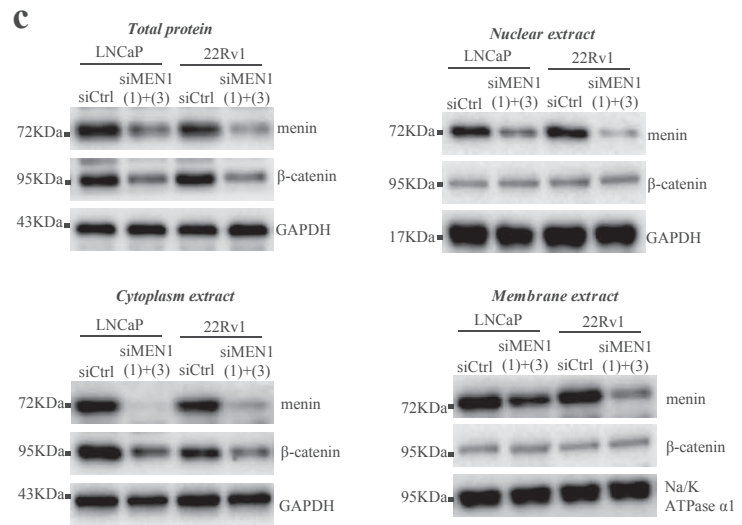
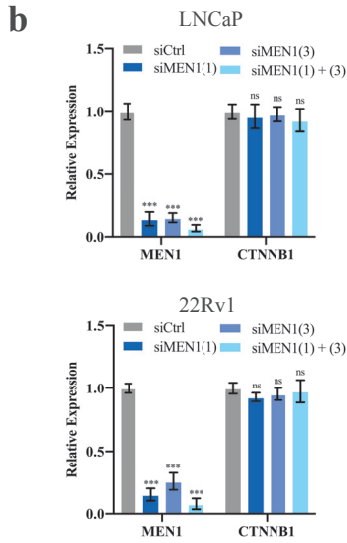
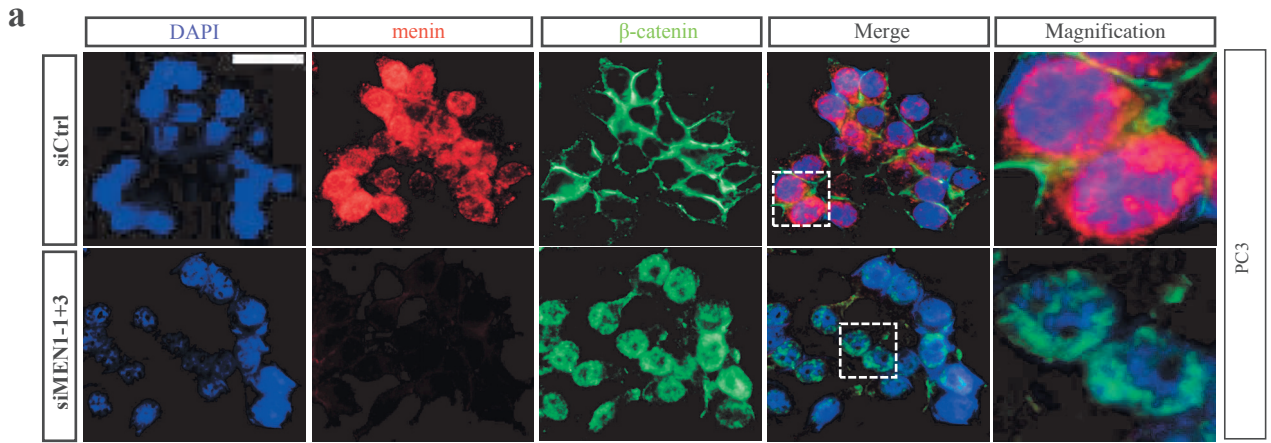


Fig. S5

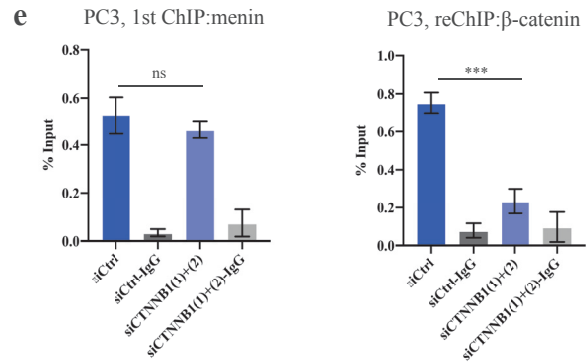
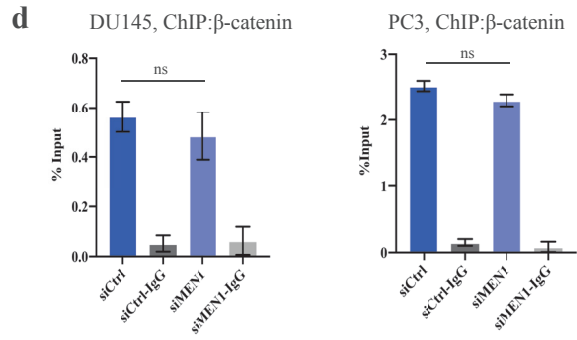
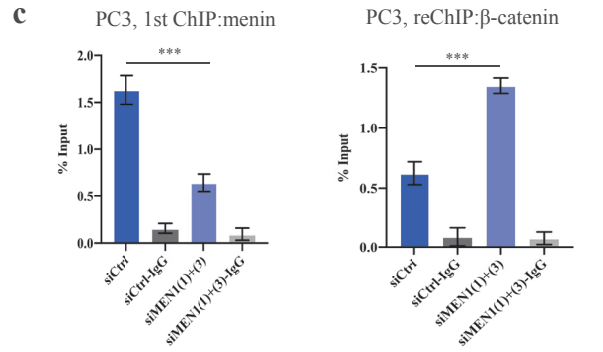
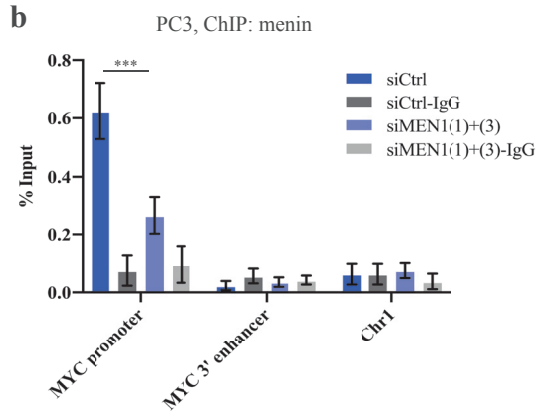
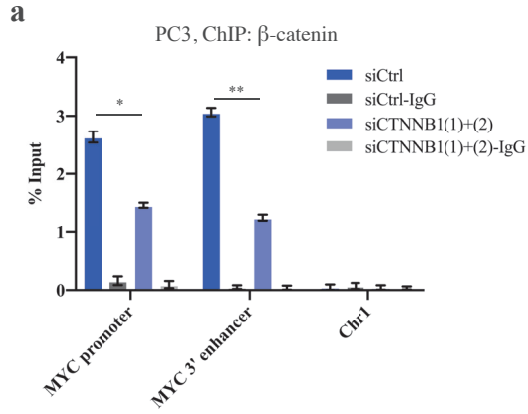
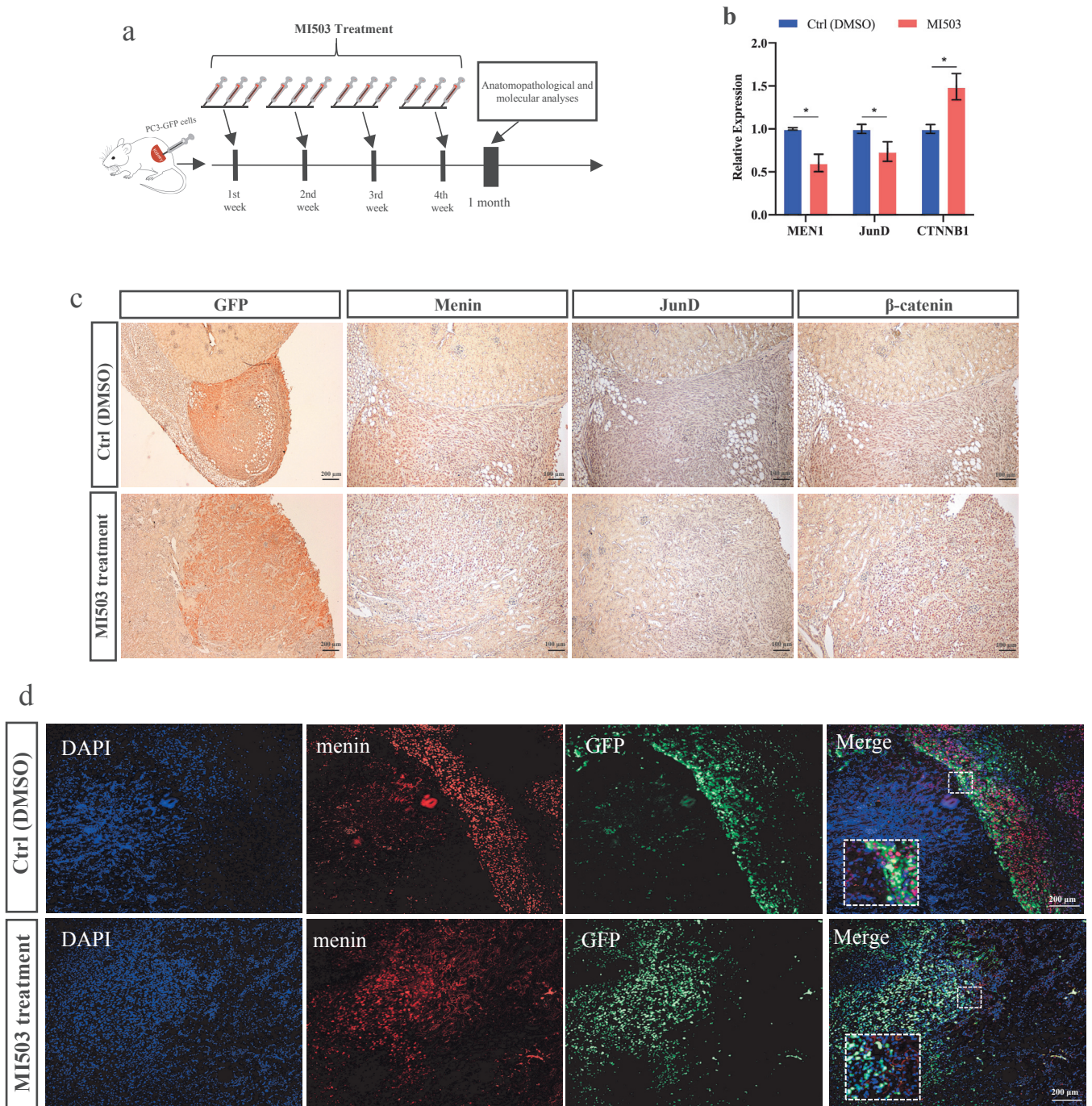


Fig. S7



SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Cell culture and treatment

LNCaP, 22RV1 and DU145 were cultured in RPMI medium, and PC3 cells in F-12 medium (Gibco Invitrogen) containing 25 mM glucose and supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-Glutamine, 100 unit/mL Penicillin, 100 µg/mL streptomycin, HEPES 10mM, Sodium Pyruvate 1 mM, at 37°C with 5% CO₂. Inhibition of the menin-MLL interaction was achieved through the use of MI-503 (Active Biochem), at 1 µM, 2.5 µM, and 5 µM.

RNA interference and transfection

MEN1 siRNA sequences, that are siMEN1(1) (MEN1HSS106462) and siMEN1(3) (MEN1HSS181079), were designed by Invitrogen. *JunD* siRNA (siJunD(1), s7664; siJunD(2), s7665) and *CTNNB1* siRNA (siCTNNB1(1), #146154; siCTNNB1(2), #146154) were purchased from ThermoFisher Scientific. A non-targeting control pool (siCtrl) from Sigma-MERCK was used as negative control for each RNA interference experiment. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and incubated for 72 h.

Cell proliferation assay

Cell proliferation assays were performed as described previously [1]. Briefly, cells were seeded onto 96-well culture plates at 5×10^3 cells for 22Rv1, DU145 or PC3, and at 1×10^4 cells for LNCaP. 24 h later, cells were treated with 20 nM of siJunD-1,

siJunD-2 or siJunD-1+2, or 20 nM of siCTNNB1-1, siCTNNB1-2 or siCTNNB1-1+2. All experiments were performed in technical and biological triplicates.

Immunofluorescence (IF) staining

For IF staining, cells were grown on glass coverslips, then fixed with methanol for 5 min at room temperature. Following fixation, cells were blocked with Dako buffer (S0809, Agilent) for 1 h, and incubated with primary antibodies overnight at 4°C, then with appropriate secondary antibodies conjugated with Alexa 555 (red) or Alexa 488 (green) (Cell Signaling Technology). Cells were counterstained with DAPI (DUO82040, Sigma-Aldrich) for 10 min and visualized by fluorescence microscopy (Eclipse-NiE NIKON microscope).

Chromosome Conformation Capture (3C) assay

3C assay potentially allows identification of physical interactions between any chromatin segments and is particularly suited to identify chromatin loops formed in genomic regions of up to several hundreds of kilobases in size. 3C was performed as described previously [2, 3] with minor modifications described here. Briefly, 2×10^7 cells (DU145 or PC3) were fixed with 1% formaldehyde for 10 min at 37°C. Cells were lysed in 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.2% NP-40 and protease inhibitor cocktail. Nuclei were pelleted and chromatin was digested overnight with 2000 U *Fat I* (NEB, Category Number R0650) and ligated with 4000 U T4 DNA Ligase (NEB) for 4 h at 16°C followed by 30 min at room temperature. 3C samples (ligation or non-ligation) were reversed cross-linked by proteinase K treatment at 65°C for 4 h and purified by phenol/chloroform extraction (Sigma). Typically, 2.5 µg of DNA was recovered at this step, and 100 ng was used for each 3C PCR amplification with specific pairs of primers and resolved on a 2.0% (w/v) agarose gel. The following primers were used for 3C assays: for the ligated fragment (311bp),

DP1: 5'-GTGGTTTCCAGTTATCTAAAC-3'and DP2:
5'-ATAGGGAGGAATGATAGAGGA-3'; for the control fragment (182bp), CP1:
5'-CAAATGCAATGGGAGTTTATT-3'and CP2:
5'-GAGAGTGGAGGAAAGAAGGGTA-3.

ChIP-3C

ChIP-3C assay was performed as described previously [4]. Following the 3C process, we obtain either cross-linked ligation or non-ligation samples. Subsequently, ChIP (see method described above) and 3C assays are combined for ChIP-3C assays.

Primers used in the study (Sequences (5' to 3'))

For qPCR

huMEN1-F:GACCTGTCCCTCTATCCTCG
huMEN1-R:TGACCTCAGCTGTCTGCTCC
huMYC-F:AACGATTCCTTCTAACAG
huMYC-R:GGCTAAATCTTTC AGTCT
huAR-F:CAGTGGATGGGCTGAAAAAT
huAR-R:GGAGCTTGGTGAGCTGGTAG
huJunD-F:TTTGGAAGAGAGAAGAACAGAG
huJunD-R:CCAAGGATTACAAACAGGAATG
huCTNNB1-F:TGTGAATCCCAAGTACCAGTGT
huCTNNB1-R:CGTCAGACAAAGGAGAAACATT
huHIF1A-F:CTCCATTACCCACCGCTGAA
huHIF1A-R:TCACTGGGACTATTAGGCTCAGGT
huBMI1-F: TGGAAAGTGACTCTGGGAGTGACAAGGC
huBMI1-R:GTCACATGTATTAGCATCTAGAAAGCTGTAATGGC
huTwist1-F:CTAGAGACTCTGGAGCTGGATAACTAAAA
huTwist1-R:CGACCTCTTGAGAATGCATGCATGAAAA
huE-cadherin-F:GCCCTGCCAATCCCGATGAAA
huE-cadherin-R:GGGGTCAGTATCAGCCGCT
huVimentin-F:GCTTCAGAGAGAGGAAGCCGAAAA
huVimentin-R:CCGTGAGGTCAGGCTTGGAAA
huKi67-F:TGACCCTGATGAGAAAGCTCAA
huKi67-R:CCCTGAGCAACACTGTCTTTT
huHPRT-F:TATGGCGACCCGCAGCCCT
huHPRT-R:CATCTCGAGCAAGACGTTTCAG

For ChIP-qPCR

MYC enhancer-F: CATCCAATAAACCTTCCTACCTGA

MYC enhancer-R: TGGCAGGTGTCCTAGAGCAT

MYC promoter 1-F: GAAGCGTAAATAAAATGTGAAT
MYC promoter 1-R: GGCTGCCTTCCAGGCATTAA
MYC promoter 2(MYC 5' enhancer)-F:AGGCAACCTCCCTCTCGCCCTA
MYC promoter 2(MYC 5' enhancer)-R: AGCAGCAGATACCGCCCCTCCT
MYC 3' enhancer-F:GCTCAGTCTTTGCCCTTTGTGG
MYC 3' enhancer-R:TAACACCTTCCCGATTCCCAAGTG
Chr 1-F:CGGGGGTCTTTTTGGACCTT
Chr 1-R:GAAACACGGCTGCCAGAAAC

For ChIP-3C-qPCR

ChIP-3C-qPCR-P1:CATAGTCTAGCTCATTCTGAC
ChIP-3C-qPCR-P2:CTATACTTTGCCAATTCTTGG
ChIP-3C-qPCR-P3:GTTAATAGATTGGCTATTGAC
ChIP-3C-qPCR-P4:CAGCGGCCTGGCAAGGAGAAG
ChIP-3C-qPCR-P5:ACGCGCTCTCCAAGTATACGT
ChIP-3C-qPCR-P6:CAGAGCGTGGGATGTTAGTGT
ChIP-3C-qPCR-P7:GCTCCAGCAGCCTCCCGCGAC
ChIP-3C-qPCR-P8:GTTCTCCTCCTCGTCGCATGA

Primary antibodies used in the study

The following primary antibodies were used for Western blot analysis: rabbit anti-menin (1/4,000) (A300-105A, Bethyl), rabbit anti-MYC (1/2,000) (#9402, Cell Signaling Tech.), rabbit anti-AR (1/2,500) (A303-965A, Bethyl), rabbit anti- β -catenin (1/2,000) (#8480S, Cell Signaling Tech.), rabbit anti-JunD (1/2,000)(#720035, Invitrogen), rabbit anti-Vimentin (1/2,000)(#5741S, Cell Signaling Tech.), rabbit anti-E-cadherin (#3195S, Cell Signaling Tech.). Blotting membranes were stripped and reprobed with mouse anti-GAPDH antibody (1/1,000) (sc-47724, Santa Cruz), anti-Na/K-ATPase α 1 (1/1,000) (#23565s, Cell Signaling Tech.) or anti-Histone H3 (1/2,000) (#4499, Cell Signaling Tech.) as a loading control.

The primary antibodies used for IF, IHC and ChIP analyses: rabbit anti-menin (1/2,000) (A300-105A, Bethyl), rabbit anti- β -catenin (1/2,000) (#8480S, Cell Signaling Tech.), rabbit anti-JunD (1/2,000)(#720035, Invitrogen), rabbit anti-MYC (1/2,000) (#9402, Cell Signaling Tech.), and goat anti-GFP (1/2,000)(ab5450, Abcam).

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3. Kodama S, Yamazaki Y, Negishi M. Pregnane X Receptor Represses HNF4 α Gene to Induce Insulin-Like Growth Factor-Binding Protein IGFBP1 that Alters Morphology of and Migrates HepG2 Cells. *Mol Pharmacol*. 2015;88(4):746-57.
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Chapter III Discussion and Perspectives

For my thesis work, I have been studying the involvement of the *MEN1* gene in the tumorigenesis of prostate cells, using different cellular and mouse models, as well as human PCa samples, with the help from my colleagues and collaborators. The data obtained showed a rather complex and sometimes even opposite role of the *MEN1* gene according to cellular and molecular context of PCa. Here, I would like to mainly discuss the questions and issues, raised from these works, that I was not able to, or not fully, address in the above mentioned articles.

1. From *Men1* mouse models and MEN1 disease to PCa

1.1 *Men1*-knock out (KO) mice: what we my learn about the role of menin in PCa ?

The first clue showing the possible involvement of the *MEN1* in prostate cancer came from our observation that aged male heterozygous *Men1* mutant mice developed cancerous lesions at low frequencies, in addition to endocrine tumors.

1.1.1 *Men1*-disruption accelerates the tumorigenesis

In the first *Men1* mouse model established by conventional knockout system, six heterozygous *Men1* mutant (*Men1*^{+/-}) mice (6/47, 12.8%) developed prostate cancer, including two adenocarcinomas and four *in situ* carcinomas, while none of the control mice developed cancerous lesions. The expression of menin was found to be drastically reduced in all carcinomas, and partial LOH of the wild-type *Men1* allele was detected in three of the five analysed lesions (Seigne et al., 2010).

Based on the above observation, more recently, our team generated a prostate-specific *Men1*-KO mice model, *Men1*^{Floxed/Floxed-Nkx3.1^{CreERT2}}, by using a inducible system with tamoxifen. We observed that the tamoxifen treated group

developed earlier and more frequently PIN lesions, and progressively developed more advanced lesions with micro invasive carcinoma (MIC) at 6 (2/7, 28.6%) and 10 months (4/6, 66.7%) of age. In addition, these carcinomas exhibited loss of the non-target *Men1* allele (LOH), therefore supporting a tumor suppressor role for the *Men1* gene in prostate glands.

Taken together, our two *Men1*-KO mouse models demonstrated that *Men1* disruption accelerates the tumorigenesis of mouse prostate cells, indicating that menin plays a tumor suppressor role in normal prostate glands in the mouse.

1.1.2 Several points remain to be elucidated

a. Difference in age of mice developing the prostate carcinoma in these two types of mouse model

For the first mouse model, heterozygous *Men1* mutant (*Men1*^{+/-}) male mice were monitored for cancer development and examined by histology analysis between 18 and 26 months. We noticed that, in the 2nd mouse model, prostate glands were subjected to pathological examination at 1.5, 4.5 and 8.5 months after tamoxifen injection. As we all know, in the first model, the occurrence of LOH is crucial for PCa development, whereas in the conditional model, the gene disruption is largely complete. Also, for the 2nd mice model, the inducible system (tamoxifen-*Nkx3.1*^{CreERT2}) is more specific and efficient to disrupt the *Men1* in the prostate luminal cell. And finally, since the *NKx3.1* gene is known as a prostatic tumor suppressor gene, the combined disruption of both *Men1* and *Nkx3.1* could accelerate the tumorigenesis. Thus, these are the reasons for which the 2nd mice model is quicker than 1st mice to develop the PCa lesions.

b. Down-regulation of AR in *Men1*-KO mice prostate cell

Indeed, pathological examination revealed that *Men1*-disruption affected the AR expression in *Men1*-KO mice prostate cells. We found that PCa from *Men1* mouse models did display heterogeneous and reduced AR expression. These results were the first indication that menin could regulate AR expression in mice prostate cell. To confirm the observation, we investigated in AR dependent human PCa cells, and found that menin regulates the expression of AR and that of its target genes such as, *PSA*, *FKBP5* and *KLK2* in these cells. We further demonstrated that, mechanistically, menin binds to the *AR* promoter and regulates *AR* transcription via H3K4me3 histone marker in these cells.

c. *Men1*-KO induce the cell dedifferentiation of mice prostate cell

Men1/Nkx3.1-deficient lesions display a AR^{low} expression and an over 60% of menin-deficient cells expressed high CD44 at a later stage in the prostate cell membrane, accompanied by reduced CK18 and E-cadherin expression. These results suggested that a shift from a luminal to a dedifferentiated epithelial phenotype (Teinturier et al., 2021). Consistently, in my thesis work, we unveiled that menin inhibition activates the JunD pathway and Wnt/ β -catenin pathway and their targets, including in particular HIF1 α , E-cadherin, Twist 1, Vimentin and BMI1, which should facilitate the cell dedifferentiation and increased tumorigenic potential observed in AR-independent PCa cells. These results further demonstrated that menin involves in the cell dedifferentiation of AR-independent prostate cells, suggesting the oncosuppressive role of the *MEN1* gene not only during the initiation of tumorigenesis of normal prostate cell but also in AR-independent PCa cells.

1.2 Why there are so few prostate cases reported in MEN1 patients ?

Even compared with the number of breast cancer cases reported in MEN1 patients, the prostate cancer cases found in MEN1 patients are very much limited. I think there are at least two points could afford some answers to this question.

On one hand, the prevalence of MEN1 disease is low, estimated to be approximately 1/10,000 to 1/30,000, whereas the incidence of PCa is very high. On the other hand, PCa is an aged disease, the average age of its occurrence being around 75 yrs (Rawla, 2019). Therefore, considering these two factors together, it would be difficult and at least challenging to statistically prove the eventual predisposition of *MEN1* mutation to PCa in MEN1 patients, like what has recently been done for breast cancer.

1.3 Any way to conceal the differences or provide more evidence ?

On the one hand, as I mentioned in the *Introduction* part, the researchers investigated the role of the *MEN1* gene in breast cancer patients by independent cohort study. I do think that we still could try to apply the cohort study to ascertain whether PCa could be part of MEN1 pathology. I hope that the coming of more studies on the role played by menin in the future could encourage MEN1 community to address the issue.

On the other hand, we could also make more effort to study the role of MEN1 in sporadic PCa cases, through more updated technology such as RNA-seq, single cell seq, ect. Meanwhile, in this thesis using human PCa cell lines, we demonstrated that menin plays an oncogenic role in AR-dependent, whereas it can play an oncosuppressor role in AR-independent PCa cells. Therefore, we should pay more attention to different subtypes in PCa patients, in order to study the dual role of menin in the future. As I mentioned in the *Introduction* part and in the second article, the incidence of AR-negative PCa is increasing and the study on the mechanisms underlying AR-independent CRPC is challenging. I believe that dissecting menin's dual role in PCa could be part of the efforts to better understand PCa development and progression, as well as to improve its treatment, especially for mCRPC.

2. Regulation of *AR* transcription by menin in PCa cells.

2.1 Whether there are other factors participating the regulation of AR by menin in prostate cancer cells?

In this thesis, we proposed a new model showing that menin regulates *AR* transcription through the MYC pathway, involving *PCAT1* and *EZH2*, in PCa cells. I believe that we still need to know whether other factors may also be involved in this regulation, in order to make this new model clearer and more completed.

Menin inhibition reduces AR and its downstream targets expression in AR-dependent PCa cells. It has been reported that SMAD3, a TGF β signal transducer, represses androgen receptor-mediated transcription (Hayes et al., 2001). It is known that menin interacts physically with SMAD3 and is also involved in the TGF β /BMP pathway in leydig cells (Kaji, et al., 2001, Hussein et al., 2008). It would be interesting to investigate whether menin activates AR signaling via inhibiting SMAD3 expression in AR-dependent PCa cells. Menin has been reported to interact with Forkhead transcription factors such as FOXA1 and FOXA2. In mouse embryonic stem cell (mESC)-derived embryoid bodies, lower *Foxa1* and *Foxa2* mRNA levels were observed in the absence of *Men1* gene expression (Zhang et al. 2011). In addition, our team previously reported that menin interacts with *Foxa2* and to regulate *Foxa2* expression in mouse pancreatic cell lines (Bonnavion et al. 2017). In ER positive breast cancer cells, menin is enriched at enhancers that contain *FOXA1*-binding motifs (Dreijerink et al. 2017). In prostate cancer, Gao *et al* have also shown that the FOXA1 protein physically interacts with the AR protein, thereby acting as an AR-collaborating cofactor (Gao et al., 2003). Furthermore, FOXA1 and FOXA2 were shown to induce the expression of AR target genes such as PSA or FKBP5 in PCa cells (Yu et al., 2005; Connelly et al., 2018). Taken together, now the question is whether menin activates AR signaling via activating FOXA1 and/or

FOXA2 in PCa cells. The detailed mechanism should be further addressed in the future.

2.2 How menin regulates AR splicing variants in prostate cancer cells ?

2.2.1 AR-Vs transcription is regulated by AR-FL signaling

The expression of AR-V7 in cell lines was shown to be dynamic, and can be rapidly suppressed by addition of androgens or induced by inhibition of AR-FL signaling (e.g., through androgen deprivation, anti-androgen enzalutamide, or AR-FL siRNA) (Hu et al., 2012), and AR-FL/AR-V expression can be dramatically upregulated upon castration in prostate cancer xenograft models (VCaP and LuCaP35) (Watson et al., 2010), or upon treatment with abiraterone in two CRPC xenografts (LuCAP35CR and LuCaP23CR) (Mostaghel et al., 2011). Increased expression of AR-FL/AR-V7 was also observed in other prostate cancer cells (e.g., LAPC4 and DuCaP cells) following long-term culture in the presence of enzalutamide (Hoefler et al., 2016). However, the detailed mechanism is still not clear yet.

2.2.2 Regulation of AR-Vs by alternative splicing

Nadiminty et al showed downregulation of heterogeneous nuclear ribonucleoproteins (hnRNPA) 1 and 2, both regulated by NF- κ B, decreased the expression of AR-V7 in CWR22Rv1 and VCaP cells (Nadiminty et al., 2015). The recruitment of hnRNPA1 and hnRNPA2 to AR-Vs including AR-V7 splicing sites were enhanced in enzalutamide resistant CRPC cell lines, and could be inhibited by chemical inhibition of hnRNPA1 (Tummala et al., 2017). Takayama et al also reported that the expression of AR-Vs in prostate cancer was affected by RNA-binding protein PSF. PSF enhances various splicing factors to promote AR expression and splicing (Takayama et al., 2017).

2.2.3 Regulation of AR-Vs by menin

Although the above studies in multiple models have established that transcription of AR-Vs can be regulated by AR gene rearrangement or the *AR* locus, the detailed mechanistic investigation should be carried out to uncover additional key regulators in PCa. Surprisingly, in my work, *MEN1* silencing reduced AR-Vs expression on the mRNA levels, including AR-FL, AR-V1, AR-V3, AR-V567es and AR-V7. Meanwhile, menin inhibition also induced down-regulation of ARV7 protein expression in VCaP and 22Rv1 cells. These results indicated that menin involves in the regulation of AR-Vs or participates in the AR splicing variants in PCa cell lines.

Basis on these results, many work are still needed to identify the detailed mechanisms. For example, a. From above literature, whether menin participates the regulation of AR-Vs by NF-kB signaling or by RNA binding proteins? b. Is it possible that menin directly regulates AR-Vs expression in PCa cells, through its binding to their promoter? c. Whether MLL complex participates in the AR-Vs regulation by menin in PCa cells. All these points are still needed to be further identified.

3. What are the mechanisms underlying the regulation of the cell growth and differentiation by menin in PCa cells ?

3.1 Menin promotes the cell proliferation through MYC-mediated AR transcription in AR-dependent PCa cells.

AR is a key factor for cell proliferation in AR-dependent PCa cells (Zhu et al., 2008). Interestingly, my thesis works provided evidence, for the first time, that menin plays an oncogenic role through its positive regulation of *AR* transcription. Recent paper reported that MYC regulates the AR expression in AR dependent PCa cells (Bai et al., 2019). Therefore, our initial question was whether and how menin participates in the regulation of AR by MYC in AR-dependent PCa cells. As my data showed, menin and MYC co-bound to the *AR* promoter to regulate its transcription in these cells. My

further analysis, such as reChIP, revealed that menin is essential for the MYC-mediated AR transcription in AR-dependent PCa cells.

Moreover, my work demonstrated that menin regulated *MYC* transcription, via its binding to the *MYC* promoter, mediated by H3K4me3. Our data indicate that MYC is a downstream target of menin in AR-dependent PCa cells. It has been reported that MYC target genes related to cell proliferation and cell differentiation, such as cyclin D, cyclin E, CDK4 and β -catenin, *ect* (Amati et al., 1998; Yochum et al., 2010). Coincidentally, menin inhibition reduced the MYC targets expression and caused cell cycle arrest in AR-dependent PCa cells in my thesis work. More interestingly, it was revealed in my study that other partners of MYC, such as EZH2 and long-noncoding RNA *PCATI*, were also involved in the regulation of AR by menin and MYC in AR-dependent PCa cells (Fig. 3.1).

In conclusion, menin plays an oncogenic role through its critical involvement in the regulation of both *MYC* transcription and MYC-mediated *AR* transcription in AR-dependent PCa cells. To my knowledge, it is the first time that such mechanisms underlying the involvement of menin as oncogenic factor in PCa cells were so far reported.

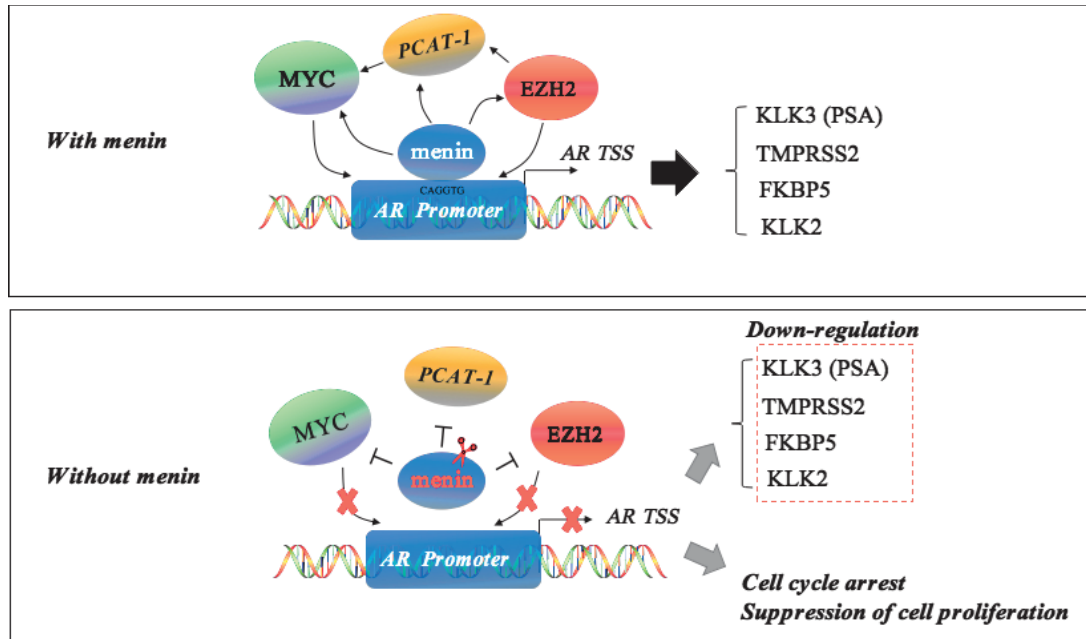


Fig. 3.1 Molecular mechanism that *MEN1* inactivation suppresses the cell proliferation of AR-dependent PCa cells.

3.2 Menin suppresses the cell growth and dedifferentiation via inactivating JunD and β -catenin in AR-independent PCa cells.

3.2.1 Menin inhibits cell growth through MYC, JunD and β -catenin in AR - independent PCa cells.

As I mentioned in the *Introduction* part, MYC proteins initiate a transcriptional program of growth, proliferation and metabolism in different cancers. In AR-independent PCa cells, we observed that menin inhibition still maintains the MYC expression, which should contribute to promote the cell growth. By further analyses, we uncovered that menin inhibition triggered JunD and β -catenin translocation in the cellular nuclear, leading to, on the one hand, increased JunD loop binding between the MYC enhancer and promoter, on the other hand, augmented β -catenin binding to the MYC promoter, both ultimately maintaining MYC expression in AR-independent PCa cells. (Fig. 3.2)

Meanwhile, menin inhibition induced the expression Hypoxia-inducible factors 1 α (HIF1 α), further accelerating the carcinogenesis of the AR-independent PCa cells (Fig. 3.2). What would be the role of HIF1 α in this context ? HIF1 α is a transcription factor regulating many pivotal pathways in normal as well as cancerous cells (Ryan et al., 2000; Elson et al., 2000). It is overexpressed in organs or tissues where oxygen level drops below threshold level (Hockel et al., 2001). High levels of HIF1 α point towards angiogenesis, cell proliferation, survival and tumor progression through regulation of growth promoters, oncogenes, glycolytic pathways and pH regulation. A large number of studies support the relation of increased level of HIF-1 α with aggressive tumor growth and poor patient prognosis (Baba et al., 2010; Semenza et al., 2013; Chen et al., 2014).

3.2.2 Menin inhibition induces the cell dedifferentiation via activation of JunD and Wnt/ β -catenin pathway in AR-independent PCa cells

Epithelial-to-mesenchymal transition (EMT) is a crucial step in metastasis initiation. Several observations from the reprogramming field also provide evidence that dedifferentiation occurs when EMT factors are expressed (Takahashi and Yamanaka, 2006; Yu et al., 2007; Takahashi et al., 2007; Park et al., 2008). EMT-related gene expression changes may also provide input to the cell differentiation state, such as E-cadherin, occludin, Twist 1, Snail1 and Traffic Jam 1 (TJ1). Interestingly, it has reported that the activation of the Wnt/ β -catenin signal pathway is the critical factor that directly controls the process of EMT induced by HIF-1 α in PCa cells (Jiang et al., 2017). In addition, as I mentioned in the 3.1, it has been known that β -catenin is a target of MYC in colorectal cancer cells (Yochum et al., 2010).

In our analysis, menin inhibition activated the Wnt/ β -catenin pathway and its targets, such as HIF1 α , E-cadherin, Twist 1, Vimentin and BMI1, leading to cell dedifferentiation in AR-independent PCa cells. (Fig. 3.2) Consistently, in our previous

Men1 knock out mouse model, we found that early-stage lesions exhibited a decreased expression of AR and its target genes, accompanied by reduced CK18 and E-cadherin expression, suggesting a shift from a luminal to a dedifferentiated epithelial phenotype. Intriguingly, over 60% of menin-deficient cells expressed CD44 at a later stage (Teinturier et al., 2021). These results suggest that menin could be a key factor inhibiting the molecular program favorite for EMT and stemness in AR-independent PCa cells or AR low expression of PCa. Nevertheless, many works are still needed to identify the similar molecular changes in human prostate cancer tissues and to detail the underlying mechanisms.

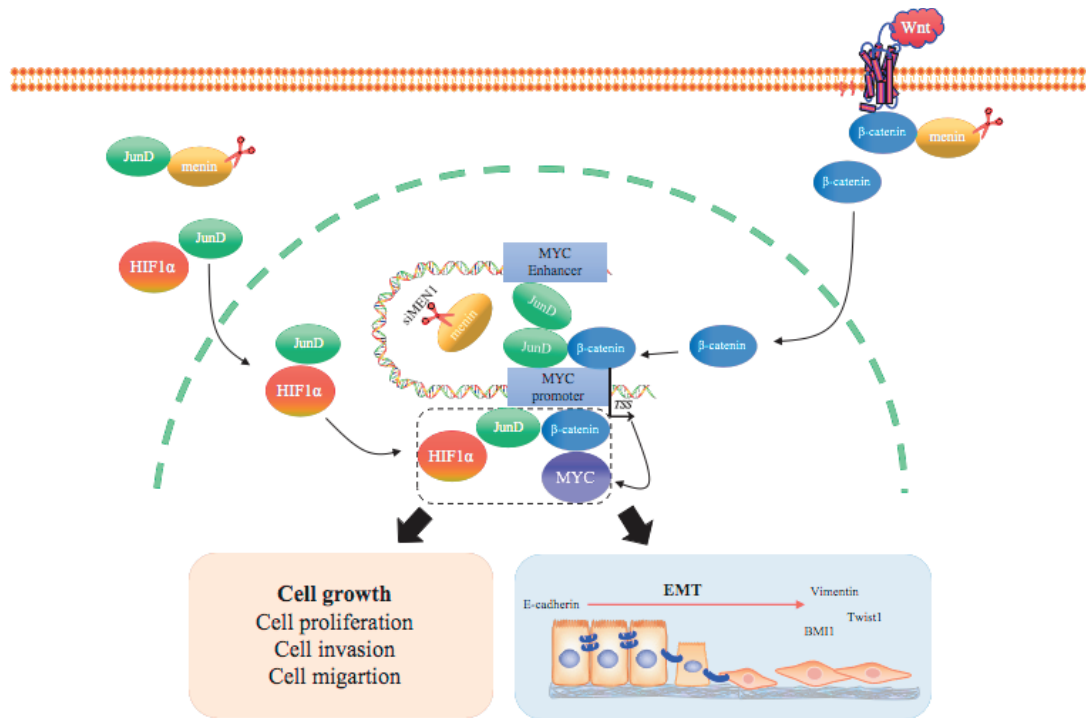


Fig. 3.2 Molecular mechanism that *MEN1* inactivation facilitates the cell growth and dedifferentiation of AR-independent PCa cells.

3.3 Mechanisms underlying the suppression of JunD and β-catenin by menin

3.3.1 Menin represses JunD expression

Menin-JunD interaction has been identified by the yeast two-hybrid method in the human brain cDNA library and has been confirmed *in vitro* and *in vivo* (Agarwal et al., 1999). Menin represses JunD-activated transcription through a histone deacetylase

(HDAC)-dependent mechanism (Gobl et al., 1999) in which mSin3A, a general corepressor, recruits HDAC to menin (Kim et al., 2003). Several naturally occurring and clustered *MEN1* missense mutations disrupted menin's interaction with JunD. These observations suggest that menin's tumor suppressing function involves direct binding to JunD and inhibition of JunD-activated transcription (Agarwal et al., 1999).

Our analysis found that menin suppresses JunD expression on the mRNA and protein level in the AR-independent PCa cells. Furthermore, we also showed that menin inhibits the JunD translocation into nuclear to function in AR-independent PCa cells. However, the detailed molecular mechanisms need to be investigated in PCa cells.

A new mechanism was reported whereby menin binds JunD and thus block c-Jun N-terminal kinase (JNK)-mediated phosphorylation of JunD at residues S90, S100 and T117, an event crucial for activating JunD function in transcriptional regulation in HEK-293 cells. Furthermore, menin had no effects on JNK binding and JNK-mediated phosphorylation of c-JUN, a close homologue of JunD that lacks a menin-binding motif (Huang et al. 2012). These results could give us a promising clue to investigate the mechanism that JunD suppression by menin in PCa cells.

3.3.2 Menin represses β -catenin expression

Our team and collaborators previously demonstrated that menin directly interacts with β -catenin and carries β -catenin out of the nucleus through its two functional nuclear export signals (Cao et al., 2009). In *Men1*-null MEFs and insulinoma tissues from β -cell-specific *Men1* knockout mice, β -catenin is accumulated in the nucleus (Cao et al., 2009). Since over expression of menin reduces nuclear accumulation of β -catenin and its transcriptional activity (Cao et al., 2009), menin may repress cell proliferation through suppression of the Wnt/ β -catenin signaling, which is pro-proliferative in many cells (Cao et al., 2009). However, in cultured rodent islet tumor cells, menin

may repress cell proliferation partly by activating the Wnt/ β -catenin signaling (Chen et al., 2008). When menin and activated β -catenin is over expressed, the Wnt/ β -catenin downstream target gene, *Axin2*, is significantly enhanced correlating with increased H3K4 trimethylation at the promoter of the *Axin2* gene (Chen et al., 2008). Indeed, it is already known that β -catenin plays as an oncogenic factor in PCa cells. However, we didn't know the relationship between menin and β -catenin in PCa cells when we started our work. In my thesis work, we found that menin inhibition by siRNA causes up-regulation of *CTNNB1* and increased nuclear expression of β -catenin protein in AR-independent PCa cells. Notably, menin also displayed the ability that inhibiting the β -catenin translocation in these cells. Nevertheless, the detailed mechanism that how menin regulates the *CTNNB1* transcripts and how menin inhibits β -catenin translocation is still needed to be investigated in AR-independent PCa cells.

3.4 Tumorigenic potential: different usage and the limit of cultured cell approaches

3.4.1 *In vitro* model for cancer research

In vitro refers to the technique of performing a given procedure in a controlled environment outside of a living organism. For decades, cultured animal and human cells are used as an important tool for cell biology and cancer research. Cells can be grown in attached to specific surfaces forming cell monolayer cultures in two-dimension (2D) system, typically surface platforms are tissue culture-plastic or surfaces with extracellular matrix components such as collagen, fibronectin among others fibrous components. However, monolayer cultures have been subject to criticism because assays still reflect a highly artificial cellular environment that may not correspond to a tissue or organ situation.

Nevertheless, researchers developed a series of methods such as foci formation assay and soft agar colony formation assay, to better characterize the tumorigenicity of cancer cells, in terms of loss of contact inhibition and anchorage independence. It cannot be denied that they provide a simple and safe way for scientists to perform early tests or basic research on new procedures and treatments. However, sometimes when we apply a single assay to research, the results may not reflect the real situation. In our analysis, menin inhibition by siRNA did not reduce the cell proliferation of PC3 cells by the Incucyte ZOOM analysis. But, when other assays were applied, I got different results. I found that the *MEN1* inactivation not only promotes the cell proliferation, but activates cell migration and cell invasion in AR-independent PCa cells by wound healing assay and soft agar assay, respectively. Taken together, we should, when possible, use several complementary culture assays in our research work.

3.4.2 *In vivo* model for cancer research

In vivo refers to experimentation using a whole, living organism as opposed to a partial or dead organism. Animal studies and clinical trials are two forms of *in vivo* research. *In vivo* testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. As I mentioned in the *Introduction* part, *Men1*-KO mice model were generated by different system. The dual role of menin has been revealed in *Men1* gene deficient of mammary gland lesions or prostate lesions.

In this thesis, owing to our collaboration with Dr. S Baron from Université Clermont Auvergne, we adopted a xenograft mouse model using PC3-GFP cells to confirm the results which we obtained *in vitro*. On the one hand, we found that MI503 treatment reduces the menin and JunD expression and triggers the β -catenin translocation in PC3-GFP cells. These results are consistent with our *in vitro* studies. On the other hand, the proliferation of MI503-treated xenografted PC3-GFP cells was increased and cells appeared more variable in cell size, disorientatedly aligned and more

invasive towards surrounding mouse tissues. Conversely, the MI503 treatment significantly inhibits the cell proliferation of PC3 cells *in vitro*. These results indicated that: a. for the technique part, sometimes, one could get different even opposite results *in vitro* and *in vivo*. I think this is not surprising, because the *in vitro* study happens in an artificial cellular environment that may not correspond to a real situation in tissue or organ. Meanwhile, cell line derived xenograft is not a “real” *in vivo* study. The PC3-GFP cells still come from *in vitro* cell culture. It would be interesting to carry out patient derived xenografts (PDX) to confirm our current data. For the function of MI503, our work or previous paper has already found that MI503 not only decrease menin expression, but that of JunD *in vitro* and in xenograft model. It would also be interesting to study whether MI503 could inhibit other factors interacting with menin or down-regulate other factors in *in vivo* environment.

3.4.3 3D culture

A new dimension to cancer research has been achieved by the advent of 3-Dimensional cell culture techniques. This approach increased many folds the ways in which cancer cell lines can be utilized for understanding complex cancer biology. 3D cell culture techniques are now the preferred way of using cancer cell lines to bridge the gap between the “absolute *in vitro*” and “true *in vivo*”. The aspects of cancer biology that 3D cell culture systems have contributed include morphology, microenvironment, gene and protein expression, invasion/migration/metastasis, angiogenesis, tumour metabolism and drug discovery, testing chemotherapeutic agents, adaptive responses and cancer stem cells. The new technique should give us a new clue to confirm our analysis *in vitro* studies at least.

4. New conception of therapeutic strategy to propose based on the findings from the current work.

4.1 MI503, inhibits the menin and JunD interaction in prostate cancer cells ?

Molecular inhibitor, MI503, blocks the menin/MLL interaction driven leukemia progression in mouse models of MLL leukemia (Borkin et al., 2015). Wu et al further demonstrated that MI503 promoted the interaction of menin with Hsp70-associated ubiquitin ligase CHIP, resulting in increased menin ubiquitination, leading to increased menin degradation (Wu et al., 2019). Interestingly, Malik et al. reported that MI503 treatment blocked AR signaling and inhibits the growth of castration-resistant tumors in vivo in mice (Malik et al., 2015). Interestingly and importantly, in our hands, *MEN1* inactivation by siRNA only inhibited the cell proliferation in AR-dependent PCa cells, but not in the AR-independent PCa cells (Teinturier et al., 2021). This finding inspired us to investigate why MI503 could inhibit the cell proliferation in AR-independent PCa cells.

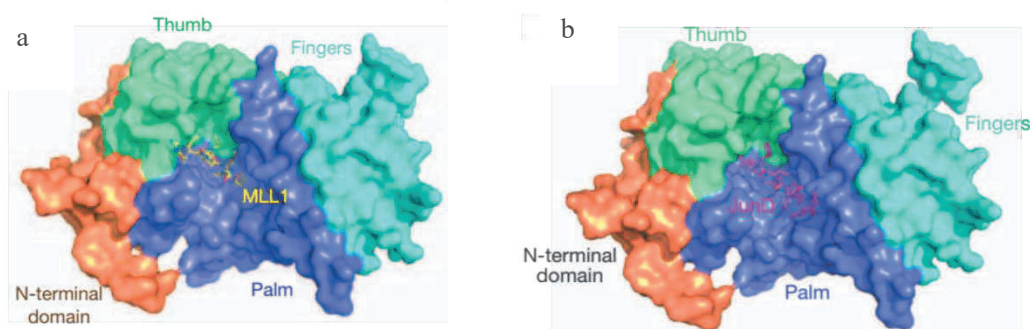


Fig 3.4 Structural studies of the menin -MLL1 and menin-JUND interaction. a. Crystal structure of the menin-MLL1 complex. **b.** Crystal structure of the menin-JUND complex. Menin is coloured as in Fig. 3.1a and JUND_{MBM} is shown as a purple stick model. *Obtained from:* Huang et al., 2012, Nature.

Huang et al established the crystal structures of human menin in its free form and in complexes with MLL1 (Fig. 3.4a) or with JUND (Fig. 3.4b) heterodimer.

These structures show that menin contains a deep pocket that binds short peptides of MLL1 or JUND in the same manner (Huang et al., 2012). Their results indicated that MI503 not only inhibits the interaction between menin-MLL, but can also block the interaction between menin-JunD. Notably, Elliott et al demonstrated recently that JunD plays an oncogenic role and is essential for MYC-mediated the cell proliferation in PCa cells (Elliott et al., 2019). Thus, our hypothesis was that MI503 treatment could deregulated menin-JunD interaction and at the same time decrease both menin and JunD expression to suppress cell proliferation in PCa cells, which could explain the difference observed in MI503-treated and siMEN1-treated AR-independent PCa cells. As we expected, MI503 treatment not only reduced the menin expression, but also decreased the JunD and MYC expression on the transcriptional and protein level in both cultured AR-dependent and AR-independent PCa cells. It has been reported that MI503 decreased menin protein stability by activating ubiquitination pathway (Wu et al., 2019). Further works are needed to determine whether MI503 affects the JunD protein stability in a similar way.

4.2 Should we use the MI503 to treat the PCa ?

Our analysis should give some clues for this question. We should be cautious when we use the MI503 to treat the PCa. On the one hand, the mechanism of MI503 is not fully understood. As I mentioned in 4.1, MI503 not only reduces the menin expression, but also inhibits the JunD expression. However, the underlying mechanism for the latter is poorly understood for the moment. More efforts should be made to better understand the mechanistic action of MI503. On the other hand, in this thesis, we revealed that menin plays as a tumor suppressor role in AR-independent PCa cells. Hence, MI503 would not be suitable for treating AR-negative or AR-independent PCa. Therefore, it will be useful in the future to distinguish the AR expression in PCa during future clinical trials.

4.3 Could we use the combine MI503 with β -catenin inhibitors to treat the PCa ?

It still depends on the the AR level in PCa patients. If the AR expression is positive, combined MI503 and β -catenin inhibitor may have the advantage to antagonise the cancer cell growth. Conversely, if the AR expression is negative, it is still too early to carry out the assay. Although according to our results, menin inhibition triggers the β -catenin activation in AR-independent PCa cells, we still don't know whether other partners of menin participates the increased tumorigenic potential of AR-indepdent PCa cells due to menin inhibition. Further studies are needed, in order to dissect the cellular and molecular events triggered by *MEN1* inactivation in these cells.

Chapter IV Conclusion

My thesis works afforded novel evidence suggesting distinct roles of menin in PCa cells, depending on different cellular and molecular contexts. On the one hand, we report here a novel mechanism underlying menin's oncogenic activity in AR dependent prostate cancer (PCa) cells. Our results demonstrate that menin is a critical cofactor for MYC-mediated *AR* transcription in AR-dependent PCa cells. Interestingly, we also showed that menin was involved in the regulation of MYC-related LncRNA *PCATI* in AR-dependent PCa cells, through both its positive regulation of *EZH2* transcription and its physical interaction with *EZH2*, as well as their co-binding on the regulatory sequence of *PCATI*. As MYC is known to be deregulated in 30-50% of human malignancies with poor prognosis (Chen et al., 2018), our novel discovery may have significant implication for cancer therapy: targeting both menin and MYC pathways might potentially work for AR-dependent PCa.

On the other hand, our findings provide evidences that menin plays a tumor suppressive role in AR-independent PCa cells. We found that *MEN1* inactivation promotes tumorigenic potential, via triggering nuclear translocation of JunD and β -catenin, in AR-independent PCa cells. Mechanistically, upon *MEN1* inactivation, both JunD and β -catenin could replace menin to bind to the *MYC* proximal promoter, and ultimately result in maintaining the *MYC* expression in AR-independent PCa cells. Moreover, the analysis of culture AR-independent cells and PC3-GFP xenografts in the mouse demonstrated that both JunD and β -catenin are necessary for the altered tumorigenic potential triggered by *MEN1* inactivation in AR-independent PCa cells. Interestingly, we also found *MEN1* inactivation alters the expression of several molecular markers of EMT and stemness, including E-cadherin, BMI1, Twist1 and HIF1 α , in these cells. Nevertheless, many works are still needed to identify the detailed mechanisms underlying the involvement of menin in the above phenomenon in AR-independent PCa cells.

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Appendices

Appendix 1. Teinturier R#, Luo Y#, Decaussin-Petrucci M, Vlaeminck-Guillem V, Vacherot F, Firlej V, Bonnavion R, Abou Ziki R, Gherardi S, Goddard I, Gadot N, Bertolino P, Le Romancer M, Zhang CX. Men1 disruption in Nkx3.1-deficient mice results in AR^{low}/CD44⁺ microinvasive carcinoma development with the dysregulated AR pathway. *Oncogene*. 2021;40(6):1118-1127. (#: co-first author)

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Men1 disruption in *Nkx3.1*-deficient mice results in AR^{low}/CD44⁺ microinvasive carcinoma development with the dysregulated AR pathway

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Abstract

Dysregulated androgen receptor (AR) plays a crucial role in prostate cancer (PCa) development, though further factors involved in its regulation remain to be identified. Recently, paradoxical results were reported on the implication of the *MEN1* gene in PCa. To dissect its role in prostate luminal cells, we generated a mouse model with inducible *Men1* disruption in *Nkx3.1*-deficient mice in which mouse prostatic intraepithelial neoplasia (mPIN) occur. Prostate glands from mutant and control mice were analyzed pathologically and molecularly; cellular and molecular analyses were carried out in PCa cell lines after *MEN1* knockdown (KD) by siRNA. Double-mutant mice developed accelerated mPIN and later displayed microinvasive adenocarcinoma. Markedly, early-stage lesions exhibited a decreased expression of AR and its target genes, accompanied by reduced CK18 and E-cadherin expression, suggesting a shift from a luminal to a dedifferentiated epithelial phenotype. Intriguingly, over 60% of menin-deficient cells expressed CD44 at a later stage. Furthermore, *MEN1* KD led to the increase in CD44 expression in PC3 cells re-expressing AR. Menin bound to the proximal AR promoter and regulated AR transcription via the H3K4me3 histone mark. Interestingly, the cell proliferation of AR-dependent cells (LNCaP, 22Rv1, and VCaP), but not of AR-independent cells (DU145, PC3), responded strongly to *MEN1* silencing. Finally, menin expression was found reduced in some human PCa. These findings highlight the regulation of the AR promoter by menin and the crosstalk between menin and the AR pathway. Our data could be useful for better understanding the increasingly reported AR-negative/NE-negative subtype of PCa and the mechanisms underlying its development.

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Introduction

Prostate cancer (PCa) is the second most common cancer in men worldwide [1]. The androgen receptor (AR) plays a crucial role not only in the development of PCa, but also in its treatment. Indeed, anti-AR therapy by second-generation

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antiandrogens is highly effective, though it ultimately results in resistance and may favor the occurrence of an AR-negative PCa subtype [2–4]. The latter has risen over last two decades [2], in particular the AR-negative and nonneuroendocrine (AR[−]NE[−]) tumor subtype, which has overtly augmented from 5 to 20%.

Aside from the previously known common genetic alterations, such as gain of AR function, loss of *NKX3.1*, and *PTEN*, recent works on metastatic castration-resistant PCa (mCRPC) detected recurrent genomic alterations implicating the factors involved in chromatin structure modification [5]. Remarkably, components of the COMPASS-like complexes, KMT2B, KMT2C, and KMT2D, as well as menin encoded by the *MEN1* gene and interacting with KMT2A/B, were shown to be mutated [5–7]. *MEN1* mutations predispose patients to multiple endocrine neoplasia type 1 syndrome (MEN1, disease identifier OMIM131100). Interestingly, it has also been shown that menin interacts with KMT2A, KMT2D, and other partners to promote leukemogenesis [8, 9]. Importantly, a recent study revealed a higher breast cancer incidence in female MEN1 patients [10]. The finding is reminiscent of our previous observation that female *Men1* mammary gland-specific *Men1* knockout mice developed breast precancerous lesions, whereas male heterozygous *Men1* mutant mice developed PCa with a low but significant frequency [11]. However, Malik et al. reported that the interaction between menin and AR is required for the growth of human PCa cell lines both in culture and in xenografts [12]. Therefore, an obvious discrepancy exists between data from spontaneous prostate tumors (in human and mouse models) and those from some PCa-derived cell lines (or xenografts), which may reflect opposite functions of menin in prostate cells in different contexts. A dual cell type-specific role for menin has been proposed in breast cancer [13]. We hypothesized that, in PCa, the effects of menin were dependent on the stage and molecular context of disease progression. To test this hypothesis we generated a novel *Men1* mutant mouse model, in which *Men1* was specifically disrupted in *Nkx3.1*-deficient prostate cells. Intriguingly, a link between *MEN1* and *AR* regulation was uncovered, highlighting the role played by menin in the regulation of AR expression and the AR signaling pathway.

Results

***Men1* disruption accelerates tumorigenesis in *Nkx3.1*-deficient prostate cells in mice**

To decipher the role played by *Men1* in the initiation of prostate cell tumorigenesis, we chose to disrupt *Men1* in a mouse model in which precancerous prostate lesions develop, in order to either accelerate or rescue tumor development. To this end, *Men1*^{F/F} mice [14] were crossed

with *Nkx3.1Cre*^{ERT2} mice, in which the activity of Cre recombinase controlled by the endogenous *Nkx3.1* promoter can be induced in prostate luminal cells upon tamoxifen injection. *Men1*^{F/F}–*Nkx3.1Cre*^{ERT2-/+} mice thus generated were subjected to tamoxifen treatment (*Men1*^{INT}) or not (*Men1*^{IN}), at 1.5 months of age, and prostate glands were subjected to pathological examination 1.5, 4.5, and 8.5 months after injection (Fig. 1a). Immunohistochemical (IHC) analysis confirmed *Men1* disruption in prostate luminal cells exclusively in *Men1*^{INT} mice (Fig. 1b, c).

Since *Nkx3.1Cre*^{ERT2} mice develop mouse prostatic intraepithelial neoplasia (mPIN) lesions at around 6 months of age at a low frequency and reach full penetrance after 10 months [15, 16], we speculated that *Men1*^{INT} mice would allow us to determine whether additional *Men1* disruption rescues or exacerbates the phenotype of *Men1*^{IN} mice. We observed mPIN lesions in dorsal, ventral, lateral, and anterior lobes in all male *Men1*^{INT} mice ($n = 7$) 1.5 months after induction of the Cre recombinase, with high-grade mPIN being observed in six of the seven *Men1*^{INT} mice and low-grade mPIN in the remaining *Men1*^{INT} mouse, whereas only one mouse displayed low-grade mPIN among the untreated age-matched *Men1*^{IN} mice ($n = 5$, Figs. 1d and S1 and Table 1). Having shown that a majority of prostate cells within lesions in *Men1*^{INT} mice lost menin expression, we then noticed that these mice progressively developed more advanced lesions with prominent pleomorphic changes (Figs. 1e and S2) and micro-invasive carcinoma (MIC) at 6 (2/7, $n = 7$) and 10 months (4/6, $n = 6$) of age (Fig. 1f, upper panel, Table 1). As experimental controls, no age-matched *Men1*^{IN} mice developed MIC ($n = 5$ for all groups of age) (Table 1), *Men1*^{F/F} mice either treated or not with tamoxifen developed no mPIN lesions within the 10-month experimental time course, and tamoxifen-treated *Nkx3.1Cre*^{ERT2-/+} mice developed no MIC lesions at 5 and 10 months of age ($n = 5$ for both groups, data not shown). Consistently, α SMA staining revealed an attenuated or broken smooth muscle cell layer around the lesions, highlighting alterations in the prostate gland structure during the process of MIC formation (Figs. 2a, upper panel, and S3). Ki67 staining of 6-month-old *Men1*^{INT} mice revealed that mPIN lesions lacking menin expression showed a significantly higher proliferative activity (Figs. 2a, lower panel, and S4). Taken together, our results demonstrated that prostate-specific *Men1* disruption in mice leads to the acceleration of tumorigenesis in *Nkx3.1*^{-/+} prostate cells.

***Men1/Nkx3.1*-deficient lesions display early AR^{low} and late membrane CD44^{high} expression**

We then sought to better characterize the prostate lesions that developed in *Men1*^{INT} mice by analyzing several major cell

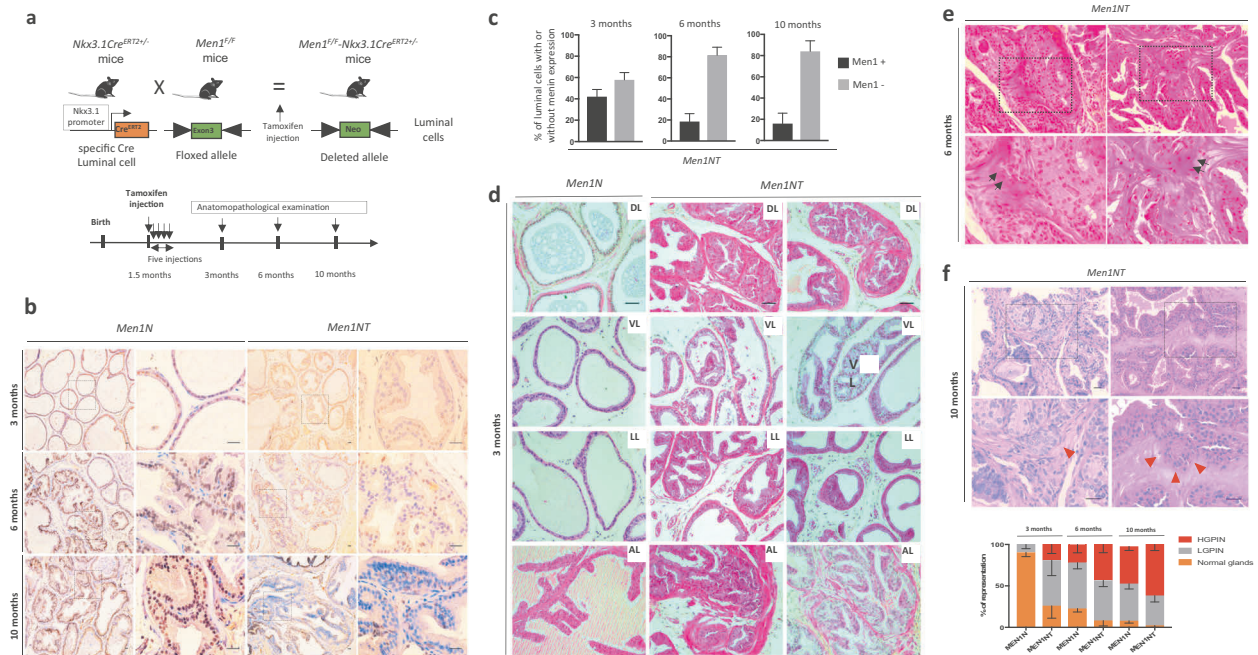


Fig. 1 Characterization of the *Men1^{F/F}-Nkx3.1Cre^{ERT2+/-}* mouse phenotype. **a** Schematic overview of the approach used for the generation of *Men1^{F/F}-Nkx3.1Cre^{ERT2+/-}* mice. **b** Depletion of menin in prostate glands and development of prostatic intraepithelial neoplasia (PIN) lesions in *Men1^{F/F}-Nkx3.1Cre^{ERT2+/-}* mice. Immunohistochemical analysis using an anti-menin antibody was performed in prostate sections of *Men1^{F/F}-Nkx3.1Cre^{ERT2+/-}* treated with tamoxifen (*Men1NT*), and in untreated control mice (*Men1N*), at 3, 6, and 10 months of age. Right columns for each condition, a twofold magnification showing *Men1* expression in the normal prostate gland or neoplastic cells is included. Scale bar = 25 μm. **c** Quantification of luminal cells positive and negative for menin expression in each group of age following tamoxifen treatment ($n = 5$). **d** *Men1* inactivation accelerates tumorigenesis of prostate cells. Prostate glands were collected from the control and mutant mice at 3 months of age.

Representative images of the prostate gland architecture for each different lobe after hematoxylin & eosin staining (H&E). DL dorsal lobe, LL lateral lobe, VL ventral lobe, AL anterior lobe. Scale bar = 25 μm. **e** Representative H&E sections of two high-grade PIN lesions in *Men1NT* at 6 months of age. The two lower panels are a twofold magnification of the upper staining. Scale bar = 25 μm. **f** Upper: Representative H&E sections of two microinvasive carcinoma (MIC) lesions in *Men1NT* mice at 10 months of age. MIC lesions were seen with nests of atypical tumor cells (arrows) on left and right panel, infiltrating the surrounding stroma and forming irregular contours. The two lower panels are a twofold magnification of the upper staining. Scale bar = 25 μm. Lower: Histological evaluation and distribution of normal gland, low-grade PIN (LGPIN), and high-grade PIN (HGPIN) lesions for each group of age. Three distant sections for each mouse were scored.

lineage markers known to be crucial for defining differentiation of prostate cells. Notably, double immunofluorescence (IF) staining using antibodies against menin (red) and AR (green) on prostate glands from 3- and 6-month-old tamoxifen-untreated or -treated double-mutant mice revealed a reduced AR expression in menin-deficient prostate cells (Figs. 2b and S5). IHC analyses also showed that this decrease was accompanied by a decrease in the expression of AR-target genes, such as FKBP5 (Fig. 2c). The strongly reduced AR expression in *Men1*-deficient lesions prompted us to further investigate the effects of menin downregulation on the expression of AR and its target genes in PCa cell lines. *MEN1* knockdown (KD) with different siRNA in LNCaP and 22Rv1 cells (AR⁺ cell lines) resulted in a significant decrease in AR and PSA (prostate-specific antigen) transcriptional expression (Fig. 2d, upper panel). To verify whether the menin-mediated effects described above were linked with the MLL complex, we treated LNCaP cells with MI503, which inhibits the interaction between menin and MLL. As anticipated, MI503 treatment led to a dose-

dependent reduction in the transcription of AR and PSA (Fig. 2d, lower panel). Moreover, a strong reduction in the protein expression of AR, PSA, and TMPRSS2 (transmembrane protease, serine 2), another AR-target, in siMEN1-treated LNCaP and 22Rv1 cells was observed by western blot analysis (Fig. 2e). Consistently, AR binding to the promoter of its known target genes, *KLK3*, *FKBP5*, and *KLK2*, was significantly reduced in siMEN1-treated LNCaP, 22Rv1, and VCaP cells (Figs. 2f and S6).

Furthermore, IHC analyses revealed a decrease in CK18 expression in menin-deficient prostate cells, whereas no increase in CK5 expression was observed (Fig. 3a). In addition, *Men1*-deficient luminal cells expressed no basal marker p63 (Figs. 3b and S7). Consistently, E-cadherin expression, prominently expressed on the membrane of normal epithelial luminal prostate cells, was reduced (Fig. 3c), whereas no vimentin expression could be detected (data not shown), suggesting a partial epithelial-mesenchymal transition. The

Table 1 *Men1* inactivation accelerates tumorigenesis of *Nkx3.1*^{+/-} prostate cells.

Genotype	Pathological classification	3 months	6 months	10 months
<i>Men1N</i>	Normal	(4/5) 80%	(0/5) 0%	(0/5) 0%
<i>Men1</i> ^{F/F} - <i>Nkx3.1</i> Cre ^{ERT2} -/-	mPIN	(1/5) 20%	(5/5) 100%	(5/5) 100%
Untreated	MIC	(0/5) 0%	(0/5) 0%	(0/5) 0%
<i>Men1INT</i>	Normal	(0/7) 0%	(0/7) 0%	(0/7) 0%
<i>Men1</i> ^{F/F} - <i>Nkx3.1</i> Cre ^{ERT2} -/-	mPIN	(7/7) 100%	(7/7) 100%	(7/7) 100%
Tamoxifen treated	MIC	(0/7) 0%	(2/7) 28%	(4/7) 57%

Prostatic lesions were classified according to the recommendation of Bar Harbor meeting of the mouse models of human cancer consortium prostate pathology committee. The number of mice displaying each pathological classification category is indicated, and the corresponding percentage is shown in parentheses. Mouse prostatic lesions were classified according to the recommendation of Bar Harbor meeting of the mouse models of human cancer consortium prostate pathology committee.

mPIN: mouse prostatic intraepithelial neoplasia, *MIC*: microinvasive carcinoma.

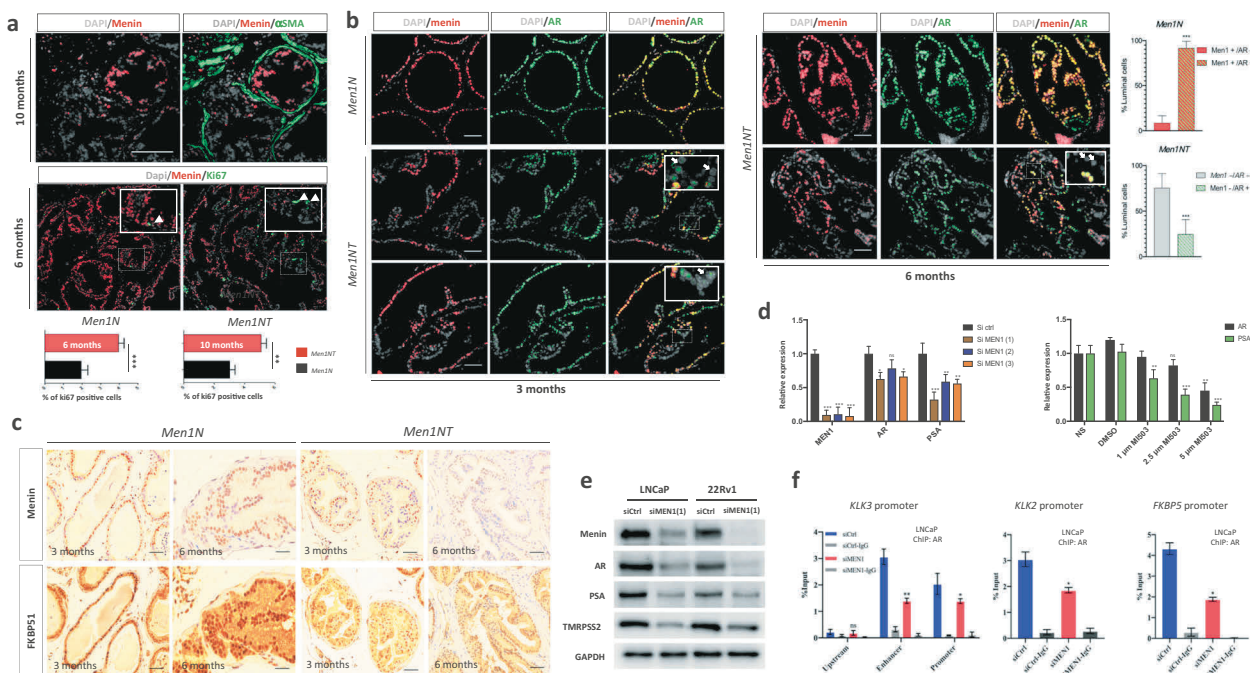


Fig. 2 *Men1* disruption in *Nkx3.1*-deficient prostate cells results in deregulated AR expression and the AR pathway. **a** Upper: α SMA immunofluorescence (IF) performed in prostate specimens of *Men1INT* mice at 10 months of age, arrows indicate discontinuous α SMA staining in surrounding stromal compartment adjacent to menin-deficient luminal cells. Scale bar = 50 μ m. Middle: IF staining against Ki67 on prostate sections from *Men1N* and *Men1INT* at 6 months of age. Scale bar = 25 μ m. Lower: Quantification of Ki67 staining. Data are presented as means \pm SEM, ***P* < 0.01; ****P* < 0.001. **b** Double IF against androgen receptor (AR) and menin in *Men1*^{F/F}-*Nkx3.1* (*Men1N*) and *Men1*^{F/F}-*Nkx3.1* + tamoxifen (*Men1INT*) mouse prostate tissues at 3 (left panel) and 6 (upper right panel) months of age. The left and right histograms represent respectively the percentage of cells with or without AR expression in menin-positive luminal cells from *Men1N* mice and menin-negative luminal cells from *Men1INT* mice (*n* = 5). Scale bar = 25 μ m. **c** Immunohistochemical (IHC) detection of

FKBP5 was performed in 6-month-old *Men1N* and *Men1INT* mice. Menin IHC detection in corresponding sections is shown in upper panel. Scale bar = 25 μ m. **d** Quantitative RT-qPCR analyses of *MEN1*, *AR*, and prostate-specific antigen (*PSA*) gene expression in LNCaP cells treated with siRNA ctrl or three different siRNAs targeting the *MEN1* gene (siMEN1 (1), (2), and (3)). Lower graph: Quantitative RT-qPCR analyses of *AR* and *PSA* gene expression in LNCaP treated with DMSO or with the menin inhibitor MI503 at three different concentrations, 1, 2.5, and 5 μ m. Data shown, normalized against GAPDH, are presented as means (\pm SEM) of technical replicates from one representative experiment out of three. **e** Western blot analysis of menin, AR, PSA, and TMRPSS2 expression in LNCaP and 22Rv1 cells treated with siRNA control (siCtrl) or siRNA targeting the *MEN1* gene (siMEN1 (1)), GAPDH was used as a loading control. **f** ChIP-quantitative PCR analyses of AR binding on the regulatory sequences of *KLK3*, *KLK2*, and *FKBP5* genes.

expression of the neuroendocrine cell marker chromogranin A was absent from menin-deficient cells (Fig. S8), suggesting that these cells were not NE⁺ cells.

A reduced AR expression has recently been linked to the appearance of prostate cell populations expressing molecular markers reminiscent of stem cells [17, 18]. We

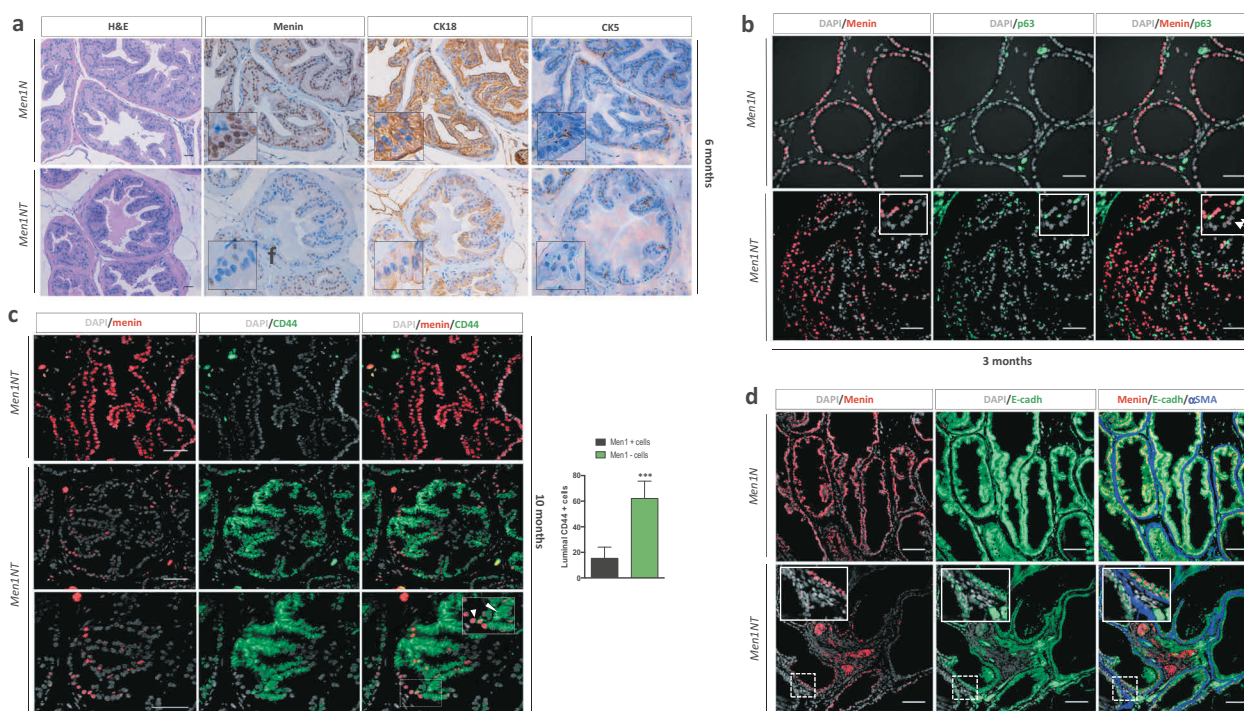


Fig. 3 *Men1* inactivation in *Nkx3.1*-deficient prostate cells led to cell dedifferentiation. **a** H&E staining (left column) and IHC against menin, CK18, and CK5 on *MenIN* and *MenINT* mouse prostate sections at 6 months of age. **b** Double IF staining against menin and p63 with DAPI counterstaining on prostate sections from *MenIN* (upper panel) and *MenINT* (lower panel) at 10 months of age, showing no evidence of p63 expression in menin-negative luminal cells. **c** Triple IF staining against menin, E-cadherin (E-cadh), and α -SMA with DAPI counterstaining on prostate sections from *MenIN* (upper

panel) and *MenINT* (lower panel) at 10 months of age. Insets show enlarged views of immunostained prostate lesions, illustrating the decrease in E-cadherin expression in some of the *Men1*-deficient cells. Scale bar = 50 μ m. **d** Double IF against CD44 and menin in *MenIN* and *MenINT* mouse prostate tissues at 10 months of age, inset being an enlarged view. The histogram presents the quantification of CD44 staining in menin-positive and -negative cells from *MenINT* mice ($n = 6$). Scale bar = 50 μ m. All of the data are presented as means \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

therefore analyzed the expression of several of these markers. IF analyses revealed that, although no CD44 expression could be detected in prostate glands of control mice, or in PIN lesions from double-mutant mice 1.5 and 4.5 months after Cre recombinase induction, surprisingly, prominent CD44 expression was detected in the majority of *Men1*-deficient prostate cells from all *MenINT* mice 8.5 months after the induction. Indeed, the percentage of CD44-expressing cells reached around 60% of *Men1*-deficient prostate cells (Fig. 3d). However, other stem cell-related markers, including CD133, α 2 β 1 integrin (CD49b), and CD49f, remained undetected (data not shown).

Menin participates in the regulation of AR expression and of cell proliferation in AR⁺ cell lines

To further study AR regulation by menin revealed by the above mentioned observations made in the mouse model and human PCa cell lines, we sought to define the occupancy of menin on the AR promoter by CHIP analysis. We uncovered a significant menin-enriched region on the proximal part of the AR promoter encompassing the -1500

to +550 bp region flanking its transcription start site, menin-occupancy signal diminishing drastically when cells were pretreated with siMEN1 (Figs. 4a, upper left panel, and S9). Importantly, we found that the same region was highly methylated at H3K4me3 and was co-occupied by one of the COMPASS-like components ASH2L, suggesting that the complex binds to this region (Fig. 4a, lower left panel). Moreover, when the cells were treated with MI503, the CHIP signals obtained with anti-menin and H3K4me3 antibodies were significantly reduced, further confirming the involvement of the menin-MLL complex in this region (Fig. 4a, right panel). Our data thus demonstrated, to our knowledge for the first time, that menin regulates AR transcription, most likely via its binding to the AR promoter region, mediated by the MLL complex.

Considering the crucial role played by AR in the differentiation and proliferation of normal prostate cells and in PCa, we further investigated the effects of menin inactivation on cell growth in AR-dependent (LNCaP, 22Rv1, and VCaP) and AR-independent (DU145 and PC3) cell lines, as well as in a PC3 cell line constitutively expressing exogenous AR (PC3-AR). Our analyses showed that *MEN1* KD with

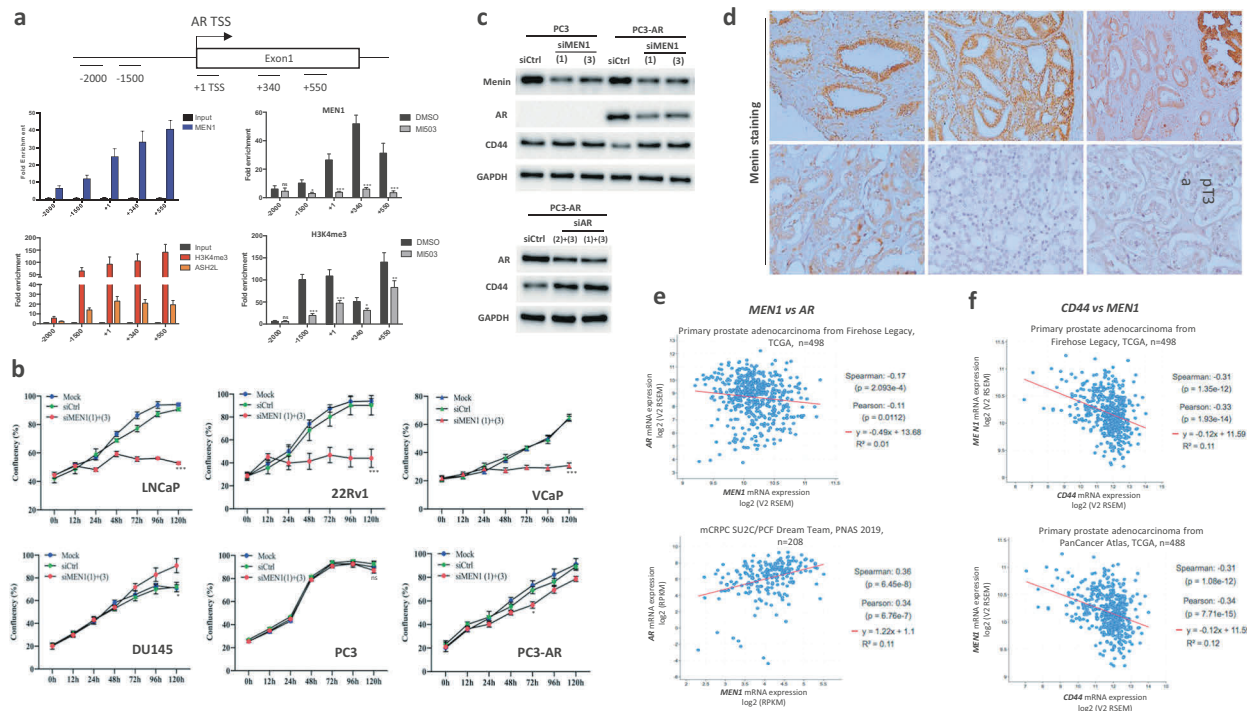


Fig. 4 Menin binds to the proximal AR promoter and is crucial to cell differentiation and proliferation of AR-dependent cells. **a** Upper graph: ChIP analysis was used to define the binding of menin and menin–MLL complex to the AR promoter. Quantitative PCR analyses of menin and H3K4me3 binding on the –2000 bp/+550 bp area flanking the AR gene transcription start site (TSS) in LNCaP cells (left panel) and in LNCaP cells treated with DMSO or MI503 at 5 μ M (right panel). **b** Cell growth of LNCaP, 22Rv1, VCaP, DU145, PC3, and PC3-AR cells treated with siRNA ctrl or siMEN1 (1 + 3). Data shown are mean (\pm SEM) of technical replicates from one representative experiment out of three. **c** Western blot analysis of menin, AR,

siMEN1 resulted in a significant decrease in cell proliferation in all three AR-dependent lines, reaching levels similar to those observed using siAR, but, surprisingly, not in the two AR-independent cell lines (Figs. 4b and S10). Even a slight increase in cell proliferation could be noted in DU145 cells: ~1.4-fold at 120 h after siMEN1 treatment. Conversely, we observed a slight but significant decrease in cell proliferation in PC3-AR cells treated with siMEN1. Our data suggest that the previously reported role played by menin in promoting cell proliferation [12] may vary among different PCa cell lines with distinct activities of the AR pathway. Intriguingly, we observed that the treatment of DU145 and PC3 cells with MI503 resulted in a decrease in cell proliferation, unlike siMEN1-treated DU145 and PC3 cells, likely due to an effect unrelated to reduced menin expression (Fig. S11).

MEN1 KD leads to increased CD44 expression in PC3 cells re-expressing AR

A previous work reported reduced CD44 expression in PC3 cells re-expressing AR, suggesting AR expression could

and CD44 expression in PC3 and PC3-AR cells treated with siRNA control (siCtrl), siRNAs targeting the *MEN1* gene (siMEN1 (1) and (3), upper panel), or siRNAs targeting the *AR* gene (lower panel). GAPDH was used as a loading control. **d** Representative images of menin IHC detection on human PCa. The upper left image shows menin staining seen in nontumoral prostate glands; other five images taken from different tested PCa with various levels of menin expression. Data mining analyses investigating the correlation between *MEN1* and *AR* mRNA expression (**e**) and between *MEN1* and *CD44* mRNA expression (**f**) using existing PCa datasets.

inhibit CD44 expression in this context [19]. Since our data revealed the role played by menin in AR regulation, we speculated that ectopic CD44 expression observed in the prostate lesions developed in double *Men1/Nkx3.1*-deficient mice could be, at least partially, due to the reduced AR expression triggered by *Men1* deficiency. To gain further evidence to support our hypothesis, we carried out *MEN1* downregulation by siMEN1 in both PC3 cells and a PC3 cell line with reconstituted AR expression (PC3-AR), and subsequently analyzed menin, AR, and CD44 expression. Unlike PC3 cells, *MEN1* KD PC3-AR cells displayed both reduced menin and AR, but interestingly, increased CD44 expression (Fig. 4c, left panel). We noticed, as expected, that AR KD using three different siAR, with two targeting coding sequence, resulted in similar CD44 overexpression in PC3-AR cells (Fig. 4c, right panel).

Menin expression diverges in different human PCa

We further analyzed menin expression in 37 human PCa with different clinical features, including 14 low-grade and

less advanced cases, 14 high-grade and more advanced cases, and 9 cases with castration resistance (CR). Our data showed that, compared to normal prostate glands, nuclear menin staining varied in the human PCa tested, with a reduction in menin expression not only frequently seen in low-grade PCa (9/14, Fig. 4d and Table 2), but also observed in high-grade (7/14) and CR-staging PCa (4/9). However, we noticed that a strong menin expression could be detected in more than half of the CR samples (5/9). To gain further insight into eventual correlations between menin and AR expression, as well as those between menin and CD44 expression in PCa, data mining analyses were carried out using different publicly available PCa mRNA expression datasets (see “Materials and methods”). Interestingly, a significant and a marginally significant positive correlation between *MEN1* and *AR* mRNA expression were seen in two mCRPC datasets, whereas a significant inverse correlation between *MEN1* and *AR* mRNA expression was found in two primary PCa datasets (Figs. 4e and S12a). Remarkably, a significant inverse correlation between *MEN1* and *CD44* mRNA expression was uncovered in two primary PCa datasets and a marginally significant inverse correlation was seen in one of two mCRPC datasets (Figs. 4f and S12b).

Discussion

The current work highlights the oncosuppressive role played by *MEN1* in the initiation of prostate cell tumorigenesis involving the dysregulated AR pathway and epithelial luminal cell dedifferentiation. The data provide unprecedented clues as to the role played by menin in regulating AR expression, and consequently, influencing the AR signaling pathway.

Specific *Men1* disruption in *Nkx3.1*-deficient prostate cells led to the acceleration of tumorigenesis, both for its initiation and development of advanced lesions. The observation is in line with our previous findings obtained in male *Men1* heterozygous mutant mice and with the *MEN1* mutations found in sporadic human PCa [5, 11]. The current work hence provides further genetic proof of a tumor suppressive role for *MEN1* in the initiation of prostate cell tumorigenesis. The characterization of *Men1*- and *Nkx3.1*-deficient PIN lesions indicates that the affected cells lose their typical luminal cell characteristics and display some dedifferentiation features. Indeed, from the early-stage onward, the lesions developed in *Men1^{INT}* mice showed downregulation of both AR and its target genes, and a general decrease in CK18 expression, as well as a reduction in E-cadherin expression. Importantly, the characteristics of these lesions are reminiscent of recent findings in human PCa in which undifferentiated PSA^{low} tumors were

Table 2 Menin expression is decreased in numerous prostate tissue sections of PCa patients.

Patient	Stage	Gleason score	Menin expression
013CHI	pT2c	6 (3 + 3)	Low
015FRE	pT2c	7 (3 + 4)	Absent
024TAP	pT2c	6 (3 + 3)	Low
027LHO	pT2c	7 (3 + 4)	Absent
031GEO	pT2c	7 (3 + 4)	Low
043F0N	pT2c	6 (3 + 3)	Low
044COU	pT2c	7 (3 + 4)	Low/Normal
054BRY	pT2c	7 (3 + 4)	Low
055SER	pT2c	7 (3 + 4)	Low
082MAD	pT2c	7 (3 + 4)	Low
005FRA	pT4	8 (4 + 4)	Absent
016BEN	pT3b	9 (4 + 5)	Absent
022SAN	pT3a	8 (4 + 4)	Low/Normal
028NAH	pT3a	8 (4 + 4)	Absent
029BAF	pT4	9 (4 + 5)	Low
034LIT	pT3a	8 (4 + 4)	Low
049SAR	pT3b	8 (4 + 4)	Absent
052PEI	pT3a	8 (4 + 4)	Low
063GIR	pT3a	8 (4 + 4)	Low
065GUI	pT3a	9 (4 + 5)	Low
179RIB	HR	N/A	Low
181MAN	HR	N/A	Absent
184GEH	HR	N/A	Normal
186MBA	HR	N/A	Absent
187FLE	HR	N/A	High/Normal
189DIO	HR	N/A	High/Normal
193CAS	HR	N/A	Normal/low
211CEV	HR	N/A	Normal/Low
212JAC	HR	N/A	Normal
213DRO	HR	N/A	Normal

Quantitation of menin intensity staining based on a high, normal, and low expression has been assessed in the sample with normal prostate and different tumor grades according to Gleason’s pattern scale. Quantitation of menin intensity staining by IF analysis based on a high, normal and low expression has been assess in the sample with normal prostate and different tumor grades according to Gleason’s pattern scale.

observed among both advanced primary PCa (GS9/10) and CRPC [20]. Interestingly, Beltran et al. described an AR-indifferent cell state as a mechanism of treatment resistance through divergent clonal evolution implying epigenetic modifiers [21]. Our mouse model may provide a useful tool for improving the characterization of AR^{low} PCa, and addressing the mechanisms involved in the development of PCa related to the complex dysregulation of the AR pathway caused by its activation and/or inactivation [22].

Our work provides compelling evidence, to our knowledge for the first time, that menin critically regulates AR

transcription, likely through its binding to the proximal AR promoter together with MLL complex. Interestingly, in different PCa cell lines, the effect of AR regulation by menin seems to be correlated with the effects of menin on cell proliferation. Of note, variable response to *MEN1* KD in different PCa cell lines was already observed [23]. Our data imply that cellular and pathological consequences of menin inactivation in prostate cells vary according to the activity of the AR pathway. It provides meaningful explanation for the dual role of menin seen in our experiments, on the one hand, as a tumor suppressor in *Men1* mutant mice, and on the other hand, as pro-oncogenic factor in AR⁺ cell lines. Although AR is known for its vital function as a transcription factor, how the transcription of its own expression is regulated remains elusive. Further investigations into the regulation of the AR pathway by menin, should shed light on this issue, as well as on the crosstalk between AR and menin in prostate cell tumorigenesis.

Intriguingly, *Men1*-deficient late-stage lesions massively expressed CD44. However, our data do not seem to suggest that *Men1*-deficient cells were converted into cancer stem cells. Firstly, the activation of CD44 was detected in the majority of the cells in more advanced prostate lesions, whereas no CD44⁺ cells were detected in early PIN lesions. Furthermore, the expression of other stem cell markers was not found in the same lesions. Instead, CD44 expression may represent a more advanced dedifferentiation of luminal cells, providing them with more proliferative and invasive capacities, as suggested by Patrawala et al. [24]. It is worth mentioning that the inverse correlation between menin and CD44 expression found in primary human PCa is in line with the observations made in our mouse model. Interestingly, our data demonstrated that the observed ectopic CD44 expression is at least partially related to the reduced AR expression triggered by *Men1* disruption. It would be interesting in the future to investigate whether the particular AR^{low}/CD44⁺ phenotype could be found among human AR^{low} PCa.

Consistent with the above observations made in *Men1* mutant mice, reduced menin expression was observed in a substantial proportion of human PCa tested. A more frequent reduction was detected in low-grade PCa arguing in favor of its putative suppressive role in some PCa at the tumor initiation stage. We concomitantly found that a considerable number of CRPC expressed high levels of menin, reminiscent of the proproliferative effects of menin observed in AR-dependent PCa cell lines. Interestingly, the significant positive correlation between *MEN1* and AR mRNA expression were found in two mCRPC datasets, reminiscent of our observations made in three AR-dependent PCa cell lines, all of them derived from mCRPC. In parallel, the inverse correlation between menin and AR in human primary PCa suggests a different role for menin in these PCa compared with mCRPC. However, we

are aware of the heterogeneous nature of primary PCa samples and the possibility that the analysis could be biased by low proportions of AR^{low} PCa present among the samples used. Together with our observations made in different experimental models, our data indicate that menin could play distinct roles in different PCa, likely in relation with the AR pathway and in a spatiotemporal-specific way.

Collectively, the specific *Men1* disruption in prostate cells of *Men1^{INT}* mice resulted in the occurrence of early AR^{low} mPIN and late AR^{low}/CD44⁺ mPIN and MIC, further suggesting the oncosuppressive role of the *MEN1* gene in the initiation of prostate cell tumorigenesis. In parallel, the current work, revealing the regulatory role played by menin on AR and the dysregulation of the AR pathway triggered by *MEN1* inactivation in AR-positive PCa cells, may have implications for new PCa treatment strategies.

Materials and methods

Human patient samples

Human paraffin embedded prostate tissues were obtained from Biological Resources Platform at Henri Mondor hospital (CPP no. 16169). The study was conducted in accordance with the guidelines in the Declaration of Helsinki.

Animals

Men1^{F/F} (on a mixed C57B:6 and 129/SvJ background) were generated, bred, and maintained as previously described [14]. *Nkx3.1Cre^{ERT2-/+}* mice were provided by the Jackson Laboratory, and bred with *Men1^{F/F}* mice to generate *Men1^{F/F}-Nkx3.1Cre^{ERT2-/+}* mice, as well as *Men1^{+/+}-Nkx3.1Cre^{ERT2-/+}* mice. Male *Men1^{F/F}-Nkx3.1Cre^{ERT2-/+}* mice were treated by intraperitoneal injection of 100 µl tamoxifen at 10 mg/ml daily for 5 consecutive days at 1.5 months of age after birth, and control mice were injected with oil. Prostatic glands were subjected to pathological examination and other analyses at 1.5, 4.5, and 8.5 months after injection. Animals were maintained in a specific pathogen-free animal facility AniCan at Cancer Research Center of Lyon at Center Léon Bérard, Lyon, France.

Cell culture and treatment

All the PCa cell lines were purchased from ATCC, except the stable cell line PC3-AR constitutively expressing an exogenous AR as described [25]. Their authentication was renewed recently and *Mycoplasma* testing was carried out regularly. LNCaP, 22Rv1, and DU145 were cultured in RPMI medium, and PC3 and PC3-AR cells in F-12 medium (ThermoFisher Scientific) containing 25 mM glucose and

supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 unit/ml penicillin, 100 µg/ml streptomycin, HEPES 10 mM, and sodium pyruvate 1 mM, at 37 °C with 5% CO₂. Inhibition of menin–MLL interaction was achieved through the use of MI503 (Active Biochem), at the concentration of 0.5, 2.5, 5, and 10 µM.

Cell proliferation assay

Cells were seeded onto 96-well culture plates at 5×10^3 cells for 22Rv1, DU145, and PC3, and at 1×10^4 cells for LNCaP. Cell morphology, attachment, spreading, and proliferation were monitored every 30 min for 0–120 h by IncuCyte ZOOM Live-Cell Analysis system (Essen BioScience), which was placed in a humidified incubator maintained at 37 °C with 5% CO₂. After 24 h cells culture, cells were also transfected with 20 nm siRNA against *MEN1* and *AR* using INTERFERin siRNA transfection reagent (#409-10, Polyplus-transfection) according to the manufacturer's protocol. All the experiments were performed in triplicate and at least three times.

Real-time reverse transcriptase-PCR

RNA were extracted using RNeasy-Kits (Qiagen, Valencia, USA). Real-time PCR analyses were carried out on a Step-One RT-System (Applied Biosystem, France) using SYBR-Green (ThermoFisher) and results of each samples normalized to *36B4*.

Immunostaining

Prostate tissues were collected and fixed in 4% PFA prior to paraffin embedding, sectioning, and staining with hematoxylin and eosin according to a standard protocol. Briefly, endogenous peroxidases were quenched in 3% H₂O₂ solution for 30 min at room temperature. Heat-induced epitope retrieval was performed by immersion in antigen-unmasking solution (catalog no. H-3300; Vector Laboratories) in a microwave oven for 15 min. After blocking with antibody diluent (Dako), sections were incubated overnight with a primary antibody. For double IF, stains were detected with a Cy3 or Cy5 tyramide amplification kit (PerkinElmer), according to the manufacturer's instructions, with prior incubation with the appropriate biotinylated secondary antibody or incubation with appropriate Alexa Fluor 488, 555, coupled secondary antibodies (Life Technologies) for 1 h.

ChIP-qPCR assay

Chromatin for ChIP was prepared from five million LNCaP cells. Briefly, cells were fixed in 1% formaldehyde for

10 min, nuclei were obtained and lysed in 300 µl ice-cold RIPA buffer prior to chromatin-DNA shearing with a Diogene Bioruptor. ChIP was performed using 5 µg of anti-menin antibody (A300-105A, Bethyl Laboratories Inc., USA), anti-H3K4me3 (C15410003, Diagenode, Belgium), and anti-ASH2L (D93F6, Cell Signaling). Dynabeads® Protein G (10003D, Life Technologies, France) was used to retrieve Immunocomplexes. DNA was then extracted with phenol/chloroform/isoamyl alcohol, following proteinase-K reverse crosslinking. Two microliters of IP-DNA or two nanograms of INPUT DNA were used for real-time PCR analysis to quantify coprecipitated chromatin DNA. Relative enrichment of a given promoter region obtained with a specific antibody was compared with Input DNA. The blocks region on Chr1 was used as a negative control for menin, ASH2L, and H3K4me3 ChIPs.

Statistical analyses

Statistical analyses were performed as described in the figure legends; unpaired Student's *t* tests were used unless otherwise indicated. All analyses were done using Prism 5 software (GraphPad, USA); *P* value < 0.05 was considered significant. Results are given as means ± standard errors of the means (SEM).

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Author contributions RT and YL conducted the experiments, analyzed and interpreted the data, and prepared figures and manuscript; MD-P, VF, and FV supervised the development of the tissue banks, and performed pathological analyses and data interpretation; VV-G, RAZ and RB provided technical and material support and participated in data interpretation; NG and IG provided material and methodological support and participated in data interpretation; PB and MLR participated in study design and provided critical revision of the manuscript; and CXZ conceived and supervised the study and manuscript preparation and obtained funding.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The specimens of patients with PCa were conducted according to French laws and regulations. All animal experiments were performed in accordance with the animal care guidelines of the European Union and were validated by the local Animal Ethic Evaluation Committee (CECCAPP: C2EA-15 agreed by the French Ministry of High School and Research, Autorisation de projet CLB-2012-053).

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Review

Involvement of the *MEN1* Gene in Hormone-Related Cancers: Clues from Molecular Studies, Mouse Models, and Patient Investigations

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Abstract: *MEN1* mutation predisposes patients to multiple endocrine neoplasia type 1 (MEN1), a genetic syndrome associated with the predominant co-occurrence of endocrine tumors. Intriguingly, recent evidence has suggested that MEN1 could also be involved in the development of breast and prostate cancers, two major hormone-related cancers. The first clues as to its possible role arose from the identification of the physical and functional interactions between the menin protein, encoded by *MEN1*, and estrogen receptor α and androgen receptor. In parallel, our team observed that aged heterozygous Men1 mutant mice developed cancerous lesions in mammary glands of female and in the prostate of male mutant mice at low frequencies, in addition to endocrine tumors. Finally, observations made both in MEN1 patients and in sporadic breast and prostate cancers further confirmed the role played by menin in these two cancers. In this review, we present the currently available data concerning the complex and multifaceted involvement of MEN1 in these two types of hormone-dependent cancers.

Keywords: breast cancer; prostate cancer; estrogen receptor alpha; androgen receptor; the *MEN1* gene

1. Introduction

The most frequently encountered hormone-dependent cancers are breast and prostate cancers. The prevalence of breast cancer (BC) has increased such that its incidence is ranked second after lung cancer among cancers occurring in women [1], with 18.1 million new cases and 9.6 million cancer deaths in 2018. Similarly, prostate cancer (PCa), with its 174,650 new cases and 31,620 deaths estimated in 2019 in the USA alone [2], continues to represent a major cause of cancer-related mortality and morbidity in men. Hence, their global health burden is enormous, especially in developed countries, where their incidence is increasing [3]. Intriguingly, several lines of evidence have recently suggested that the tumor suppressor gene *MEN1*, the mutation of which predisposes patients to multiple endocrine neoplasia type 1 (MEN1, OMIM131100), may be involved in the development of these two cancers. In this review, we present the currently available data concerning the seemingly complex and multifaceted implications of *MEN1* in these two types of hormone-dependent cancers. We believe that a better understanding of the role played by *MEN1* should provide useful insights,

not only into the mechanisms underlying the development of these two cancers, but also into their treatment, and may provide new markers for their diagnosis and prognosis.

2. Background about Breast and Prostate Cancers

2.1. Histopathology and Classification

2.1.1. Breast Cancer

BC is histologically divided into two subtypes based on its invasive features—in situ carcinoma or invasive (infiltrating) carcinoma. BCs can also be divided into ductal or lobular types, depending on the tissue of origin, whether arising from the inner wall of the mammary ducts or the mammary glands, respectively [4]. More recently, a classification based on molecular markers such as estrogen receptor alpha (ER α), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) was purposed to facilitate the diagnosis and treatment of the four main subtypes. These include: (i) Luminal A, which represents approximately 40% of diagnosed BCs, is ER α -positive, PR-positive, or both; HER2-negative; Ki-67-low; and is associated with a slow proliferation and a significantly good prognosis, being sensitive to hormonotherapy. (ii) Luminal B is ER α -positive, PR-positive, or both; either HER2-positive or -negative and Ki-67-high; and has a worse prognosis than the luminal A subtype. (iii) The HER2-enriched subtype is ER α - and PR- negative, HER2-positive, and more aggressive than luminal subtypes. (iv) Triple-negative breast cancers (TNBCs) are ER α -negative, PR-negative, and HER2-negative [5], and are the most aggressive subtype, with the worst prognosis. Recent studies have further attempted to divide this classification into six subtypes by including basal-like and androgen receptor (LAR) subtypes, the latter displaying a high level of androgen receptor (AR) expression and an enrichment in AR signaling [6]. Treatments for BC including surgery, radiotherapy, chemotherapy, hormonotherapy, and rapidly developing targeted therapies, depend on the BC subtypes. ER α -positive BC subtypes are the most sensitive to hormonotherapy using either selective estrogen receptor modulators (SERMs) or selective estrogen receptor downregulators (SERD), whereas the treatment for HER2-enriched BCs has been greatly improved owing to therapies targeting the HER2 receptor. Unfortunately, there are very limited therapeutic options for TNBCs, although inhibitors of poly (ADP-ribose) polymerase (PARP) have shown promising results [7].

2.1.2. Prostate Cancer

PCa classifications mainly revolve around the Gleason grading system, based entirely on the histological pattern of carcinoma cells in Hematoxylin and Eosin (H&E)-stained prostate tissue sections [8,9] and the local disease state [10]. Aberrant signaling in the androgen pathway is critical in the development and progression of PCa. Androgen deprivation therapies (ADT) are the frontline treatment for PCa [11,12]. Although highly effective, ADT are characterized by the predictable emergence of resistance, termed castration-resistant PCas (CRPCs) [13,14], with a high mortality rate [15]. Genomic characterizations of CRPCs have led to the subdivision of CRPCs into two subtypes: (1) AR-dependent CRPCs, containing alterations in the *AR* gene, such as amplification, point mutations, and generation of splice variants; and (2) AR-independent CRPCs, in which resistant cells or metastatic CRPC (mCRPC) lack AR expression or signaling. The latter subtype has recently been reported to be associated with cellular plasticity and neuroendocrine (NE) molecular features. Importantly, there are mCRPCs that neither express the AR nor markers of NE differentiation (“AR null–NE null”) [16,17]—their incidence has risen over the past 2 decades from 5% in 1998–2011 to 23% in 2012–2016 [18]. Neuroendocrine prostate cancer (NEPC) displays a more complex spectrum of phenotypes, ranging from anaplastic carcinomas to pure small-cell carcinomas (SCCs) [18,19]. Several studies [20–22] have shown that 10–20% of lethal PCa display SCC features with a very poor prognosis [23,24].

2.2. Estrogen Receptor-Alpha and Androgen Receptor

2.2.1. Estrogen Receptor-Alpha (ER α)

Structure

ER α belongs to the steroid-stimulated nuclear receptors, which are transcriptional factors involved in regulating the transcription of hundreds of target genes [25]. The gene encoding ER α is called *ESR1*. This gene is highly conserved, localized on chromosome 6q25.1, and composed of 8 exons on a 140 kb genomic locus (Figure 1, upper panel) [26]. ER α consists of 595 amino acids, with two transactivation domains AF1 and AF2 located in the N-terminal domain (NTD) and domain E, respectively. The NTD is involved in both inter-molecular and intra-molecular interactions, as well as in the regulation of gene transcription, while the DNA-binding domain (DBD) allows ER α to dimerize and to bind to specific estrogen response element (ERE) sequences on DNA. The hinge domain (D region) containing the nuclear localization sequence (NLS) plays a role in receptor dimerization and in binding to chaperone heat shock proteins (Hsp). The ligand-binding domain (LBD, E/F region, C-terminal) comprises the E₂-binding domain and works synergistically with the NTD in the regulation of gene transcription (Figure 1, upper panel) [27]. At least 2 isoforms of ER α have been identified: ER α -46, lacking the AF1 domain [28]; and ER α -36, devoid of both transcriptional activation domains (AF1 and AF2) and localized in both the plasma membrane and cytoplasm, where it mediates non-genomic ER α signaling [29,30]. *ESR1* mutations, such as *ESR1* amplifications or point mutations, were found in endocrine-therapy-resistant breast tumors, and occur predominantly in the LBD, leading to constitutive hormone-independent activation of ER α [31,32].

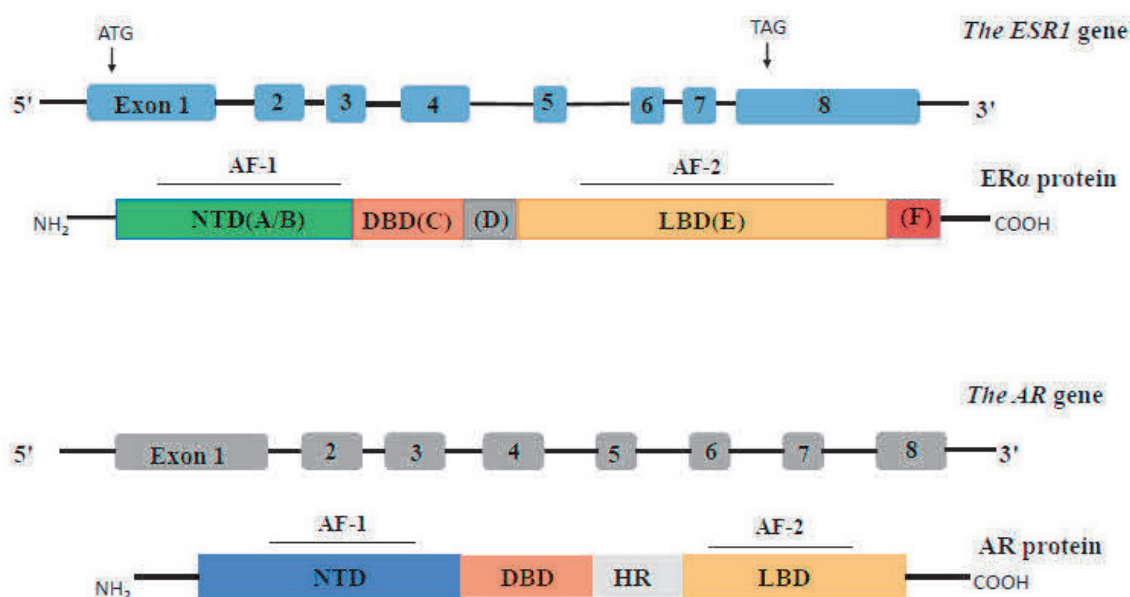


Figure 1. Schematic representation of the structure of the *ESR1* gene and the *AR* gene and their proteins. Different protein domains are indicated, including the N-terminal domain (NTD), the DNA binding domain (DBD), the hinge domain (D for ER α protein and HR for AR protein), and the ligand binding domain (LBD).

ESR1 Gene Regulation

Several studies have demonstrated that the *ESR1* promoter is positively or negatively regulated by epigenetic factors. In 1994, Ottaviano et al. showed that the lack of ER α expression in ER α -negative BC cell lines was due to the hypermethylation of *ESR1* CpG islands [33]. It was subsequently shown that the *ESR1* promoter was occupied by several complexes with inhibitory components such as DNA methyltransferases (DNMTs) and histone modifiers such as HDAC1 and msin3A [34,35].

Many studies have also reported that the *ESR1* promoter was occupied by several transcription factors, including members of the AP1 [36] and Forkhead box (FOX) family (FOXO3A [37], FOXM1 [38]), as well as metastasis-associated protein 1 (MTA1) and Twist [39]. The most extensively described ER α -associated transcription factors are GATA3 [40] and FOXA1 [41], which activate the transcription of the *ESR1* gene and are necessary for its proper functioning [40,42,43]. Recently, a study also revealed a regulation of the *ESR1* distal promoter by a loop-like complex involving GATA3, FOXA1, and menin [44]. In this study, they showed that menin binds to the *ESR1* enhancer region at sites that are also bound by FOXA1 and GATA3, and recruits the mixed lineage leukemia (MLL) compass-like complex containing MLL1/2, menin, ASH2L, RBBP5, and WDR5 [45] to these sites, thus forming a complex and regulating its expression.

Gene Targets and Gene Functions

Estrogens, through the ER α signaling pathway, play important and various developmental, physiological, and pathological roles. ER α is essential for the normal development of the female reproductive tract, including the uterus and the ovaries, as well as the proliferation and differentiation of mammary glands [46]. Furthermore, ER α plays a role in male fertility and in other non-reproductive organs, such as the neuroendocrine and cardiovascular systems and bone metabolism [46,47]. Estrogens can bind to ER α in the cytoplasm and causes their release from bound chaperones, dimerization, and their nuclear translocation, where they bind to ERE and regulate transcription of downstream ER α genes, triggering the “genomic signaling pathway”. ER α can also indirectly bind to promoters via protein–protein interactions, activating a variety of transcription factors, such as the activator protein (AP)-1 or the nuclear factor- κ B (NF- κ B) [48]. Finally, estrogens can bind to ER α in the plasma membrane, thus inducing the “non-genomic pathway”.

Among the thousands of ER α target genes, one of the earliest identified was *pS2/TFF1* [49,50], followed by many genes that were discovered by monitoring the global expression changes upon estradiol induction [51–55]. ER α target genes display a wide variety of functions, such that they can be divided into (i) pro-proliferative genes, such as *Cyclin D1* [56], *cMyc* [57,58], and *IGF-1* [59]; (ii) anti-apoptotic factors, such as *TIT-5* and *EIT-6* [55]; (iii) enzymes, such as the lysosomal proteinase cathepsin D [60]; (iv) and nuclear receptors, such as progesterone receptor [61], in addition to many other genes of as yet unknown function. Interestingly, these global expression experiments indicated that approximately half of ER α target genes are downregulated upon estrogen induction, reinforcing the view that estrogen promotes cell survival by downregulating pro-apoptotic genes.

2.2.2. Androgen Receptor (AR)

Structure

The *AR* gene is located on chromosome X (Xq11–12) and consists of 8 exons coding a protein of about 110 kDa (Figure 1, lower panel). The full-length AR has four domains, namely from the N-terminal, the NTD, the DBD, the hinge domain, and the LBD [62,63]. The NTD includes the transcriptional regulatory domain AF1, while the LBD includes AF2. Over 20 splice variants of the AR have been reported in the last 2 decades [64]. Most of them are lacking the C-terminal region containing the LBD [65,66] and are, therefore, functionally active independently of the presence of androgens. Among them, AR-V1 and AR-V7 are the most abundant variants [65]. Somatic AR mutations may occur selectively in response to androgen deprivation [67]. A review of 27 clinical studies revealed that AR mutations in androgen-dependent tumors ranged from 2 to 25%, while the incidence in CRPC tumors was slightly higher at 10–40% [67,68]. Furthermore, the AR LBD was described as a mutational hotspot, placing the incidence of its point mutations in CRPC at ~15–20% [69,70].

AR Gene Regulation

A better understanding of the regulation of *AR* transcription is crucial for studying prostate cell tumorigenesis. SP1, a zinc finger transcription factor, binds to GC-rich motifs of the *AR* promoter and activates the transcription of *AR*, whereas the associated antagonistic transcription factor pur- α can bind to the same region and inhibit *AR* transcription [71]. More recently, Deng et al. demonstrated that PRMT5 promotes prostate cancer cell growth by epigenetically activating the transcription of *AR* in prostate cancer cells. PRMT5 binds to the proximal promoter region of the *AR* gene and mainly contributes to the enriched symmetric dimethylation of H4R3 in the same region. Mechanistically, PRMT5 is recruited to the *AR* promoter upon its interaction with Sp1, forming a complex with Brg1, an ATP-dependent chromatin remodeler [72]. In addition, Grad and colleagues found that *AR* is regulated by *AR* itself in osteoblast-like U2OS cells. Indeed, two androgen response elements (AREs) were identified in exons 4 and 5 of the *AR* gene that were responsible for the androgen-mediated upregulation of *AR* mRNA [73].

Gene Targets and Functions

AR, playing a key role in both normal prostate development and prostate cancer, is a hormonal transcription factor. Upon binding to androgens, testosterone, or dihydrotestosterone (DHT), the AR localizes to the nucleus [74,75]. There, the receptor dimers bind to AREs in the promoter regions of target genes, such as prostate-specific antigen (PSA) and transmembrane protease serine 2 (TMPRSS2), to regulate transcription [76]. Similarly to other transcription factors, AR-enhanced transcription depends on the recruitment of RNA polymerase II to its target gene promoter. Some dynamic changes in the state of covalent histone modifications, relying on methyltransferase activities, are related to androgen-stimulated transcription. Fu et al. demonstrated direct interactions between p300, CBP, P/CAF, and AR. Moreover, several signaling pathways are known to enhance AR activity [77], including the EGF, IGF, IL6, Wnt, Ras-Raf-MAP kinase, PI3K/AKT, and MAPK/ERK pathways [75,78–83]. Mounir et al. reported that PMRT5 display inhibitory effects on the transactivation of differentiated genes by AR via AR methylation [84].

For prostate cancer cells, studies have shown that AR is a critical regulator of the G1-S transition in AR-dependent cell cycle progression. Indeed, Xu et al. demonstrated that androgen induces Cyclin D expression via mTOR-dependent enhancement of translation [85]. The p21^{ciP} has been validated as a direct AR target [86], consistent with the findings revealing that p21^{ciP} expression is enhanced in tumors and is correlated with a higher proliferative index and Gleason grade [87,88]. Furthermore, Knudsen et al. showed that androgen depletion induces p27^{Kip1}, which likely contributes to the observed reduction in CDK2 activity [89].

Rokhlin et al. found that androgen and AR signaling could directly regulate p53 to suppress apoptosis. Mechanistically, androgen suppresses TNF- α /Fas-induced apoptosis through the inhibition of p53 expression and caspase-2 activation [90]. Interestingly, Frezza et al. reported that a significant decrease in AR expression leads to an increase in caspase-3 activity in LNCaP and PC-3AR cells, suggesting that AR might suppress caspase-3 expression [91]. Liao et al. also showed that knockdown of AR via siRNA leads to apoptotic death in PCa cells [92]. Blockade of AR degradation and ectopic expression of Bcl-2 or selected caspase inhibitors can suppress this pro-apoptotic activity [93].

Zhao et al. demonstrated that AR can act as a transcriptional repressor to directly inhibit gene expression. This repression is mediated by the binding of AR to AREs, and is facilitated by EZH2-mediated repressive chromatin remodeling [94]. More recently and interestingly, Song et al. revealed that AR upregulated EZH2 expression by binding to the *EZH2* promoter and stimulating its transcriptional activity in hepatocellular carcinoma (HCC) cells. EZH2 overexpression increased H3K27me3 levels, thereby silencing the expression of Wnt signal inhibitors, resulting in the activation of Wnt/ β -Catenin signaling and subsequent induction of cell proliferation and tumorigenesis [95].

3. The Involvement of the *MEN1* Gene in Breast and Prostate Cancers

Multiple endocrine neoplasia type 1 (MEN1) is a hereditary syndrome characterized by the multiple occurrence of endocrine tumors of the parathyroid, pancreas, and anterior pituitary. The large tissue spectrum of the disease, affecting a dozen different endocrine cell lineages [96], indicates that the predisposition gene, *MEN1*, possesses a relevant role in all of the endocrine tissues affected. The *MEN1* gene, the mutation of which predisposes patients to MEN1 syndrome, was first identified in 1997 [97,98], and functional studies have since further improved our understanding of the gene. In particular, both genetic and biochemical experiments suggest that the *MEN1* gene has a large spectrum of expression and that the menin protein encoded by the gene plays multifaceted biological functions in a broad range of different tissues and cells, likely through physical and functional interactions with its numerous protein partners. Menin primarily has a nuclear localization, although it can also be located in the cytoplasm and membranes [99]. Menin may act as an adaptor protein involved in the regulation of gene expression via its physical interaction with several transcription factors, such as JunD, Smad1/3/5, β -Catenin, MafA/B, Foxa2, and P53, as well as epigenetic factors, including KMT2A/2B, Sin3A, and EZH2 [44,100–107]. The interactions between menin and several nuclear receptors were recently unveiled (see below). Importantly, various analyses demonstrated that menin is involved in different cellular activities controlled by many signaling transduction pathways, in particular cell proliferation, cell cycle, and cell death. Finally, the experiments using various *in vivo* models have also revealed that the biological functions of menin extend far beyond endocrine cells to hematopoiesis, adipogenesis, myogenesis, fibrogenesis, or even osteogenesis [108–110].

3.1. Molecular Studies

The first clues as to the possible involvement of menin in BC came from the observation that the menin protein binds physically to ER α . In 2006, Dreijerink et al. revealed that menin, owing to an evolutionarily conserved amino acid sequence LXXLL, could physically interact with several nuclear receptors, such as the vitamin D receptor, RXR, and ER α , and played the role of a cofactor. In the same study, they showed that menin binds to the AF2 domain of ER α and coactivates the transcription of *TFF1*, an estrogen-responsive ER α target gene, through the recruitment of the compass-like complex trimethylating H3K4me3 on the *TFF1* promoter [111]. In 2009, Imachi et al. confirmed the previous results by showing that menin coactivates ER α in an estrogen-dependent manner in the ER α -positive MCF7 BC cell line [112]. A recent study conducted by Dreijerink et al. demonstrated that menin regulates the expression of the *ESR1* gene (as described above) through an upstream enhancer via a looping mechanism that connects the TSS bound menin&MLL1/2 to the enhancer-bound transcription factors GATA3 and FOXA1 [44].

Almost a decade after discovering the interaction between menin and ER α , menin was identified as an important cofactor for AR signaling due to its physical interaction with AR-NTD and the recruitment of the MLL histone methyltransferase complex to AR target genes [113]. Inhibition of menin–MLL interaction with a small-molecule inhibitor (MI) impaired AR signaling and inhibited the growth of castration-resistant tumors in xenograft experiments in mice [113]. Hence, these results suggest that menin can facilitate oncogene activation through AR signaling in PCa (Figure 2).

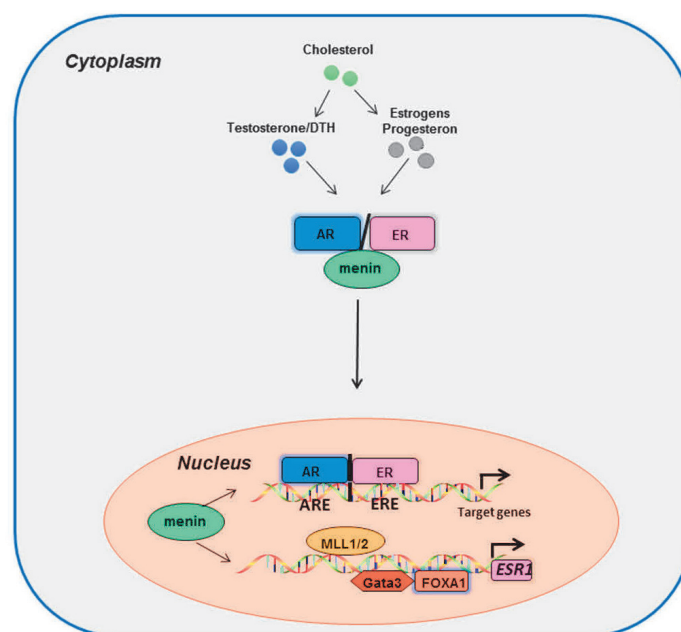


Figure 2. The menin protein interacts physically and functionally with ER α and AR, and is involved in the regulation of ESR1 transcription and the transactivation of the target genes of both ER α and AR.

3.2. Mouse Models

3.2.1. Mammary Gland Lesions in Mouse *Men1* Models

Our team is at the forefront of studies on the role of *MEN1* using *Men1* mutant mouse models. We have observed that aged heterozygous *Men1* mutant mice, in addition to endocrine tumors, developed mammary gland carcinomas in female and prostate cancers in male mutant mice at low frequencies [114]. To further confirm and understand the role of menin in the development of mammary lesions, we generated a conditional mammary-specific *Men1* knock-out mouse model by crossing the mice carrying floxed *Men1* alleles (*Men1^{F/F}*) with *WapCre* transgenic mice expressing Cre recombinase under the control of the whey acidic protein (*Wap*) promoter, which is known to be expressed in luminal mammary epithelial cells. Our results demonstrated that female *Men1^{F/F}-WapCre* mice developed substantially higher amounts of early mammary intraepithelial neoplasia (MIN), which are precursor lesions, in comparison with control *Men1^{+/+}-WapCre* mice. Interestingly, we found that ER α expression and the number of ER α -positive cells were clearly reduced in MIN lesions of mutant mice compared with normal mammary glands. In addition, cell membrane expression of β -Catenin and E-Cadherin was almost absent in the mammary lesions of *Men1^{F/F}-WapCre* mice compared with control mice; neither β -Catenin nor E-cadherin were detected in the TS1 cell line derived from a mouse *Men1* BC [115].

3.2.2. Prostate Lesions in Mouse *Men1* Models

By following a cohort of 47 male heterozygous *Men1* mutant mice (*Men1^{+/-}*) and 23 male wild-type (*Men1^{+/+}*), age-matched littermate mice from 18 to 26 months of age, our group found that six *Men1^{+/-}* mice (6/47, 12.8%) developed prostate cancer, including two adenocarcinomas and four in situ carcinomas, while none of the control mice developed cancerous lesions. No prostate carcinoma was found in age-matched *Men1^{+/+}* littermates (0/23). In addition, these carcinomas exhibited loss of the non-target *Men1* allele (LOH), therefore supporting a tumor suppressor role for the *Men1* gene in prostate glands. Moreover, the AR and p27 expression decreased in tumor lesions, likely facilitating prostate cell tumorigenesis due to *Men1* inactivation [116].

Taken together, all of the data obtained from mouse models suggest a tumor-suppressive role for menin during the initiation and development of murine breast and prostate cancers.

3.3. Human Studies

3.3.1. *MEN1* in Human Breast Cancer

Over the last two decades, several case reports have described breast cancer cases related to *MEN1*. In 2004, a 44-year-old Japanese woman was diagnosed with *MEN1* syndrome, having hyperparathyroidism, primary aldosteronism, and also scirrhous breast carcinoma. The DNA taken from her parathyroid adenoma and breast cancer tissues showed germline *MEN1* mutation at codon 451 in exon 10, which resulted in alanine-to-tyrosine substitution (A541T), as well as LOH [117]. Another study by Jeong et al. reported a case of a patient with both *MEN1*-associated tumors and breast cancer. They found a germline *MEN1* mutation manifested as a 5-bp duplication in exon 3, named c.196_200dupAGCCC, which resulted in a frameshift mutation. In addition, the tested exon 10 showed a polymorphism at codon 423 with substitution of a cytidine to a thymidine (C423T), causing a change of amino acid [118]. More recently, a 41-year old patient with no familial history of breast cancer but with a mother with primary hyperparathyroidism (PHP) was found carrying a variant p.C421R/p.426R in the *MEN1* gene. The patient's histopathological study revealed hormone receptor negativity, as well as HER-2 and p53 negativity. A family study showed positive findings for *MEN1* in a sister, two maternal nephews, and one of the patient's daughters, with no record of breast cancer development in any of these people [119].

Evidence of the likely involvement of menin in BC arose from the observation that female *MEN1* patients were at a higher risk of developing BC [120]. In this study, Dreijerink et al. referred to the Dutch longitudinal *MEN1* database to assess the incidence of BC in *MEN1* patients, and found that out of 190 female patients, the relative risk of invasive BC was 2.83 ($p < 0.001$) and the mean (\pm SD) age at diagnosis of essentially luminal-type BC was 48 ± 8.8 years, compared with an age range of 60 to 65 years in the general population. This feature is often observed in the patients harboring a genetic predisposition. The authors validated their results using 3 other independent *MEN1* patient cohorts from the United States ($p = 0.11$), Tasmania ($p = 0.22$), and France ($p = 0.03$), which provided similar values for relative risk as those obtained in the Dutch cohort, with an average age at diagnosis of 51 years. Furthermore, 8 out of 10 BC samples obtained from Dutch *MEN1* patients displayed more than 50% reduction of menin expression in the nucleus, and subsequent analysis showed loss of heterozygosity at the *MEN1* locus in 3 of 9 tumors. Overall, these observations strongly suggest that *MEN1* mutations could be involved in human breast tumorigenesis as a tumor suppressor.

Concomitantly to our work carried out in mice, we also observed that a substantial proportion of human sporadic BCs displayed reduced menin expression, as observed through the analyses of two series of human BCs [115]. More recently, a study in which the whole-genome sequences of 560 BCs were analyzed highlighted sporadic *MEN1* mutations, albeit at low frequency, as being among driver mutations (such as *BRCA1*, *TP53*, *PIK3CA*, *MYC*, *CCND1*, *PTEN*) in BC [121]. In addition, several other case reports identified *MEN1* mutations among sporadic BC patients, independent or not of germline mutations in *BRCA1* and *BRCA2* genes that are usually associated with hereditary BC [118,122–124].

However, in a clinical study conducted by Imachi et al. with 65 ER α -positive BC samples treated with tamoxifen for 2–5 years as adjuvant therapies, they observed that menin-positive tumors (20 patients) had a worse clinical outcome and were more resistant to tamoxifen than menin-negative tumors (46 patients) [112]. They, therefore, proposed that menin could be a predictive factor of resistance to tamoxifen. Furthermore, they found that raloxifene could inhibit the binding of menin to the AF2 domain of ER α and proposed raloxifene as the therapeutic options for menin-positive and ER α -positive BC [125]. Their works suggest an oncogenic role for menin, which raised the controversy as to its precise role in BC.

3.3.2. *MEN1* in Human Prostate Cancer

Perakakis et al. reported two cases of PCa seen in a *MEN1* family with atypical tumor spectrum [126]. The DNA sequencing analysis revealed a novel mutation—Ser38Cys (TCC > TGC) in exon 2, located in a region of menin that is responsible for interaction with the transcription factor JunD. The latter has recently been associated with prostate cancer.

Only limited sporadic *MEN1* mutations have so far been reported in human sporadic PCa [127]. Manson-Bahr et al. found that missense mutations of the *MEN1* gene were detected in 2 of 8 formalin-fixed prostate needle biopsy materials [125]. Interestingly, Grasso et al. analyzed 58 human CRPC samples by aCGH and found that 17.2% of all samples (10 of 58) harbored mutations in the *MLL* complex, including the *MEN1* gene [69]. *MLL* functions as part of a multi-protein complex containing menin [128]. Many members of the complex have different levels of aberrations in CRPC [69]. Noticeably, Chen et al. analyzed 150 cases for advanced and metastatic human PCa. They observed that the percentage of *PTEN* and *MEN1* co-loss was almost the same as the co-loss of *PTEN* and *PML* (*Promyelocytic Leukemia*), which is around 11% in all cases [129]. Conversely, Paris et al. reported that the *MEN1* locus was amplified in some patients and was predictive of post-operative recurrence [130]. The similar observation was made Kerstin et al. [131]. Moreover, *MEN1* knockdown resulted in a decrease in cell proliferation in DU145 cells [132,133], but curiously not in the PC3 cell line [132].

In total, the current data obtained from human studies suggest that the *MEN1* gene could play a complex even opposite role in the development of human breast and prostate cancers.

4. Further Clues for the Role of Menin in Breast and Prostate Cancers

As we mentioned above, many different factors and signaling pathways are involved in mammary and prostate cell tumorigenesis. By investigating the possible molecular links between the former and menin, we speculated that we might gain further insight into the possible role played by menin in these cancers (Figure 3).

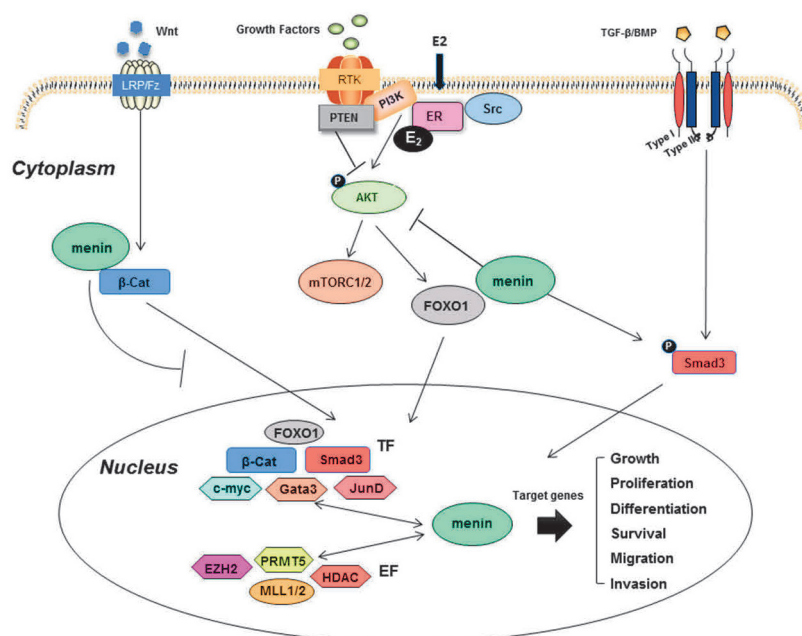


Figure 3. Mechanistic clues underlying the involvement of menin in mammary and prostate cell tumorigenesis. Menin interacts with numerous menin-interacting factors, consequently participating in the regulation of many target genes and interfering with different signaling pathways strongly implicated in breast and prostate cancers. EF: epigenetic factors; TF: transcriptional factors.

4.1. Epigenetic Factors

Interestingly, several epigenetic factors reported to be involved in mammary cell tumorigenesis are known to be partners of menin. Histone methylase MLL1 (*KMT2A*) and MLL4 (*KMT2B*), which are the most characterized partners of menin, were shown to act synergistically with ERs (ER α and ER β) to mediate the estrogen-induced transcriptional activation of the *HOXB9* gene, which is critical for mammary gland development and BC [134]. Menin was also shown to upregulate several members of the same family (mainly *HOXA9* gene) in leukemia by associating with the compass-like complex and lens-epithelium-derived growth factor (LEDGF) [45,135]. The HDAC family, which contains known partners of menin [54,55], is implicated in the regulation of ER α expression, mainly by silencing the *ESR1* gene. It was proposed that HDAC may be responsible for loss of ER α expression in ER-negative BC [34,35]. EZH2 and PRMT5 are two shared partners of menin [100,136,137] and the ER α pathway. Indeed, EZH2 inhibits the transcription of estrogen-responsive genes through its association with the transcriptional corepressor repressor of estrogen receptor activity (REA) [138]. Although there is no direct evidence of the interaction between PRMT5 and ER α , PRMT5 plays an important role in BC by methylating programmed cell death 4 (PDCD4), a tumor suppressive protein with anti-proliferative functions on arginine residue 110 [139].

4.2. Transcription Factors

JunD, a member of the AP-1 family that interacts physically with menin [140,141], has a higher level of expression in BCs [142]. JunD and menin co-expression was found in the mouse submandibular gland, an AR-responsive tissue, with their expression pattern and localization changing with cell differentiation status [143]. Moreover, JunD physically binds to ER α and facilitates its binding to target genes [36]. Intriguingly, it has been shown that prostaglandin E₂ (PGE₂) induces JunD and JunB expression, resulting in the activation of the aromatase promoters I.3/II, while JunD and c-Jun mediate the suppression of the aromatase promoter I.4, leading to high levels of local estrogen, and thus to BC progression [144]. JunD is crucial for cell proliferation in PCa cells, as it controls cell cycle regulatory genes [145,146]. Their analyses further suggest that the essential role played by JunD in prostate cancer cell proliferation is mediated by *MYC* signaling [147]. Furthermore, Mehraein-Ghomi et al. highlighted JunD as an AR co-activator, as it triggers the oxidative stress pathway in prostate cancer cells by regulating the *SSAT* promoter, which produces large amounts of metabolic reactive oxygen species (ROS) [148].

Another important factor is *cMyc*, a well-known estrogen-regulated oncogene [149,150], which is overexpressed in approximately 20–30% of BCs [151] and has also been shown to interact with ER α to modulate estrogen-mediated signaling [152]. The *cMyc* overexpression in PCa has been a well-recognized phenomenon since 1986, when Fleming et al. showed a significantly higher level of its expression in adenocarcinoma of the prostate than in benign prostate hyperplasia by Northern blotting [153]. Furthermore, Sato et al. reported that *cMyc* amplification is strongly associated with higher histopathological grades and Gleason scores, as well as with earlier disease progression and cancer-associated death [154]. It is now known that *cMyc* is a partner of menin and that they collaborate to either activate or repress the expression of certain genes. The most recent report shows that menin can directly interact with the transactivation domain (TAD) of *cMyc*, and that they in turn bind to E boxes to enhance the transcription of *cMyc* target genes [155]. Interestingly, menin can interact with the *cMyc* promoter to regulate its transcription in HEK293 cells [156].

Finally, menin was recently shown to interact with GATA3 and FOXA1 [44] in BC to regulate the *ESR1* promoter (see details above), both of which are markers of luminal BC, especially for the luminal A subtype [42,43,146], and which are highly associated with ER α and are required for the proper function of most of its target genes [40,157,158]. Menin interacts with GATA3 to activate Th2 cell maturation in primary human peripheral blood T cells [159] and to physically interact with a member of the FOXA family, namely FOXA2 [103]. It is worth mentioning that FOXA1 plays a crucial role in the AR signaling, and possibly in CRPC occurrence [160].

4.3. Signal Transduction Pathways

Menin is known to interfere with different signaling pathways that play important roles in breast and prostate cancers.

4.3.1. The PI3K/PTEN/AKT/mTOR Pathways

Activation of the PI3K/PTEN/AKT/mTOR pathways occurs in 70% of BCs overall [161]. *PIK3CA* (a subclass of the PI3K family of genes) is the most commonly mutated gene in ER-positive BCs [162]. This mutation is present in approximately 35% of HR-positive BCs, 20–25% of HER2-overexpressing BCs, and with a lower frequency (8.3%) in TNBCs [163].

PTEN is one of the most commonly deleted and mutated genes in human breast and prostate cancers. Loss of *PTEN* in BC is negatively correlated with ER α and PR status, and is associated with the basal-like phenotype [164,165], with more aggressive behaviors (tumor size, lymph node metastasis, etc.), and with worse outcome (disease-free survival DFS and overall survival OS) [166]. Accumulating evidence has highlighted an association between loss of *PTEN* and the development of CRPC, likely due to AR phosphorylation [167,168]. Moreover, loss of *PTEN* and AR expression has been clinically correlated with increased mortality in CRPC patients [169]. More recently, Wong and colleagues generated mouse models with insulin-specific biallelic inactivation of *Men1* and *Pten* in β -cells, and showed that concomitant loss of *Pten* and *Men1* accelerated islet cell tumorigenesis. Co-mutations of *MEN1* and *PTEN* were observed in a small percentage of human PanNETs [170,171], suggesting that menin and Pten may function synergistically to suppress tumorigenesis.

Several studies have focused on the relationship between the PAM (PI3K/Akt/mTOR) and resistance to endocrine therapy in pre-clinical BC models [172], in which the authors showed that Akt can activate the ER α pathway independently of estrogen availability and that the combination of mTOR inhibitors and endocrine therapy can overcome this resistance [173,174]. In addition, the PAM pathway has also been implicated in trastuzumab resistance in HER2-overexpressing BCs [175]. Interestingly, menin interacts with AKT1, downregulates its kinase activity and suppresses both AKT1 induced proliferation and anti-apoptosis in endocrine and non-endocrine cells, mainly by reducing the translocation of AKT1 from the cytoplasm to the plasma membrane during growth factor stimulation [176]. Another study showed that menin can interact with FOXO1, a downstream effector of Akt, in the hepatocytic cancer cell line HepG2 and in MEFs [177]. In the same year, a study also showed that *MEN1* and genes from the mTOR pathway are frequently altered in pancreatic neuroendocrine tumors [170]. A recent study revealed that menin regulates milk protein synthesis through mTOR signaling in normal mammary epithelial cells [178]. According to the authors, menin overexpression caused significant suppression of factors involved in the mTOR pathway, as well as milk protein κ -casein (CSNK). All of the abovementioned data suggest that menin may regulate the PI3K/Akt/mTOR pathway in mammary cells.

4.3.2. Cell Cycle, Growth, and Death Control

Kaji et al. demonstrated that menin could suppress cell proliferation via the transforming growth factor- β (TGF- β) pathway in the rat pituitary cell line by interacting with Smad3 [179]. Agarwal et al. reported that menin is essential for JunD-mediated inhibition of cell proliferation [140]. Ratineau et al. showed that menin represses cell proliferation in rat intestinal epithelial cells [180] by inhibiting the expression of Cyclin D1, Cyclin D3, and CDK4. Based on a transcriptomic study of differentially expression genes, our team demonstrated that *Men1* ablation in mouse islet cells greatly affected the expression of factors involved in cell cycle and cell growth control, such as Cyclin A2, B2, and D2 for the former; and IGF2, IGFBP3, and 6 for the latter [181]. Menin can also repress cell proliferation by interacting and inhibiting ASK (S-phase kinase) [182]. In addition, menin was reported to upregulate the expression of Cyclin-dependent kinase inhibitors p18^{ink4c} and p27^{kip1} with the help of the MLL compass-like complex, which adds H3K4 trimethylation marks on their promoters, thus activating

gene expression [183–185]. Interestingly, p18 has recently been shown to be a downstream target of GATA3 in luminal BC and to suppress luminal progenitor cell proliferation and tumorigenesis [186]. P27 is ranked as one of the 18 most significantly mutated genes in luminal A BC, and loss of p27 was associated with poor outcome in BC patients [187].

Schnepp et al. revealed that the infection of cells using menin-expressing adenoviruses could trigger apoptosis in MEFs [188,189] by activating an apoptotic pathway that depends on Bax [186]. They also highlighted that *Men1* disruption in vivo increased resistance to TNF α -induced apoptosis, further supporting a vital role for menin in regulating apoptosis.

4.3.3. Wnt Signaling

It is well known that the Wnt pathway plays a crucial role in the development of breast and prostate cancers, in particular at late stages [190]. We observed that in *Men1*-deficient mice insulinomas, β -Catenin expression switched from a membrane expression to a cytoplasmic or even nuclear expression [187]. Along with our collaborators, we also showed that menin physically interacts with β -Catenin, and menin overexpression reduced the nuclear accumulation of β -Catenin and suppressed its transcriptional activity in *Men1*-null MEFs [104]. Jiang et al. further demonstrated that β -Catenin ablation leads to the suppression of tumorigenesis and significantly improved hypoglycemia and the survival rate of *Men1*-deficient mice [105]. Applying the small molecule inhibitor, PKF115–584, in *Men1*-deficient mice to antagonize β -Catenin signaling suppressed tumor cell proliferation in vitro and in vivo [105]. Kim et al. reported that menin promotes ubiquitin-mediated degradation of β -Catenin and menin overexpression downregulates the transcriptional activity of β -Catenin and target gene expression, as well as the proliferation of human renal carcinoma cells with an activated β -Catenin pathway [191].

5. Finishing Words

5.1. The Dual Role of Menin

The abovementioned data provide clues on the complex and sometimes paradoxical role of the *MEN1* gene in mammary and prostate cell tumorigenesis (Figures 2 and 3). Dreijerink et al. proposed a hypothesis on the dual role of menin in BC, which may shed light on these discrepancies and the surrounding confusion [44]. They proposed that menin could act as a tumor suppressor in normal luminal mammary epithelial cells and as an oncogene in sporadic ER-positive BCs, the key point being its essential role in the regulation of the *ESR1* gene mediated by the MLL–menin complex via H3K4me3 sites. Therefore, when *MEN1* is mutated or inactivated in normal mammary and prostate cells, it could result in dysregulated ER α and AR pathways, leading to aberrant cell proliferation and differentiation, and to tumor development with the participation of other oncogenic alterations. Conversely, in ER-positive BC and AR-positive prostate cancer cells, menin could act as a co-activator of these two nuclear receptors, playing a crucial role in promoting cell proliferation by the latter.

5.2. Remaining Questions

The currently available data and the abovementioned molecular clues suggest that menin may play a multifaceted but non-negligible role in the tumorigenesis of both mammary and prostate cells. However, concerning the detailed mechanisms underlying its involvement, many questions remain. Among them, one may wonder about the molecular pathophysiological consequences of *MEN1* inactivation in these two tissues during the initiation of tumorigenesis. In addition, since menin interacts and regulates the ER α and AR pathways, does menin play different roles in HR-positive than in HR-negative cancers? Last but not least, as menin acts as a scaffold protein, what are the other factors, in particular interacting partners, involved in the process?

To further understand the involvement of menin in these two cancers, there is an urgent need to generate adequate cell, tissue, and animal models in order to better investigate the distinct roles played

by menin during the initiation of carcinogenesis on the one hand, and during cancer progression on the other hand. Concurrently, strengthening *MEN1* mutation detection and menin expression analysis for breast and prostate cancer samples collected from young and aged patients or in different subtypes would be informative. The availability of more relevant models and crucial data from clinical samples, together with the rapidly improved tools in molecular study, should be of great help in obtaining rightful answers for the abovementioned questions.

6. Summary

Even though the role of menin in the development of neuroendocrine cancers is well known, its role in human breast and prostate cancers is slowly emerging. Based on the literature presented above, we speculate that future research could unveil further crosstalk between menin and the ER α and AR pathways. Finally, a better understanding of the mechanisms underlying its role in the mammary and prostate cell tumorigenesis could also make menin a potential therapeutic target for the treatment of these cancers, as well as a new marker for their diagnosis and prognosis.

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Abbreviations

ADT	Androgen deprivation therapies
AP-1	Activator protein 1
AR	Androgen receptor
AREs	Androgen response elements
ASK	Activator of S-phase kinase
BAX	BCL2 Associated X
BC	Breast cancer
BRCA1&2	Breast cancers 1 and 2
CDK	Cyclin-dependent kinase
CRPC	Castration-resistant prostate cancer
DBD	DNA-binding domain
DHT	Dihydrotestosterone
DNMTs	DNA methyltransferases
EIT-6	Estrogen Induced Tag-6
ER	Estrogen receptor
ERE	Estrogen response element
EZH2	Enhancer of zeste homolog 2
FOX	Forkhead box
H3K4me3	Tri-methylation at the 4th lysine residue of the histone H3 protein
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HOX	Homeobox

Hsp	Heat-shock proteins
IGF-1	insulin-like growth factor-1
IGFBP-3	Insulin-like growth factor-binding protein 3
LAR	Luminal-androgen receptor
LBD	Ligand-binding domain
LEDGF	Lens epithelium-derived growth factor
LOH	Loss of heterozygosity
mCRPC	Metastatic CRPC
MEF	Mouse embryonic fibroblast
MEN1	Multiple Endocrine Neoplasia type 1
MI	Molecule inhibitor of menin-MLL interaction
MIN	Mammary intraepithelial neoplasia
MLL1&2	mixed lineage leukemia 1&2 (KMT2A and 2B)
MTA1	metastasis-associated protein 1
mTOR	Mammalian target of rapamycin
NEPC	Neuroendocrine prostate cancer
NF- κ B	nuclear factor- κ B
NLS	Nuclear localization sequence
NTD	N-terminal Domain
PCa	Prostate cancer
PR	Progesterone receptor
PRMT5	Protein arginine N-methyltransferase 5
PSA	Prostate-specific antigen
PTEN	Phosphatase and TENsin homolog
ROS	Reactive oxygen species
SCC	Small cell carcinomas
SERDs	Selective estrogen receptor downregulators
SERMs	Selective estrogen receptor modulators
TGF- β	Transforming growth factor beta
Th2	T- helper type 2
TIT-5	Tamoxifen Induced Tag-5
TMPRSS2	Transmembrane protease serine 2
TNBC	Triple negative breast cancer
TNF α	Tumor necrosis factor alpha
TSS	Transcription start site
Wap	Whey acidic protein

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Generation and characterization of *Men1* mutant mouse models for studying MEN1 disease

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Abstract

Patients with multiple endocrine neoplasia type 1 (*MEN1*) mutations are predisposed to MEN1 syndrome affecting various endocrine cell lineages. Following its identification in the late 1990s, laboratories around the world, including our own, used gene-targeting approaches in murine models to study the *MEN1* gene and its related diseases. Subsequently, this field of research witnessed an upsurge in the use of *Men1* mutant mouse models to dissect MEN1 functions. These studies led to unraveling the natural history of MEN disease, and highlighted cellular and molecular mechanisms underlying the development of the disease. In this review, we present the currently available data concerning the generation and characterization of *Men1* mutant mouse models in connection with MEN1 syndrome.

Keywords: Mouse models, The *MEN1* gene, Tumorigenesis

Introduction

The multiple endocrine neoplasia type 1 (*MEN1*) gene, mutations of which are observed in patients with MEN1 syndrome,^[1] was identified in 1997, although its precise biological function initially remained unknown.^[2,3] This led to debates among scientists around the world, including about the normal biological functions of the gene in different cells and tissues, the cellular and molecular consequences of its inactivation, and whether the *MEN1* gene also plays a role in other tissues and cells? Needless to say, addressing these issues was deemed highly relevant for improving the general understanding of diseases related to its inactivation and for seeking adequate therapeutic strategies for treating MEN1.

Since then, concomitantly to MEN1 studies using diverse approaches and models, different laboratories around the world, including our own, have adopted gene-targeting approaches in murine models to study the *MEN1* gene, and have been engaged in the generation and characterization of *Men1* mutant mouse models. This approach has coincided with the broad-scale and worldwide development of experimental mouse models for a variety of contexts, as evidenced by the fact that every single gene within the entire mouse genome has now been targeted. Of the

mutant mouse strains available, currently exceeding 20,000, no less than 4500 models recapitulate human diseases. Extensive investigations based on these mouse models, especially using continually developing systems biology approaches, is rapidly changing our knowledge on and our strategies to deal with human diseases. Indeed, with the establishment of various *Men1* mutant mouse models, an increasing number of laboratories are involved in the use of these models to dissect *MEN1* functions. Here, we exclusively present data on the generation and characterization of *Men1* mutant mouse models in connection with the MEN1 syndrome.

Men1 mutant mouse models

Current technological advances in gene-targeting approaches enable scientists to perform either germ-line (conventional mutant mice) or cell type-specific (conditional mutant mice) disruptions of a given gene.

Conventional *Men1* mutant models

This type of *Men1* mutant mouse model mimics genetic events occurring in MEN1 patients, hence facilitating studies on the natural history of the disease.

The first conventional *Men1* mutant mouse model was reported in 2001.^[4] Authors demonstrated that homozygous *Men1* mutant embryos died mid-gestation, whereas heterozygous *Men1* mutant mice developed multiple endocrine tumors from the age of 9 months onwards, affecting islet cells, parathyroid, pituitary, and adrenal glands, all associated with hyperinsulinemia. Interestingly, thyroid tumors, considered to be a coincidental finding in MEN1 patients were detected in mutant mice, with loss of *menin* expression, suggesting that the MEN1 disease may encompass thyroid tumors.

In 2003, a second conventional *Men1* mutant model was established following our collaboration with the laboratory of Dr Zhao-qi Wang.^[5] Examination of over 150 heterozygous *Men1* mutant mice, divided into 3 age groups, 8 to 12 months, 13 to 18 months, and >18 months, for the emergence of tumors, revealed that around 1 year after birth, some heterozygous *Men1*

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mice developed multiple endocrine tumors observed in the MEN1 pathology, concomitantly to hyperinsulinemia and increased levels of serum parathyroid hormone. Importantly, we were able to document the presence of gastrinomas, the most common functional pancreatic neuroendocrine tumor (PanNET) in MEN1 patients, and glucagonomas. Intriguingly, *Men1* mutant mice also developed gonadal tumors with complete menin loss in both male and female mice, which are not reported in MEN1 patients, except for 1 Leydig tumor case.^[6] The significance of this observation with regards to the human MEN1 pathology remains to be determined.

Two additional conventional *Men1* mutant models were later established by the teams of Dr N. Steward and Prof Raj Takker.^[7,8] The observations derived from these 2 models largely confirmed data reported by the previous models, with one of the models also reporting the occurrence of adrenocorticotropinomas with hypercorticosteronemia.^[8]

The analyses carried out on homozygous *Men1* mutant embryos revealed that complete *Men1* ablation led to neural tube closure and abnormal development of fetal liver and heart.^[9] Cranial and facial developmental defaults were also observed.^[4] These findings thus indicate the essential role played by the gene in controlling cell proliferation and differentiation in these tissues during the embryonic mid-gestation stage. Of note, comparison of conventional *Men1* mutant models set-up in different genetic backgrounds highlighted significant differences in terms of timing and severity of developmental phenotypes.^[10]

Conditional *Men1* mutant models

Cell type- or tissue-specific *Men1* mutant mouse models were generated by crossing mice carrying the floxed *Men1* allele, *Men1^{F/F}*, with different *Cre* recombinant mice for given cell types or tissues. These models played a vital role in dissecting cellular and molecular mechanisms involved in the development of the MEN1 disease.

β-Cell Men1 mutant mice. Two conditional *Men1* knockout mice specifically targeting pancreatic β cells were reported in 2003, using different *RipCre* mice to generate *Men1^{F/F}-RipCre⁺* mutant mice.^[4,11] A similar model was described in 2004.^[12] Histological analyses revealed that, as early as 2 months of age, hyperplastic islets composed of *Men1*-deficient β cells appeared in some of the *Men1^{F/F}-RipCre⁺* mice. At 4 months, the number of conditional *Men1* mice with hyperplastic and/or dysplastic islets increased significantly, and at 6 months, insulinoma was detected in a substantial proportion of these mice. At 10 months, all *Men1^{F/F}-RipCre⁺* mice developed multiple insulinomas, accompanied with hypoglycemia and hyperinsulinemia. In addition, we and others observed that mouse *Men1* insulinomas displayed certain characteristics of tumor progression, including dedifferentiation, loss of cell-cell adhesion and angiogenesis.^[11,12]

α-Cell Men1 mutant mice. The incidence of glucagonoma is low in MEN1 patients, whereas α -cell pre-tumorous lesions are rather common.^[13] To better understand the role played by α cells in MEN1 PanNET development, we carried out a project aiming at specifically disrupting the gene in α cells using *GluCre⁺* mice.^[14] Characterization of *Men1^{F/F}-GluCre⁺* mice enabled us to fully document the appearance of α -cell proliferation, α -cell pre-tumorous lesions, the lesions expressing both glucagon and

insulin, and the development of both glucagonomas and insulinomas as early as 7 months of age. The data provided evidence, for the first time, of the involvement of cell trans-differentiation in the histogenesis of PanNET. The second α -cell-specific *Men1* mutant mouse model was reported shortly after.^[15] Intriguingly, only insulinomas, but no glucagonomas, were observed in the model, presumably due to a cross communication between these 2 islet cell populations according to the authors.

Pan-pancreatic Men1 mutant mice. Shen et al^[16] generated a *Men1* mouse mutant model targeting pancreatic progenitors using *Pdx1Cre* mice. Indeed, they observed insulinomas in aged mutant mice, whereas no neoplastic lesions were detected in exocrine tissues, albeit *Men1* disruption was documented in the latter. Neither glucagonoma, nor pancreatic gastrinoma were observed, and data concerning menin inactivation in non- β -cell lineages was not provided. More recently, we generated a novel *Men1* pan-pancreatic progenitor mutant mouse model using *pTF1Cre* mice, which allowed us to follow *Men1* pancreatic gastrinoma development.^[17] In these mutant mice, we did observe the occurrence of neoplastic lesions not only from β cells, but also α -, γ -, and pancreatic polypeptide cells (unpublished data).

Gastrointestinal epithelium cell Men1 mutant mice. Veniaminova et al^[18] initially reported that *Men1* deletion in gastrointestinal epithelium cells using Villin-Cre resulted in antral G cell hyperplasia and a hyperproliferative epithelium with hypergastrinemia, but no gastrinomas. Later, the same mutant mice were placed on a somatostatin null genetic background, leading to the development of gastric carcinoids (GCs) the occurrence of which could be accelerated by suppressing acid secretion.^[19] The authors reported that the GCs developing in this mouse model were reminiscent of human gastrointestinal neuroendocrine tumors, and accompanied by altered p27kip1 subcellular localization and stability.

Parathyroid Men1 mutant mice. A mouse model in which the *Men1* gene was specifically disrupted in parathyroid glands was generated by crossing the mice carrying *Men1^{F/F}* allele with *PthCre* mice.^[20] Mutant mice displayed parathyroid lesions with neoplastic changes and systemic hypercalcemia.

Mammary gland Men1 mutant mice. Some of the female mutant *Men1* heterozygous mice developed breast cancers, with a small but substantial frequency.^[5] In an attempt to determine the role played by the gene in controlling mammary cell proliferation, we performed *Men1* disruption specifically in murine mammary glands by generating *Men1^{F/F}-WapCre⁺* mutant mice.^[21] This led to mammary intraepithelial neoplasia with a high incidence (>50%) in mutant mice aged 12 months. We concomitantly performed analyses of menin expression in sporadic breast cancers and found that, among 124 analyzable cancers, 95 (78%) showed a decrease in menin expression compared with normal controls. The data were corroborated by a recent report revealing the significant predisposition of female MEN1 patients to breast cancer development.^[22]

Prolactin-secreting-cell Men1 mutant mice. Due to the leakage of *RipCre*^[12] and *WapCre*^[21] in the pituitary, highly hemorrhagic pituitary adenomas developed in the *Men1* mutant mice generated using these 2 *Cre* mice, at the age of 12 months. The tumor cells were shown to be prolactin-secreting cells, with a

2-fold increase in the level of serum prolactin in mutant mice compared to control mice, indicating prolactinomas. Subsequent analyses demonstrated that *Men1* ablation in prolactin-secreting cells triggered prolactinoma development in mice, leading to the premature death of these mice.

Craniofacial osteogenic cell and osteoblast *Men1* mutant mice. Recently, 2 laboratories published their studies on the function of *Men1* in bone. Kanazawa et al carried out *Men1* ablation in mature osteoblasts using osteocalcin-Cre mice. They found that the mutant mice displayed a significant reduction in bone mineral density, trabecular bone volume, and cortical bone thickness, whereas no tumor developed. Interestingly, in another study,^[23] the *Men1* gene was disrupted in craniofacial lineages of osteogenic cells using *Runx2Cre* mice.^[24] The mutant mice developed multiple ossifying fibromas (OF) in the mandible with 100% penetrance, generating the first genetic mouse model of OF.^[25] The study provided not only interesting clues for better understanding the occurrence of gingival papules in MEN1 patients, but also for dissecting molecular mechanisms of OF development, such as dysregulated *Cdkn1a* expression due to *Men1* inactivation.

Inducible *Men1* mutant models

Schnepf et al analyzed the effects of *Men1* ablation in an inducible *Men1* mutant mouse model by crossing mice carrying floxed *Men1* alleles and mice with a tamoxifen-inducible Cre transgene. They observed a slight increase in islet volume and a higher proliferation of islet cells shortly after the induction of Cre activity (2 weeks to 1 month).^[26] Interestingly, by either using the same mouse model or an inducible adult β -cell-specific *PdxCreER* line, the same laboratory reported that acute and temporally controlled *Men1* disruption improved pre-existing hyperglycemia in both streptozotocin-treated mice or mice fed with high fat diet, and glucose intolerance in genetic db/db diabetic mice.^[27] Based on a similar approach, using a mouse line with tamoxifen-inducible Cre-ER driven by the rat insulin promoter, Line et al examined histological changes in *Men1^{EF}-RIP2Cre^{ER}* mice 2 to 5.5 months after the induction of Cre activity by tamoxifen. They revealed that all of the tamoxifen-treated mice developed PanNETs originating from β cells.^[28]

Defining the cell of origin of PanNETs using *Men1* mutant mouse models

The cells at the origin of the development of PanNETs have been the object of a long-standing debate. Previously, multihormonality was observed in at least 35% to 50% of islet tumors.^[29] Several pioneering studies provided interesting finely documented observations. One study proposed that the histogenesis of these tumors arose from the differentiation of ductal pancreatic progenitor cells.^[30] Another study surprisingly demonstrated that early pancreatic lesions observed in MEN1 patients mainly affected α cells, whereas almost all of the advanced islet tumors observed in these patients were insulin-secreting or non-functional types.^[13] The histogenesis of these tumors remains to be elucidated, since it is crucial for the improvement of patient management and the treatment of the disease.

In order to determine whether α cells could be at the origin of PanNETs in MEN1, we carried out *Men1* disruption specifically in α cells (see above). The data obtained from our analyses indicate that α -cell-specific *Men1* disruption first leads to

proliferation of these cells and to the development of glucagonomas. Simultaneously, *Men1*-deficient α cells transdifferentiate into insulin-secreting cells, leading to the occurrence of insulinoma. The tumor phenotype observed in mutant mice reflects one of the most important features of islet tumors described in humans, multihormonality. Data obtained from this model revealed, for the first time, the involvement of transdifferentiation of α cells in the PanNET development and provide us with crucial clues to better understand the histogenesis of these tumors. In addition, they will make it possible to better understand the biology of α cells and the control of their cell proliferation and differentiation for other diseases affecting islet cells.^[14]

The cells of origin of pancreatic gastrinomas, a rather common PanNET in MEN1 patients, remain an enigma, since no gastrin-expressing cells are found in the normal adult pancreas. We addressed the issue by further characterizing previously described transient pancreatic gastrin-expressing cells using cell lineage tracing in a pan-pancreatic progenitor and a pancreatic endocrine progenitor model. In effect, we provided evidence that pancreatic gastrin-expressing cells, found from embryonic day 12.5 until postnatal day 7, are derived from pancreatic *Ptf1a*- and neurogenin 3-expressing progenitors, the majority of which coexpress glucagon, and the remaining coexpress insulin. Based on these observations and fine analyses of both *Men1* progenitor mutant mice, and *Men1* α - or β -cell mutant mice, we demonstrated that pancreatic gastrinomas related to *Men1* inactivation originated from pancreatic endocrine cells themselves.^[17]

It is worth mentioning that the hypothesis concerning the ductal origin of PanNETs has so far not been supported or confirmed.

Summary

Huge efforts have been made to generate and characterize *Men1* mutant mouse models by different laboratories. It is worth noting that conventional *Men1* mutant models have succeeded in genuinely recapitulating clinical features of MEN1 syndrome, whereas conditional *Men1* mutant mouse models have enabled scientists to carry out mechanistic studies to unravel cellular and molecular mechanisms underlying the development of the disease, from deciphering the cells of origin of PanNETs to the identification of different factors and/or cell signaling pathways involved in tumorigenesis upon *menin* inactivation. Interestingly, many of the studies using *Men1* mutant mouse models concomitantly revealed the importance of dysregulated cell differentiation and cell proliferation, as well as the factors involved in the corresponding alterations, although, unfortunately, they are beyond the scope of this review. These advances have improved our understanding of the spectrum of tumors and their evolution in MEN1 patients.

The knowledge acquired may also be valuable for designing treatments by finding potential MEN1 targets, as demonstrated by Jiang et al.^[31] We believe that a wider use of *Men1* mutant mice for drug discovery is thus imminent.

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Author contributions

None.

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Conflicts of interest

The authors declare no conflicts of interest.

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