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Razan Abou Ziki

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The role played by *MEN1* in breast cancer and the underlying mechanisms: Crosstalk between menin, ER α and MYC

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Abstract

The *MEN1* gene, initially known to be associated with the development of multiple endocrine tumors (MEN1 disease), has been shown over the recent years to play a role in hormone dependent cancers like prostate and breast cancer. Studies have shown that menin, encoded by *MEN1*, can play a dual role in breast cancer. While *MEN1* mutations cause higher risk of breast cancer initiation, menin is also shown to interact with and positively regulate the expression of estrogen receptor alpha (ER α) in ER+ breast cancer. Thus, menin plays a tumor suppressive role in normal mammary cells during tumor initiation procedure, and an oncogenic role in ER+ breast cancer initiated by other factors. However, the underlying mechanisms behind this regulation remain elusive.

To further address this issue, we carried out both clinical and experimental investigations and found that *MEN1* silencing led to reduced ER α expression on the transcriptional and protein levels in ER α -positive cell lines. While Dreijerink *et al.*, showed that menin regulates the *ESR1* enhancer and promoter via MLL complex, we demonstrated that menin also binds and regulates the proximal *ESR1* promoter, probably in an MLL-independent way. Interestingly, by analyzing the expression of menin in a cohort of 354 human breast cancers, our clinical data showed that reduced menin expression was correlated more with the luminal B breast cancer subtype and ER- breast cancer. Consistent with our clinical data, we further demonstrated that menin interacts with GATA3 and FOXA1, co-factors in *ESR1* regulation and known marker of the luminal A subtype. Taken together, our data provide relevant clues to the important role of menin in the formation of breast cancer subtypes, likely related to its regulatory role on ER α .

In parallel, we also showed that menin inactivation in BC cells led to reduced cell proliferation in 2 ER α -positive BC cell lines. Searching for molecular explanations, we found that, though *MEN1* knockdown activated the mTORC1 pathway, it led to reduced MYC expression on both transcriptional and protein levels in ER+ BC cell lines. We uncovered that menin bound to the *MYC* promoter and enhancer and regulated the expression of its target genes in these cells. Clinically, we also found that menin expression was negatively correlated with the expression of mTOR and its downstream genes, and more interestingly, with two inhibitory MYC-related genes.

Taken together, my thesis work provides relevant clues to the important role of menin in the formation of breast cancer subtypes, and, for the first time, to the role of menin in the regulation of the mTORC1 and MYC pathways in BC cells.

Résumé

Le gène *MEN1* codant pour menin a d'abord été associé au développement de Néoplasies endocriniennes multiples de type 1 (NEM1 syndrome), avant d'être récemment impliqué dans des cancers hormono-dépendants tels que le cancer de la prostate et du sein. Des études ont montré que menin peut jouer un double rôle dans le cancer du sein. En effet, si les mutations germinales du gène *MEN1* prédisposent les patientes au développement du cancer du sein, menin interagit avec le récepteur des œstrogènes alpha ($ER\alpha$) et régule positivement son expression dans le cancer du sein $ER\alpha$ -positives. Ainsi, menin joue un rôle de suppresseur de tumeur dans les cellules saines ou précancéreuses, et un rôle d'oncogène dans les cellules de tumeurs mammaires $ER+$ initiées par d'autres facteurs. Cependant, les mécanismes sous-jacents de cette régulation restent inconnus.

Afin de mieux comprendre ces mécanismes, nous avons mené des recherches cliniques et expérimentales et avons constaté que l'inhibition de l'expression de *MEN1* entraînait une diminution de l'expression de $ER\alpha$ aux niveaux transcriptionnels et protéiques dans les lignées cellulaires $ER\alpha$ -positives. Alors que Dreijerink *et al.* ont montré que menin régule le promoteur et l'enhancer *ESR1* via le complexe MLL, nous avons découvert que menin se lie et régule le promoteur *ESR1* proximal, vraisemblablement indépendamment du MLL. Il est intéressant de noter qu'en analysant l'expression de menin dans une cohorte de 354 cancers du sein humains, nos données cliniques ont montré que la réduction de l'expression de menin était davantage corrélée au sous-type de cancer du sein luminal B et au sous-type de $ER\alpha$ -négatif. Ces données cliniques sont en cohérence avec nos données expérimentales, puisque nous avons aussi démontré que menin interagit avec GATA3 et FOXA1, des cofacteurs de la régulation de *ESR1* et des marqueurs connus du sous-type luminal A. Dans l'ensemble, nos données mettent en évidence sur le rôle important de menin dans la formation des sous-types de cancer du sein, probablement lié à son rôle de régulateur de l' $ER\alpha$.

En parallèle, nous avons également montré que l'inactivation de menin dans les cellules du cancer du sein entraînait une réduction de la prolifération cellulaire dans deux lignées $ER\alpha$ -positives. D'un point de vue moléculaire, nous avons découvert que, bien que l'inhibition de l'expression de *MEN1* active la voie mTOR, elle entraîne une réduction de l'expression de MYC aux niveaux transcriptionnel et protéique dans les lignées cellulaires du cancer du sein $ER\alpha$ -positives. Nous avons également constaté que menin se lie au promoteur et à l'enhancer *MYC*, et régule ainsi l'expression de ses gènes cibles dans ces cellules. Sur le plan clinique, nous avons également observé que l'expression de menin est négativement corrélée avec mTOR et ses gènes en aval, mais également avec deux gènes inhibiteurs de MYC.

Dans l'ensemble, mes travaux de thèse fournissent des indices pertinents sur le rôle important de menin dans la formation des sous-types du cancer du sein, et mettent en évidence pour la première fois un rôle de menin dans la régulation des voies mTOR et MYC dans les cellules du cancer du sein.

List of abbreviations

4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1

AF1: activation function 1

AF2: activation function 2

AKT1: serine/threonine kinase 1

AP1: Activator Protein-1

AR: androgen receptor

BC: breast cancer

BL1: basal-like 1

BL2: basal-like 1

BMI: body mass index

BMP: bone morphogenetic pathway

BRCA1& BRCA2: breast cancer gene 1

BRCA2: breast cancer gene 2

CCNE1: cyclin E1

Cdc7: Cell division cycle 7-related protein kinase

CDH1: Cadherin-1

CDK2: cyclin-dependent kinase 2

CDK4: cyclin-dependent kinase 4

CDK6: cyclin-dependent kinase 6

CDKIs: cyclin-dependent kinase inhibitors

CK14: cytokeratin 14

CK17: cytokeratin 17

CK5: cytokeratin 5

CK6: cytokeratin 6

DBD: DNA-binding domain

DCIS: ductal carcinoma in situ

DFS: disease-free survival
DNA: Deoxyribonucleic acid
DNMT1: DNA methyltransferase 1
DNMT3B: DNA methyltransferase 3B
E2: estradiol
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
eIF4A: The eukaryotic initiation factor-4A
eIF4E: Eukaryotic translation initiation factor 4E
eIF4F: Eukaryotic initiation factor 4F
eIF4G: Eukaryotic translation initiation factor 4G
ERE: estrogen responsive elements
ERK: extracellular signal-regulated kinase
ER α : estrogen receptor α
ER β : estrogen receptor β
EZH2: Enhancer of zeste homolog 2
FBP1: Fructose-1,6-bisphosphatase 1
FOXA1: forkhead box A1
FOXO1: Forkhead box protein O1
FOXO3A: Forkhead box O3
FUSBP1: Far upstream element-binding protein 1
GATA 3: GATA binding protein 3
GCN5: general control nonderepressible-5
GPER1: G protein-coupled estrogen receptor 1
H3K27me3: lysine 27 trimethylation of histone 3
H3K4: lysine 4 trimethylation of histone 3
H3K79: Lysine 79 methylation of histone 3

H4R3me2: arginine 3 dimethylation of histone 4
HDAC1: histone deacetylase 1
HER2: human epidermal growth factor receptor 2
HIV-1: Human immunodeficiency virus 1
HMTs: histone methyl transferases
HOXA10 : Homeobox protein 10
HOXA7 : Homeobox protein 7
HOXA9: Homeobox protein 9
HOXB9: Homeobox protein Hox-B9
Hsp: chaperone heat-shock proteins
IDC: infiltrating ductal carcinoma
IFI16: Gamma-interferon-inducible protein
IGF: insulin-like growth factor
IGF1-R: insulin-like growth factor receptor
IM: immunomodulatory
JAK: Janus kinase
JNK: c-Jun N-terminal kinase
KMT2A: Histone-lysine N-methyltransferase 2A
KMT2B: Histone-lysine N-methyltransferase 2B
KMT2C: Lysine N-methyltransferase 2C
LAR: luminal androgen receptor
LBD: ligan-binding domain
LCIS: lobular carcinoma in situ
LEDGF: lens epithelium-derived growth factor
LXR α : liver X receptor
M: mesenchymal
MAPK: mitogen-activated protein kinase
MDM2: Mouse double minute 2 homolog

MEF: mouse embryonic fibroblast
MEN1: multiple endocrine neoplasia type 1
MI: menin/MLL inhibitor
MIN: mammary intraepithelial neoplasia
MLL: mixed lineage leukemia
MLL3: Mixed lineage leukemia 3
MLL-FPs: MLL1 fusion proteins
mRNA: messenger Ribonucleic acid
MSL: mesenchymal-stem-like
MTA1: metastasis associated protein 1
mTOR: mammalian target of rapamycin
mTORC1: mammalian target of rapamycin complex 1
mTORC2: mammalian target of rapamycin complex 2
MYCBP2: myc-binding protein 2 or Probable E3 ubiquitin-protein ligase
NF1: Neurofibromatosis type 1
NFIB: Nuclear factor 1 B-type
NFkB: nuclear factor-kappa B
NLS: nuclear localization signal
NTD: N-terminal domain
OS: overall survival
PAM: PI3K/ PTEN/AKT/MTOR
PARP: Poly (ADP-ribose) polymerase
PDK1: Pyruvate Dehydrogenase Kinase 1
PI3K: phosphatidyl inositol 3 kinase
PIK3CA: phosphoinositide-3-kinase catalytic alpha polypeptide
PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
PKA: protein kinase A
PKC: protein kinase C

PLC: phospholipase C

PPAR γ : peroxisome proliferator-activated receptor

PR: progesterone receptor

PRC2: polycomb repressive complex 2

PRMT5: Protein arginine N-methyltransferase 5

P-TEFb: positive transcription elongation factor

PTEN: Phosphatase and tensin homolog

PTN : pleiotrophin

RB1 : retinoblastoma protein 1

RTKs: receptor tyrosine kinases

S6K1: Ribosomal protein S6 kinase beta 1

S6RP: S6 Ribosomal Protein

SAGE: Serial Analysis of Gene Expression

SBR: scarff-Bloom-Richardson

SERD: Selective estrogen receptor down regulators

SERM: Selective estrogen receptor modulators

SiRNA: Small interfering RNA

SKIP: SKI-interacting protein

SP1: specificity protein 1

SPDEF: SAM pointed domain-containing Ets transcription factor

SREBP1: Sterol regulatory element-binding transcription factor 1

STAT: signal transducer and activator of transcription proteins

TFAP2C: Transcription factor AP-2 gamma

TFF1: Trefoil factor 1

TGF- β : tumor growth factor β

Th2: T helper 2

TNBC: triple negative breast cancer

TNF: tumor necrosis factor

TNM: tumor node metastasis

TP53: tumor protein 53

TRRAP: transactivation/transformation-associated protein

TSS: transcription start site

UNS: unstable cluster

VDR: vitamin D receptor

XPB1: X-box binding protein 1

YBX1: Y box binding protein 1

Introduction

I. Breast cancer:

1) Generalities (incidence):

Cancer is defined as a disease that results from the uncontrolled proliferation of normal cells after acquiring one or several gene mutations forming a tumor mass of cells (*NIH Curriculum Supplement*). Breast cancer originates from the breast tissues, mainly the mammary ducts and glands (*NCI Dictionary of Cancer Terms*). It is a very common and frequent malignancy among women and one of the major causes of morbidity worldwide. Its prevalence has expanded such that it ranked in the second place after lung cancer in incidence considering all cancers occurring in women (*Ferlay et al., 2009*). There were over 2 million new cases in the world in 2018, where France ranked 4th with an incidence Age-standardized rate of 99.1 per 100,000, followed by 93.6 in the UK, and 84.9 in US (*Bray et al., 2018*).

Despite the increasing efforts to raise awareness and encourage early detection all over the world for breast cancer (for example, many countries like France recommend regular mammography tests for women above 50 years), many women still discover this disease late at its advanced stages, which makes it even harder to treat. Late detection often is characterized by the tumor spread and metastasis, in addition to its histological and molecular heterogeneity and complexity.

2) Risk Factors:

There are a lot of risk factors associated with breast cancer development. Taking into consideration that women who live to the age of 85 will have a 1 in 9 lifetime chance of developing breast cancer, many women are at higher risk than others and these are usually advised to take several measures from the simple annual checkups and surveillance to the more complex (if necessary) prophylactic surgery (oophorectomy or mastectomy) and chemoprevention.

a) *Age:*

Like many diseases, especially cancers, age is one of the most documented associated risk factors for breast cancer as well. Breast cancer incidence is extremely low before age 30 with incidence of less than 25 cases per 100,000, after which it increases linearly to reach 500 cases per 100,000 at the age of 80. Thus, the incidence rate being augmented especially between the age of 55 and 70 (*Ries et al.,2002*) (*Anders et al.,2010*).

b) *Genetic factors:*

About 20% of breast cancer patients show genetic predisposition by having a positive family history of breast cancer, and 5% of patients showed specific germline mutations (*Easton et al.,1995*). Familial breast cancer was first described over 135 years ago (*Broca et al.,1866*). Since then, several studies have followed to define levels of risk associated with varying degrees of positive family history. The results have showed that compared to individuals with no family history of breast cancer, the estimated relative risk associated with a first-degree relative who developed breast cancer at 50 years of age or older is 1.8 compared with 3.3 for a first-degree relative who developed breast cancer at an age less than 50 years. The relative risk associated with having a second-degree relative with breast cancer is 1.5, two first-degree relatives (e.g., mother and daughter) is 3.6 (*Goldgar et al.,1996*) (*Kelsey et al.,1990*). The *BRCA1* and *BRCA2* genes are perhaps the most famous genes associated with inherited susceptibility to breast and ovarian cancers (*Brody et al.,1998*). Studies have showed that 55 to 70% of women with *BRCA1*&*BRCA2* mutations eventually develop breast cancer (*Nielson et al.,2016*). In addition, 13 polymorphisms in 10 additional genes were discovered most of them are responsible for rare genetic syndromes like Li Fraumeni syndrome, Cowden syndrome, ataxia-telangiectasia and MEN1 (multiple endocrine neoplasia syndrome) (please see III 5, page) (*Brody et al.,1998*) (*De Jong et al.,2002*).

c) *Reproductive or hormonal factors:*

Studies have shown that women who began menstruating before the age of 12 had a relatively higher risk for invasive breast cancer compared to those who began after the

age of 15. However, at the other end of the reproductive period, those who did not reach menopause until age 55 or after showed a relatively higher risk compared with those who experienced menopause before the age of 45 (*Vogel et al.,1998*). Based on these data, it was suggested that the risk of breast cancer observed from these gynecologic variables was a simple function of the number of ovulatory menstrual cycles that a woman undergoes during her lifetime (*Collaborative Group on Hormonal Factors in Breast Cancer 2012*). As women who had both ovaries removed before the age of 40 showed 45% reduction in risk compared with women who undergo a longer period of exposure to estrogen was used to explain the increase in the risk of breast cancer development (*Brinton et al.,1983a*). Breast cancer risk also increased if a woman was nulliparous or experienced her first live birth at or after the age of 30 compared to a woman with a first live birth at an age less than 20 (*Brinton et al.,1983b*) (*Collaborative Group on Hormonal Factors in Breast Cancer 2002*). Having at least one child as well as breast feeding is associated with a decrease in the long-term risk of developing breast cancer compared with risk among the nulliparous, and this protective effect increases with number of children (*Collaborative Group on Hormonal Factors in Breast Cancer 2002*).

d) *Obesity:*

Epidemiological studies have also shown that body mass index (BMI) could affect the incidence and outcomes of breast cancer. While in premenopausal women BMI was not that important risk factor, in postmenopausal women, however, it is strongly associated with increased incidence of breast cancer (*Tretli et al.,1989*). This observation was explained by the fact that postmenopausal women rely on their adipose tissue as an important extragonadal source of bioavailable estrogens and long exposure to estrogen increases the risk of initiation and promotion of breast cancer (*Clemens et al.,2001*) (*Verkasalo et al.,2001*). In addition, several studies also suggested that high BMI is associated with increased levels of insulin and insulin like growth factors, which have been associated with increased risk of breast cancer (*Suga et al.,2001*) (*Goodwin et al.,2002*).

Other lifestyle and environmental factors could also be mildly associated with breast cancer development like smoking, alcohol consumption, diet, stress, lack of exercise, hormone replacement therapy, and exposure to carcinogens or radiation.

3) Mammary gland anatomy and histology:

The mammary gland is a milk-producing gland regulated by the endocrine system and is a main characteristic of all female mammals, although it's also present in males but however in a rather primitive or nonfunctional form. Mammary gland is composed of a conical disk of glandular tissue covered in fat to give it its characteristic shape. The glandular tissue itself is made up of 15–20 lobes composed of solid cords of ductal cells; each lobe is subdivided into many smaller lobules, separated by broad fibrous suspensory bands (Cooper's ligaments), which connect the skin with the fascia, or sheet of connective tissue, that covers the pectoral muscles beneath the breast. Each lobe is drained by a separate excretory duct which converge beneath the nipple and widen into milk reservoirs, before narrowing again to emerge as pinpoint openings at the summit of the nipple. The areola, a circular disk of roughened pigmented skin surrounding the nipple, contains circular and radiating muscles (to help erect the nipple and facilitate lactation) as well as sebaceous glands to provide lubrication for the nipple during nursing. Under the influence of estrogens from the maturing ovary at puberty, the ductal cells proliferate and form branches. After ovulation, progesterone causes the terminal ductal cells to differentiate into the milk-producing cells, which form acini. Interspersed with these cells are smooth muscle cells, which can contract and assist in the ejection of milk. The acini are collapsed or filled with desquamated epithelium (epithelium that has been shed), until the stimulus of pregnancy causes proliferation of all the epithelial cells. The actual secretion of milk is induced by prolactin from the pituitary and somatomammotropin from the placenta (*Encyclopaedia Britannica 2018*). **Fig1**

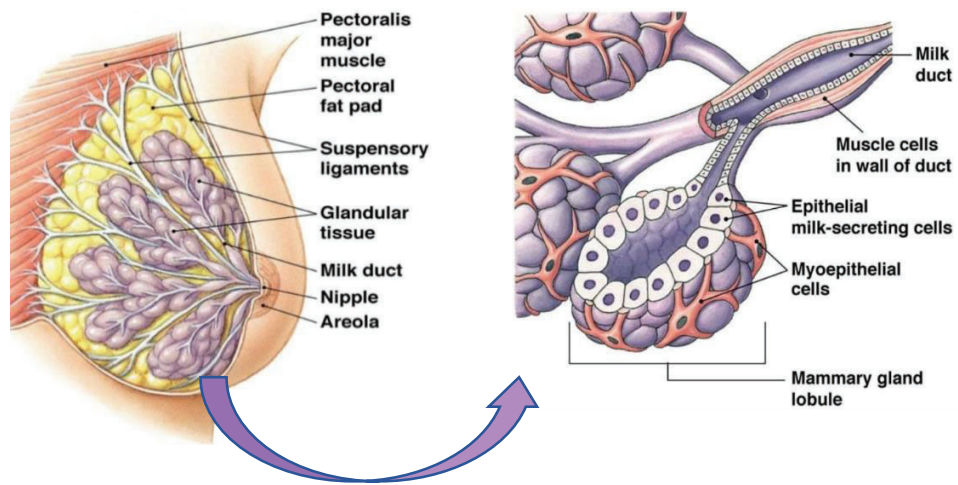


Figure 1: Mammary gland anatomy

Adapted from austincc.edu

4) Breast cancer different classifications:

a) *Histological classification:*

Breast cancer was initially histologically divided into several subtypes based on its invasive or not growth features, namely *in situ* carcinoma and invasive (infiltrating) carcinoma. The carcinomas can be further divided into ductal or lobular depending on its origin, if it rises from the inner wall of the mammary ducts or the mammary glands respectively (Malhotra et al.,2010). The ductal carcinoma in situ (DCIS), being more common than the lobular carcinoma in situ (LCIS), can be further sub-classified into five subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid. The invasive carcinoma can also be divided into several subtypes such as: infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), and tubular, medullary and papillary carcinomas (Malhotra et al.,2010). With the infiltrating ductal carcinoma (IDC) being the most common subtype accounting for 70–80% of all invasive lesions (Li et al.,2005). IDC is further sub-classified as either well-differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3) (Lester et al.,2009). **Fig 2**

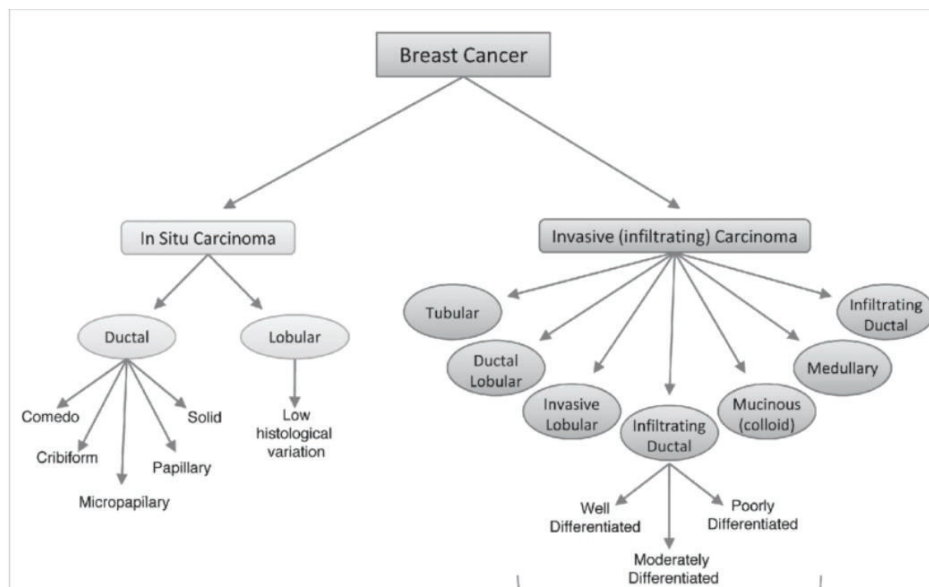


Figure 2: Histological classification of breast cancer

Adapted from Malhotra et al., 2010

b) *TNM classification:*

Due to the complexity of the previous histological classification and to make it easier for doctors to identify the stage of breast cancer, the American Joint Committee on Cancer (AJCC) created a new classification or staging system called TNM. TNM stands for Tumour, Node, Metastasis. Tumour (T) describes the size of the tumour (area of cancer), node (N) describes whether the cancer has spread to the lymph nodes, Metastasis (M) describes whether the cancer has spread to a different part of the body (*American Cancer Society 2019*). **Table 1**

Primary Tumor (T)	Histological Characteristics
Tx	Primary tumor can't be assessed
T0	Primary tumor not clear or evident
Tis	Carcinoma in situ: <i>invasion of lamina propria</i>
T1	Tumor invades submucosa (<i>still less than 2cm size</i>)
T1mic	T1 with less than 1mm microinvasion
T2	Tumor invades muscularis propria (<i>size between 2 and 5cm</i>)
T3	Tumor invades into subserosa or non peritonealized pericolic or perirectal tissues (<i>size more than 5cm</i>)
T4	Tumor perforates visceral peritoneum and invades other organs
Regional Lymph Node (N)	degree of invasion
Nx	Regional lymph node invasion can't be assessed
N0	Absence of regional lymph node invasion
N1mi	Presence of micro metastases (<i>less than 2mm</i>)
N1	Metastases to 1 to 3 regional lymph nodes
N2	Metastases to 4 or more regional lymph nodes
N3	Metastases to at least 10 regional lymph nodes
Distant metastases (M)	Absence or presence of metastasis
Mx	Distant metastasis can't be assessed
M0	No distant metastasis
M1	Distant metastases
Extent of resection (R)	Absence or presence of residual tumor
Rx	Presence of residual tumor can't be assessed
R0	No residual tumor
R1	Microscopic residual tumor
R2	Macroscopic residual tumor

Table 1: TNM classification

c) *SBR classification:*

SBR (Scarff-Bloom-Richardson) classification is based on the histological and cytological characteristics of a tumor in which tumors are graded by adding up scores for tubule formation (percent of the tumor forms normal duct structures) nuclear pleomorphism (irregular breast duct epithelial cells) and mitotic count (the number of dividing cells seen in 10x high power microscope field), each of which is given 1 to 3 points. The scores for each of these three criteria are then added together to give an overall final score and corresponding grades (*Bloom & Richardson 1975*) (*Genestie et al., 1998*). **Table 2**

Characteristics	Score
Tubules formation (%)	
Majority or tumoral cells (>75%)	1
Moderate (10-75%)	2
Rare or absence of tubules (<10%)	3
Nuclear pleomorphism	
Normal cell size and shape, small nucleus	1
Average increase in nuclear size	2
Important increase in nuclear size and form	3
Mitotic index	
0-5 (histo) or 0-1 (cyto)	1
6-10 (histo) or 2-4 (cyto)	2
>11 (histo) or >5 (cyto)	3
Grade	
Grade 1: cells well differentiated	3-5
Grade 2: cells moderately differentiated	6-7
Grade 3: cells with little or no differentiation	8-9

Table 2: SBR classification

d) *Molecular classification:*

More recently in attempts to better reflect the genetic heterogeneity of breast cancer and improve prediction of tumor behavior and therapy, classification into molecular subtypes depending on molecular markers such as the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 HER2 was purposed to facilitate diagnosis and treatment, though it should still be used in association with other characteristics such as the tumor size, grade, and lymph node invasion (*Makki et al.,2015*) (*Perou et al.,2000*).

There are four main molecular subtypes:

- i. Luminal A: is ER positive and or PR positive, HER2 negative and Ki 67 low, causing it to grow slowly and have a significantly good prognosis, being vulnerable to hormone therapy. About 50% of the diagnosed breast cancers are luminal A (*Rosai 2011*).
- ii. Luminal B: is ER positive and or PR positive, either HER2 positive or negative, and Ki67 high, having a worse prognosis than luminal A type and variable response to endocrine and chemotherapy (*Rosai 2011*).
- iii. HER2 enriched: is ER and PR negative, HER 2 positive (amplification mutations in the coding region of HER2 gene), high Ki-67 expression and common *TP53* mutations. They are more aggressive than luminal subtypes but have good response to the therapies that target HER2 receptor (*Rosai 2011*).
- iv. Triple negative / basal-like: characterized by molecular expression of basal cytokeratines like (CK5,6,14,17) and/or EGFR positive, ER negative, PR negative, HER2 negative, high Ki-67 and *TP53* mutation. This type has the worst prognosis and frequently seen in BRAC1 mutated patients. It has no response to endocrine therapy or trastuzumab but appears to be sensitive to

special kinds of chemotherapy (platinum-based) (Rosai 2011) (Shawarby et al.,2013).

Recently, due to the heterogeneity found among this group of tumors, attempts have made to divide it into seven subtypes: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal-stem-like (MSL), luminal androgen receptor (LAR), and an unstable cluster (UNS). The Androgen Receptor (LAR) subtype display a luminal phenotype, characterized by high expression of Androgen receptor (AR) and luminal lineage-driving transcription factors (MDAMB453 cell line) (Collins et al.,2011) (Lehmann et al.,2011). **Fig 3**

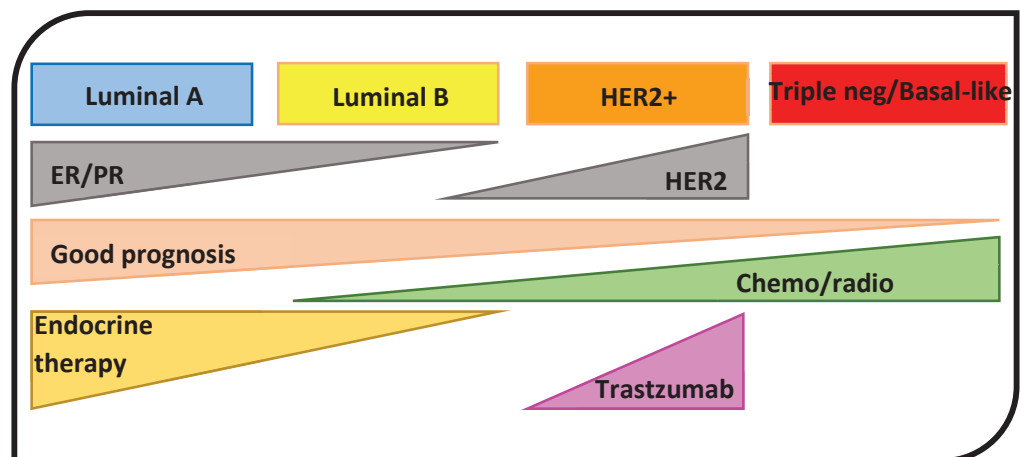


Figure 3: Molecular classification of breast cancer and their characteristics

5) Brief overview of molecular mechanisms underlying different subtypes of sporadic BC:

Breast cancer development is a multi-step process that occurs over an extended period of time, arising from early pre-invasive lesions to invasive metastatic carcinomas. However, not all subtypes present the same malignant phenotypes, such as self-sufficiency in growth signaling, resistance to apoptosis, invasion, and metastasis and abnormal angiogenesis, meaning that the molecular mechanisms behind these phenotypes could vary according to the subtype. Generally, alterations in signal transduction pathways for mitogenic signals, cell cycle control, DNA repair and epigenetic gene expression modification systems happen in all subtypes but not in the same variation. For example, among the TNBCs, different patterns of molecular alterations exist in terms of RNA expression, somatic mutations and copy number variations, which tend to cluster in genes implicated in specific pathways. The BL1 subtype shows high levels of DNA-damage response and cell-cycle regulation genes like TP53 mutations (highest rate 92%), high gain/amplifications of MYC, CDK6, or CCNE1 and deletions in BRCA2, PTEN, MDM2, and RB1 (*Bareche Y et al., 2018*); the BL2 subtype possess enriched growth factor signaling and strong metabolic activity and displays a highly proliferative phenotype. JAK/STAT, TNF and NFkB genes that are involved immune cell and cytokine signaling are highly expressed in the IM subtype. Mesenchymal-like TNBC subtypes, M and MSL, display similar expression profiles with the previous subtypes with a unique enrichment in MSL of angiogenesis- and stem cell-associated genes, and low claudin expression. As for the LAR subtype, they display a luminal pattern of gene expression like high levels of FOXA1, GATA3, SPDEF, and XBP1 with elevated mRNA and protein levels of androgen receptor (AR), overlapping in 82% of cases with luminal-A- or luminal-B-intrinsic subtypes. As a result, LAR tumors display high number of mutations in PIK3CA, KMT2C, CDH1, NF1, and AKT1 (*Bareche Y et al., 2018*). Studies on HER2+ BC showed in addition to HER2 (also called EGFR2 or ERBB2) mutations, mutations in TP53, PIK3CA, MLL3, *MT-ND2*, FAT2, NF1 and GATA3 genes (*Zongbi et al.,2020*) (*Lesurf et al.,2017*). Multiple signaling pathways are also enriched such as: cell cycle, PI3K-Akt signaling, p53 signaling, Wnt signaling, focal adhesion, TGF-beta signaling, ErbB signaling, and immune function (*Lesurf et al.,2017*). In another study conducted on Chinese HER2+ breast cancers, they also showed high number of

mutations in CDK12 in addition to TP53(highest) and PI3KCA (*Li et al.,2020*). As for ER+ BCs molecular mechanisms will be discussed in detail in part III, 5.

II. ER α : functions and role in breast cancer

1) Generalities:

Estrogens are steroid hormones that are derived from cholesterol and exist as estrone, estradiol (E2), and estriol. 17 β -Estradiol, however, is the most dominant estrogen hormone in the circulation and plays an important role in different tissues like the cardiovascular, musculoskeletal, immune, and central nervous system in addition to its main role in the development and maintenance of reproductive system. Estradiol can also contribute to the initiation and development of target tissue malignancies like breast cancer (*Gruber et al.,2002*) (*Nelson et al.,2001*).

The effects of E2 are mediated by the estrogen receptors ER α and ER β (*Evans et al.,1988*) (*Ribeiro et al.,1995*) which are nuclear receptors that act as transcription factors capable of promoting the transcription of hundreds of target genes, especially those associated with cell differentiation, proliferation and survival (*Kos et al.,2001*) (*Harris et al.,2007*). Studies over the years have showed that while ER β plays a minor role in the uterus, mammary glands, pituitary gland, skeletal muscle, adipose tissue, and bone, it was found to be critical in mediating E2 signaling in the ovary, prostate, lung, cardiovascular and central nervous systems (*Harris et al.,2007*) (*Hamilton et al.,2014*) (*Hewitt et al.,2014*) (*Deroo & Buensuceso 2010*). Although significant progress has been made towards understanding the mechanism of ER β signaling in response to E2, ER β 's actions and exact physiological role remain not clear due to limitations of studies (*Deroo & Buensuceso 2010*) (*Hamilton et al.,2014*) (*Haldosén et al., 2014*) (*Marino et al., 2006*). In other words, current knowledge indicates that ER α predominates ER β actions in most tissues and is widely studied in the malignancies of these tissues like mammary gland tumors. ER α and ER β though encoded by different genes on

different chromosomes, share high sequence homology and structural similarities (*Marino et al., 2006*) (*Green et al., 1986*).

2) ER α (from gene and protein to transduction pathway)

a) *Structure of the ESR1 gene*

The complete complementary DNA of the estrogen receptor (ER α cDNA) was first cloned and sequenced in 1986 (*Green et al., 1986*). The gene coding for ER α , *ESR1*, is of 140 kb in size, localized on chromosome 6q25.1 and composed of 8 exons. Comparative analysis of the gene between species showed that gene structure is highly conserved (*Koike et al., 1987*). In breast cancer, studies using exome sequencing have shown that the mutations in *ESR1* gene were more chromosomal amplification than point mutations (*Vincent-Salomon et al., 2008*) (*Stephens et al., 2012*).

b) *ER α structure and localization:*

ER α consists of 595 amino acids, composed of two domains of functional activity AF1 & AF2 located in the N-terminal domain (NTD) and C-terminal Ligand binding domain (LBD) respectively. These 2 domains are separated by a connecting DNA binding domain (DBD) and work together to mediate the transcriptional activity of the protein. The NTD or AF1(A/B) domain is involved in both inter-molecular and intra-molecular interactions as well as in the activation of gene transcription in a ligand independent manner, opposite to the LBD or AF2 (E/F) domain which requires the binding of a ligand (*Kumar et al., 2011*). LBD domain also allows ER α to dimerize and to bind to the specific ERE sequence situated on the promoters of its target genes (*Klinge et al., 2001*). The estrogen receptor includes 2 more domains: the centrally situated hinge domain (D region) that has a role in receptor dimerization and in binding to chaperone heat-shock proteins (Hsp), containing a nuclear localization signal (NLS) that guides the estrogen-ER α homodimer transfer from the cytoplasm to the nucleus, and the C-terminal F domain where the ER α transcriptional activity could be modulated (*Kumar et al., 2011*). As all

nuclear receptors, ER α is mainly found in the nucleus with and without a ligand (Monje et al.,2001) (Kocanova et al.,2010). However, in the recent years, ER α and its several isoforms have been found to play a certain role in the cytoplasm (Thomas&Gutafsson 2015). **Fig 4**

c) *ER α isoforms:*

ER α isoforms exist due to several splice variants in both normal mammary tissues and breast cancer (Taylor et al.,2010) (Leygue et al.,1996) (Pfeffer et al.,1995). However, the levels of expression of these variants are lower in normal than in tumor tissue and increase with breast tumor progression, likely involved in the shift from hormone dependence to independence in breast cancer (Leygue et al.,1996). Alternative splicing of pre-mRNA being the most occurring event, as most *ESR1* splice variants are of the exon skipping variety (Hirata et al.,2003) (Poola et al.,2000). Other methods of splice variant generation include exon duplication (Pink et al.,1996) (Murphy et al.,1996) and insertion of additional coding material (Hirata et al.,2002). In addition, different tissues can utilize different upstream promoters and so form pre-mRNA with a variable length non-coding 5' untranslated region, also some somatic mutations lead to altered stop codons and/or inclusion of intronic material (Flouriot et al.,1998) (Weickert et al.,2008). There are many names and terms to classify the splice variants: the most frequently used system, with exon deletions numbered and prefixed by a delta (Δ) sign, e.g., ER $\alpha\Delta 5$, ER $\alpha\Delta 7$. Partial exon deletions are denoted by a preceding Asterix, e.g., ER $\alpha\Delta 4^*7^*8$, has a full deletion of exon 4 and partial deletions of exons 7 and 8 (Taylor et al.,2010). Perhaps the most simple nomenclature is ER α splice variant proteins named after their size. ER α 36, a 36 kDa protein, that lacks both transcriptional activation domains (AF-1 and AF-2) but it retains the DNA-binding domain, and partial dimerization and ligand-binding domains of hER- α 66 (total ER α), and is localized in both plasma membrane and cytoplasm where it mediates non-genomic ER α signaling in the presence(since it retains its ability to bind E2) and absence of ligand (Wang et al.,2006) (Omarjee et al.,2017) increasing the aggressiveness and the anti-estrogen resistance in breast cancer cells (Chaudhri et al.,2012) (Deng, et al.,2014). ER α -

46, a 46 kDa protein, that lacks only the AF-1 domain (Flouriot *et al.*, 2000) and is membranously localized such that it mediates the membrane-associated estrogen signaling and estrogen-dependent growth of breast cancer cells (Marquez & Pietras 2001). Fig 4

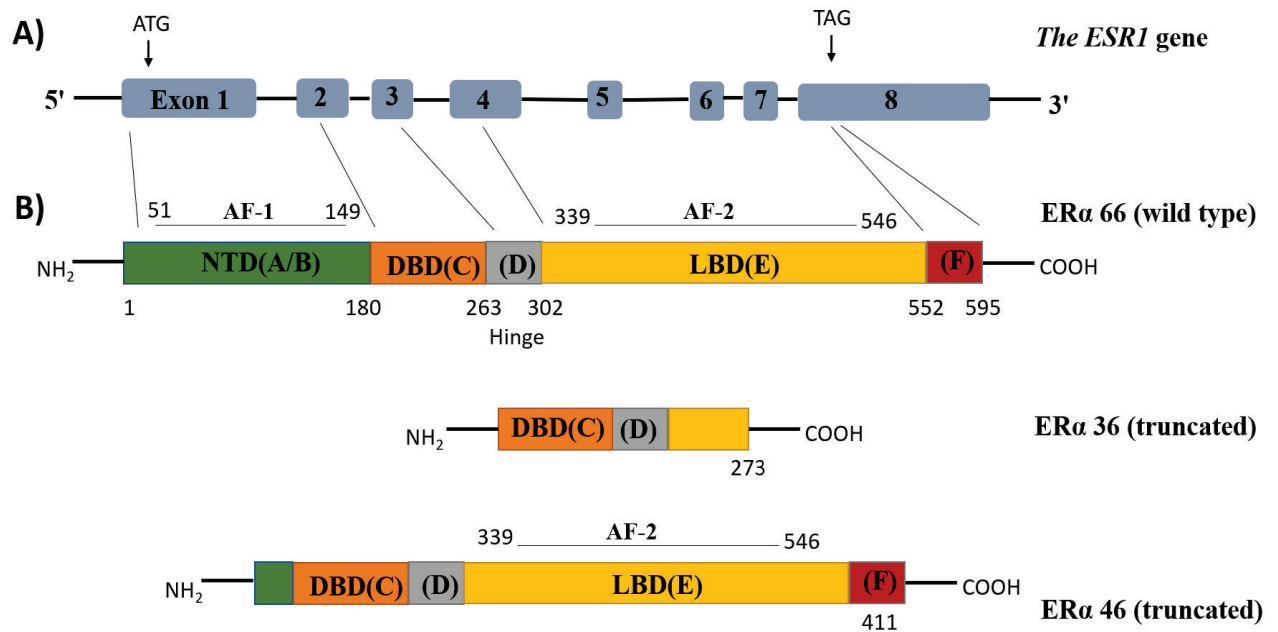


Figure 4: Schematic representation of *ESR1* gene and ERα and its isoforms: (A) *ESR1* structure composed of 8 exons (grey boxes) separated by introns (black lines), the vertical lines show the coding sequences leading to the functional domains of ERα protein. (B) Different ERα domains, A/B region composing the AF1 domain, C region or DNA binding domain, D region which serves as hinge region, and region E which forms the AF2 domain. Underneath are the 2 ERα isoforms showing the missing regions compared to the full-length ERα.

d) *ER α function and targets:*

A. ER α signaling pathways:

i. Direct ligand-dependent genomic pathway of ER α :

The genomic or “classical” estrogen mediated ER α signaling pathway starts when estradiol binds the LBD of ER α in the cytoplasm, causing it to dissociate from the chaperone protein Hsp90 (heat-shock protein 90), dimerize, and translocate to the nucleus where it binds, via DBD, to the estrogen responsive elements (ERE) sequences situated on the promoters of its target genes (*Klinge et al., 2001*). Bound to ERE, ER α can then activate or repress gene expression by recruiting coactivators or corepressors like transcription factors and histone modifiers (*Thomas & Gutafsson 2015*). Genome-wide studies have identified that ERE are usually located near mRNA transcriptional start sites and share a high degree of similarity in sequence that can alter the affinity of the receptor to bind DNA. Other studies have also shown that ER α can also bind the upstream enhancer (distal) part of the regulatory sequences of its target genes (*Carol et al., 2006*). **Fig 5**

ii. ERE independent or indirect genomic pathway of ER α :

ER α is also capable of regulating several genes that do not contain ERE in their promoters (35% of estrogen target genes lack ERE-like sequences) by interacting with other transcription factors and their response elements (*Marino et al., 2006*) (*Aranda & Pascual 2001*) (*Göttlicher et al., 1998*), like SP-1 (*Saville et al., 2000*), NF-kB (*Biswas et al., 2005*), c-Jun/Fos (AP1) sites (*Kushner et al., 2000*), STAT5 (*Fox et al., 2009*) and others. This mechanism is known as the indirect genomic signaling or transcriptional crosstalk that allows ER α to regulate a wide number of genes. **Fig 5**

iii. Ligand independent genomic pathway of ER α :

Interestingly, ER α can also be activated in the absence of estrogen or other agonists, through its phosphorylation on specific residues (serine, tyrosine) by several protein kinases (PKA, PKC), growth factors (IGF, EGF), cell cycle regulators, and others (Thomas & Gustafsson 2015) (Nilsson et al.,2001). **Fig 5**

iv. Non genomic pathway:

A non-genomic pathway of estrogen signaling was discovered, owing to the observation of a quick response to estrogen treatment, as fast as 15 seconds compared to what was usually known of a 2-hour time lag after the treatment. This observation showed that the nuclear receptor ER α could also be found in the plasma membrane (Pedram et al.,2006) and interfere in cytosol signaling pathways such as phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/AKT, and cAMP/protein kinase A (PKA) (Marino et al.,2006). Additionally, a membrane bound G protein-coupled estrogen receptor 1 (GPER1) was discovered which promotes estrogen-dependent activation of adenylyl cyclase and epidermal growth factor receptor (EGFR) (Yu et al.,2018). Moreover, several ER α isoforms, like ER α 36 and ER α 46, are also found in the plasma membrane of several breast cancer cell lines (Omarjee et al.,2017) (Li et al.,2003). These isoforms are capable of activating proliferative and anti-apoptotic pathways like Src, PI3K/AKT, MAPK/ERK pathways in response to estrogen and sometimes without (in case of ER α 36) (Kim& Bender 2009) (Rao et al.,2011). **Fig 5**

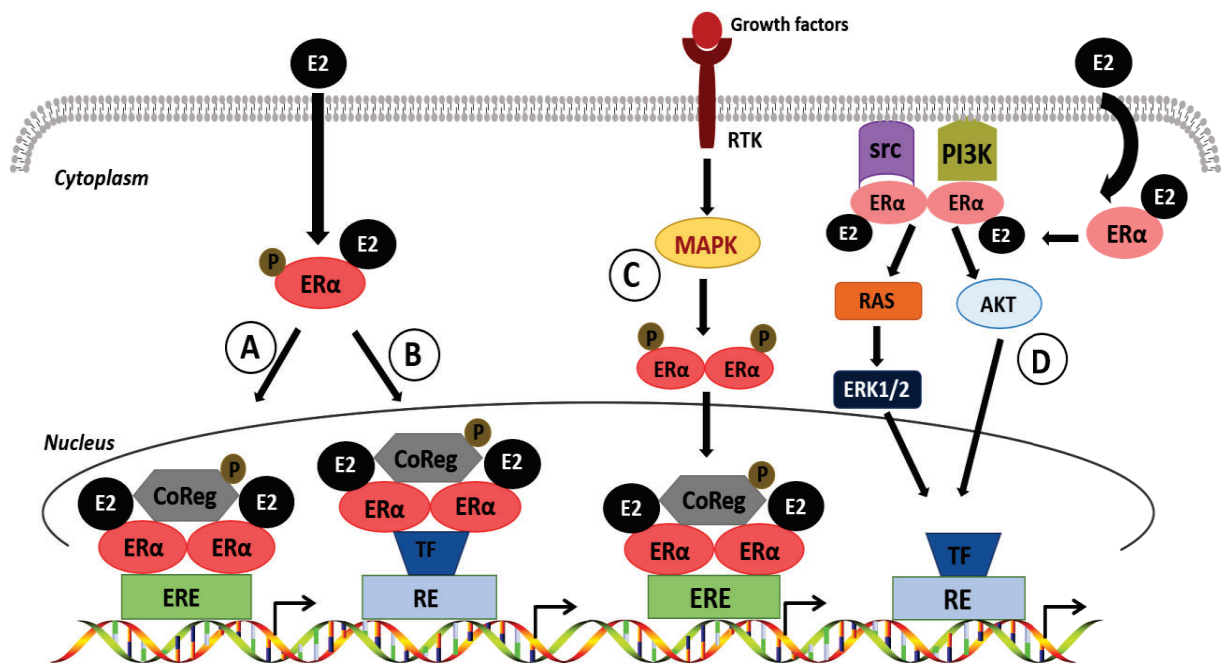


Figure 5: ER α signaling pathways: A: Classical ligand-dependent genomic pathway, B: Non-classical ERE independent genomic pathway, C: Ligand independent genomic pathway, D: Non genomic pathway.

B. Hormonotherapy: ER α targeting in breast cancer treatment

Breast cancer treatments usually include surgery, radiotherapy, chemotherapy, and endocrine or hormonotherapy. ER⁺ breast cancer types are the most benefited from the latter, because they depend on ER α signaling for survival as it regulates several genes required for proliferation and metastasis. Different types of endocrine therapies exist that can block ER α 's actions at different levels. Selective estrogen receptor modulators (SERM) have been used for over than 30 years now and have shown great efficiency in treating ER⁺ breast cancer in premenopausal women. Unlike aromatase inhibitors that block the synthesis of estrogen from testosterone, SERMs' mechanism of action involves blocking the ER action in the cancer cells by binding, instead of estrogen, to the receptor, SERMs have proven their efficiency in treating postmenopausal women. (*Maximove et al.,2013*) (*Ma et al., 2015*). Cells in other tissues in the body, such as bones, liver and the uterus, also have estrogen receptors but the structure of estrogen receptor slightly varies depending on cell type, so the advantage of SERMs compared to aromatase inhibitors is their selectivity which allows them to differentiate the estrogen receptors in breast cells from that of the bone and other tissues increasing the efficiency of the treatment and minimizing the side effects. Three SERMs exist: tamoxifen, raloxifene (Evista), toremifene (Fareston). Tamoxifen being the most commonly used SERM that exist in pill form under the brand name "Nolvadex" and in liquid form under the brand name "Soltamox" (*Clemons et al.,2002*). Recently and due to the acquired resistance to the previous endocrine treatments, a third type of therapy was discovered and named as the selective estrogen receptor downregulators (SERD) or anti-estrogens that blocks and damages estrogen receptors by decreasing their expression and activity. Fulvestrant (Faslodex) is the drug of use and is currently approved only for use in post-menopausal women (*McDonnell et al.,2015*). **Fig 6**

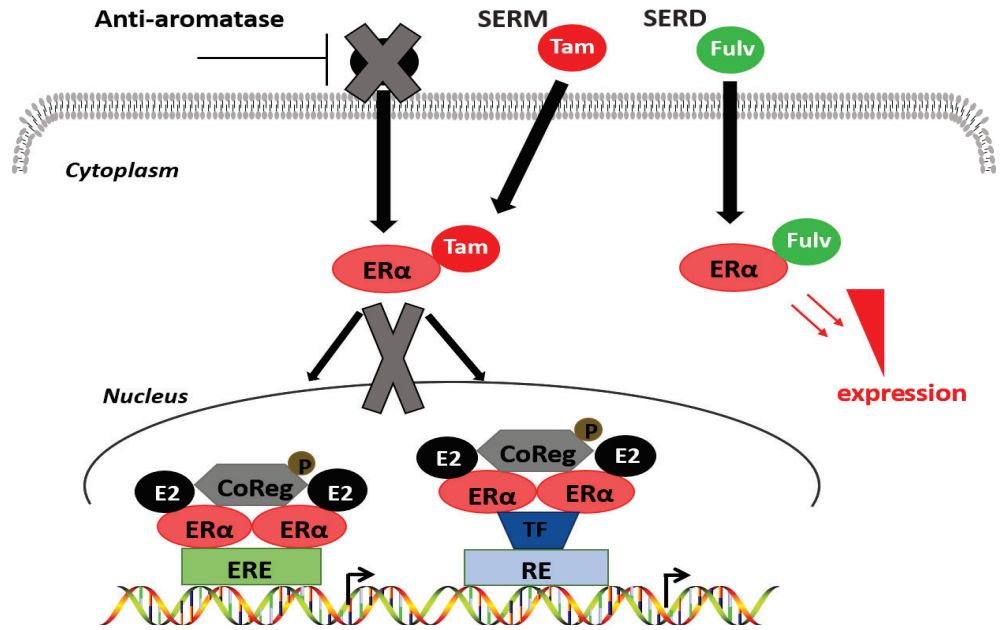


Figure 6: Hormonotherapy: mechanisms of action:

Tam=Tamoxifen ; Fulv=Fulvestrant

C. ER α function in normal cells:

Estrogens, through the ER α signaling pathway, play important developmental and physiological and pathological roles both in the normal and tumor development. Studies from animals and humans have demonstrated that ER α is essential for the normal development of female reproductive tract, like the uterus and the ovaries, as well as the proliferation and differentiation of the mammary gland (*Korarch et al.,2003*). These studies have further showed that ER α is also important for male fertility and is implicated in other non-reproductive organs such as regulation of the neuroendocrine and cardiovascular systems and bone metabolism (*Korarch et al.,2003*) (*Lin et al.,2004*). Over the last decade, the research focus has been being mainly on the role of ER α in breast cancer initiation, progression, and response to treatment (*Ali et al.,2000*) (*Persson I. 2000*).

D. ER α and its target genes' functions in breast cancer:

There are thousands of ER α target genes. One of the earliest ER α identified target genes was pS2/TFF1 (*Brown et al.,1984*) (*Jakowlew et al.,1984*) followed by many genes that were discovered by monitoring the global expression changes upon estradiol induction by SAGE or microarray strategies in BC cells (*Charpentier et al.,2000*) (*Cunliffe et al.,2003*) (*Frasor et al.,2003*) (*Inoue et al.,2002*) (*Seth et al.,2002*). ER α target genes are of wide and different functions, such that they can be divided into either: pro-proliferative genes, like cyclin D1(*Altucci et al.,1996*), MYC (*Dubik et al.,1987*)(*Dubik &Shiu 1988*) and IGF-1 (*Umayahara et al.,1994*), or ant-apoptotic factors , like TIT-5, and EIT-6 (*Seth et al.,2002*), enzymes like the lysosomal proteinase cathepsin D (*Elangovan & Moulton 1980*); or even nuclear receptors like progesterone receptor (*Yu et al., 1981*), in addition to many other genes of yet unknown function. Interestingly, these global expression experiments indicated that around half of ER α target genes are down regulated upon induction with estrogen, knowing that many of these down-regulated genes are cell cycle inhibitors, pro-apoptotic factors or cytokines and growth factors that inhibit proliferation,

explaining the view that estrogen promotes cell survival by down regulating pro-apoptotic genes.

ER+ positive tumors have the advantage of benefiting from endocrine therapy, which results in positive outcomes. However, in many the cases, resistance to endocrine therapy and metastasis occur instead (*Davies et al., 2011*) (*Ma et al., 2015*). Resistance to endocrine therapy is a complex process that could be due to many factors, like alterations in growth factor receptor tyrosine kinases (RTKs) such as EGFR and HER2, or insulin-like growth factor receptor (IGF1-R) that increase ER transcriptional activity leading to reduced sensitivity to ER targeted therapies and poor outcomes (*Osborne et al., 2003*) (*Arpino et al., 2004*) (*Song et al., 2004*). Deregulation of the PI3K-AKT-mTOR pathway (*PIK3CA* being the most frequently mutated gene in ER+ breast cancer) (*Ellis&Perou 2013*) has also been involved in the process of resistance, in which the use of mTOR inhibitors has recently shown to improve progression-free survival (PFS) for patients failing previous endocrine therapies (*Sanchez et al., 2011*) (*Baselga et al., 2012*). Dysregulation in cell cycle components is common in ER+ breast cancer and CDK inhibitors are now FDA approved for use in combination with endocrine therapy to treat advanced stage ER+ tumors (*Cancer Genome Atlas N. 2012*) (*Thangavel et al., 2011*).

In addition to the non-ER-related alterations (tumors expressing wild-type ER) listed above, mutations in the *ESR1* gene itself such as *ESR1* amplifications or point mutations were found in endocrine therapy resistant breast tumors. The latter mainly happening in the ligand-binding domain (LBD) and leading to constitutive hormone-independent activation of ER (*Zhang et al., 1997*) (*Basudan et al., 2019*). Some mutations cause estrogen hypersensitivity like (K303R, E380Q) (*Fuqua et al., 2000*) (*Toy et al., 2017*) or neutral, retaining hormone dependent activation function (S432L, V534E) (*Toy et al., 2017*). The most common and well characterized point mutations are the activating mutations occurring at the Y537 and D538 residues which induce changes in protein structure, resulting in reduced ligand affinity. This may be a potential mechanism for their ligand-independent activity even upon antagonist binding and resistance to endocrine therapy (*Toy et al., 2013*) (*Pavlin et al., 2018*). Several studies have also shown that these mutations could also recruit

coactivators like SRC-1 and SRC-3 in the absence of ligand to further potentiate ER transcription (*Gates et al.,2018*) (*Merenbakh-Lamin et al.,2013*). Moreover, several studies have shown that WT and mutant ERs share classical ER signaling signatures in addition to the mutant-specific transcriptional regulation (*Jeselson et al.,2018*) (*Bahreini et al.,2017*) (*Martin et al., 2017*). There was a high overlap between ER chromatin binding sites of estrogen-stimulated WT receptor and hormone-deprived mutant receptors, indicating that the exact mechanism of how these constitutive activating mutations regulate transcription should be studied further.

3) Estrogen receptor gene (ESR1) regulation:

As I discussed above, ER α expression and levels in the tumor cells is a limiting factor that predicts the progression of the tumor and response to endocrine therapy. The availability of the receptor in the cell is a result of a fine balance between the synthesis and degradation of the receptor (protein turn over) (*Nonclercq et al.,2004*). The expression of ER α is regulated by several epigenetic and transcriptional factors present on the *ESR1* promoter.

a) *The structure of the ESR1 promoter*

The human *ESR1* gene, whose genomic organization was described in 1986 (*Ponglikitmongkol et al.,1988*), is 300 kb in size, located on chromosome 6q25.1 locus, and includes a 160 kb 5'-region containing regulatory sequences for *ESR1* transcription. The comparison between ER α cDNA sequence in human and other species have showed a high level of conservation except at the 5'- end (*Koike et al.,1987*) (*Krust et al.,1986*) (*White et al.,1987*), indicating that *ESR1*, just like other steroid hormone receptors, can have multiple promoters (*Hodin et al.,1989*) (*Kastner et al.,1990*). To date, several exons encoding 5'-untranslated regions (UTRs) of *ESR1* mRNAs have been identified, alternative splicing of these exons have resulted in the identification of different *ESR1* mRNA transcripts that vary in their 5'UTR length in different breast cancer cell lines (*Flourirot et al.,1998*) (*Grandien et al.,1996*) (*Thompson et al.,1997*). Different terminologies have been used by researchers to describe the different promoters used in

ESR1 expression, which was confusing until Kos et al in 2001 suggested a simpler nomenclature, based on the chronological order of the discoveries of human ER α exons upstream of the translational start site (Ponglikitmongkol et al.,1988). At least seven *ESR1* promoters, situated upstream exon 1, have been identified that can control *ESR1* expression differently in different cellular contexts or tissues (Kos et al.,2001). **Fig 7**

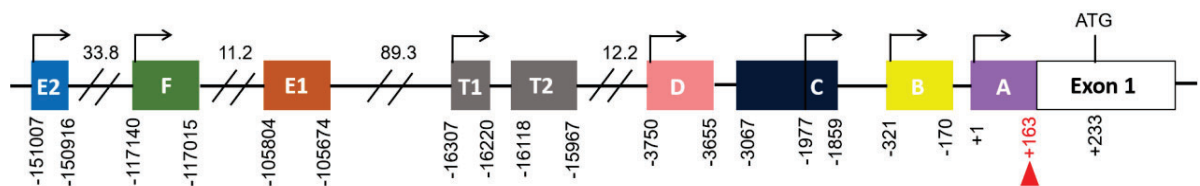


Figure 7: ESR1 genomic promoter organization based on Ros et al.,2001 nomenclature: The promoter region of the *ESR1* gene contains 9 non-coding exons (boxes), annotated from A to F-T1, T2, and 7 alternative promoters each having its own TSS (arrows), Exons E1 and T2 expression depends on the promoters E2 and T1, respectively. Exons A to D constitute the proximal part of the ER α promoter. Expression of exons T1 and T2 is restricted to the testicles while exons E1 and E2 in the liver. The numbers below represent the distance in (bp) from the transcription start site (+1), and the numbers above represent the distance in (kb) between exons. All exons represented in this scheme undergo alternative splicing due to a splice acceptor site located at position +163 bp from +1 transcription start site.

b) *Epigenetic regulation of ESR1:*

A lot of studies have demonstrated that the *ESR1* promoter is epigenetically regulated by many factors that can either activate or silence the gene transcription thus activating or silencing ER α expression. In 1994, Ottaviano et al., have showed that the lack of ER α expression in ER⁻ breast cancer cell lines was due to hypermethylation of the *ESR1* CpG islands (Ottaviano et al.,1994). Then, in the following years other studies showed that the *ESR1* promoter was occupied by several complexes with inhibitory components like DNA methyltransferases (DNMTs) and histone deacetylases like HDAC1 and mSin3A (Ferguson et al.,1997) (Ellison-Zelski et al.,2009) which explained the mechanisms behind ER α regulation and loss of expression in ER⁻ tumors. HDAC1 and mSin3A were shown to associate together at the +1 transcription start site of the *ESR1* promoter, the exact function of this complex is not well understood but it has been shown to correlate with the decrease in certain acetylation marks (AcH3K14) and increase in methylation marks (H4K20me3) thus repressing ER α expression in a hormone dependent manner **Fig 8A** (Ellison-Zelski et al.,2009). DNA hypermethylation was also thought to be responsible for the resistance to anti-hormonal therapy (Lapidus et al.,1998) , so the use of 5-aza-2'-cytidine (5-aza-dC) inhibitor that inhibits the action of DNMT1 have restored ER α expression in the ER⁻ MDAMB231 cell line (Ferguson et al.,1997). Another study has showed that 2 molecular complexes pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 regulate *ESR1* in ER α -positive and ER α -negative breast cancer cells respectively (Macaluso et al.,2003). **Fig 8B**

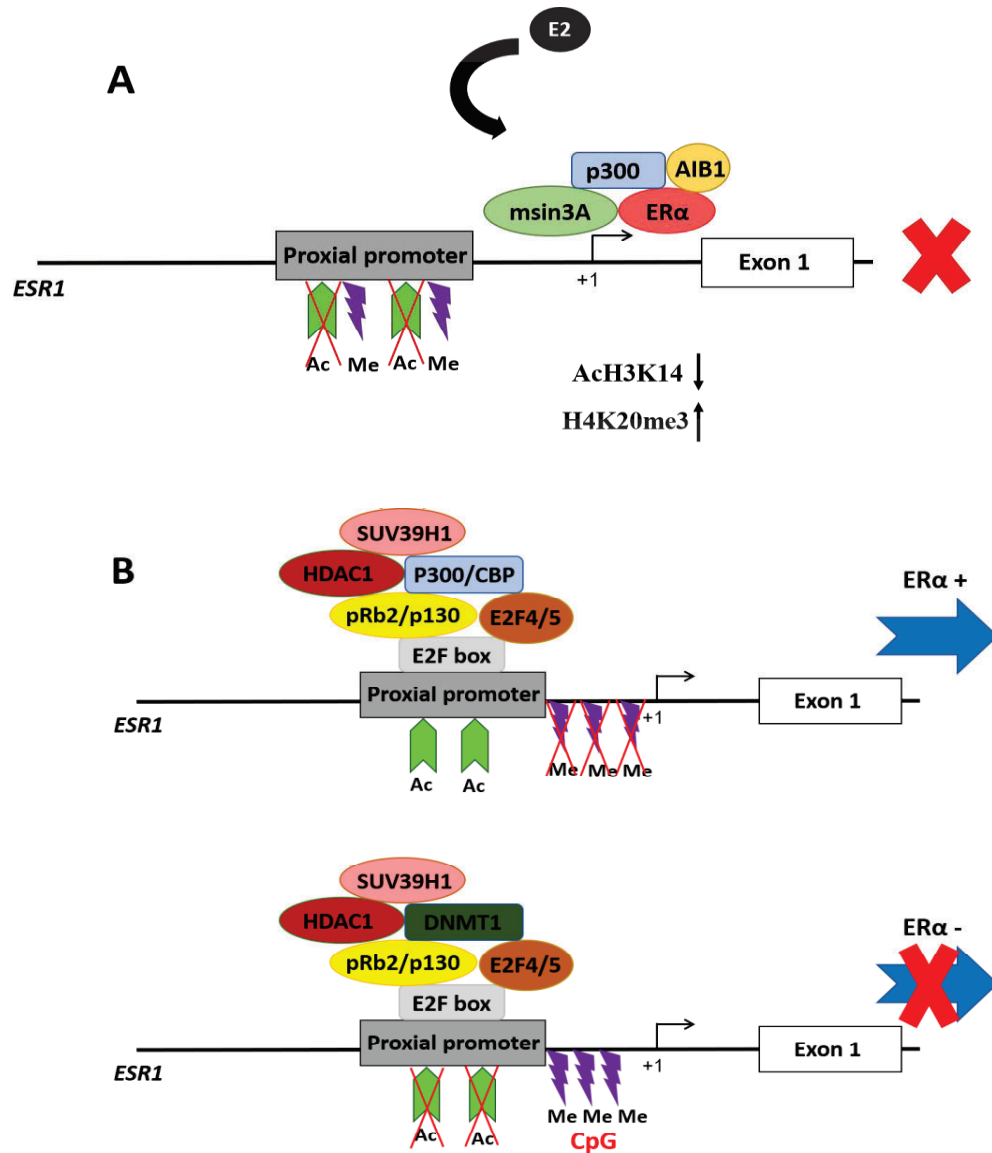


Figure 8: Epigenetic regulation of ESR1: (A): In response to estrogen, msin3A and ERα are recruited to ESR1 proximal promoter mainly on the +1 position from transcription start site to repress ERα expression. (B) (upper panel): In ER+ cells pRb2/p130 recruits HDAC1, SUV39H1 and p300/CBP to ESR1 proximal promoter and increase acetylation marks opening the chromatin and activating its expression. In ER- lines however, (lower panel), DNMT1 is recruited instead of p300/CBP decreasing acetylation marks and increasing methylation marks (CpG islands) on the promoter, leading to more compact chromatin and thus repressing ERα expression.

c) *ESR1* regulation by transcription factors:

Several studies showed that the *ESR1* promoter was occupied by several transcription factors, like members of the AP1 (Wang *et al.*,2004) and FOX (Forkhead box) family (FOXO3A (Guo & Sonenshein 2007) (FOXM1(Madureira *et al.*,2006). However, perhaps the most described ER α associated transcription factors are GATA3 (Lacroix *et al.*,2004) and FOXA1 (Bernardo *et al.*,2010) which activate the transcription of the *ESR1* gene and are necessary for its proper functioning (Vijver *et al.*,2002) (Oh *et al.*,2006) (Lacroix *et al.*,2004). Recently, a study has also revealed a regulation of the *ESR1* between the proximal (promoter) and distal regulatory sequences (enhancer) by a loop-like complex involving GATA3, FOXA1 and the *MEN1* (multiple endocrine neoplasia 1) gene product: menin (Dreijerink *et al.*,2017) **Fig 9B**. In this study, they showed, that menin binds the *ESR1* enhancer region (about 70 kb upstream TSS) at sites that are also bound by FOXA1 and GATA3 and that menin recruits MLL compass-like complex to these sites thus forming a complex between *ESR1* enhancer and the promoter region and regulating its expression. Another transcription factors that rather negatively regulate ER α expression are MTA1 (metastasis associated protein 1) which can either activate or suppress *ESR1* expression depending on the cellular context (ER $^{+}$ or ER $^{-}$) by recruiting a complex to its proximal promoter and exon 1. In the ER $^{+}$ MCF7 cell line MTA1 associates with TFAP2C to positively regulate ER α , while in the ER $^{-}$ MDAMB231 cell line MTA1 associates with IFI16 to mediate its repressive effects (Kang *et al.*,2014) **Fig 9A**. Twist has also been shown to recruit HDAC1 and DNMT3B to the *ESR1* promoter and suppress its transcription (Vesuna *et al.*,2012). NFIB and YBX1 transcription factors physically interact with ER α to repress its activity, and drive breast cancer cells toward a less estrogen-dependent cancer phenotype (Campbell *et al.*,2017).

Studies have shown that ER α loss is a common event in metastasis of the tumors, leading to resistance to endocrine-therapy and thus worse prognosis (Kuukasjarvi *et al.*,1996) (Broom *et al.*,2009). Not only is *ESR1* transcriptionally controlled by several epigenetic and transcription factors that regulate and mostly inhibit the *ESR1* gene, it is important to note that ER α can also be regulated by other post translational mechanisms such as phosphorylation, palmitoylation, and ubiquitination that contribute in ER α activation,

diminishment, and loss (*Le Romancer et al., 2011*). More specifically, studies have revealed that Ser118, Ser167, and Ser305 sites are the most frequently phosphorylated sites of ER α , in which Ser305 phosphorylation activates Ser118 phosphorylation (*Oladimeji et al., 2016*), which in turn contributes to tumor progression, metastasis, and endocrine therapy resistance (*Anbalagan et al., 2016*) (*Park et al., 2017*). ER α is also palmitoylated at Cys-447, and studies have demonstrated that the palmitoylation of ER α is essential for its location and function in the plasma membrane (*Pedram et al., 2007*) (*Acconcia et al., 2005*). After estrogen binding, the palmitoylation of ER α allows the activation of downstream signaling pathways (*Adlanmerini et al., 2014*). At last, although ubiquitination is the primary way of degrading ER α , different studies have showed that the functions of ER α ubiquitination is rather more complicated (*Wu et al., 2017*) (*Yang et al., 2018*) (*Xue et al., 2019*). For example, while ER α ubiquitination promotes tumorigenesis in hepatocellular carcinoma, it results slower growth of breast cancer cells (*Yang et al., 2018*) (*Xue et al., 2019*).

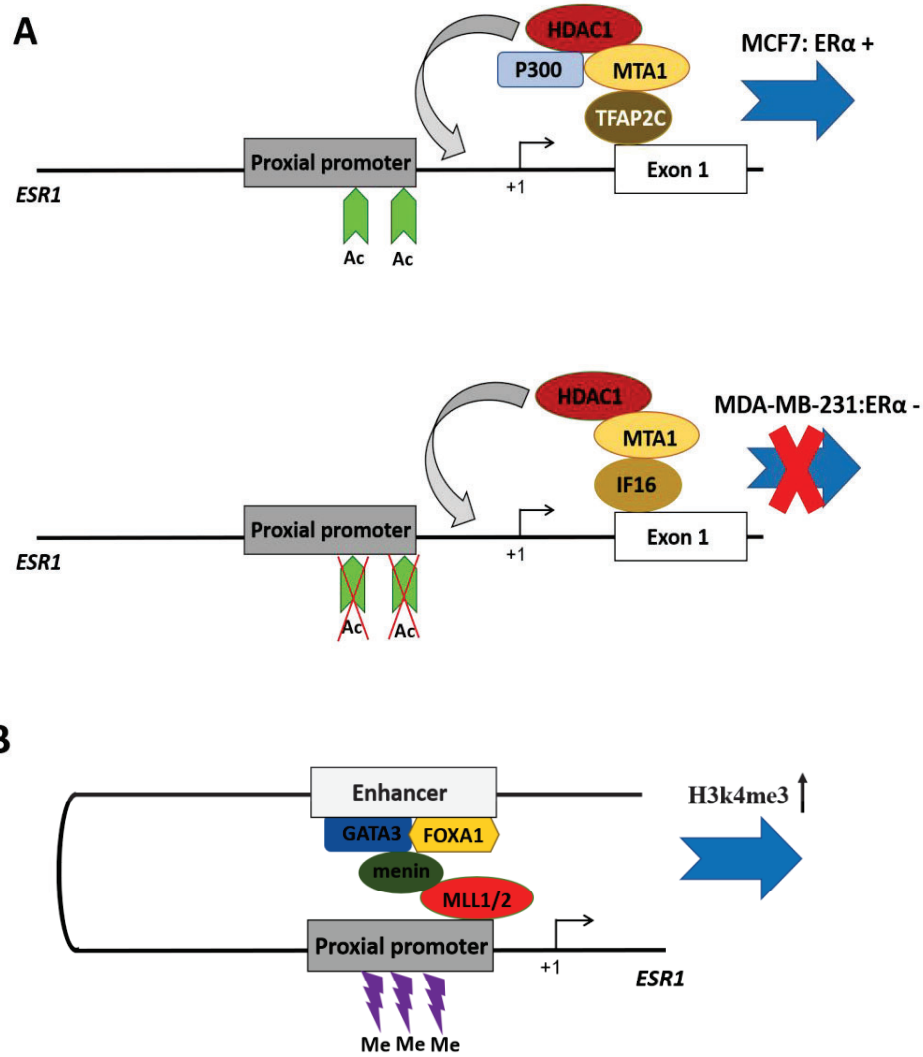


Figure 9: ESR1 regulation by transcription factors: (A): differential regulation of ESR1 by MTA1 in ER⁺ and ER⁻ breast cancer cells. (B): Schematic representation (as proposed by Dreijerink et al.,) of the regulation of ESR1 through formation of a loop between its distal (enhancer) promoter bound by GATA3 and FOXA1 and its proximal bound by menin and the MLL complex that activates gene transcription by increasing the H3K4me3 marks.

III. **MEN1 syndrome (Multiple endocrine neoplasia type 1) and *MEN1* gene:**

1) Generalities:

Multiple endocrine neoplasia syndrome type 1 (MEN1, OMIM#131100) is a hereditary disease, transmitted in an autosomal dominant trait, characterized by the multiple occurrences of neuroendocrine tumors mostly seen in the parathyroid glands, endocrine pancreas and anterior pituitary gland (*Brandi et al.,2001*). MEN1 syndrome whose prevalence ranges from 3–20/100,000 people and equally in men and women, could also occur sporadically (*Francesca et al.,2018*). One of the most prevalent manifestations of the disease is the hyper secretion of hormones such as hypergastrinemia, hyperparathyroidism and sometimes hyperinsulinemia. Many MEN1 tumors can be benign with an only clinical outcome of hypersecretion of hormones, while other MEN1 tumors can be leading to malignancy (*Francesca et al.,2005*). Tumors developed by patients show mutations and frequent loss of heterozygosity at the locus of the *MEN1* gen, indicating that the *MEN1* gene is a tumor suppressor. *MEN1* inactivation is also involved less frequently with the development of foregut endocrine carcinoids, adrenal cortical and thymus tumors, as well as non-endocrine tumors, such as angiofibroma and lipomas (*Lemos et al.,2008*). **Fig 10**

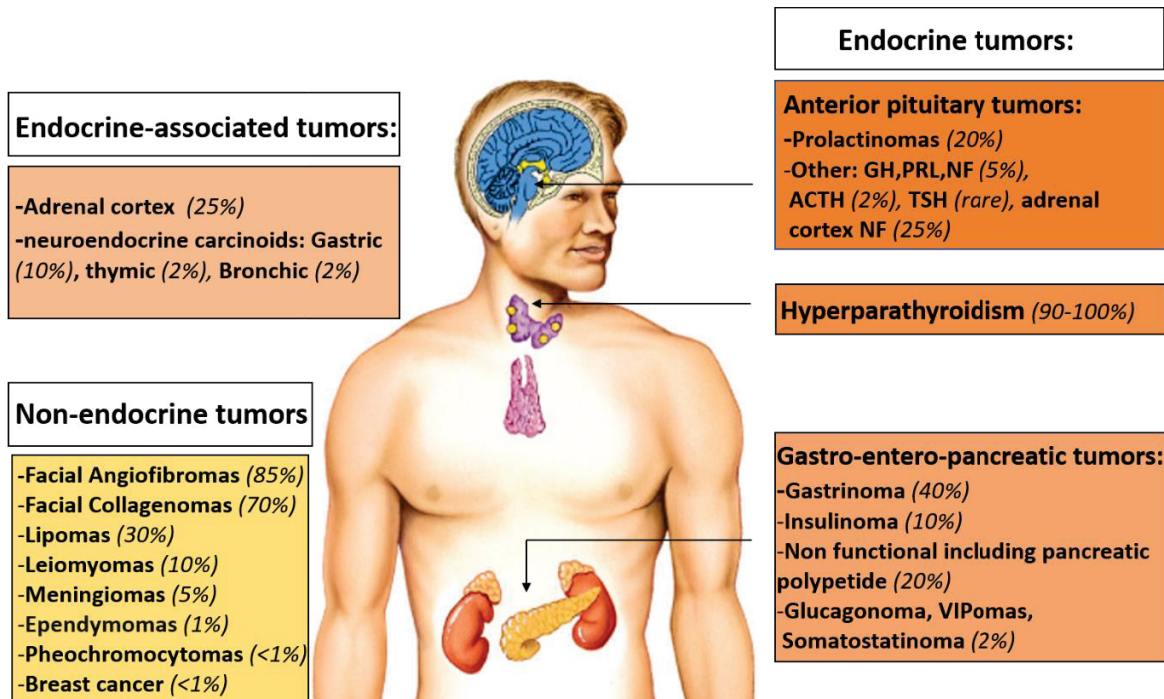


Figure 10: Clinical manifestations of MEN1 syndrome and their penetrance estimated at 40 years of age.

2) The *MEN1* gene:

The *MEN1* gene was identified in 1997 (*Chandrasekharappa et al., 1997*) (*Lemmens et al., 1997*), the cytogenetic location of this gene being on chromosome 11q13. It is known for years now that the *MEN1* is a tumor suppressor in endocrine tissues but oncogenic in certain types of leukemia (*Yokoyama et al., 2005*). Recently, the role played by this gene in mammary and prostate tissues has elicited some debates, because as different studies showed some distinct effects of these gene in these types of tumors.

a) *Structure, evolution, and significance:*

The human *MEN1* gene comprises 10 exons distributed over 7.2 kb in the chromosome 11q13 region and encodes an mRNA of approximately 2.8 kb. The coding sequence starts from exon 2 to 10 forming a total of 1830 bp and encoding the 610-amino acid protein called menin. In humans, another *MEN1* transcript of 4.2 kb exist and specifically expressed in thymus and pancreas (*Lemmens et al., 1997*). The fact that *Men1* orthologs exists in species like zebrafish and *Drosophila* (*Manickam et al., 2014*) (*Guru et al., 2001*) but absent in *Saccharomyces cerevisiae* and *C.elegans* indicates that the gene is relatively new or “young” compared to others in terms of gene evolution. The *MEN1* is highly conserved between mammals especially human and mice that show high sequence homology (*Guru et al., 1999*). This fact was a useful tool in studying the pathology of *MEN1* mutations and MEN1 syndrome through the generation of *Men1*- deficient mouse models (*Crabtree et al., 2001*) (*Bertolino et al., 2003a*).

b) *MEN1 mutations:*

More than a thousand *MEN1* sequence abnormalities have been identified to date (1,133 germline and 203 somatic mutations) (171). Loss of heterozygosity (LOH) of *MEN1* is a common observation in familial MEN1 patients as well as sporadic tumors (*Lemos et al., 2008*). The disease follows Knudson's "two hit" model in which the first hit is inherited from a parent in the familial case or sporadically as a consequence of any sudden inactivating mutation in the *MEN1* gene during early embryogenesis. These mutations are scattered throughout the entire 1,830-bp coding region and splice sites and can be

either frameshift deletions or insertions (41%), nonsense mutations (23%), missense mutations (20%), splice site mutations (9%), in-frame deletions or insertions (6%), and whole or partial gene deletions (1%), leading in all cases to a truncated and/or inactivated protein (*Francesca et al., 2006*) (*Lemos et al., 2008*). Unfortunately, despite this wide range of mutations discovered, it was not possible to correlate between each mutation type (genotype) and the phenotype or characteristics seen in MEN1 patients (*Wautot et al., 2002*), suggesting that menin needs its different domains to exert its biological functions. Genetic polymorphism in the *MEN1* gene have also been reported, a total of 24 different polymorphisms (12 in the coding region, nine in the introns, and three in the untranslated regions). It is important to differentiate between these polymorphisms and mutations, as some studies have reported that between 5% and 10% of MEN1 patients didn't show mutations in the coding region or adjacent splice sites indicating that these patients may have had mutations in non-coding sequences, including the promoter, untranslated regions and introns (*Lemos et al., 2008*).

3) Mouse models: **Table 3**

In order to study the normal biological function of the *MEN1* gene in different cells and tissues and the mechanisms underlying its involvement in MEN1 disease, different laboratories around the world, including our own, have adopted gene-targeting in the mouse as approach through the generation of *Men1* mutant mouse models. In general, these approaches carry out either germline (conventional mutant mice) or cell type-specific (conditional mutant mice) disruptions of a given gene.

Conventional *Men1* mutant models allowed mimicking the genetic events occurring in MEN1 patients, facilitating studies on the natural history of the disease. Results from these mouse models showed that homozygous *Men1* mutant embryos (complete menin loss) died in mid-gestation due to neural tube disclosure, abnormal development of fetal liver and heart (*Bertolino et al., 2003b*), and cranial and facial developmental defaults (*Crabtree et al., 2001*). These findings thus indicated the essential role played by the gene in controlling cell proliferation and differentiation in these tissues during the embryonic mid gestation stage.

Heterozygous *Men1* mutant mice, on the other hand, developed multiple endocrine tumors from the age of 9 months onwards, affecting islet cells, parathyroid, pituitary, and adrenal glands, associated with hyperinsulinemia and hyperparathyroidism. In addition, thyroid follicular tumor was also found in some of the mutant mice, although it has been previously considered as a coincidental association in MEN1 patients. all, in MEN1 patients (*Crabtree et al., 2001*) (*Bertolino et al., 2003a*). 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. Importantly, at a small but substantial proportion of the mutant mice also developed breast and prostate tumors, thus suggesting that the gene could also have relevant function in these two hormone-related tissues.

Conditional *Men1* mutant models were generated by crossing mice carrying the floxed *Men1* allele *Men1*^{F/F} with different transgenic mice expressing Cre recombinase in given cell types or tissues and helped understand the cellular and molecular mechanisms involved in MEN1 disease and. Many models have been created: β -Cell and α -Cell *Men1* mutant mice (*Bertolino et al., 2003c*)(*Biondi et al., 2004*)(*Lu et al., 2010*)(*Shen et al., 2010*), Pan-pancreatic *Men1* mutant mice (*Shen et al., 2009*) (*Bonnavion et al., 2015*), Gastrointestinal epithelium cell *Men1* mutant mice (*Veniaminova et al., 2012*), Parathyroid *Men1* mutant mice(*Libutti et al., 2003*), Prolactin-secreting-cell *Men1* mutant mice (*Biondi et al., 2004*)(*Seigne et al., 2013*), Craniofacial osteogenic cell and osteoblast *Men1* mutant mice (*Kanazawa et al., 2015*) (*Liu et al., 2017*) (*Lee et al., 2018*), and more recently, mammary and prostate gland *Men1* mutant mice (*Seigne et al., 2013*)(*Seigne et al., 2010*). The last 2 models were significant tools to understand whether and how menin is involved in hormone dependent cancers (see below in part III section 5)

Conventional <i>Men1</i> mutant models:	Conditional <i>Men1</i> mutant models: (cell type specific)	Inducible <i>Men1</i> mutant models:
<p>-mimics MEN1 patients</p> <p>-germline mutation</p> <p>References:</p> <ul style="list-style-type: none"> - Crabtree et al., 2001 - Zhao-qi Wang et al.,2003 - Loffler KA et al.,2007 - Harding B et al.,2009 	<p>1. β-Cell <i>Men1</i> mutant mice (<i>Men1^{F/F}-RipCre⁺</i>)</p> <ul style="list-style-type: none"> - Crabtree et al., 2001 - Bertolino P et al.,2003 - Biondi CA et al.,2004 	<p>1. Crossing <i>Men1^{F/F}</i> mice with transgenic mice carrying tamoxifen-inducible Cre</p> <p>Schnepf RW et al., 2006</p>
	<p>2. α-Cell <i>Men1</i> mutant mice (<i>Men1^{F/F}-GluCre⁺</i>)</p> <ul style="list-style-type: none"> - Lu J et al., 2010 - Shen HC et al., 2010 	<p>2. inducible adult β-cell-specific PdxCreER line</p> <p>Yang Y et al., 2010</p>
	<p>3. Pan-pancreatic <i>Men1</i> mutant mice</p> <ul style="list-style-type: none"> -(Pdx1Cre mice) Shen HC et al., 2009 - (pTF1Cre mice) Bonnavion R et al.,2015 	<p>3. mouse line with tamoxifen-inducible Cre-ER driven by the rat insulin promoter.</p> <p>(<i>Men1^{F/F}-RIP2Cre^{ER}</i>)</p> <p>Lines KE et al.,2017</p>
	<p>5. Parathyroid <i>Men1</i> mutant mice (<i>Men1^{F/F}-PthCre</i>)</p> <p>Libutti SK et al., 2003</p>	
	<p>6. Mammary gland <i>Men1</i> mutant mice (<i>Men1^{F/F}-WapCre⁺</i>)</p> <p>Seigne C et al.,2013</p>	
	<p>7. Craniofacial osteogenic cell and osteoblast <i>Men1</i> mutant mice</p> <ul style="list-style-type: none"> -(osteocalcin-Cre mice) Kanazawa I et al., 2015 - (Runx2Cre mice) Liu P et al.,2017 	

Table 3: Summary of different *Men1* mutant mouse models

4) Menin protein:

a) Generalities:

MEN1 gene encodes a 610 amino acid protein menin that is highly conserved among species from drosophila to human and shares no homology with other proteins or protein domains (*Wautot et al., 2002*). Menin is ubiquitously expressed and its functions can vary in a tissue specific manner, such that it plays a tumor suppressive role in endocrine organs, whereas it promotes leukemogenesis (*Matkar et al., 2013*). Menin is mainly a nuclear protein but it is also present in the cytoplasm and even in the cell membrane where it may interact with several cell signaling pathways.

Although menin has been so far only linked to MEN1 syndrome and leukemogenesis, it is important to note that its involvement in several other cancers emerges recently, since it interacts with a variety of proteins from different signaling pathways, causing either transcriptional activation or repression (*Matkar et al., 2013*).

b) Structure of the protein:

Menin has two classic nuclear localization signals NLS1 and NLS2 (*Guru, et al., 1998*) on its C-terminus which are indispensable for its nuclear localization. In the same C-terminus region a novel accessory NLS: NLSa was also identified. These NLSs allow menin to stay in the nucleus, a basis for its binding to the DNA of its target genes (*La et al., 2006*). Menin have two functional nuclear export signals, NES1 that extend from amino acid 33 to 41 and NES2 that extend from amino acid 258 to 267 in human sequence of menin. These NESs aid in its translocation between the cytoplasm and nucleus and vice versa (*Cao et al., 2009*). Menin also contain a SID domain extending from amino acid 371 to 387, with which it interacts with the histone deacetylase (HDAC) corepressor msin3A. Five guanosine triphosphatase (GTPase) sites: G1 to G5 were found in menin. Finally, menin also contain the LXXLL motif, extending from amino acid 263 to 267, that is usually present in many transcription factors. This motif allows menin to interact with nuclear receptors like ER α . **Fig 11**

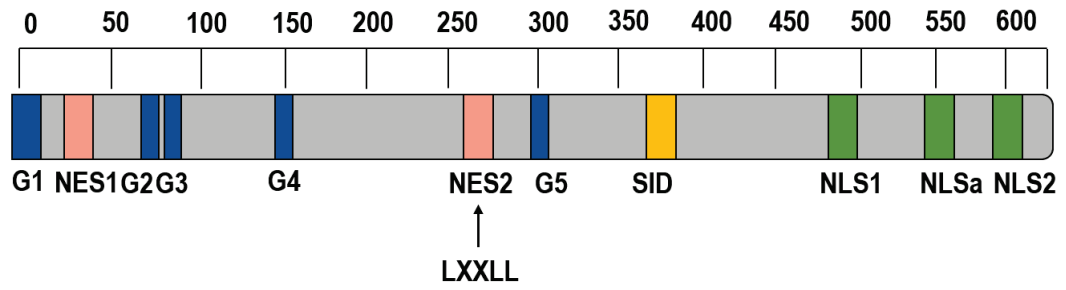


Figure 11: Menin protein structure

c) *Menin: a scaffold protein that regulates gene transcription:*

Because menin lacks homology with other proteins, it was challenging to elucidate its exact molecular and cellular functions. Substantial efforts have been made by many groups to identify menin-interacting proteins, in the hopes of finding clues about how menin biochemically suppresses tumorigenesis. These studies, together with uncovering menin's crystal structure, provided valuable information regarding the biochemical function of menin and its ability to activate or repress gene expression. Menin, that is present at many sites of the chromatin, has been shown to interact with transcription activators or repressors, and cross talks with various signaling pathways and proteins involved in controlling different cellular activities (*Balogh et al., 2006*) (*Poisson et al., 2003*).

A. Menin interacts with epigenetic factors:

i. Menin and the MLL/COMPASS-like complex:

The first interaction between MLL/COMPASS-like and menin was identified in 2004, indicating that menin's partnership with this complex is an activating one (*Yokoyama et al., 2004*), then other studies have shown that this partnership could have activating or suppressive roles depending on the tissue.

In acute leukemia the *MLL1* (mixed lineage leukemia) gene situated on chromosome 11q23 can undergo chromosomal translocations with one of various partner genes, resulting in the expression of MLL1 fusion proteins (MLL-FPs) (*Slany 2005*) that can induce leukemia through activating the HOX genes (*Hess et al., 2004*). Several studies have shown the involvement of menin in the positive regulation of HOX genes. Yokoyama et al have shown that menin binds to different MLL-FP to induce myeloid transformation and the upregulation of genes like HOXA7, HOXA9 and HOXA10 (*Yokoyama et al., 2005*). The same group then showed that menin links MLL proteins with

LEDGF and that this complex recruited histone methyl transferases to the promoter of HOXA9 and Meis1 genes in the mouse hematopoietic cells to regulate their expression (*Yokoyama & Cleary 2008*). Another oncogenic role of menin/COMPASS-like roles was demonstrated by Thiel et al. They showed that menin recruits both wild-type MLL and oncogenic MLL-AF9 fusion protein to the loci of HOX genes to activate their transcription through both H3K4 and H3K79 methylation (*Thiel et al., 2010*).

In the endocrine pancreas menin interacts with MLL1 to upregulates the expression of cyclin-dependent kinase (CDK) inhibitors (CDKIs) p18 and p27, thereby reducing beta cell proliferation. In pancreatic MIN6 and MEFs cells, menin was found to bind to the *p18* and *p27* promoters and recruit the compass like compass like complex that adds tri-methylation to histone H3 at lysine 4 (H3K4me3), a chromatin modification associated with transcriptional activation (*Schnepp et al., 2006*) (*Karnik et al., 2005*) (*Milne et al., 2005*). A decreased expression of P18 and P27 was observed in mouse and human MEN1 insulinomas (*Karnik et al., 2005*). These results afford the molecular mechanisms indicating that, while menin/COMPASS-like complex plays an oncogenic role in leukemia, it plays a tumor suppressive role in the endocrine pancreas.

Menin's crystal structure could be represented as a curved left hand where the C- terminus resembles the curved fingers, the middle region resembles the palm, and the transglutaminase-like domain resembles the thumb. In addition, a long β hairpin forms the N-terminal domain. The N-terminal region of MLL1 binds the deep central pocket of menin, and the further downstream sequence of MLL1 loops around the N-terminal part of menin, forming an alpha helix that directly contacts the menin N terminus (*Huang et al., 2012*) (*Murai et al., 2011*). It is important to note that MLL1 and JunD compete to bind menin in the same pocket of its structure however MLL1 has more affinity (*Huang et al., 2012*). Based on the crystal structure of menin and MLL1/2, several inhibitors that block efficiently the interaction between the two were produced,

including MI-2-2 which has been shown to inhibit HOX genes expression in leukemic cells (*Shi et al., 2012*), MI-503 and MI-463 which have also shown some positive results in murine models (*Borkin et al., 2015*). **Fig 12**

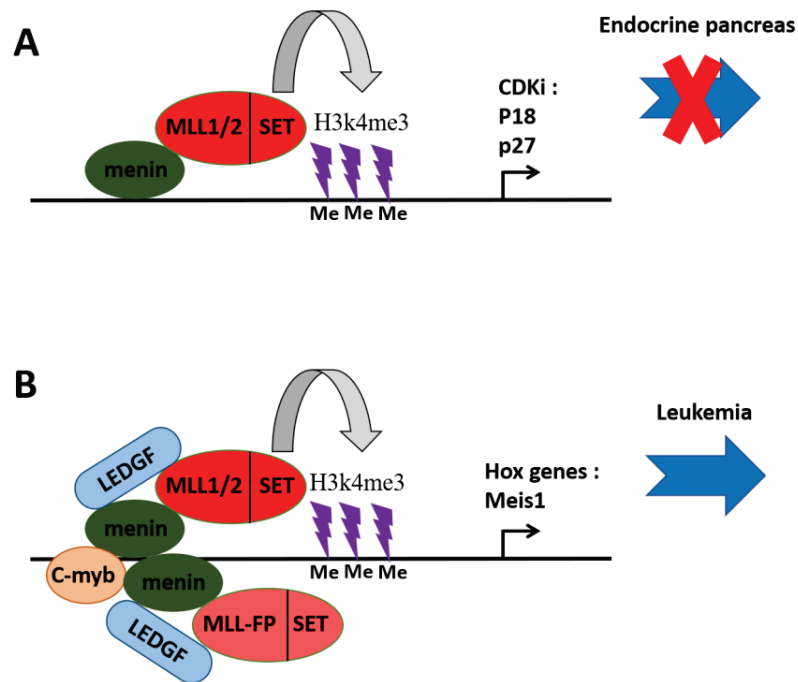


Figure 12: Menin and the MLL complex: (A) In the endocrine pancreas menin interacts with MLL1/2 and play a tumor suppressive role by positively regulating the expression of cyclin inhibitors p18 and p27. (B) in leukemic cells, however, menin plays an oncogenic role by recruiting both wild type and fused MLL proteins (MLL-FP) to increase the expression of Hox genes. Inspired by Matkar et al.,2013

ii. PRMT5:

Menin binds the negative regulator PRMT5 of the Hedgehog pathway. By using *Men1*-deficient mouse embryonic fibroblasts (MEF) and primary islets derived from *Men1* mutant mice, Gurung et al provided compelling data showing that menin plays a crucial role in inhibiting the Hedgehog pathway through its physical interaction with PRMT5 and its binding to its downstream genes *Gli* and *Gas1* promoters increasing the H4R3me2 inhibitory marks (Gurung et al., 2013). They also showed that, menin plays a tumor suppressive role by inhibiting the shh pathway via PRMT5 in mouse *Men1* insulinomas (Gurung et al., 2013).

iii. EZH2:

EZH2 belongs to the polycomb repressive complex (PRC)2 which is known to add the repressive H3K27me3 histone trimethylation marks on their targets' promoter (Margueron & Reinberg 2011). Menin was first reported to bind to the pleiotrophin (*PTN*) promoter together with polycomb repressive complex (PRC)2 in lung cancer cell lines, where it plays a tumor suppressor role by increasing the repressive H3K27me3 marks and thus repressing the expression of this proliferative gene (Gao et al., 2009). Also, in *Men1*^{-/-} MEFs, two members of PRC2, EZH2 and SUZ12, were diminished from the *PTN* promoter, confirming the importance of menin in allowing these factors to bind to the *PTN* promoter and regulate its expression. Gherardi et al recently showed in vivo and in vitro that menin recruits EZH2 via an indirect mechanism involving Akt-phosphorylation, without physically binding to EZH2, to the *Inhbb* promoter (gene) and repress its expression by adding H3K27me3 repressive marks (Gherardi et al., 2017). Taking into consideration that menin loss in β -cell-specific *Men1* mutant mice developing insulinomas resulted in increased Activin B encoded by the *Inhbb* gene, these

results uncovered detailed mechanisms underlying the interaction between menin and EZH2.

B. Menin interacts with transcription factors:

i. Jun D:

Jun D belongs to the AP1 family of transcription factors and is the first identified menin partner (Agarwal *et al.*, 1999). Jun D has been shown to activate proliferation of menin-deficient MEFs (Agarwal *et al.*, 2003). Opposite to menin/MLL interaction (which binds menin in the same pocket as JunD), menin has been shown to play a repressor role through its association with JunD and mediate this effect on different levels. First, menin was indispensable for the recruitment of HDAC1 and mSin3A repressors on the promoters of JunD target genes to inhibit their expression (Kim *et al.*, 2003). Second, menin was shown to compete with c-Jun N-terminal kinase (JNK) binding to JunD, which normally phosphorylates JunD leading to its activation (Huang *et al.*, 2012), thus hindering the pro-proliferative activity of JUND. Menin has repressive effects on JunD-mediated transcriptional activation, however the function of menin and JunD interaction in tumorigenesis remains elusive. Agarwal *et al.* hypothesized that, though JunD inactivation has so far not been implicated in the pathogenesis of any tumors, it is possible that tumor suppressor-like properties of JunD are due to the functional synergy between the suppressive effects of menin and JunD. Inversely, when menin is inactivated, JunD may exert an oncogenic role, since some *MEN1* missense mutations were shown to disrupt menin–JunD interaction.

ii. NFkB:

Menin has been shown to inhibit the transcription of NFkB target genes in hepatic cells. In these cells, menin has been shown to physically interact with

NFkB and repress its activity by interacting with Sirt 1, a histone deacetylase (HDAC), that deacetylates lysine 310 of p65 subunit of NFkB, thus inhibiting the downstream inflammatory response and cytokine secretion (*Heppner et al., et al.,2001*) (*Gang et al., 2012*).

iii. MYC (c-Myc):

MYC belongs to the *MYC* oncogene family that is called “super-transcription factors” and consists of two other members, N-Myc, and L-Myc (*Adhikary et al., 2005*) (*Pelengaris et al.,2002*) (*Dang et al.,2012*). This family regulates the transcription of at least 15% of the entire genome (*Dang et al.,2006*). The major downstream effectors of MYC include those involved in DNA repair, ribosome biogenesis, protein translation, cell-cycle progression and metabolism, thus controlling a wide range of biological functions, such as cell proliferation, differentiation, and immune surveillance (*Dang et al.,2006*) (*Meyer et al.,2008*). MYC mediates its functions by binding to Max that shares a common structural homology (BR/HLH/LZ motif) which is required for DNA–protein interactions (*Adhikary et al., 2005*) (*Pelengaris et al.,2002*) (*Meyer et al.,2008*). The MYC/Max heterodimer then activates transcription by binding to a conserved E-box DNA sequence (CACGTG) located in the transcriptional regulatory region of target genes where it recruits chromatin-modifying complexes, like GCN5 and TIP60 (histone acetyltransferases), TIP48 (ATP-binding protein), and TRRAP (transactivation/transformation-associated protein) (*Adhikary et al., 2005*) (*Pelengaris et al.,2002*) (*Meyer et al.,2008*).

Myc family is frequently deregulated in human cancers (>50%). Myc deregulated expression can be induced either by retroviral promoter insertion, chromosomal translocation/amplification, activation of super-enhancers within the *MYC* gene, and/or mutation of upstream signaling pathways that enhance MYC stability (*Meyer et al.,2008*). Targeting MYC has been challenging for a long time, first due to its “undruggable” protein structure and its nuclear localization. For these reasons alternative approaches that indirectly inhibit

MYC oncogenic functions have been achieved such as *MYC* transcription and/or translation inhibition, and MYC destabilization, or MYC/Max complex disruption like such as the MYC/Max inhibitor 10058-F4 (*Soucek et al., 2008*) (*Chen et al., 2018*) (*Huang et al., 2006*).

MYC is another menin partner that collaborates with menin to both activate or repress the expression of certain genes. However, unlike Menin / MLL-mediated H3K4me3 regulation on the promoter of the target gene (*Jin et al., 2010*), the latest report shows that menin can directly interact with the transactivation domain (TAD) of MYC and then bind to E boxes to enhance the transcription of MYC target genes (*Wu et al., 2017*). The enhancement of MYC target gene transcription depends on P-TEFb, which is a key factor in promoting the regulation of MYC transcription in HT1080 and HepG2 cells. Menin and MYC can also bind to SKI-interacting protein (SKIP) co-activator at the *HIV-1* promoter (*Bres et al., 2009*). Interestingly, it is reported that menin can interact with MYC to regulate its own expression in HEK293 embryonic kidney cells, where menin and the MLL complex interact with the transcription factor FUSE binding protein 1 (FBP1/FUSBP1) to bind the FUSE site (box) on the *MYC* promoter and stimulate *MYC* transcription (*Zaman et al., 2014*). However, the binding of menin on the MYC promoter has not been clearly defined.

C. Menin interferes in several signaling pathways:

As mentioned above, menin is mainly a nuclear protein which allows it to interact with several transcription factors and histone modifiers and play an important role in regulation of gene transcription. However, menin also exists in the cytoplasm and cell membrane, where it interacts with several signaling pathways such as:

i. Canonical and non-canonical TGF- β pathway:

Menin interacts with SMAD3, the downstream effector of the TGF- β canonical pathway. Menin inactivation inhibited Smad 3 binding to the regulatory transcriptional sites of its target genes, thus leading to abortion of the TGF- β induced inhibitory signal of cell proliferation (*Kaji et al., 2001*).

Menin also interacts with Runx2 and Smad1/5, the downstream effectors of the TGF- β non canonical pathway BMP (bone morphogenetic pathway) that is responsible for the commitment of the multipotent mesenchymal stem cells into osteoblasts (*Sowa et al., 2004*). This may explain the abnormalities in facial and cranial bone development in Men1-null mutant embryos (*Bertolino et al., 2003b*).

ii. Wnt/ β -catenin:

Our team together with our collaborators found that menin physically interacts with beta-catenin, and the overexpression of menin reduces nucleus accumulation of beta-catenin and suppresses its transcriptional activity in *Men1*-null MEFs, whereas nuclear beta-catenin accumulation was found in insulinomas from β -cell-specific *Men1* knockout mice (*Cao et al., 2009*). Jiang et al also found that beta-catenin ablation led to the suppression of tumorigenesis and significantly improved hypoglycemia and the survival rate of *Men1*-deficient mice. Applying the small molecule inhibitor, PKF115-584, in *Men1*-deficient mice to antagonize the β -catenin signaling, also suppressed tumor cell proliferation *in vitro* and *in vivo* (*Jiang et al., 2014*). These results showed that menin negatively regulates the Wnt/ β -catenin pathway in pancreatic β -cells, making this pathway a clinical or therapeutic interest to treat MEN1 patients.

iii. PI3K/ PTEN/AKT/MTOR or PAM pathway:

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signaling pathways are often regarded as a single unique pathway, since they are much interconnected. They are involved in several

processes that lead to cellular growth and survival especially during stress (*Datta et al., 1999*). Since tumors exist in an intrinsically stressful environment (limited nutrient and oxygen supply, low pH), this pathway plays important role in cancer. Mutations affecting this pathway exist in a number of different solid and hematological tumors (breast, colon, neuroendocrine tumors (NETs), kidney cancer, and some lymphomas). The activation of the PI3K/Akt/mTOR pathway disturbs the control of cell growth and survival, which ultimately leads to a competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance. These facts made this pathway a very good target for anticancer agents and many inhibitors have been designed that inhibit this pathway, some which showed great results in different cancers (*Hennessy et al., 2005*) (*Chen et al., 2005*).

The PI3K/AKT/mTOR pathway is stimulated by RTK (receptor tyrosine kinase) and cytokine receptor activation. Tyrosine residues are then phosphorylated and provide anchor sites for PI3K translocation to the membrane. Activated PI3K catalyzes the formation of PIP3, which then recruits PDK1 and AKT to the cell membrane (*Simioni et al., 2018*) (*Tuncel & Kalkan 2018*) (*Yu et al., 2015*). AKT is regulated by a variety of hormones, including insulin and growth factors (*Simioni et al., 2018*). PDK1 phosphorylates at Thr308 in the AKT kinase catalytic region (*Szymonowicz et al., 2018*). Activated AKT mediates the regulation of cell growth, proliferation, the cell cycle and glucometabolism by further phosphorylating the GSK-3, FoxOs, Bad, Caspase 9, nuclear transcription factor-kappa B (NF-kappa B), mTOR and p21 proteins (*Risso et al., 2015*) (*Kumar et al., 2013*).

mTOR, a serine/threonine protein kinase activated downstream the PI3K-associated kinase protein family, interprets nutritional signals and regulates cell growth and proliferation (*Wei et al., 2019*). mTOR includes mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1, sensitive to rapamycin, composed of mTOR, Raptor and mLST8, mainly regulates cell growth and energy metabolism. mTORC2, not sensitive to rapamycin,

composed of mTOR, Rictor, Sin1 and mLST1, is mainly involved in reconstruction of the cytoskeleton and cell survival (*Murugan 2019*) (*Kim & Guan 2019*). mTORC1 is a downstream molecule of AKT and is activated by phosphorylated AKT. The downstream effectors of mTORC1 include two signaling pathways: 4EBP1 and S6Ks. 4EBP1 inhibits the assembly of the eukaryotic translation initiation complex eIF4F. High levels of eIF4F are frequently observed in cancer, and could be related with poor prognosis, drug resistance. The eIF4F complex is composed of eIF4A which forms the enzymatic core, eIF4E which is responsible for cap dependent translation of all nuclear encoded mRNA, and eIF4G that act as a scaffold protein to link them together and associates with 43S preinitiation complex and eIF3 (*Chu et al., 2016*). mTORC1 can phosphorylate 4EBP1 (*Rivera-Calderon et al., 2019*). Hypo-phosphorylated 4E-BP1 has a high affinity for eIF4E, while highly phosphorylated 4E-BP1 releases eIF4E, allowing the binding of eIF4G to eIF4E to initiate the translation of relevant mRNAs (*Mahoney et al., 2016*). Studies have also shown that mTORC1 can activate SREBP1 through S6K1 affecting cell lipid production (*Yaguchi et al., 2019*) and mTOR overactivation enhances lipid anabolism in mouse models of obesity and diabetes (*Zhang et al., 2017*).

Menin has been shown to interact with AKT1 and downregulate its kinase activity in pancreatic cells. Menin 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 reported that menin can interact with FOXO1, a downstream effector of Akt, in the hepatocytic cancer cell line HepG2 and MEFs (*Wuescher et al., 2011*). In the same year, a study showed that *MEN1* and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors (*Jiao et al., 2011*). All these publications being focused mainly on endocrine cells. Interestingly, a recent study demonstrated that menin regulates milk protein synthesis through mTOR signaling in the normal mammary epithelial cells (*Honghui et al.,*

2017). In this study they showed that menin overexpression caused significant suppression of factors involved in the mTOR pathway, as well as milk protein κ -casein (CSNK), which suggests that menin can also regulate the PI3K/akt/mTOR pathway in mammary cells.

PTEN is a tumor suppressor gene mutated in many types of cancer. As a lipid phosphatase and negative regulator of the PI3K/AKT/mTOR pathway, PTEN controls several cellular processes, including proliferation, growth, metabolism, migration, and survival. Recently, Wong et al generated MPR ($Men1^{flox/flox} Pten^{flox/flox} RIP-Cre$) and MPM ($Men1^{flox/flox} Pten^{flox/flox} MIP-Cre$) mouse models with insulin-specific biallelic inactivation of *Men1* and *Pten* in β -cells. Their results showed that concomitant loss of *Pten* and *Men1* accelerated NE tumorigenesis (Wong et al., 2019). Co-mutations of *MEN1* and *PTEN* have also been observed in a small percentage of human sporadic PanNETs (Jiao et al., 2011) (Scarpa et al., 2017). All these results suggest that menin can play important roles in the PAM pathway at different levels.

D. Menin interacts with nuclear receptors:

As I mentioned above, menin's protein structure contains a putative nuclear receptor interaction motif (LXXLL) that allows it to bind to nuclear receptors, including ER α , AR, peroxisome proliferator-activated receptor (PPAR γ), liver X receptor (LXR α) and vitamin D receptor (VDR) (Dreijerink et al., 2006) (Malik et al., 2015) (Cheng et al., 2011) (Dreijink et al., 2009a) (Cheng et al., 2015) (Dreijerink et al., 2009b). Dreijink et al showed that menin acts as a cofactor of ER α in which it interacts with ER α ligand-binding domain in a hormone-dependent fashion to regulate its expression by recruiting MLL-HMT to the *ESR1* promoter (Dreijerink et al., 2006). Malik et al have also shown that menin bound MLL (COMPASS like complex) physically interacts with AR and act as a coactivator of AR targets in castration resistant prostate cancer (Malik et al., 2015). Menin was also found to physically interact and regulate the transcriptional activity of PPAR γ and LXR α in the hepatocytes (Cheng et al., 2011) (Cheng et al., 2015) (Dreijerink

et al., 2009a). While menin enhances PPAR α trans activity and prevent triglyceride accumulation in the liver, it inhibits the transcriptional activity of LXR α and repress hepatic lipogenesis. Accordingly, while menin overexpression results in reduced expression of LXR α target genes (such as lipogenic enzymes), menin on the other side acts synergistically with the nuclear receptor PPAR α to control gene expression of fatty acid oxidation in the liver. These results show the importance of menin in maintaining the balance between lipid synthesis and metabolism and protecting the liver from steatosis. Menin can also interact directly with the vitamin D receptor (VDR) and enhance its transcriptional activity. mRNA levels of VDR target genes were also significantly lower in MEN1 parathyroid adenomas compared to normal tissue (*Dreijerink et al., 2009b*).

d) Menin is inhibited by several signaling pathways:

In addition to the various roles that menin plays in different signaling pathways, menin itself is also regulated by many other factors and signaling pathways, either activated or repressed. For example, menin expression is thought to be stimulated by somatostatin to inhibit the expression of gastrin in the stomach (*Mensah-Osman et al., 2008*). Another activating regulation involves TGF β pathway that menin normally stimulates via its interaction with the downstream effector SMAD3. It has also been shown that TGF- β increases menin's expression in parathyroid, MLL-AF9-transformed leukemia cells and mouse hepatocytes (*Sowa et al., 2004*), (*Zhang et al., 2011*). Menin can also be repressed, such as in the case with K-ras that down regulates menin expression through recruiting the DNA methyl transferase 1 (DNMT1) on the *MEN1* promoter (*Wu et al., 2012*). It was reported that glucose, via PI3K/Akt pathway, inhibited menin expression in the endocrine pancreas (*Zhang et al., 2012*). Furthermore, menin was found to be prone to post-translational modifications such as phosphorylation, ubiquitylation and SUMOylation, and to be post translationally repressed by miR24-1 (*Matkar et al., 2013*).

All menin interactions mentioned above are summarized by **Figure 13**

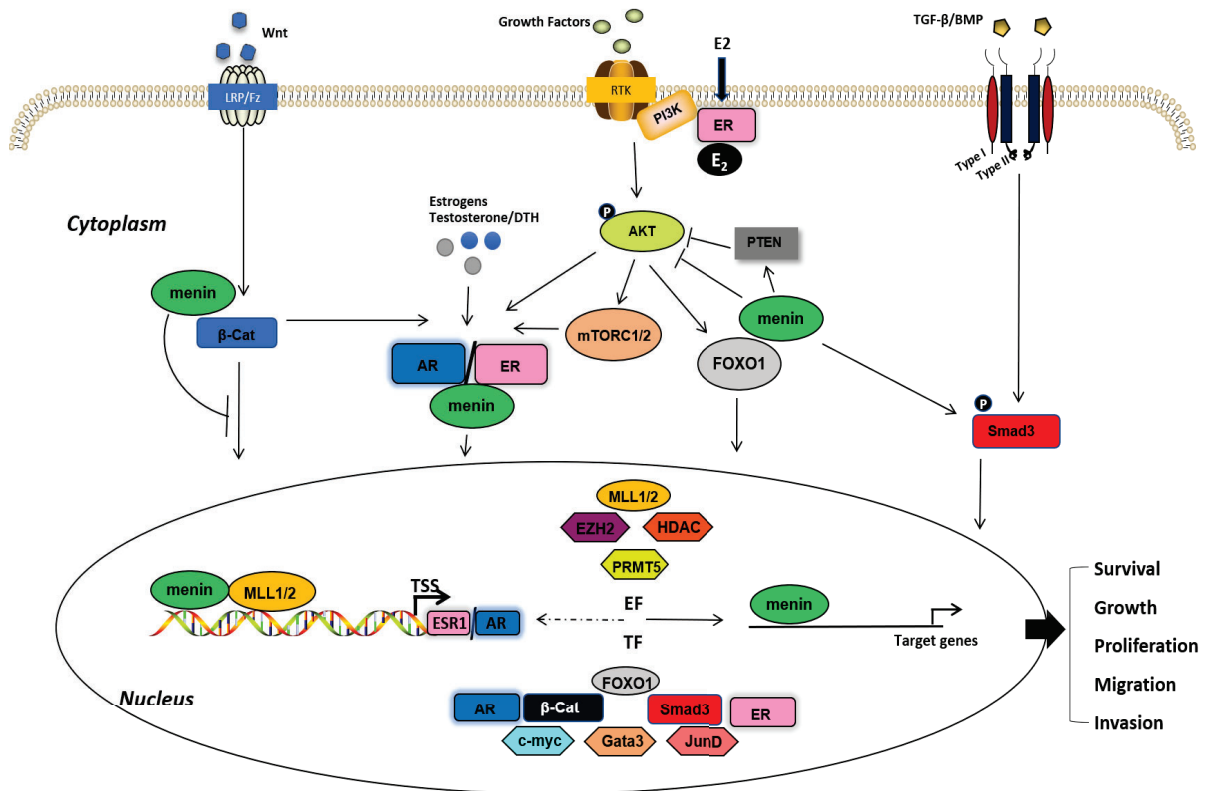


Figure 13: Menin a scaffold protein that regulates gene transcription: Menin interacts with numerous menin-interacting factors, consequently participating in the regulation of many target genes and interfering with different signaling pathways strongly implicated in breast and prostate cancers. EF: epigenetic factors; TF: transcriptional factors.

5) Menin and breast cancer:

a) *Molecular studies:*

The first molecular clues of the possible involvement of menin in breast cancer came from the observation that menin protein binds physically to ER α . In 2006, Dreijerink *et al.* revealed that menin could physically interact with several nuclear receptors, including ER α , playing the role of a cofactor. In the same study, they detailed that menin binds to the AF2 domain of ER α and coactivates the transcription of TFF1, the estrogen-responsive ER α target gene through recruitment of compass-like complex that trimethylating H3K4 on the *TFF1* promoter (Dreijerink *et al.*, 2006). Then, in 2009, Imachi *et al.* confirmed the above work in the ER α ⁺ MCF7 breast cancer cell line, by demonstrating that menin binds and coactivates ER α in a hormone (estrogen) dependent manner. They also documented that menin could be a predictive factor of tamoxifen resistance. For that, they conducted a clinical study with 65 ER-positive breast cancer samples—all of which had been treated with tamoxifen for 2–5 years as adjuvant therapies. They observed that menin positive tumors had worse clinical outcome and were more resistant to tamoxifen treatment than menin negative tumors in these samples (Imachi *et al.*, 2010). Thus, these results suggested an oncogenic role for menin, raising the controversy about the exact role of menin in breast cancer. A recent study conducted by Dreijerink *et al.*, in 2017 demonstrated that menin regulates the expression of the *ESR1* gene (as I described above) through an upstream enhancer, via a looping structure connecting the TSS bound menin&MLL1/2 (adding H3K4 trimethylation marks), to the enhancer bound transcription factors GATA3&FOXA1 (Dreijerink *et al.*, in 2017).

b) *Mouse models:*

Meanwhile, we have observed that aged heterozygous *Men1* mutant mice developed, in addition to endocrine tumors (pancreas, pituitary parathyroid, etc.), mammary gland carcinomas in female and prostate cancers in male mutant mice at low but substantial frequencies (Bertolino *et al.*, 2003a). To further confirm and understand the role played by menin in the development of mammary lesions, we generated a conditional mammary-

specific *Men1* knock-out mice 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, known to be expressed in luminal mammary epithelial cells. The results showed that female *Men1^{F/F}-WapCre* mice developed high incidence of early mammary intraepithelial neoplasia (MIN), precursor lesions of mammary cancer, in comparison with control *Men1^{+/+}-WapCre* mice (Seigne *et al.*, 2013). 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. Also, cell membrane expression of beta-catenin and E-cadherin was nearly absent in the mammary lesions of *Men1^{F/F}-WapCre* mice compared with control mice; neither E-cadherin nor beta-catenin was detected in the TS1 cell line derived from the mouse *Men1* breast cancer (Seigne *et al.*, 2013). Consistent with the observations made in the mouse, through the analyses of two series of human breast cancers, we demonstrated that a substantial proportion of breast cancers displayed reduced menin expression.

c) *Human studies:*

The decisive evidence of possible involvement of menin in breast cancer came from the observation that female MEN1 patients were at higher risk to develop breast cancer (Dreijerink *et al.*, 2014). In this study, Dreijerink *et al.* referred to the Dutch longitudinal MEN1 database to assess the incidence of breast cancer in MEN1 patients, and they found that out of 190 female patients, the relative risk of invasive breast cancer was 2.83 (P<0.001) and the mean (\pm SD) age at diagnosis of mostly luminal-type breast cancer was about 48 years \pm 8.8 years as compared with an age of 60 to 65 years in the general population, a feature being often observed in the patients carrying genetic predisposition. They validated their results using 3 independent cohorts from United States, Tasmania, and France which gave almost the same incidence risk values as those of the Dutch and the average age at diagnosis was 51 years. Furthermore, 8 out of 10 breast-cancer samples obtained from MEN1 Dutch 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. All these observations suggested strongly that *MEN1*

mutations could be involved in human breast carcinogenesis. More recently in 2016, a study that has analyzed the whole-genome sequences of 560 breast cancers has shown sporadic *MEN1* mutations (though at very low frequency) to be one of the many other driver mutations (like *BRCA1*, *TP53*, *PIK3CA*, *MYC*, *CCND1*, *PTEN*, etc.) in breast cancer (Nik-Zainal et al., 2016). In addition, several other case reports have identified *MEN1* mutations among breast cancer patients, some being independent of germline mutations in *BRCA1* and *BRCA2* genes which are usually associated with hereditary breast cancer (Zorka et al., 2012) (Jeong et al., 2014), and others being combined with BRCA mutations (Papi et al., 2009) (Ghataorhe et al., 2007).

d) *Other clues menin's role in breast cancer:*

As mentioned above, many different factors and signaling pathways are involved in mammary cell carcinogenesis. By investigating the possible link between the formers and menin, one may gain clues into the eventual role played by menin in breast cancer.

Epigenetic factors:

Interestingly, several epigenetic factors reported to be relevant in the carcinogenesis of mammary cells are known menin interacting partners. Histone methylase MLL1 (KMT2A) and MLL4 (KMT2B), menin's most known partners, have been shown to coordinate with ERs (ER α &ER β) to mediate the estrogen-induced transcriptional activation of HOXB9 gene that is a critical player in mammary gland development and breast cancer (Ansari et al., 2011). The HDAC family, mainly HDAC1, known partners of menin (Kim et al., 2003) (Wu et al., 2010), has also been implicated in the regulation of ER α expression, mainly silencing the *ESR1* gene, and even been proposed that this is how ER α expression might have been lost in ER negative breast cancer (Kawai et al., 2003) (Macaluso et al., 2003). The HMTs PRMT5 and EZH2 are other examples of common partners between menin and the ER α pathway (Gurung et al., 2013) (Gao et al., 2009). EZH2 has been shown to inhibit the transcription of estrogen-responsive genes through its association with the

transcriptional corepressor REA (repressor of estrogen receptor activity) (*Hwang et al., 2008*). Although there is currently no clear evidence of interaction between PRMT5 and ER α , PRMT5 still plays an important role in breast cancer by methylating programmed cell death 4 {PDCD4, a tumor suppressive protein having many anti-proliferative functions} on arginine residue 110 (*Powers et al., 2011*).

Transcription factors:

JunD, menin's first identified partner, shows higher expression in breast cancers compared to adjacent non-tumor tissues (*Kharman-Biz et al., 2013*) and binds ER α and facilitate its binding to target genes (*Paech et al., 1997*). Prostaglandin E₂ (PGE₂)- induced JunD and JunB have been shown to activate the aromatase promoters I.3/II, while JunD and c-Jun mediated suppression of aromatase promoter I.4, leading to high local levels of estrogen and thus breast cancer progression (*Chen et al., 2011*). JunD activation has also been shown to reduce the proliferation of Δ^9 -tetrahydrocannabinol (THC) (cannabinoids) treated breast cancer cells (*Caffarel et al., 2008*).

Another important factor is *MYC*, a well-known estrogen-regulated oncogene (*Shang et al., 2000*) (*Shang et al., 2002*), that is overexpressed in approximately 20%–30% of breast cancers (*Bièche et al., 1999*) and has also been shown to interact with ER α to modulate estrogen-mediated signaling (*Cheng et al., 2006*).

At last, 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 Foxa 1 being markers of luminal breast cancer especially luminal A (*Perou et al., 2000*) (*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*) (*Carrol et al., 2005*) (*Carrol et al., 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*)

Signaling pathways and cell cycle regulators:

Menin is involved in the regulation of several factors in the cell cycle and cell growth control. Indeed, the altered expression of a substantial numbers of these factors, including cyclins, CDKs and CDK inhibitors was observed in mouse *Men1* insulinomas (Fontaniere et al., 2006). Menin represses cell proliferation in rat intestinal epithelial cells (Ratineau et al., 2004), by inhibiting the expression of cyclin D1, cyclin D3 and cyclin CDK4, and promoting cell cycle transition from G1 to S phase. Menin can also repress cell proliferation by interacting and inhibiting ASK (S-phase kinase), an essential regulatory component of cdc7 protein kinase (Schnepp et al., 2004a). In addition, menin was reported to be able to upregulate the expression of cyclin-dependent kinase inhibitors p18^{ink4c} and p27^{kip1} with the help of the MLL compass like complex that adds H3K4 trimethylation marks on their promoters activating gene expression (Schnepp et al., 2006) (Karnik et al., 2005) (Milne et al., 2005). Interestingly, p18 has recently been shown to be a downstream target of GATA3 in luminal breast cancer, and to suppress luminal progenitor cell proliferation and tumorigenesis (Pei et al., 2009). P27 has recently been ranked as one of the 18 most significantly mutated genes in luminal A breast cancer and loss of p27 was associated with poor outcome in breast cancer patients (Stephens et al., 2012).

The PAM pathway is another common pathway between menin and breast cancer, as the activation of the PI3K/PTEN/AKT/mTOR pathways has been estimated to be in as frequent as 70% of breast cancers overall (Castaneda et al., 2010). PIK3CA (subclass of the PI3K family of genes) is the most commonly mutated gene of significance in estrogen receptor (ER) positive breast cancer (Ellis et al., 2013), occurring in approximately 35% of HR-positive breast cancers, 20%-25% of HER2-overexpressing breast cancers, and with a lower frequency (8.3%) in triple-negative breast cancers (Stemke-Hale et al., 2008). PTEN loss in breast cancer is negatively correlated with estrogen receptor (ER) and progesterone receptor (PR) status and associated with the basal-like phenotype (Jones et al., 2013), more aggressive behavior (tumor size, lymph node metastasis, etc..) and worse outcomes (worse DFS&OS) (Li et al., 2017). Several studies have focused on the relation between the PAM (PI3K/Akt/mTOR) and resistance to endocrine therapy in preclinical breast cancer models where they showed that Akt can activate the ER α pathway

independent of estrogen availability and that the combination of mTOR inhibitors with endocrine therapy can overcome this resistance (*Boulay et al., 2005*) (*Miller et al., 2010*). In addition, the PAM pathway has also been implicated in trastuzumab resistance in HER2-overexpressing breast cancers (*Crowder et al., 2009*) (*Nagata et al., 2004*).

Results:

I. Article 1: Reduced menin expression leads to decreased ER α expression and is correlated with the occurrence of human luminal B and ER-negative breast cancer subtypes

My contribution: The study was initiated by my colleague Romain Teinturier (former PhD student in our lab), especially on the regulation of ER α expression by menin and menin's binding to the *ESR1* promoter. I took part in this study by confirming his results, then, further elaborating the work especially for the MLL1/2, GATA3 and FOXA1 part. I conducted the related experiments, analysed and interpreted the data, prepared the figures and contributed to the manuscript writing.

Status: Submitted in the journal of Breast Cancer Research and Treatment

II. Article 2: Menin downregulation triggers the deregulated mTORC1 pathway and *MYC* transcription in ER-positive BC cell lines

My contribution: Based on some preliminary data from my colleague Romain Teinturier who found that menin inhibition caused altered protein expression of members of the Akt/mTOR family, I went on further and investigated more in detail the relation between menin and the mTORC1 pathway, especially the translation initiation complex, downstream targets of mTORC1, in ER+ breast cancer cell lines. Given the contradiction between the molecular alteration and cell growth behavior of these cells, I developed the new axis on the role of menin in regulating the MYC pathway breast cancer. I conducted the experiments, analysed and interpreted the data, prepared the figures and contributed to manuscript writing.

Status: Submitted in the journal of Breast Cancer Research.

I.

Reduced menin expression leads to decreased ER α expression and is correlated with the occurrence of human luminal B and ER-negative breast cancer subtypes

Running title Menin affects occurrence of breast cancer subtypes through *ESR1* regulation

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Abstract

Purpose: Menin, encoded by the *MEN1* gene, was recently reported to be involved in breast cancers, though the underlying mechanisms remain elusive. In the current study, we sought to further determine its role in mammary cells.

Methods: Menin expression in mammary lesions from mammary specific *Men1* mutant mice was detected using immunofluorescence staining. qRT-PCR and western blot were performed to determine the role of menin in ER α expression in human breast cancer cell lines. CHIP-qPCR and reporter gene assays were carried out to dissect the action of menin on the proximal *ESR1* promoter. Menin expression in female patients with breast cancer was analyzed and its correlation with breast cancer subtypes was investigated.

Results: Immunofluorescence staining revealed that early mammary neoplasia in *Men1* mutant mice displayed weak ER α expression. Furthermore, *MEN1* silencing led to both reduced *ESR1* mRNA and ER α protein expression in MCF7 and T47D cells. To further dissect the regulation of *ESR1* transcription by menin, we next examined whether and in which way menin could regulate the proximal *ESR1* promoter, which has not been fully explored. Using CHIP analysis and reporter gene assays covering -2500 bp to +2000 bp of the TSS position, we showed that the activity of the proximal *ESR1* promoter was markedly reduced upon menin downregulation independently of H3K4me3 status. Importantly, by analyzing the expression of menin in 354 human breast cancers, we found that a lower expression was associated with ER-negative breast cancers ($p = 0.041$). Moreover, among the 294 ER α -positive breast cancer samples, reduced menin expression was not only associated with larger tumors ($p = 0.01$) and higher SBR grades ($p = 0.005$), but also with the luminal B breast cancer subtype ($p = 0.006$). Consistent with our clinical data, we demonstrated

that GATA3 and FOXA1, co-factors in *ESR1* regulation, interact physically with menin in MCF7 cells and *MEN1* knockdown led to altered expression of Foxa2 and GATA3, the latter being a known marker of the luminal A subtype, in MCF7 cells.

Conclusion: Taken together, our data provide clues to the important role of menin in ER α regulation and the formation of breast cancer subtypes.

Key words

menin, the *ESR1* gene, ER α , GATA3, breast cancer, luminal B subtype

Introduction

Breast cancers are among the most common malignancies worldwide and remain the leading cause of cancer-related mortality in women¹. Previous receptor expression analyses enabled their classification into 4 major clinical subtypes, including luminal A, luminal B, HER2-enriched and triple negative². The luminal A subtype encompasses approximately 44% of breast cancers. This subtype is ER α -positive and/or PR-positive and HER2-negative, displays a reduced expression of proliferation-related genes³ and is sensitive to endocrine therapy and has an overall favorable prognosis. The luminal B subtype represents around 20% of breast cancers and displays lower expression of ER α -related genes, a variable expression of HER2, and a higher expression of proliferation-related genes⁴. This subtype harbors more genomic instability and has a poorer prognosis than the luminal A subtype⁵. The HER2-enriched subtype is ER α -negative, PR-negative, HER2-positive and highly sensitive to therapies targeting the HER2 receptor. The triple negative breast cancer (TNBC) subtype is negative for all three receptors⁶ and is the most aggressive and has the worst prognosis.

Patients harboring *MEN1* mutations are predisposed to multiple endocrine neoplasia type 1 (MEN1) syndrome, which is associated with multi-occurring endocrine tumors⁷, as well as several types of non-endocrine tumors⁸. Numerous studies have revealed that menin is a multifaceted protein involved not only in the development and control of cell growth of endocrine cells, but also in a variety of biological processes, including hematopoiesis and osteogenesis^{9–11}. The wide range of biological functions regulated by menin results from its interaction with numerous proteins¹². These menin-interacting proteins include transcription factors (the components of AP1, NF κ B and the TGF β signaling pathways) and chromatin modifying proteins (mixed lineage leukemia, MLL, and Sin3A, HDAC)^{12,13}. Notably, menin physically interacts with a range of

nuclear receptors, including ER α and Androgen Receptor (AR), to regulate their corresponding pathways¹⁴⁻¹⁶.

Over the last few years, evidence has emerged, *in vivo*, to suggest that menin may play a role in breast cancers¹⁷. 1) Female heterozygous *Men1* knockout mice develop cancers of mammary cells with a low frequency¹⁸, and conditional mammary gland-specific *Men1* disruption leads to the development of mammary intraepithelial neoplasia (MIN) in over 50% of female mutant mice¹⁹. 2) Importantly, an exhaustive analysis of several cohorts of MEN1 patients revealed a significant predisposition to breast cancer²⁰. 3) Menin downregulation was detected in a substantial proportion of human sporadic breast cancer samples¹⁹, and *MEN1* mutations were found, although rarely in sporadic breast cancers, justifying its addition to the list of driver mutations/genes of this pathology^{21,22}. Of note, the above-mentioned analyses all highlight the suppressive role of menin in mammary cell tumorigenesis. However, Imachi *et al.* found that, among 65 ER α + breast cancer samples treated with tamoxifen, menin-positive tumors (20 patients) had worse clinical outcome and were more resistant to tamoxifen than menin-negative tumors, suggesting that menin exerts oncogenic effects in these cases¹⁵. Interestingly, a recent publication, revealing the role of menin in the regulation of the enhancer of the *ESR1* gene, suggests distinct functions for menin in primary normal mammary cells and in breast cancers²³. The authors showed that, although menin possesses a crucial tumor suppressive role in normal mammary cells, it acts as an oncogenic factor in ER α + breast cancer cell lines through an enhancer-mediated regulation of *ESR1* transcription. Notably, they showed that, by using ZR75-1 BC line which does not express menin, the re-expression of menin led to enhanced ER α expression. Given the heterogeneous nature of breast cancers, we sought to further investigate the regulation of *ESR1* by menin and assess the putative relationship

between *ESR1* dysregulation due to menin inactivation and the occurrence of human breast cancer subtypes.

Results

Men1-deficiency in mice leads to ER α downregulation in early mammary lesions

We have previously reported the occurrence at a high incidence of mammary intraepithelial neoplasia (MIN) lesions, displaying weak ER α expression, in *Men1* mammary conditional mutant mice¹⁹. To further determine the causative role of menin deficiency in reduced ER α expression, we carried out double IF analysis of menin and ER α expression in normal and young mutant mice with MIN lesions. Three mice per control or mutant group were analyzed. As shown in Fig. 1a, all of the mammary luminal cells in *Men1^{F/F}-WapCre⁻* control mice expressed menin. Conversely, menin expression was lost in 80% of mammary cells in the 10-month-old *Men1^{F/F}-WapCre⁺* mice (Fig. 1a). ER α was expressed in approximately 40% of luminal cells expressing menin in the former group (Fig. 1a, upper panel), whereas immunofluorescence revealed that ER α expression was lost in *Men1*-deficient cells in *Men1^{F/F}-WapCre⁺* mice (lower panel). The merged images of menin and ER α staining clearly highlight that ER α is less expressed specifically in the nuclei of menin-deficient luminal cells (Fig. 1a).

Menin downregulation in human ER α ⁺ mammary cells leads to reduced ER α expression

Next, we further dissected the regulation of ER α expression by menin using different approaches in ER α + breast cancer cell lines. To achieve this, we first performed *MEN1* knockdown (KD) using an SiRNA approach. As shown in Fig. 1 b, *MEN1* KD MCF7 and T47D cells displayed reduced ER α protein expression by Western blot analysis, unlike the menin-negative ER α + line, ZR75-1. Moreover, *MEN1* knockdown led to a twofold decrease in *ESR1* mRNA levels by RT-qPCR analysis in MCF7 and T47D cell lines (Fig. 1c). We then verified the effects of *MEN1* KD on *ESR1* mRNA and ER α expression levels under estrogen stimulation. Western blot and RT-qPCR analyses showed that *MEN1* silencing further abrogated ER α expression under E2 stimulation but had no additional effect on *ESR1* transcription (Fig. 1d-e). All the above data thus confirm our *in vivo* analyses, indicating that, menin is essential in maintaining *ESR1* transcription and ER α expression.

Menin binds to the proximal region of the ESR1 promoter

Dreijerink *et al.* reported that menin plays a crucial role in the regulation of *ESR1* transcription in an enhancer-mediated way²³. We noticed that, although the study revealed the binding of menin at the transcription start site (TSS) of the *ESR1* promoter, no further analyses were reported on this region. We thus carried out ChIP analyses to evaluate the binding of menin to the -2500 bp to +2000 bp around the TSS previously defined (Fig. 2a)²⁴, to fully decipher the regulation of *ESR1* transcription by menin at the proximal *ESR1* promoter region. Menin was significantly enriched in the *ESR1* promoter region encompassing the TSS to +2000 bp in MCF7 (Fig. 2b, left panel) and T47D (Fig. 2b, right panel) cells, and more specifically in the promoter area C in MCF7 cells. Importantly, we confirmed by using luciferase reporter assays that the transcriptional activity of

the proximal *ESR1* promoter region A/B, and C was markedly reduced when *MEN1* was knocked down (Fig. 2c).

Considering the data published by Dreijerink et al²³, showing that H3K4me3 marks are enriched in the proximal part of the *ESR1* promoter, we sought to investigate the involvement of MLL complex, a major actor modifying H3K4me3 marks¹³, in the regulation of the *ESR1* promoter. By treating MCF7 and T47D cells with an inhibitor of the menin-MLL interaction, MI503, RT-qPCR analyses unveiled a more than two-fold decrease in *ESR1* transcription (Fig. 3a). Western blot analyses in MCF7 and T47D cells using the same inhibitor also revealed a decrease in ER α expression at the protein level (Fig. 3b). We then verified the potential alteration of H3K4me3 marks at this region upon inhibition of the MEN1/MLL complex. CHIP analysis with anti-H3K4me3 antibodies showed that, while MI503 treatment led to a markedly reduced binding of menin (Fig. 3c left panel), H3K4me3 methylation was not altered upon menin/MLL inhibition in the tested region (Fig. 3c right panel). Furthermore, RT-qPCR analyses also showed that neither SiMLL1, nor SiMLL2, nor their combination affected *ESR1* transcription (Fig. 3d left panel) and ER α expression (Fig. 3d right panel). Taken together, our data provide evidence that menin regulates the proximal *ESR1* promoter, likely via mechanisms involving other factors part from the compass-like complex.

Lower menin expression is associated with luminal B and basal-like breast cancer subtypes

Our above observation prompted us to perform a thorough investigation of the expression of the menin protein in a cohort of breast cancer patients having surgery at the Centre Léon Bérard (CLB) hospital from 2001 to 2003. For sake of correlations and survival analyses, the whole cohort of

patients was divided into high menin expression (H score > 100) and low menin expression (H score \leq 100). Accordingly, among the whole cohort of 354 patients, 151 (42.7%) had low menin expression, while 203 (57.3%) had high expression. Among the 294 patients with ER+/HER2- tumors, 116 patients (39.5%) had a low nuclear menin expression and 178 patients (60.5%) had a high expression. In this cohort of 354 patients, we found that lower nuclear menin (H score \leq 100) expression was significantly associated with ER-negative breast cancers ($P = 0.041$) and with the HER2-enriched subtype ($P = 0.049$, Table 1). Moreover, among the 294 ER+/HER2- patients, we observed that low menin expression was associated with the luminal B breast cancer subtype ($P = 0.006$), larger tumors ($P = 0.016$), and higher SBR grades ($P = 0.005$, Table 2).

Interestingly, among the ER+/HER2- cohort, we found that low menin expression was associated with worse distant metastasis-free survival (DMFS), with a 10-year DMFS of 71.5% *versus* 81.2% in patients with high menin expression, $P = 0.053$ (Fig. 4a). Furthermore, low menin expression was also associated with a trend for worse disease-free survival (DFS), with a DFS of 65.7% at 10 years *versus* 75.0% in patients expressing high levels of menin ($P = 0.088$, Fig. 4b). Finally, lower expression of menin was also associated with a tendency towards worse OS (10-year OS of 77.5% *versus* 85.2%, $P = 0.092$, Fig. 4c).

The abovementioned data obtained in human patients, reminiscent of the observations made in *Men1*-deficient mutant mice, highlighted a relationship between reduced menin expression and weaker ER α expression, suggesting that decreased ER α expression triggered by *Men1*-defficiency could be related to the occurrence of luminal B and basal-like breast cancer subtypes.

Menin downregulation alters GATA3 and FOXA1 expression in MCF7 cells

Having demonstrated a clinical correlation between menin inactivation and breast cancer subtypes, we wondered whether the factors important for luminal cell differentiation could be affected by menin. GATA3 is known to be a major factor involved in the regulation of *ESR1* expression and is ubiquitously present in luminal A breast cancers²⁵. Western blot analysis revealed that GATA3 expression was markedly reduced in MCF7 cells at the protein level after *MEN1* KD, although its level of mRNA was not impacted (Fig 5a,b). In parallel, we investigated the expression of FOXA1, which plays an important role in mammary cell differentiation and tumorigenesis, and found that its protein expression was increased upon *MEN1* KD in MCF7 cells, whereas no transcriptional alteration could be detected (Fig. 5a,b). Since GATA3 has been reported to interact with menin in lymphocytes²⁶, and menin is known to interact with one member of the FOXA family, FOXA2²⁷, we performed immunoprecipitation (IP) and PLA analyses, to determine whether menin could interact with GATA3 and FOXA1 in breast cancer cells. The data obtained demonstrated that they indeed interacted with menin in MCF7 cells, as evidenced by IP at the endogenous level (Fig. 5c), by GST pull-down (Fig. 5d) and by PLA (Fig. 5e).

Discussion

The current work provides both clinical and experimental data showing that menin is critically involved in ER α expression and that its inactivation in normal mammary cells is correlated with the occurrence of luminal B and basal-like breast cancer subtypes. Our data highlighted cellular and molecular consequences of reduced menin expression in mammary cells, which may affect not only cell proliferation, but also other hallmarks of cancer cells, in particular, cell differentiation.

We previously observed that the mammary lesions developing in mammary cell-specific *Men1* mutant mice displayed low ER α expression¹⁹. Our current study further demonstrates that the decrease is an early event, suggesting that menin inactivation favors the occurrence of mammary lesions with weak ER α expression. Interestingly, by analyzing this expression in a large cohort of breast cancer patients, we found that reduced menin expression is significantly correlated with both ER α -negative and luminal B breast cancer subtypes. Consistently, low levels of menin correlated with larger tumors, more advanced SBR grades and worse prognosis, all of which are major features of these two breast cancer subtypes⁴. Moreover, while searching for luminal cell factors likely interacting with menin, we unveiled that menin binds physically to GATA3 and FOXA1 in mammary cells, and that *MEN1* silencing reduces GATA3 expression but upregulates FOXA1 expression. Of note, reduced GATA3 expression is often seen in the luminal B breast cancer subtype but not in luminal A²⁸. However, the mechanisms leading to the occurrence of both luminal breast cancer subtypes remain elusive. The current work may provide useful insight and generate interest for further studies. In the meantime, considering the retrospective nature of the study and the heterogeneity of the therapies received by the patients included, the clinical analyses should be confirmed in other cohorts, preferably through prospective studies.

Dreijerink et al first described the capacity of menin to regulate *ESR1* transcription by binding to the remote upstream part of regulatory sequences of the *ESR1* gene, through an enhancer-mediated looping mechanism, involving GATA3²³. Moreover, the occupancy of this enhancer sequence by GATA3 has been reported to play an important role in the regulation of ER α expression upon estradiol stimulation²⁵. Our findings provide complementary information related to the role of menin in *ESR1* regulation through its proximal promoter. Intriguingly, our data showed neither altered H3K4me3 methylation on the proximal *ESR1* promoter with MI503

treatment, nor affected *ESR1* transcription after single MLL1, MLL2, or their combined knockdown with SiRNA. Since MI503 has been demonstrated not only to be capable of inhibiting the interaction between menin and MLL1/MLL2, but also reducing menin expression itself²⁹, our data suggest that the factors other than the MLL complex may also participate in this regulation. It would be interesting in the future to identify the factors or cofactors that interact, positively or negatively, with menin to regulate this gene. In addition, our data seem to support the oncogenic role played by menin in ER α + breast cancer cell lines, the proliferation of which is highly ER α -dependent. Therefore, by combining the data obtained from our experimental and clinical analyses, we consider that menin most likely plays the opposite role in luminal A breast cancer subtype.

Conclusions

The emerging role for the *MEN1* gene in mammary cell tumorigenesis appears to be multifaceted. Our current results provide further data showing that menin may play different, even opposite, roles in the development of different breast cancers, in agreement with the findings reported by Dreijerink et al. Taken together, these results may explain seemingly controversial data reported so far, in particular when comparing data obtained from naturally occurring tumors and those of cultured cancer cells. Furthermore, our findings may also raise awareness to the breast cancer subtypes selected when designing new therapeutic strategies involving the eventual use of menin and MLL inhibitors.

Materials and Methods

Patients

We screened a total of 433 consecutive female patients with breast cancer who underwent surgery and (neo)/adjuvant therapy at the Centre Léon Bérard (CLB) between January 2001 and December 2003 (Additional file 1). Patients with complete data and with adequate samples assessable for menin by IHC were 354, among which 294 patients had ER+/HER2- tumors. The study was conducted in accordance with the guidelines in the Declaration of Helsinki and the use of all patient tissues was approved by local IRB and carried out according to French laws and regulations.

TMA analysis of human breast cancers

Formalin fixed paraffin embedded breast cancers were prepared and processed for immunostaining as previously described¹⁹. Tissue micro-array (TMA) block preparation, menin nuclear expression assessment using IHC, and statistical analyses were performed as previously described¹⁹. The percentage of stained cells was multiplied by the intensity of staining to obtain the H score³⁰. For sake of correlations and survival analyses, the whole cohort of patients was divided into high menin expression (H score > 100) and low menin expression (H score ≤ 100).

Animal breeding

Men1^{F/F}-WapCre⁺ and *Men1^{+/+}-WapCre⁺* mice previously generated in our lab were used¹⁹. All animal experiments were conducted in accordance with accepted standards of animal care and were approved by the Animal Care and Use Committee of the University Lyon 1.

Cell culture, transfections and luciferase assays

Three breast cancer cell lines expressing ER α , namely MCF7, T47D and ZR75-1 were used in this study. Transient transfections were carried out in phenol red-free medium supplemented with 10% charcoal-stripped serum (Biowest), in order to remove steroid hormones (steroid depletion). Cells were transfected with 20 nM SiRNA of, respectively, control SiRNA (5 nmoles, Eurogentec), two different SiRNA targeting human *MEN1* transcript (SiMEN1 hs1 (HSS106462) and hs2 (HSS181079), ThermoFisher Sci.), SiRNA targeting human *MLL1* (SiKMT2A: siRNA 107890 ThermoFisher Sci.), SiRNA targeting human *MLL2* (SiKMT2B: siRNA s18833 ThermoFisher Sci.), using Jetprime[®] transfection reagent (Polyplus) for 72h according to the manufacturer's instructions. Inhibition of the menin-MLL interaction was achieved by MI503 (Active Biochem) at different concentrations. Prior to performing treatment with E2 and MI503, cells were grown in phenol red-free medium supplemented with 10% charcoal-stripped serum (Biowest), in order to remove steroid hormones (steroid depletion). Cells were then treated for 3 hours with E₂ (Sigma) 10⁻⁸M and MI503 for 48h. The treatment was repeated after 24h due to the degradation of the inhibitor with time. Please also see Additional file 2 - Supplemental Materials & Methods.

Construction of luciferase constructs

We used genomic DNA extracts from MCF7 cells to generate regions of the human *ESR1* promoter, PrAB (genomic location: Chr6q25.1; 152127793-152129027) and PrC (genomic location: Chr6q25.1; 152124474-152127509) (Additional file 2). The resulting fragments of the proximal *ESR1* promoter were cloned into the pGL3 Basic vector (Promega, Madison WI).

Luciferase assays

For luciferase assays, MCF7 cells were cultured in 24-well plate. 48h after transfection with 250 ng of the reporter plasmid PrAB or PrC, and 5 ng pRL-TK internal control vector, cell lysates were prepared and analyzed using a dual-luciferase reporter assay system (Promega, Madison, WI), as previously reported²⁷. Comparisons between mean values were assessed using the two-tailed Student *t*-test.

Real-time reverse transcription and qPCR analyses

RNAs 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 (Life-Technology, France) and corresponding primers (Additional file 2). Results of each sample were normalized.

Protein extraction, immunoprecipitation, GST pull-down and immunoblotting

Total protein extracts from cells and immunoprecipitation were prepared and analyzed as described previously²⁷. For GST pull-down assays, 1.25 µg purified GST-menin protein or GST control protein was incubated with 1 mg or 2 mg of nuclear cell extracts prepared from MCF7 cells, as previously described²⁷. The co-sedimented proteins were detected by Western blot using standard conditions.

Immunostaining

Tissue preparation, immunostaining and statistical analyses were performed as previously described¹⁹. 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 (Additional file 2). For immunofluorescence (IF) staining, signals were detected with a Cy3 or Cy5 tyramide amplification kit (PerkinElmer), with prior incubation with the appropriate biotinylated secondary antibody according to the manufacturer's instructions. Images were acquired on an Eclipse-NiE NIKON microscope, using the NIS-Elements Software.

Proximity ligation assay (PLA), image acquisition and analysis

MCF7 cells were fixed in methanol for 5 min and washed twice in PBS, then treated and analyzed according to the manufacturer's instructions (Duolink II Fluorescence, Olink Bioscience, Sweden). Images were acquired on an Eclipse- NiE NIKON microscope, using the NIS-Elements Software. For each sample, at least one hundred cells were counted. Analysis and quantification of these samples were performed using the Image J software (free access). PLA dots were quantified on 8-bit images using the 'Analyse Particles' command, while cells were counted using the cell counter plugin.

ChIP-qPCR assay

Chromatin for ChIP analysis was prepared from 5 million MCF7 or T47D 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 primary antibodies. Dynabeads® Protein G (10003D, Life Technologies, France) was used to retrieve immunocomplexes according to manufacturers' instructions.

Statistical analyses

For molecular biology experiments, statistical analyses were performed as described in the Fig. legends; unpaired Student t-tests were used unless otherwise indicated. All analyses were conducted using the Prism5 software (GraphPad, USA); a P value of < 0.05 was considered to be significant. Results are expressed as means +/- standard errors of the means (SEM). For the patient samples, numerical variables were compared using Student's T test while categorical variables compared using Chi-square test. Distant metastasis-free survival (DMFS) was defined as time from diagnosis to the date of distant metastasis or death or last follow-up. Disease-free survival (DFS), defined as the time from diagnosis to death or progression or to date of last follow-up (for censored patients), was also calculated. Survival rates were estimated using the Kaplan-Meier method, and comparisons between menin expression groups were performed using the log-rank test. All statistical tests were two-sided, and the p-value was considered statistically significant if lower than 5%. Statistical analyses were performed using SPSS 20.0 statistics package.

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Author contribution statement

RT and RAZ conducted the experiments, analysed and interpreted the data, prepared figures and manuscript; LK participated in the design of the clinical study, performed the statistical analysis and participated in manuscript preparation; YL participated in the molecular and cellular studies and interpreted the data; LM participated in the molecular and cellular studies and interpreted the data; SG participated in the design of the molecular study and established methods; LC participated in the design of the molecular study and data interpretation, and helped to draft the manuscript; PB provided technical and material support, and participated in data interpretation and manuscript preparation; TB conceived the design of the clinical study and participated in clinical data collection and data interpretation; IT conceived the design of the clinical study, participated in clinical data collection, data interpretation and manuscript preparation, 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.

Conflict of interest statements

Nothing to disclose

Consent for publication

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

Ethics approval and consent to participate

The specimens of patients with breast cancer were conducted according to French laws and regulations and written informed consent was obtained from each patient. 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).

Data availability statement

The data that support the findings of this study are available from the corresponding author, [CXZ for basic research and IT for clinical data analyses], upon reasonable request.

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Legends

Fig. 1 Reduced menin expression leads to decrease in ER α expression. **a**, Co-immunofluorescence against menin and ER α on mammary gland sections from *Men1^{F/F}WapCre⁻* mice (upper panel) and *Men1^{F/F}WapCre⁺* mice (lower panel) at 10 months of age. Arrows in the inset illustrate that menin expression is correlated with ER α expression. **b**, Western blot analyses using antibodies against menin and ER α in MCF7, T47D and ZR75-1 cells treated with SiRNA control (SiCtrl) or SiRNA targeting the *MEN1* gene (SiMEN1 hs1). **c**, Quantitative RT-qPCR

analyses of *MEN1* and *ESR1* gene expression in MCF7 and T47D cells treated with SiCtrl or two different SiMEN1 (hs1 or hs2). **d**, Western blot analyses using antibodies against menin and ER α in MCF7 and T47D treated with SiRNA control (SiCtrl) or SiMEN1 hs1, then subjected to estradiol (E₂) stimulation at a concentration of 10 nM. **e**, Quantitative RT-qPCR analyses of the *MEN1* and *ESR1* gene expression in MCF7 and T47D cells treated with SiCtrl or SiMEN1 hs1, then subjected to estradiol (E₂) stimulation at a concentration of 10 nM. All data are expressed as mean +/- SEM, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****.

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Fig. 5 Menin interacts with GATA3 and FOXA1 and influences their expression. **a**, Western blot analyses using antibodies against menin, GATA3 and FOXA1 in MCF7 cells treated with SiCtrl or SiMEN1 hs1. **b**, Quantitative RT-qPCR analyses of the *GATA3* and *FOXA1* transcription in MCF7 cells treated with SiCtrl or SiMEN1 hs1. **c**, Co-immunoprecipitation analyses were carried out by incubating nuclear lysates of MCF7 cells with either anti-IgG, or anti-GATA3 or FOXA1 antibodies and subjected to Western blot analyses. **d**, GST pull-down using GST-full-length (FL) menin and nuclear fraction of protein lysates of MCF7 cells, detected by Western blot using the anti-GATA3 or FOXA1 antibodies. Coomassie blue stained gel showing levels of recombinant GST proteins used in GST pull-down assay. **e**, Upper: PLA analysis with anti-menin

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Table 1 Correlation between menin expression and the clinico-pathological factors of 354 breast cancer patients. *Correlation by Pearson's Chi square test unless otherwise specified † Difference between means analyzed using the Student t-test.

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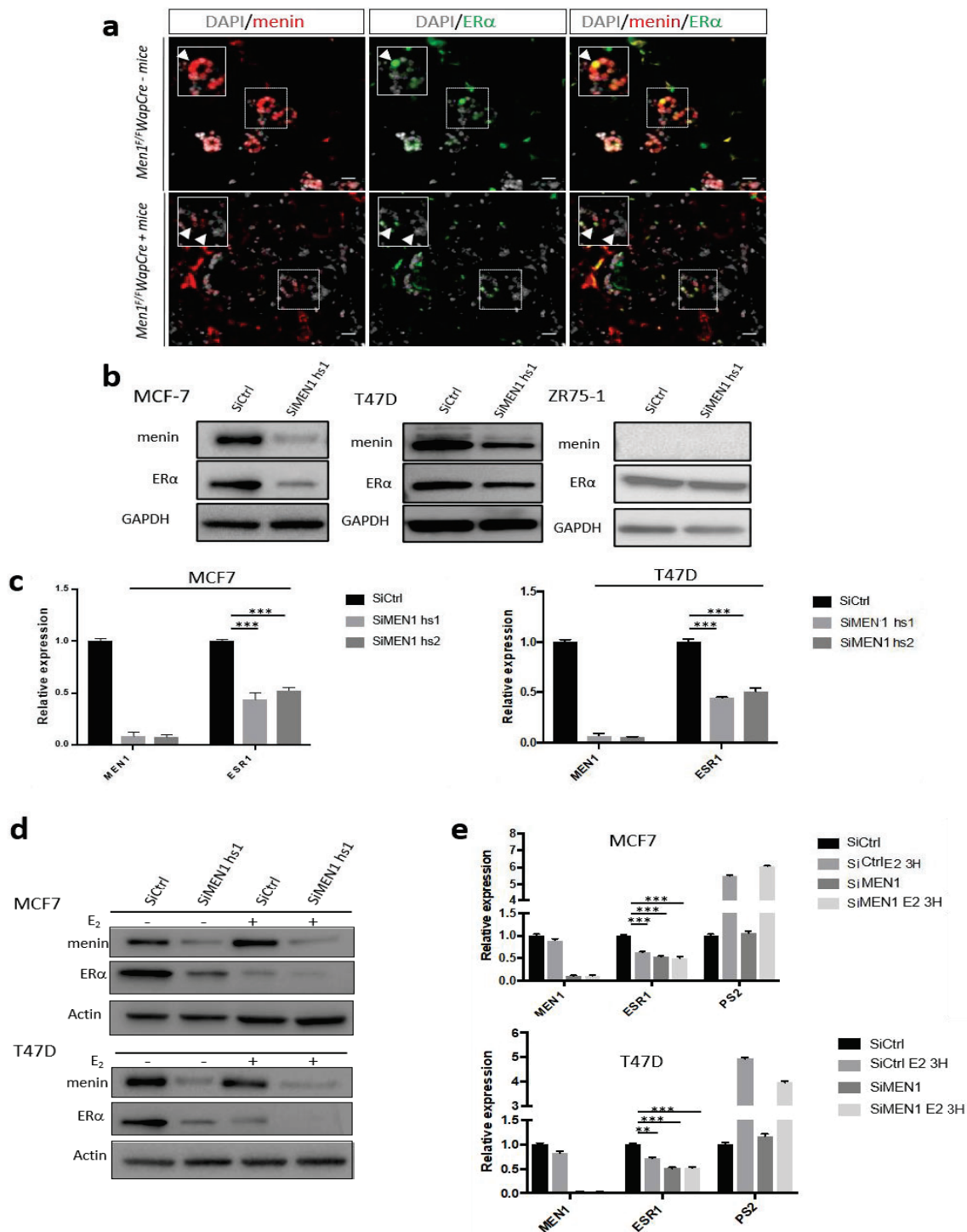


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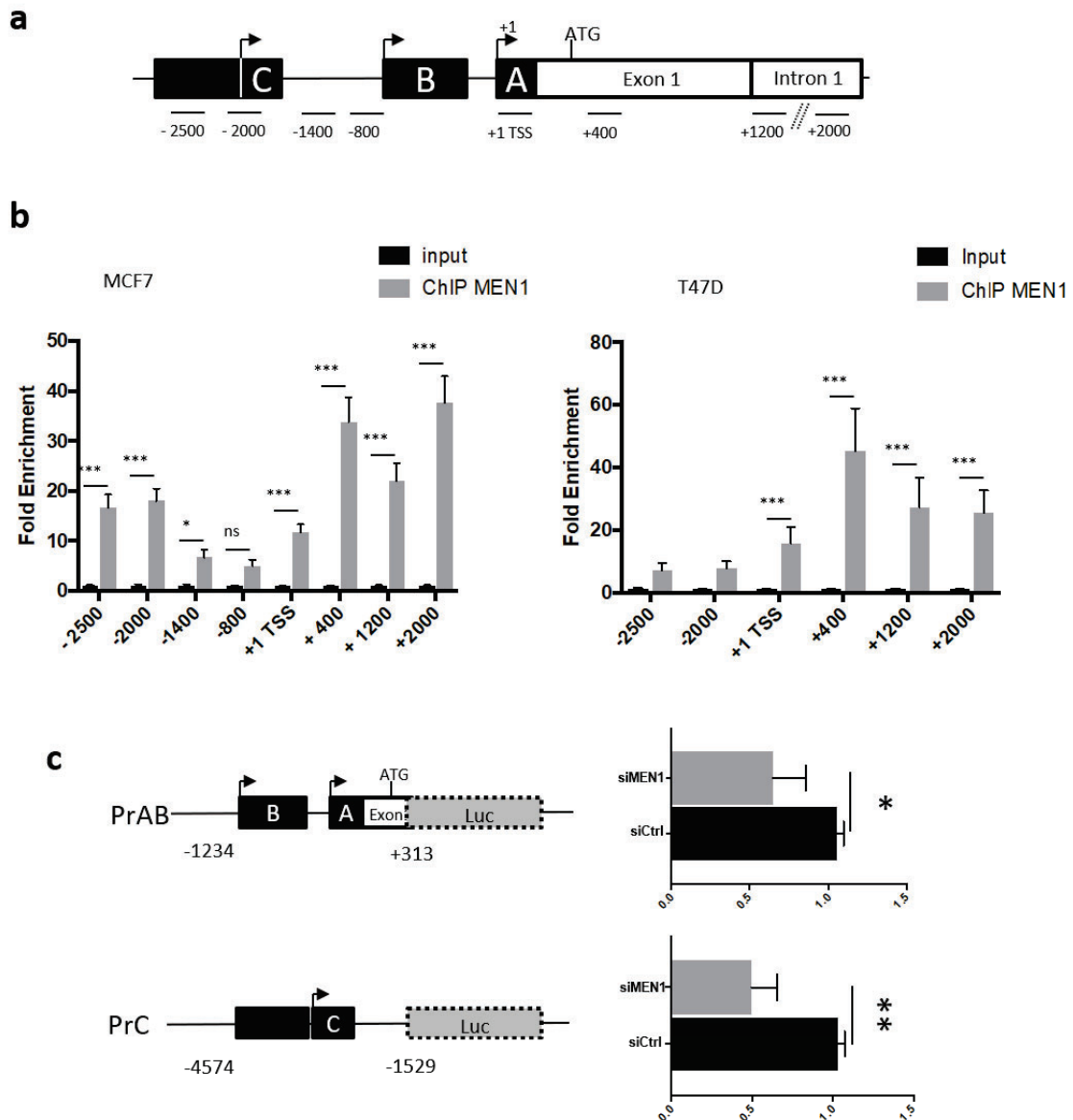


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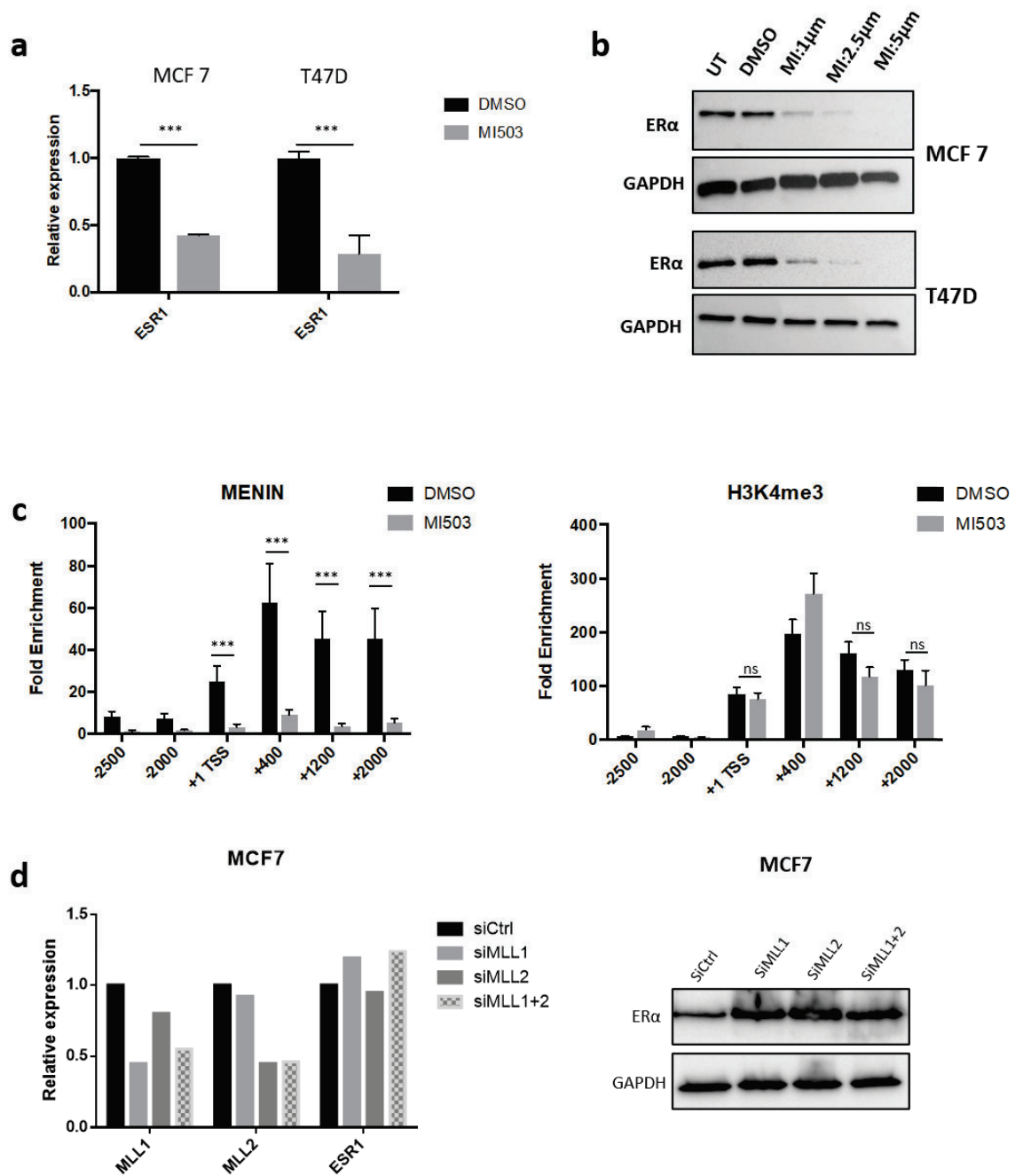


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Variable		Menin low (≤ 100)		Menin high (> 100)		P*
		No.	(%)	No.	(%)	
		151	(42.7)	203	(57.3)	
Age (Yr)	- <i>Mean (\pm SD)</i>	59.2	(± 13.3)	58.6	(± 11.2)	0.081†
Age groups	- $\leq 50y$	31	(20.5)	58	(28.6)	0.085
	- $> 50y$	120	(79.5)	145	(71.4)	
BMI	- ≤ 25	78	(53.4)	133	(67.9)	0.007
	- > 25	68	(46.6)	63	(32.1)	
T. size	- $\leq 2cm$	72	(47.7)	131	(64.5)	0.002
	- $> 2cm$	79	(52.3)	72	(35.5)	
LN invasion	- <i>No</i>	64	(42.4)	84	(41.1)	0.85
	- <i>Yes</i>	87	(57.6)	119	(58.6)	
SBR grade	- <i>Gr 1</i>	16	(10.6)	46	(22.7)	0.001
	- <i>Gr 2</i>	68	(45.0)	102	(50.2)	
	- <i>Gr 3</i>	67	(44.4)	55	(27.1)	
ER status	- <i>Negative</i>	26	(17.2)	20	(9.9)	0.041
	- <i>Positive</i>	125	(82.8)	183	(90.1)	
PR status	- <i>Negative</i>	44	(29.1)	44	(21.7)	0.108
	- <i>Positive</i>	107	(70.9)	159	(78.3)	
Her 2 status	- <i>Negative</i>	84	(89.6)	100	(95.2)	0.049
	- <i>Over-expressed</i>	12	(10.4)	5	(4.8)	

Table 1: Correlation between menin expression and the clinico-pathologic factors of from 354 breast cancer patients. *Correlation by Pearson's Chi square test unless otherwise specified † Difference between means by Student's T test.

Variable		Menin low		Menin high		P*
		No.	(%)	No.	(%)	
		116	(39.5)	178	(60.5)	
Age groups	- $\leq 50y$	21	(18.1)	51	(28.7)	0.040
	- $> 50y$	95	(81.9)	127	(71.3)	
Menopausal Status	-Premenopausal	22	(19.9)	56	(32.6)	0.053
	-Postmenopausal	92	(80.1)	118	(67.4)	
BMI	- ≤ 25	64	(57.1)	116	(67.8)	0.045
	- > 25	48	(42.9)	55	(32.2)	
T. size	- $\leq 2cm$	62	(53.4)	120	(67.4)	0.016
	- $> 2cm$	54	(46.6)	58	(32.6)	
LN invasion	-No	46	(39.7)	74	(41.6)	0.744
	-Yes	70	(60.3)	104	(58.4)	
SBR grade	-Gr 1	16	(13.8)	46	(25.8)	0.005
	-Gr 2	62	(53.4)	99	(55.6)	
	-Gr 3	38	(32.8)	33	(18.5)	
PR status	-Negative	18	(15.5)	21	(11.8)	0.358
	-Positive	98	(84.5)	157	(88.2)	
Breast Ca. Subtype	-Luminal A	67	(57.8)	130	(73.0)	0.006
	-Luminal B	49	(42.2)	48	(27.0)	
Type of adjuvant hormonal	-Tamoxifen	51	(45.5)	81	(45.8)	0.970
	-AI	61	(54.5)	96	(54.2)	
Type of adjuvant chemo	-Anthracyclin	51	(96.2)	77	(88.5)	0.509
	-Anthra/Taxane	2	(3.8)	9	(10.3)	
	-Other	0	(0)	1	(1.1)	

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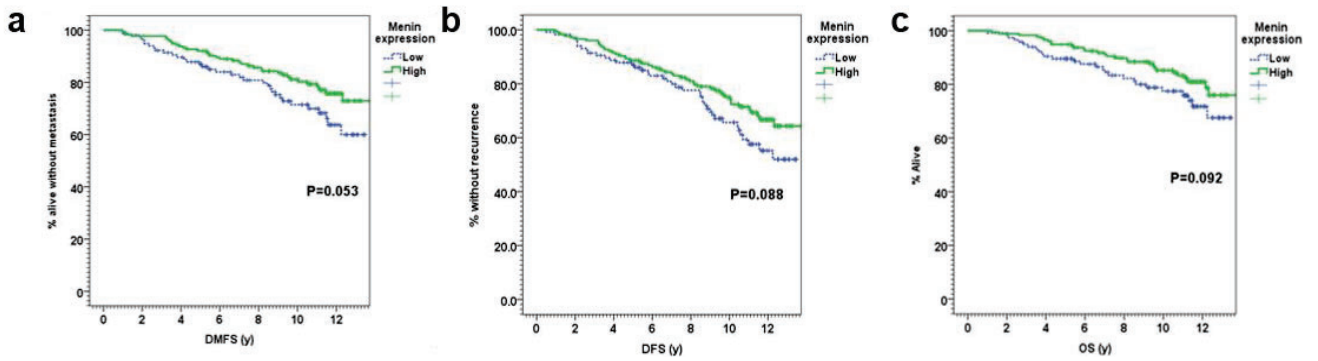


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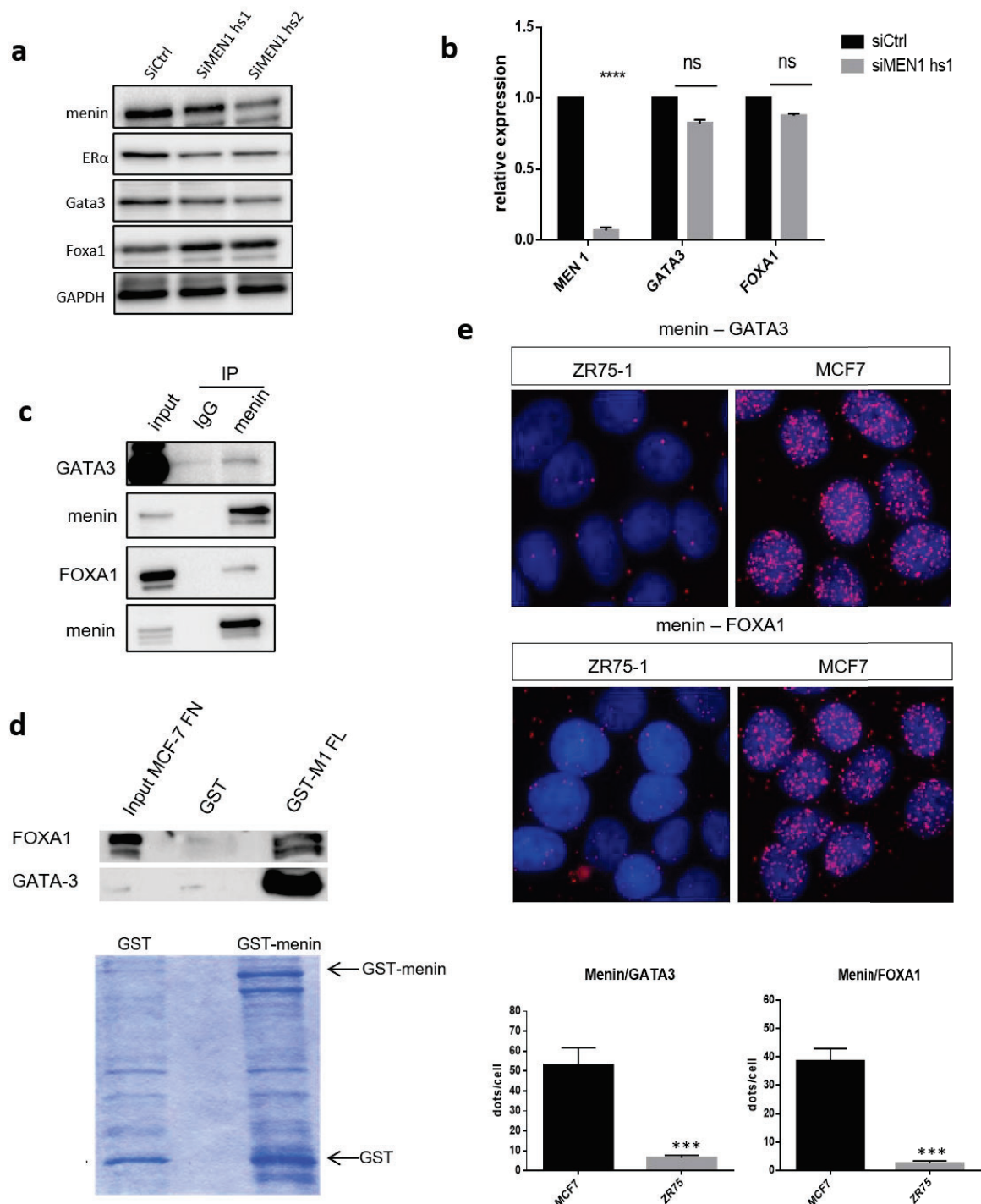


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Characteristic		Num	percent
Age group	<i>≤ 50 years</i>	113	26.1%
	<i>> 50 years</i>	320	73.9%
Menopausal status	<i>Premenopausal</i>	121	28.5%
	<i>Post-menopausal</i>	303	71.5%
	<i>Unknown</i>	9	
BMI	<i>≤ 25 kg/m²</i>	258	61.9%
	<i>> 25 Kg/m²</i>	159	38.1%
Tumor size	<i>≤ 2cm</i>	252	58.2%
	<i>> 2cm</i>	181	41.8%
Axillary LN metastasis	<i>No</i>	184	42.5%
	<i>Yes</i>	249	57.5%
SBR grade	<i>I</i>	82	18.9%
	<i>II</i>	207	47.8%
	<i>III</i>	144	33.3%
ER status	<i>Negative</i>	56	12.9%
	<i>Positive</i>	377	87.1%
PR status	<i>Negative</i>	109	25.2%
	<i>Positive</i>	324	74.8%
HER2 status	<i>Negative</i>	397	92.8%
	<i>Positive</i>	31	7.2%
	<i>Missing</i>	5	
Breast cancer subtype	<i>Luminal A</i>	243	56.1%
	<i>Luminal B</i>	134	30.9%
	<i>HER2 enriched</i>	11	4.6%
	<i>TNBC</i>	45	10.4%
Adjuvant hormonal regimen	<i>Tamoxifen</i>	173	46.6%
	<i>Tam-AI</i>	198	53.4%
	<i>Missing</i>	62	

Supplementary 1: Clinico-pathological characteristics of the tested patient cohort (433 patients)

II.

*Menin downregulation triggers the deregulated mTORC1 and MYC pathways
in ER-positive breast cancer cells*

Running title Menin activates *MYC* transcription in ER-positive BC cells

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Abstract

Menin, encoded by the *MEN1* gene, has been identified as a critical factor regulating *ESR1* transcription and playing an oncogenic factor in ER-positive (ER+) breast cancer (BC) cells. In order to dissect in detail the cellular and molecular consequences of menin inactivation in BC cells, we sought to search for factors participating in the process, and analysed eventual alteration of two major pathways in these cells, namely mTOR and MYC. Intriguingly, *MEN1* silencing in ER+ BC cells resulted in an increase in phosphor-P70S6K1, phosphor-P85S6K1 and phosphor-4EBP1 expression and increased formation of eIF4E and 4G complex, suggesting the activation of the mTORC1 pathway. Interestingly, *MEN1* knockdown (KD) in ER+ BC cells also led to reduced MYC expression on both transcriptional and protein levels. Furthermore, ChIP analysis demonstrated that menin bound not only to the *MYC* promoter but also to its 5' enhancer. Moreover, E2-treated *MEN1* KD MCF7 cells displayed a decrease in MYC activation, suggesting its important role in regulating estrogen-mediated *MYC* transcription. Furthermore, expression data mining in tumors evidenced a significant inverse correlation between *MEN1* and mRNA expression of 2 MYC inhibitory factors, *MYCBP2* and *MYCT1*, in ER+ BC. Finally, combined treatment of MCF7 cells with SiMEN1 and MYC inhibitor 10058-F4 showed synergic effects for cell proliferation inhibition. The current work revealed the altered mTORC1 and MYC pathways after menin inactivation in ER+ BC cells, providing clues into the relevance of the crosstalk between menin and MYC in ER+ BC.

Key words

Menin, MYC, mTORC1, breast cancer, ER-positive subtypes

Introduction

Breast cancer (BC) is the most common cancer occurring in women¹. A classification based on molecular markers and gene expression studies subdivides BC into four major subtypes, namely luminal A, luminal B, HER2-enriched and triple negative BC (TNBC). Although both luminal A and B subtypes, representing around 40% and 20% of BC respectively, express ER α , the latter harbors more genomic instability and has more proliferative features and displays a poorer prognosis. The HER2-enriched subtype behaves more aggressively than luminal subtypes but is sensitive to therapies targeting the HER2 receptor. The TNBC subtype is known to be heterogeneous, and also the most aggressive and has the worst prognosis^{2,3}. Albeit the progress made in identifying factors responsible for BC development and progression, it is still challenging to better understand the underlying mechanisms involved in the development of the different BC subtypes.

ER α , a nuclear receptor, plays major functions in the development and physiology of normal mammary gland. It also plays a crucial role not only in the development of luminal subtypes of BC, but also in their treatment, since its presence is essential for cell proliferation and cell survival of ER-positive (ER+) BC cells. Hormonotherapy using either selective estrogen receptor modulators (SERMs) or selective estrogen receptor downregulators (SERD) has, therefore, been developed since more than 20 years, and has met a great success in the treatment of luminal subtype BC. However, resistance to these treatments ultimately occurs in a substantial proportion of the cases. Consequently, a better understanding of the mechanisms and factors involved in the regulation of the ER α pathway is urgently needed to propose new concepts of strategies in BC treatment. As such, MYC, overexpressed in approximately 20-30% of BCs⁴, is not only a well-known estrogen-regulated oncogene⁵, but has also been shown to interact with ER α to modulate

estrogen-mediated signaling⁶. Identifying the factors influencing the expression and functions of MYC, in order to find ways to control its oncogenic effects, could thus be a promising approach. The PI3K/PTEN/AKT/mTOR pathways are also considered having close relation with the ER α signaling pathway. It has been reported that the activation of the PI3K/PTEN/AKT/mTOR pathways can be found in 70% of BCs⁷. *PTEN* mutations and inactivation are negatively correlated with ER α and PR status in BC^{8,9}, associated with more aggressive behaviors and worse outcome¹⁰. The activated Akt is known to be capable of promoting the ER α pathway¹¹. Therefore, the combination of mTOR inhibitors and endocrine therapy has been proposed, in order to overcome the resistance to endocrine therapy¹².

Menin, encoded by the *MEN1* gene, is a multifaceted cofactor, interacting physically or functionally with numerous transcription and epigenetic factors, including Smad3, JunD, AKT, MYC, KTM2A/B and Ezh2¹³. Interestingly, it has been identified as an ER α interacting partner¹⁴ and a crucial factor that binds to the regulatory sequences of the *ESR1* gene and participates in the positive regulation of its transcription in ER+ BC cells¹⁵. Over the recent years, increasing evidence have been published highlighting the complex and important role of menin in BC. For instance, we have observed that heterozygous *Men1* mutant mice develop mammary gland tumors in females and prostate cancers in males at advanced age at low frequencies¹⁶. Moreover, conditional mammary-specific *Men1* knock-out female mice displayed higher amounts of early mammary intraepithelial neoplasia (MIN), that represent precursor cancer lesions¹⁷. Importantly, a comprehensive analysis of several cohorts of MEN1 patients revealed that female MEN1 patients were at a higher risk of developing BC¹⁸. In human sporadic BCs, *MEN1* mutations, albeit at low frequency, was found as being among driver mutations and reduced menin expression was reported¹⁹. Interestingly, Dreijerink et al. have demonstrated that menin acts as a tumor suppressor

in normal mammary cells, through regulating different sets of genes in primary luminal progenitor cells from normal individuals, compared with those found in ER+ BC cells, but plays an oncogenic factor in the latter cells through its positive regulation of the *ESR1* gene⁹. Moreover, Imachi et al. observed that, with 65 ER+ BC samples treated with tamoxifen for 2-5 years as adjuvant therapies, menin-positive tumors (20 patients) had a worse clinical outcome and were more resistant to tamoxifen than menin-negative tumors (46 patients)²⁰. Intriguingly, the AKT/mTOR pathways have been reported to be related to the occurrence of resistance to endocrine therapy, whereas menin is known to have close relation with the former. Wang et al. demonstrated that menin interacts with AKT1, inhibits its kinase activity and AKT1 induced proliferation and anti-apoptosis in endocrine and non-endocrine cells²¹. Moreover, a recent study reported that menin is involved in milk protein synthesis through mTOR signaling in normal mammary epithelial cells²², by suppressing the mTOR pathway components and milk protein κ -casein (CSNK). The above data suggest that menin may play an important but complex, even opposite role in mammary cells and in different BC subtypes. In the current study, in order to clarify the role of menin in BC, we investigated the above mentioned MYC and mTOR pathways in human BC samples and derived cell lines.

Materials and Methods

Cell culture, estrogen treatment and SiRNA transfections

MCF7, ZR75-1 and T47D, derived from human invasive breast ductal carcinomas, are ER+ breast cancer cell lines displaying luminal mammary epithelium features. MCF7 and ZR75-1 cells were

cultured in DMEM (Gibco Thermofisher Scientific) containing 25 mM glucose supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-Glutamine, 100 unit/ml Penicillin, 100 µg/ml streptomycin, 1X MEM non-essential amino acids solution (all being supplied by Gibco by life technologies), 100 mM β-mercaptoethanol, at 37°C with 5% CO₂. T47D cells cultured in RPMI medium (Gibco by life technologies) containing 25 mM glucose and supplemented with 10% (v/v) FCS, 2 mM L-Glutamine, 100 unit/ml Penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 1 mM Sodium Pyruvate, at 37°C with 5% CO₂. MDA-MB-231 and MDA-MB-453 cell lines are human metastatic ER- breast cancer cell lines, which were cultured in the same medium as MCF7 and ZR75-1 except non-essential amino acids.

MCF7 and ZR75-1 cells were seeded at 250,000 cells/well and T47D cells were seeded at 300,000 cells per well in 6-well plates and cultured for 24h. Then, cells were cultured in white complete phenol-red free medium containing 10% charcoal stripped serum (Biowest) and treated with 10 nM E2 (β-estradiol, E2758-1G, SIGMA) for 30 mins, 1h, 2h, 4h, 8h, 24h (depending on the experiment) or transfected with 20 nM SiRNA of either control SiRNA (5 nmoles, Eurogentec) or SiRNA against the targeted gene using Jetprime® transfection reagent (polyplus) for 72h. Two different SiRNA targeting human MEN1 transcript (SiMEN1 hs1 (HSS106462) and hs2 (HSS181079), ThermoFisher Sci.), two SiRNA against MYC (SiMYC1 (SASI_HS01_00222676) and SiMYC2 (SASI_HS01_00222677), Sigma-Aldrich), One SiRNA targeting eIF4G (eIF4G SiRNA (h): sc-35286, SANTA CRUZ BIOTECHNOLOGY, INC) were used.

Protein extraction and Western blot

Protein extracts were prepared by washing the plates twice with 1X PBS, then, 200-1000 μ l of complete RIPA buffer (50 mM Tris HCL pH=8; 150 mM NaCl; 0.1% SDS; 0.5% Sodium Deoxycholate, 1% NP40 + Protease Inhibitors (Roche) were added and left for at least 20 mins. Then, the cell lysate was transferred to 1.5 ml tubes and centrifuged at 13,000 rpm for 10 minutes at 4°C, in order to precipitate genomic DNA and other cellular debris. The supernatant was retained, and the proteins dosed by Bradford assay at 595 nm.

20 μ g or 30 μ g (depending on experimental conditions) of cell lysate was boiled in 5 μ l Laemli buffer and loaded on 8 or 10 or 12% acrylamide gel depending on protein size. Proteins were then rapidly transferred on a 0.2 μ m PVDF membrane (Biorad) by electrotransfer carried out at 25 V for 10 mins. The membranes were then saturated with 0.1% TBS-Tween, 5% milk for 30 min and incubated with the appropriate primary antibody overnight at 4°C. Membranes were washed three times for 10 mins with 1X TBS -0.1% Tween solution and were incubated for 45 mins with an appropriate secondary antibody coupled to HRP (horseradish peroxidase). Membranes were washed again and were revealed using Roche® Lumi-light Plus Western blotting substrate and Chemiluminescence Film (GE Healthcare). GAPDH was used as an internal control for normalization of the results.

The following primary antibodies were used for western blot: rabbit anti-menin at 1/8000 (Bethyl laboratories, A300 105A), rabbit Anti-ER α 60c at 1/2000 (Millipore, 04-820), mouse Anti GAPDH at 1/25000 (Santa cruz, sc-47734), rabbit Anti MYC at 1/1000 (cell signaling, 9402), rabbit Anti-CDK2 at 1/1000 (Santa Cruz, sc-163) ,rabbit Anti-CDK4 at 1/1000 (Santa Cruz, sc-260) , mouse Anti-cyclin E at 1/1000 (Santa Cruz, sc-377100) , mouse Anti-cyclin D at 1/1000 (Santa Cruz, sc-450) ,rabbit Anti-p53 at 1/1000 (cell signaling , 9282) , rabbit Anti- caspase 3 at 1/1000 (cell signaling 8G10), rabbit Anti- PARP at 1/1000 (cell signaling, 46D11) , rabbit Anti-

eIF4G at 1/1000 (cell signaling, C45A4), mouse Anti- eIF4E at 1/1000 (Santa Cruz, A-10), mouse Anti-eIF4A at 1/1000 (Santa Cruz,H-5), rabbit Anti-phospho 4EBP1 ser 65 at 1/1000 (Cell Signaling, S65), rabbit Anti-Phospho p70 S6K1 at 1/1000 (Cell signaling, 49D7), rabbit Anti-AKT total at 1/1000 (Cell signaling), rabbit Anti-Phospho 4EBP1 Thr37/46 at 1/1000 (cell signaling, 236B4), rabbit Anti-Phospho AKT Ser 473 at 1/1000 (Cell signaing,D9E), rabbit Anti-Phospho 4EBP1 total at 1/1000 (Cell signaling, 53H11), rabbit Anti-Phospho p60 S6K1 (T389) at 1/1000 (Cell signaling, 9205S), rabbit Anti-Phospho S6RP Ser 235/236 at 1/1000 (Cell signaling, 2211S) , rabbit Anti-Phospho p85 S6K1 (T389) at 1/1000 (Cell signaling, 9205S), rabbit Anti-S6K1 total at 1/1000 (Cell signaling).

Real-time reverse transcriptase and qPCR analyses

RNAs were extracted using RNeasy-Kits (Macherey-Nagel). Real-time PCR analyses were carried out on a Step-One RT-System (Applied-Biosystem, France) using SYBR-Green (Life-Technology, France) and results of each sample were normalized with the 28S house keeping gene used here as a control. Primers used were as following: 28s Fw: 5'-CGATCCATCATCCGCAATG-3', Rev: 5'-AGCCAAGCTCAGCGCAAC-3'; MEN1 Fw: 5'-CATCCAGGACTACA ACTACT -3', MEN1 Rev: 5'-ATGACATCATTGGCTACTTC -3'; ESR1 Fw: 5'GGCTGCAAGGCCTTCTTCAA- 3', ESR1 Rev: 5'-CCTGGCAGCTCTTCCTCCTG -3'; 4-EBP1 Fw: 5'-GGCGGCACGCTCTTCA-3',Rev: 5'-GAAATTTCTGATGGAGTGT-3';S6K1 Fw: 5'-AGAAGATGCAGGCTCTGA-3', Rev: 5'-TTACCAAGTACCCGAAGTA-3'; eIF4G Fw: 5'-GCCATTTTCAGAGCCCAACTTCTC-3', Rev: 5'-CGGAAGTTCACAGTCACTGTTGG-3'; eIF4A: Fw: 5'-AAGCCGTGGATTCAACGACCAG-3',Rev: 5'-CACCTCAAGCACATCAGAAGGC-3';

eIF4E Fw: 5'-ATGCCTGGCTGTGACTACTCAC-3', Rev: 5'-GAGGTCACCTTCGTCTCTGCTGT-3'; MYC Fw:5'-TCGGATTCTCTGCTCTCCTC-3', Rev: 5'-GAGCCTGCCTCTTTTCCAC-3'; cyclin A2 Fw: 5'-CTGCATTTGGCTGTGAACTAC-3',Rev: 5'-ACAAACTCTGCTACTTCTGGG-3'; cyclin B1 Fw: 5'-GGCTTTCTCTGATGTAATTCTTGC-3',Rev: 5'-GTATTTTGGTCTGACTGCTTGC-3'; cyclin E Fw: 5'-ATCAGCACTTTCTTGAGCAACA-3', Rev: 5'-TTGTGCCAAGTAAAAGGTCTCC-3'; CDk2 Fw: 5'-GCTAGCAGACTTTGGACTAGCCAG-3', Rev: 5' AGCTCGGTACCACAGGGTCA-3'; CDK4 Fw: 5'-CTGGTGTTTGAGCATGTAGACC-3',Rev: 5'-AAACTGGCGCATCACATCCTT-3'; p53 Fw: 5'-CAGCACATGACGGAGGTTGT-3', Rev: 5'-TCATCCAAATACTCCACACGC-3'; p21 Fw: 5'-GCCCAGTGGACAGCGAGCA-3', Rev: 5'-GCCGGCGTTTGGAGTTGGTAGA-3'; p27 Fw: 5'-CCGGCTAACTCTGAGGACAC-3', Rev:5'-AGAAGAATCGTCGGTTGCAG-3'; BCL2 Fw: 5'-CGGAGGCTGGGATGCCTTTG-3', Rev:5'-TTTGGGGCAGGCATGTTGAC-3'.

Proximity Ligation Assay (PLA)

PLA assays were performed using Duolink In Situ PLA detection kit (orange) and Probes: Anti-Goat MINUS and Anti-Rabbit PLUS ((#DUO92006, #DUO92002, Sigma-Aldrich), according to the recommendation of the supplier. Briefly, MCF-7 cells were seeded in a 12-well plate containing cover slips for 72h, on the second day cells were transfected with SiCtrl, SiMEN1 (hs1), SieIF4G, or SiMEN1 (hs1) + SieIF4G as described above. The last day, cells were treated with Estrogen for 2h. Cells were fixed with methanol for 2 mins and washed 3 times with 1X PBS, then, blocked with Blocking Solution and incubated at 37°C for 30 min. 40 µl/slip of solution containing primary antibodies of interest and secondary antibodies (anti-Rabbit + anti-Goat or anti-

mouse+anti-rabbit) coupled to oligonucleotides were added consecutively with 1 h incubation each at 37° after one time wash with buffer A. Slips were washed twice with Buffer A with shaking for 10 min, ligation reaction was performed for 30 min, and the signal was amplified for 100 min at 37°C . Cover slips were then washed twice with 1X Buffer B for 10 min with shaking, then once with 0.01x Buffer B. Cells were mounted with mounting solution containing DAPI and observed under florescence microscopy. The primary antibodies used for PLA assays in this study were goat anti-menin (A300 106A, bethyl laboratories) at 1/1000, rabbit anti-MYC at 1/500, rabbit anti-ER at 1/500, mouse anti-eIF4E at 1/8000, mouse anti-eIF4A at 1/2000, rabbit anti-eIF4G at 1/2000 (Same antibodies used in Western blot),

Cell proliferation assays:

MCF7, T47D, and MDA453 cells were seeded in white complete medium at 15000 cells per well for MCF7 and MDA453 and 20000 cell per well for T47D in a 48-well plate. 24h after seeding, cells were transfected with SiRNA (same as described before) or treated with 10058-F4 inhibitor (abcam, ab145065) at different concentrations. The plate was then left for 144h in the Incucyte™ device (Essen Bioscience), allowing the observation of cell growth in kinetics. The device is connected to the software Incucyte™ that determines the cell confluence in real-time.

ChIP-qPCR assay

Chromatin immunoprecipitation (ChIP) was performed with the Millipore ChIP Assay Kit (17-295) according to the recommendations of manufacturer. Cells were crosslinked with 1% formaldehyde for 10 min at 37°C. Chromatin was prepared 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. 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). Rabbit Anti-menin (same used for W.B) was used to immunoprecipitate menin with a concentration of 2 µg/sample, and equal amount of rabbit IgG was used as control. Primers used for MYC promoter: TSS: Fw: 5'-ACTCACAGGACAAGGATGCG-3', Rev: 5'-TGCTCCTCCGTAGCAGTACT-3'; +300: Fw: 5'-CCAACAAATGCAATGGGAGT-3'; Rev: 5'-ATGATAGAGGCATAAGGAG-3'; Enhancer: Fw:5'-CATCCAATAAACCTTCTACCTGA-3', Rev:5'-TGGCAGGTGTCCTAGAGCAT; Proximal promoter: Fw:5'-ACAAGGATGCGGTTTGTC-3', Rev: 5'-CGCCTACCATTTTCTTTTGC-3'; Chromosome 1 negative control primers: F: 5'-CGGGGGTCTTTTGGACCTT-3', Rev: 5'-GAAACACGGCTGCCAGAAAC-3'.

Mining of public gene expression data

RNA-seq data (Illumina HiSeq 2000, version 2 analysis) obtained in human BC samples in The Cancer Genome Atlas (TCGA) project were previously collected from the Genomic Data Commons data portal (<https://portal.gdc.cancer.gov>). Expression data were available for 1095 tumors as RNA-Seq by Expectation Maximization (RSEM) normalized counts. Clinical data including ER status were available as well, allowing the identification of ER+ (n=807) and ER-negative (ER-), (n=237) tumors. Co-expression between *MEN1* and all of the 20531 genes,

including *MYC*, was examined using Pearson correlation coefficient. Genes showing significant correlation coefficient with absolute value greater than 0.2 were taken as correlated with *MEN1*. Gene set enrichment analysis (GSEA, <http://www.gsea-msigdb.org/>) was used to identify Gene Ontology (GO) gene sets enriched in *MEN1* positively correlated genes.

In addition, using the cBioPortal (<https://www.cbioportal.org/>), a recognized and widely used online portal for cancer genomics, other BC data sets could be investigated for *MEN1* co-expression. Among the most recent ones, we selected the SMC 2018 study focusing on young Asian patients²³.

Statistical analyses

Statistical analyses were performed as described in the figure legends; unpaired Student t tests were used unless otherwise indicated. All analyses were done using Prism5 software (GraphPad, USA); a *P* value of < 0.05 was considered significant. Results are given as means +/- standard errors of the means (SEM).

Results:

***MEN1* knockdown decreases cell proliferation in ER+ BC cell lines**

It is known now that menin regulates ER α expression in ER+ BC cells and menin is critical for cell growth of ER+ BC cell lines^{8,9}. To better study the role of menin in BC cells, we studied the effects of *MEN1* KD on the proliferation of two ER+ BC cell lines, MCF7 and T47D, and two ER- BC cell lines, MDA231 and MDA453. As shown in Fig 1a (2 upper panels), the proliferation of MCF7 and T47D cells started to decrease 48h after *MEN1* KD and continued to decrease even

after 120h of transfection, compared to the untreated and SiCtrl cells. In parallel, our data demonstrated that *MEN1* KD had the same effects on the proliferation of MDA231 cells, but no effect at all on that of MDA453 cells (Fig 1a, 2 lower panels). Our data confirmed that *MEN1* is critical for cell proliferation in tested ER+ cells, but showed that it is differentially needed in ER- cell lines, suggesting the role of menin in the proliferation of mammary cancer cells could be context-specific. In our following work, we, therefore, mainly focused on the role of menin in ER+ BC cells.

***MEN1* knockdown activates the mTORC1 pathway in ER+ BC cell lines**

To investigate the molecular mechanisms triggered by *MEN1* silencing in ER+ cells, we first studied its effects on the mTORC1 pathway, which is critical for cell growth and breast cancer development, as well as involved in resistance to endocrine therapy²⁴. Hence, we knocked down menin expression in MCF7 and T47D cells treated with insulin (to activate the mTORC1 pathway) and/or the mTOR inhibitor RAD001. We observed, surprisingly, that *MEN1* KD in these cells increased the expression of the active form of mTORC1 components, namely phosphorylated p70, and p85 (T389) of S6K1 and S6RP Ser 235/236, after insulin treatment, which was inhibited by RAD001 treatment (Fig 1b). The analysis was further carried out in MCF7 cells treated with essential amino acids (EAA), another mTOR stimulator, and similar result was obtained (Fig S1a). *MEN1* silencing also led to increased phosphorylated 4EBP1(ser 65) in T47D cells (Fig 1c). 4EBP1 is a repressor of the downstream events of mTOR pathway, and while S6K1 and S6RP phosphorylations have an activating effect, 4EBP1 phosphorylation has an inactivating one. To further confirm these unexpected results, we used a reverse approach by overexpressing menin in MCF7 cell line. The results showed that menin overexpression led to the decrease in the expression

of phosphorylated S6K1 and 4EBP1, reversing the effects seen by *MENI* KD (Fig 1d). We also noticed that there was an increase in the expression of total 4EBP1 protein in MCF7 cells with menin overexpression (Fig 1d).

Previous studies have reported a relation between menin and AKT in other types of cancers²¹, where deregulated AKT was found in sporadic pancreatic endocrine tumors, in which menin is frequently inactivated²⁵. For that we thought it was important to check if the role played by menin on the mTORC1 pathway is AKT dependent or not in our breast cancer cell lines. We thus analyzed AKT expression after menin knockdown in MCF7 cells and found that *MENI* KD increased slightly the phosphorylated AKT without altering the total protein level (Fig 1e), and the use of AKT inhibitor inhibited the activation of S6K1 and S6RP triggered by *MENI* KD (Fig S1b), suggesting that the effect of menin knockdown on mTORC1 activation is AKT-dependent.

***MENI* KD increases the activity of the translation initiation complex**

To further dissect the downstream effects of mTORC1 activation triggered by reduced menin expression, we studied the consequences of *MENI* silencing on the translation initiation complex eIF4F (one of the downstream targets of the mTORC1 pathway) composed of 3 factors: eIF4A, the enzymatic core, eIF4E, responsible for cap-dependent translation of all nuclear encoded mRNA, and eIF4G, the scaffold protein that holds the complex together²⁶. We first checked whether the expression of these factors was altered after *MENI* KD. The data showed a reduced expression of eIF4A and eIF4E, and a stable expression of eIF4G on mRNA levels, but no significant change for all 3 factors on the protein levels (Fig 2a). We then analyzed the formation of eIF4F by assessing the interaction between eIF4G/eIF4E and eIF4G/eIF4A using Proximity

ligation assay (PLA). We found that *MEN1* silencing resulted in an increase in the interaction between eIF4G and eIF4E, but not that between eIF4G and eIF4A in *MEN1* KD MCF7 and T47D cells (Fig 2b), compared to the menin negative ZR75 cell line with same treatment (Fig S2a).

In addition, mining *MEN1* gene co-expression in BC TCGA data provided a correlation coefficient for each of the genes available in the data set. Gene set enrichment analysis identified the GO term Translational initiation as enriched in genes positively correlated with *MEN1* in ER+ tumors (Fig 2c). As shown in Fig 2d and S2b, among the so-annotated genes, *EIF4EBP1* encoding 4EBP1, as well as *EIF4BP3* were positively correlated with *MEN1*, in agreement with the increased expression of 4EBP1 protein observed in MCF7 cells with menin overexpression. Inversely, four other genes encoding components related to the eIF4F complex showed inverse correlation with *MEN1*: *EIF4E3*, *EIF4G2*, *EIF4G3*, and *EIF4E* that was slightly below the correlation threshold. These results corroborate at the mRNA level the increased formation of the translation initiation complex eIF4G/eIF4E after *MEN1* KD shown above. A similar inverse relationship was observed for *MTOR* (Pearson correlation coefficient = -0.236). These data mining results appeared in agreement with the above experimental data obtained in ER+ BC cell lines.

Menin regulates MYC expression and binds to the *MYC* promoter and enhancer in ER+ breast cancer cells:

The above results showing the activation of the mTOR pathway could not explain the reduced cell proliferation in *MEN1* KD ER+ breast cancer cells. We thus chose to study *MYC* which is an important estrogen regulated oncogene and is known to play a major role in proliferation, metabolism, and apoptosis of different cancer cells, including BC cells, and analyzed the effects

of *MEN1* silencing on *MYC* expression. The results showed that menin inactivation decreased *MYC* expression at both the mRNA (Fig 3a, left panels) and protein levels (Fig 3a, right panels) in *MEN1* KD MCF7 and T47D cells. However, our data showed heterogeneous results in 2 tested ER- cell lines, with reduced *MYC* transcription but increased *MYC* protein levels in MDA231 cells, and no effect at all in MDA453 cells (Fig 3b). To understand how menin regulates *MYC* expression on transcriptional level, we sought to investigate the binding of menin to both the *MYC* proximal promoter and the *MYC* 5' enhancer previously reported²⁷ by ChIP analysis. We found that menin bound indeed the *MYC* 5' enhancer and the proximal *MYC* promoter, including the areas involving transcription start site (TSS) and +300bp sites of the *MYC* promoter in MCF7 cells (Fig 3c). We also analyzed the influence of *MYC* expression on the expression of ER α and menin. Our data demonstrated that *MYC* KD had no effect on ER α expression, but, interestingly, resulted in decreased *MEN1* expression both on transcription and protein levels in MCF7 and T47D cells (Fig S3a, b).

We observed that *MEN1* knockdown also showed decrease in protein and mRNA expression of several known *MYC* target genes, which are either involved in cell cycle control, including Cyclins, Cyclin-dependent kinases (CDKs), p53, and cyclin-dependent kinase inhibitors (CKI) like p21 and p27, or in the apoptosis, like cleavage of PARP and caspase 3, in MCF7 cells (Fig 3d). These data are in total consistency with reduced *MYC* expression detected in *MEN1* KD MCF7 and T47D cells.

Menin is needed for estrogen mediated *MYC* activation and physically interacts with *MYC* in BC cells

Since *MYC* is an estrogen regulated oncogene, whereas all our above findings have been conducted in the absence of estrogen (Charcol stripped media), we thought it would be relevant to investigate whether the regulation of *MYC* expression is influenced by estrogen stimulation. We thus first treated MCF7 cells with β -estradiol (E2) at different time points (Fig S4). We found that while the expression of ER α protein was decreased after E2 stimulation, the expression of *MYC* was significantly increased at both protein and mRNA levels, starting 1h after treatment, whereas menin expression did not change (Fig 4a). Interestingly, *MEN1* silencing resulted in decreased *MYC* expression under E2 stimulation in MCF7 cells, suggesting that menin expression is critical for estrogen mediated *MYC* activation) (Fig 4b).

In order to better understand the interaction between menin and *MYC*, previously reported in HeLa cells²⁸, in BC cells, we analyzed whether there was a physical interaction between menin and *MYC* in ER+ breast cancer cells used in the current work, and, if yes, whether this interaction was estrogen dependent. Our data showed that menin interacts indeed with *MYC* in MCF7 cells, but their interaction was reduced upon E2 stimulation (Fig 4c left panel), We also noticed that E2 treatment in MCF7 cells led to increased interaction between menin and ER α (Fig 4c right panel), suggesting menin's role in estrogen-mediated *MYC* activation functions likely through its physical interaction with ER α .

Co-expression of menin and *MYC* in some breast cancers and their combined inhibition has better effects on cell proliferation reduction

Data mining analyses were then carried out using different publicly available BC mRNA expression datasets to further investigate co-expression between menin and *MYC* in BC. Although no positive correlation was detected between *MEN1* and *MYC* mRNA expression in TCGA BC

samples in all tumors and in ER+ tumors, an interesting significant positive correlation was seen in all tumors from the SMC 2018 dataset, as well as in ER+ tumors only (Fig 5a). With regards to the TCGA data, a positive correlation was observed for ER- tumors (Pearson correlation coefficient = 0.256) (Fig 5b).

Next, we focused on the relationship between *MEN1* and MYC-related genes. Interestingly, we found that two genes, *MYCBP2* and *MYCT1* described as negative regulating factors in the MYC pathway, displayed a significant negative correlation with *MEN1* in ER+ tumors (Fig 5c). Knowing that both of these MYC-related proteins play an inhibitory role in the MYC pathway, the data seem to support the inconsistent relationship between *MEN1* and *MYC* uncovered above.

Based on these findings, we tested whether *MEN1* KD could provide or not synergistic effects with MYC inhibition to reduce cell proliferation. Although separate inhibition of MYC and menin using SiRNA or 10058-F4 (MYC/MAX inhibitor) decreased cell proliferation in MCF7 cells, the combined use of *MEN1* KD and SiRNA and MYC inhibitor 10058-F4 did not decrease further cell proliferation in MCF7 line, suggesting that the effects of menin depends mainly on MYC expression (Fig 5d).

Discussion

Our current work revealed that *MEN1* silencing dysregulates mTORC1 and MYC pathways, specifically in ER+ BC cell lines, providing useful clues into molecular mechanisms underlying the oncogenic effects of menin observed in these cells.

We noticed that *MEN1* silencing led to reduced cell proliferation in ER+ BC cell lines as previously reported^{15,20}, whereas it triggered a distinct cell growth reaction in an ER- BC cell line,

indicating the effects exerted by the menin protein are dependent on cellular and molecular context. Since it is known that ER- BC is a particularly heterogeneous subtype, which has recently been suggested to be further divided into at least 6 subgroups², it is not surprising that menin could play different roles in ER- BC cell lines. In fact, Dreijerink et al. reported that menin did not regulate ER α target genes in ER- BC cell lines. It would, therefore, be interesting to further dissect the role of menin in responsive or non-responsive ER- BC cells, beyond the ER α pathways, in order to gain insight into the factors and/or pathways participating in the growth and survival of these cells.

Menin is known to physically and functionally interact with the AKT/mTOR pathway in pancreatic endocrine cells, mainly by the retention of AKT in the cytoplasm²¹. However, its role in the mTOR pathway, in particular on the downstream effectors remains elusive. Our data showed that menin expression is crucial for mTORC1 activity in BC ER+ cells, since its inactivation led to mTORC1 activation. Moreover, the latter seemed to mainly result in the activation of cap-dependent translation, since 4EBP1 expression decreased and its phosphorylated form increased after *MEN1* KD, whereas 4EBP1 normally represses the assembly of the eIF4F complex and its phosphorylation allows the components of eIF4F complex to interact and thus initiate mRNA translation. Importantly, our data show that menin inactivation in ER+ BC cells specifically favored the formation of eIF4E and eIF4G complex, which is considered to be the rate-limiting determinant of cap-dependent protein synthesis²⁷ and one of the characteristic activities of cancer cells, including BC cells³⁰. Data mining of gene mRNA expression in ER+ human tumors corroborate the effects of menin inactivation on 4EBP1 and on the formation of the eIF4F complex observed in ER+ BC cells. Notably, our clinical data analysis on TamRad trial also supports the possible activation of the mTOR pathway in menin-low BC ER+ patients, even though the trend observed in the trial needs to be further confirmed by other studies with more cases. We noticed

that a recent study reported the reduced ER α signaling pathway upon PI3K/AKT activation¹¹. Their data showing increased ER α expression after the treatment with PI3K and/or AKT inhibitor is reminiscent of the data reported in the current study. It is, therefore, plausible that the activation of AKT/mTORC1 triggered by *MEN1* silencing contributes to the reduced ER α expression and cell proliferation observed in ER+ BC cells in our works, along with the inhibition of the MYC pathway. Another possibility would be that the activation of the mTORC1 pathway triggered by menin inactivation could lead to oncogenic stress in these cells, as formerly reported³¹.

Interestingly, *MEN1* knockdown led to reduced MYC expression in ER+ cells. Our analyses demonstrated, for the first time, that menin is a critical factor regulating *MYC* transcription in these cells, through its binding not only to the *MYC* promoter, but also to the *MYC* 5' enhancer. Furthermore, we observed that *MEN1* silencing in these cells triggered altered expression of factors known to be MYC targets and responsible for cell growth, cell cycle and cell death. The novel role of menin uncovered in the current work in regulating *MYC* transcription and the MYC pathway is, therefore, consistent with molecular alterations observed in these cells. Our finding is also in agreement with the recently emerged notion that menin could play a dual role in the tumorigenesis of BC cells. The fact that menin is essential for *ESR1* and *MYC* transcription revealed in the previous study¹⁸ and the current work on ER+ BC cells makes it a powerful oncogenic factor in these cells, by regulating two main actors. Imach *et al* previously reported that menin expression in BC patients is clinically correlated with resistance to tamoxifen²⁰. It would be relevant to verify whether the role of menin in regulating MYC expression could be one of the molecular bases conferring such resistance. In parallel, we observed that, in ER- BC cells, the role of menin in regulating MYC and cell proliferation varies among different cell lines tested, suggesting more diverse molecular elements may participate in the carcinogenesis of these cancers.

In *MEN1* KD ER- BC cells showing reduced *MYC* transcription and reduced cell proliferation without altered *MYC* protein expression, as in MDA-MB-231, menin may also be relevant to its post-transcriptional regulation via factors, such as *MYCBP1* and *MYCT1*, and/or to the expression of other genes that are critical for the growth of these cells. Future works on ER- BC cells could provide useful answers to explain these findings. We noticed that the positive correlation between menin and *MYC* expression in some BC samples. Moreover, even in the absence of *MYC-MEN1* co-expression, a clear inverse relationship was detected between *MEN1* and *MYCT1* or *MYCBP2* expression in ER+ as well as ER- tumors, suggesting the involvement of menin in post-transcriptional regulation of *MYC*. Considering the heterogeneous nature of BC³², it would be relevant to further dissect and validate the molecular crosstalk unveiled in the current work between menin and its related pathways, including *MYC* and mTORC1, in different BC subtypes.

DECLARATIONS SECTION

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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Author Contributions

R.A.Z conducted the experiments, analysed and interpreted the data, prepared figures and manuscript; R.T and YL provided technical and material support and participated in data interpretation; T.B and I.T conducted clinical study design, collection and interpretation of data; C.C and J.M.V realized bioinformatic analyses and data interpretation, as well as participated in manuscript preparation; M.L.R and C.X.Z conceived and supervised the study and manuscript preparation, and obtained funding.

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Legends:

Fig 1. Menin downregulation leads to mTORC1 activation in ER+ cell lines. **a** Cell proliferation curves of MCF7, T47D (2 upper panels), and that of MDAMB231 and MDAMB453 cell lines (2 lower panels) transfected with SiCtrl or SiMEN1 hs1 at 24h after cell culture. Western blot showing menin expression in the above cells treated with different siRNAs as indicated. Data shown are mean (\pm SEM) of technical replicates from one representative experiment out of three. **b** Western blot analysis showing the protein expression of phosphorylated and total S6K1 and S6RP in MCF7 (left panel) and T47D (right panel) cells transfected with SiCtrl or SiMEN1 hs1 and treated with insulin and/or the mTOR inhibitor RAD001 as indicated. **c** Western blot analysis detecting the phosphorylated and total 4EBP1 in T47D cells transfected with SiCtrl or SiMEN1 hs1 and treated with insulin and/or the mTOR inhibitor RAD001 as indicated. **d** Western blot analysis showing the protein expression of S6K1, S6RP, and 4EBP1 in MCF7 cells transfected with the control vector PCI-Neo or the vector PCI-MEN1 overexpressing menin and treated with insulin and/or RAD001 as indicated. **e** Western blot analysis showing the protein expression of

phosphorylated (Ser 473) and total AKT in MCF7 cells transfected with SiCtrl or SiMEN1 hs1 and treated with insulin and/or the AKT inhibitor MK2206 as indicated.

Fig 2. *MEN1* silencing results in activation of eIF4F formation. **a** Quantitative RT-PCR (left panel) and Western blot (right panel) showing the mRNA and protein expression respectively of eIF4E, eIF4A, eIF4G in MCF7 cells transfected with SiCtrl or two different SiMEN1 hs1 or hs3. Data shown, normalized against GAPDH, are presented as means (\pm SEM) of technical replicates from one representative experiment out of three. **b** Proximity ligation assay (PLA) showing the interaction between either eIF4G/eIF4E or eIF4G/eIF4A in MCF7 (Upper panel) and T47D (lower panel) cell lines transfected with SiCtrl, SiMEN1 hs1, SiEIF4G and SiEIF4G+MEN1 hs1, respectively. Quantification of PLA signals, represented by dots/cell, for each condition is shown on the right panels. **c** GO term Translational initiation gene set was identified among *MEN1* correlated genes with Gene Set Enrichment Analysis. **d** Clinical correlation from TCGA data between *MEN1* and *mTOR*, *MEN1* and *4EBP1*, *MEN1* and *EIF4E*, *MEN1* and *EIF4G2* in ER+ BC.

Fig 3. Menin regulates MYC expression and binds to the MYC promoter in ER+ BC cells. Quantitative RT-PCR (left panels) and Western blot (right panels) showing MYC expression in ER+ MCF7 and T47D cell lines (**a**) and ER- MDAMB231 and MDAMB453 cell lines (**b**) transfected with SiCtrl or two different SiMEN1 hs1 or hs3. Quantitative RT-PCR data shown, normalized against GAPDH, are presented as means (\pm SEM) of technical replicates from one representative experiment out of three. **c** Schematic representation of the primers used ChIP-quantitative PCR analysis (left panel). ChIP-qPCR analyses analysis showing menin binding (normalized with input) on different regions of the *MYC* promoter (right panel). Results were compared to IgG and an unrelated region on chromosome 1 (Chr1) used as controls for ChIP. Cells were also transfected with SiMEN1 hs1 to confirm the specificity of binding. **d** Quantitative RT-

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Fig 4. Menin is needed for estrogen-mediated MYC activation and physically interacts with MYC in BC cells. **a** Quantitative RT-PCR (left panel) and Western blot (right panel) showing the protein and mRNA expression of menin, ER α , and MYC in MCF7 cells after estrogen treatment for 1 or 2h. **b** Left panel: Quantitative RT-PCR showing the expression of menin, ER α , and MYC in MCF7 cells transfected with SiCtrl or SiMEN1 hs1 or hs3 in the absence or presence of 1h estrogen stimulation; Right panel: Western blot showing the expression of menin, ER α , and MYC in MCF7 cells transfected with SiCtrl or SiMEN1 hs1 or hs3 in the absence or presence of 2h estrogen stimulation. **c** Upper panel: PLA showing the physical interaction between menin and MYC (left panel) or menin and ER α (right panel) in MCF7 cells before and after estrogen treatment. SiMEN1 hs1 and SiMYC were used as controls; Lower panel: Quantification of PLA signals measured in terms of numbers of dots/cell.

Fig 5. Correlation between *MEN1* and *MYC* mRNA expression in breast cancer and effects of cell proliferation inhibition with combined inactivation of *MEN1* and *MYC*. **a** Left panel: Data mining analysis from the SMC 2018 dataset showing the positive correlation between *MEN1* and *MYC* mRNA expression in BC including all subtypes. Right panel: same analysis from the SMC 2018 dataset in ER+ BC subtypes. **b** Correlation of mRNA expression between *MEN1* and *MYC* observed in ER+ (left panel) and ER- (right panel) tumors from TCGA dataset. **c** Inverse correlation between the expression of *MEN1* and 2 inhibitory factors in the MYC pathway, *MYCB2* and *MYCT1*, in ER+ BC from the TCGA dataset. **d** Proliferation curve of MCF7 cells transfected with either SiMEN1 hs1 or SiMYC or both (left panel), and transfected with SiMEN1 hs1 or

treated with 10058 F4 (MYC/MAX inhibitor) or both (middle panel). Western blot showing MYC protein expression in MCF7 cells treated with DMSO or 20 and 50 μ M of 10058 F4 inhibitor (right panel). 10058 F4 (MYC/MAX inhibitor) or both (middle panel). Western blot showing MYC protein expression in MCF7 cells treated with DMSO or 20 and 50 μ M of 10058 F4 inhibitor (right panel).

Supplementary Fig 1. Effects of reduced menin expression on mTORC1 activation: **a** Western blot analysis showing the protein expression of the different phosphorylated S6K1 in MCF7 cells transfected with SiCtrl or SiMEN1 and treated with insulin and/or essential amino acids (EAA), stimulators of AKT. **b** Western blot analysis showing the protein expression of the different phosphorylated S6K1 and S6RP in MCF7 cells transfected with SiCtrl or SiMEN1 and treated with insulin and/or AKT inhibitor MK2206.

Supplementary Fig 2. Menin is involved in the regulation of translation initiation complex in ER+ BC. **a** Proximity ligation assay (PLA) showing the interaction between either eIF4G/eIF4E or eIF4G/eIF4A in ZR75 cell line transfected with SiCtrl, SiMEN1, SiEIF4G and SiEIF4G+MEN1, respectively. Quantification of PLA signals, represented by dots/cell, for each condition is shown on the right panels. **b** Clinical correlation from TCGA datasets between *MEN1* and 4EBP3 (positive correlation) and eIF4G2, eIF4G3, and eIF4E3 (negative correlation) in ER+ BC.

Supplementary Fig 3. MYC KD affects menin but not ER α expression: **a** Quantitative RT-PCR (upper panel) and Western blot (lower panel) showing the mRNA and protein expression of *ESR1* and ER α respectively in MCF7 cells treated with SiCtrl or 2 different SiMYC. **b** Quantitative RT-PCR (upper panel) and Western blot (lower panel) showing the mRNA and protein expression of menin in MCF7 cells treated with SiCtrl or SiMYC1.

Supplementary Fig 4. MYC, menin and ER α expression under estrogen stimulation. Western blot analysis showing the protein expression of MYC, menin and ER α in MCF7 cells treated with E2 for 1, 2, 4, 8, or 24h.

Supplementary Fig 5. Significant positive clinical correlation between *MEN1* and *MYC* mRNA expression in ER+ and ER- BC. **a** Positive correlation of mRNA expression from SMC 2018 dataset between *MEN1* and MYC in ER+ BC. **b** Positive correlation of mRNA expression between *MEN1* and MYC observed in ER- tumors from TCGA dataset.

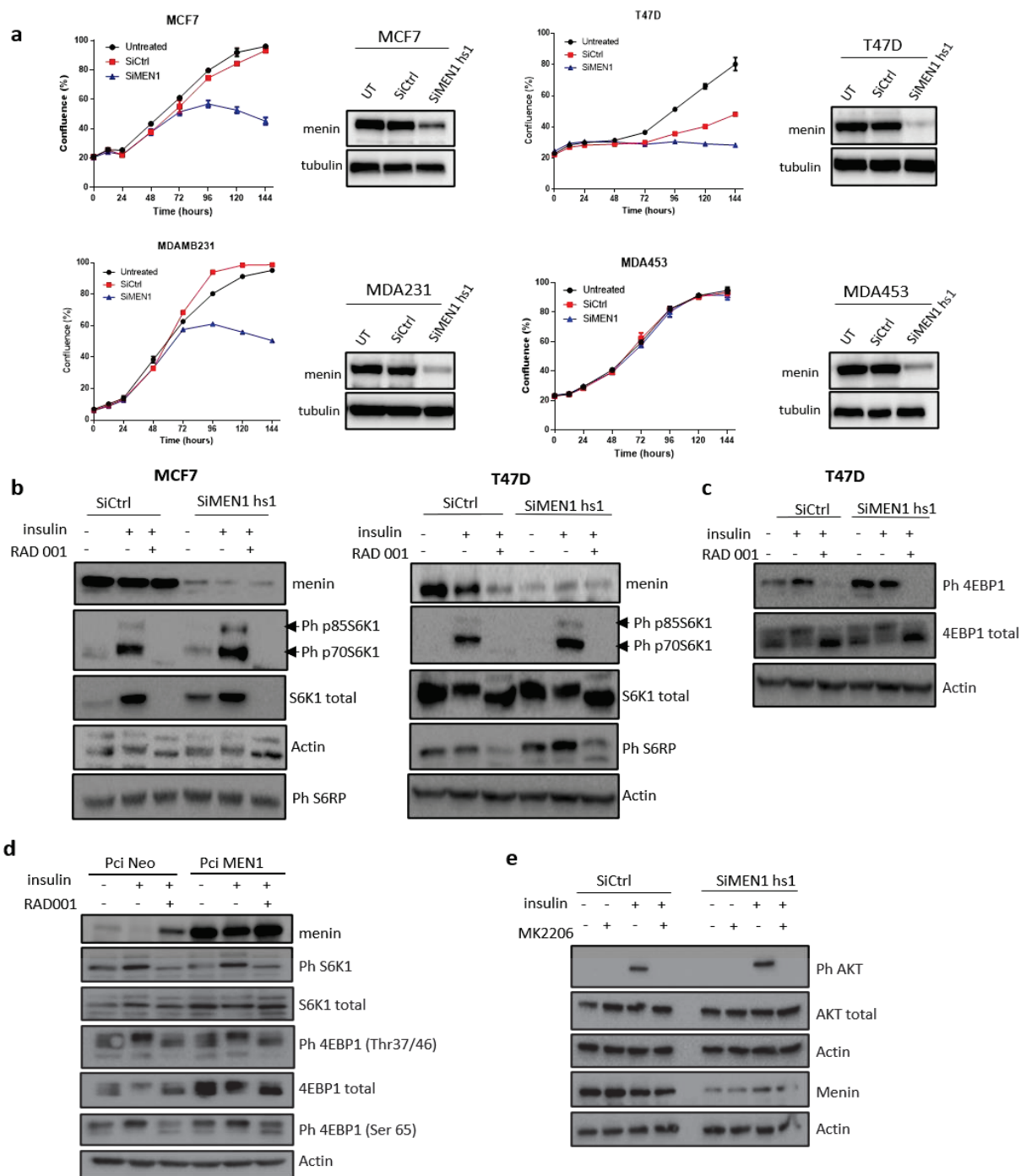


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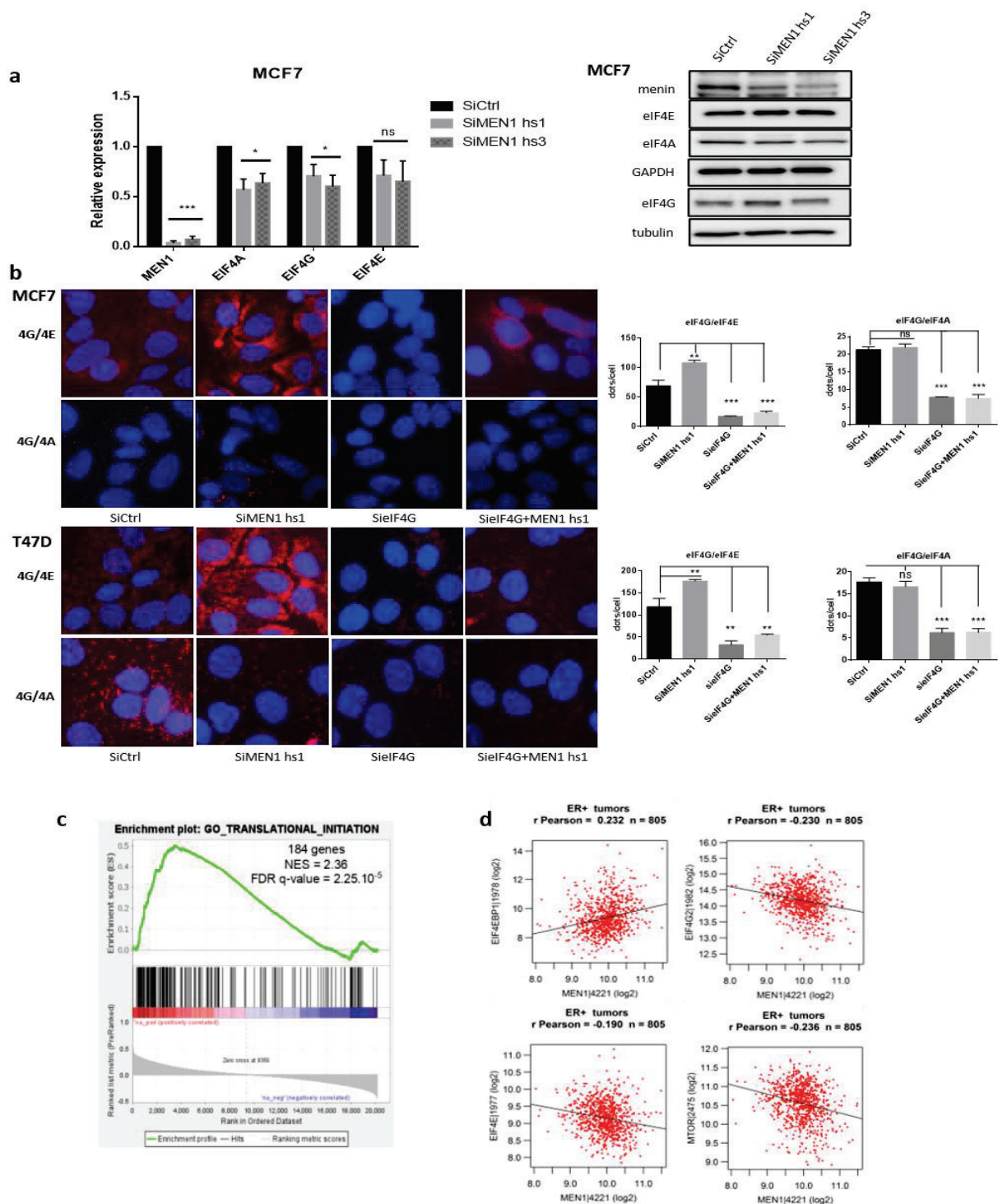


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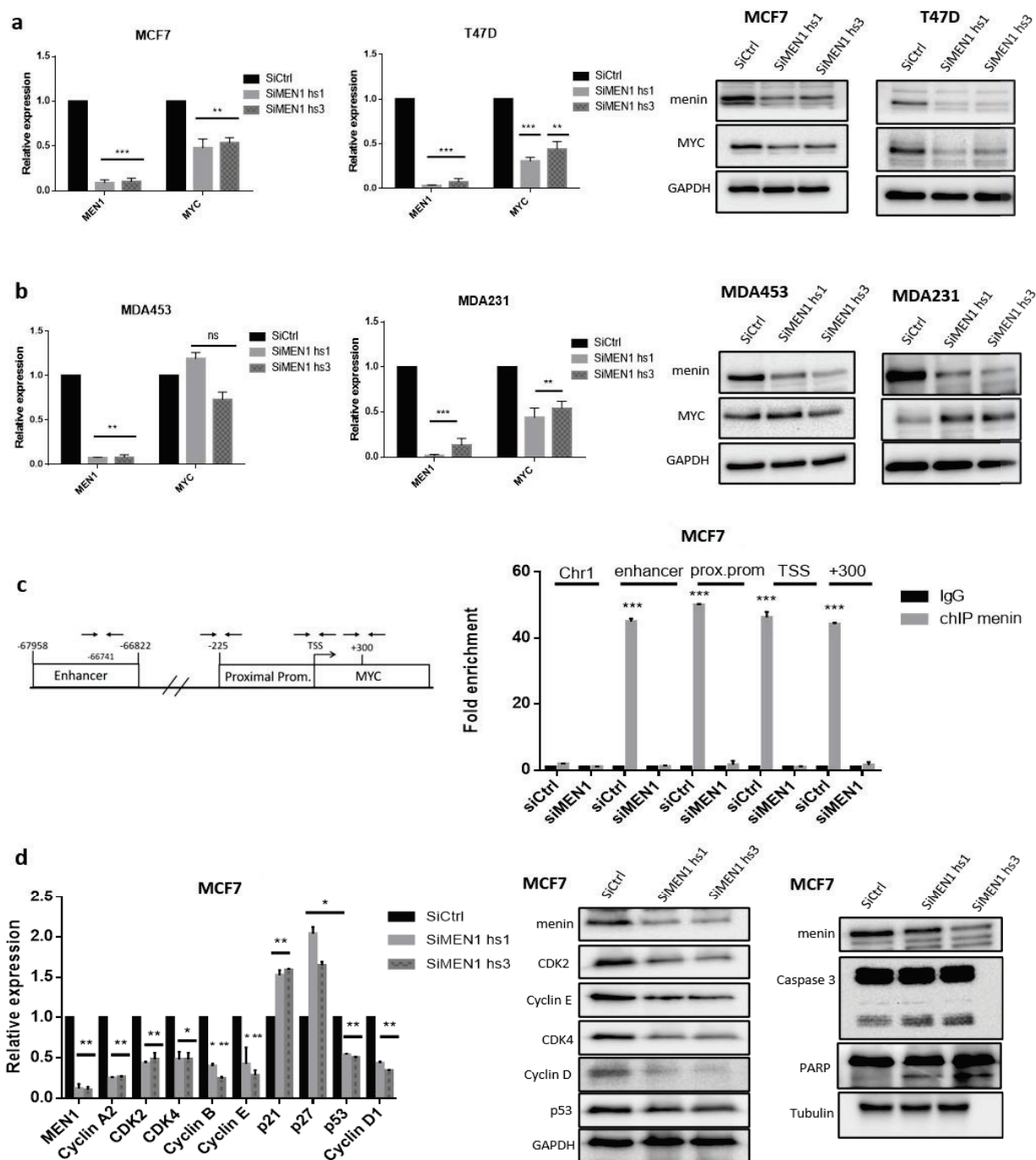


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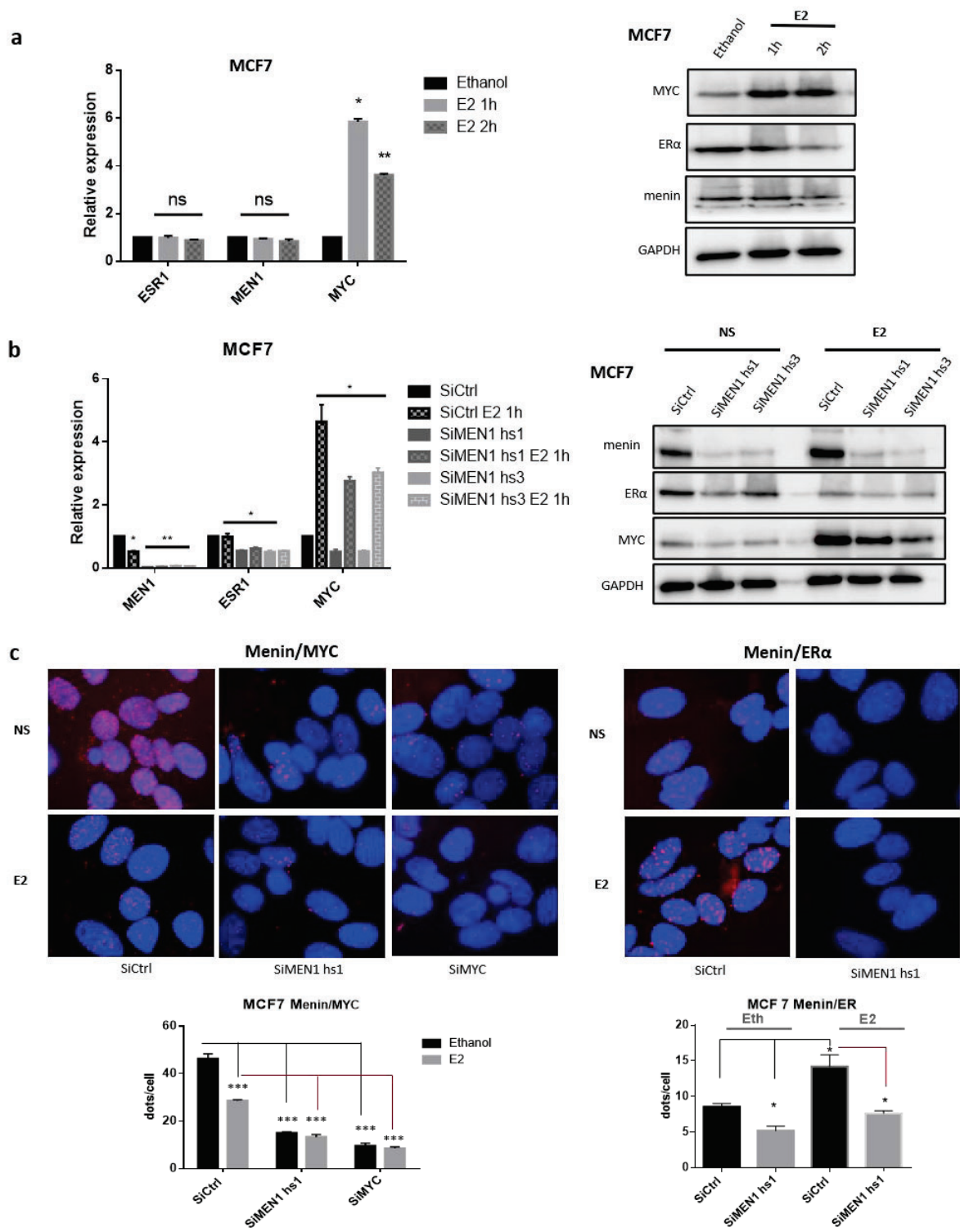


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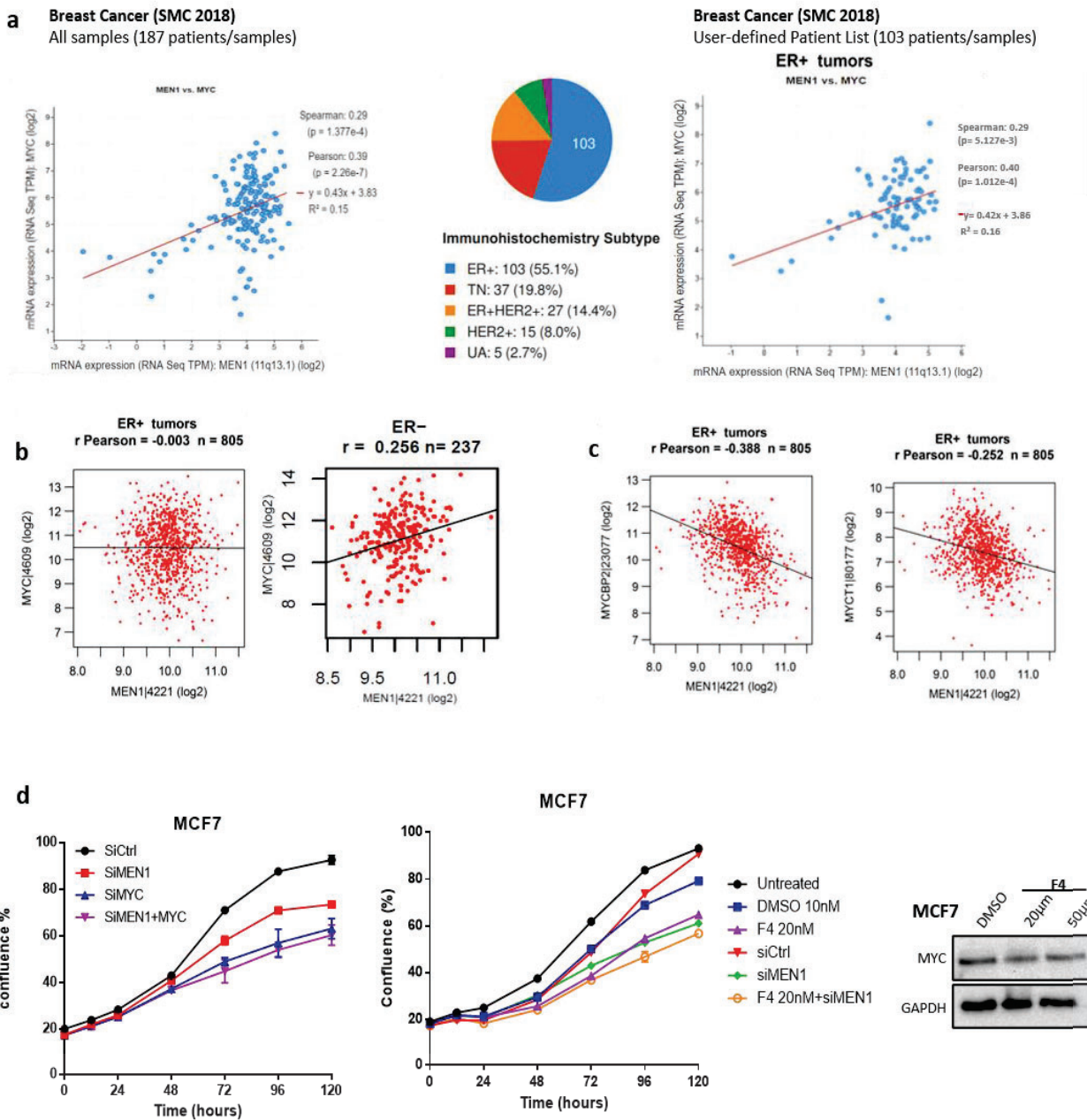
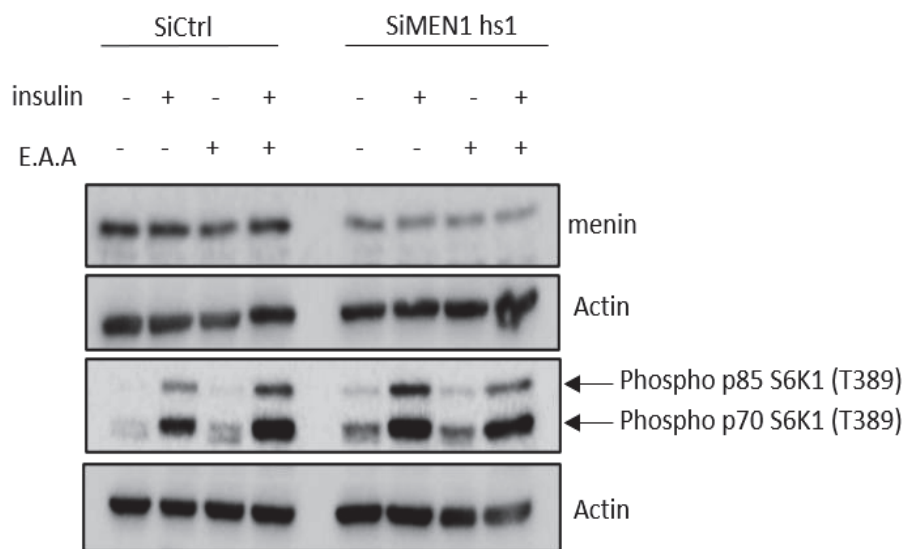
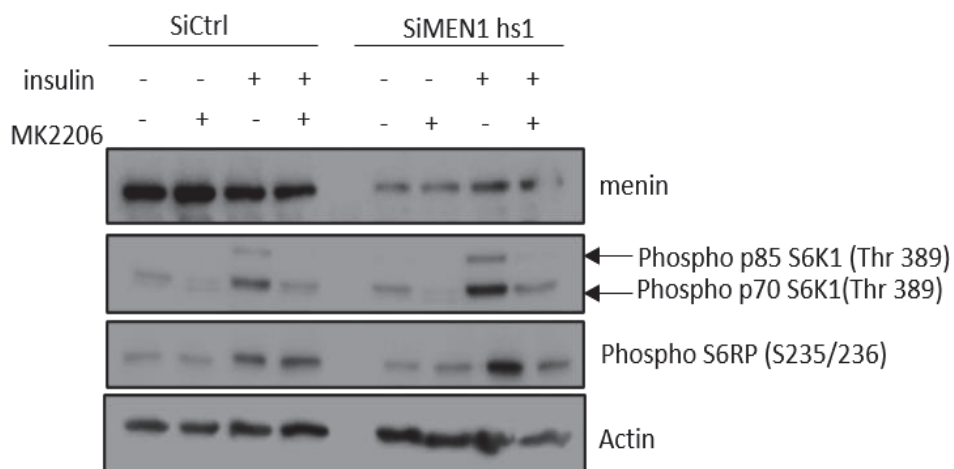


Fig 5. Correlation between *MEN1* and *MYC* mRNA expression in breast cancer and effects of cell proliferation inhibition with combined inactivation of *MEN1* and *MYC*. **a** Left panel: Data mining analysis from the SMC 2018 dataset showing the positive correlation between *MEN1* and *MYC* mRNA expression in BC including all subtypes. Right panel: same analysis from the SMC 2018 dataset in ER+ BC subtypes. **b** Correlation of mRNA expression between *MEN1* and *MYC* observed in ER+ (left panel) and ER- (right panel) tumors from TCGA dataset. **c** Inverse correlation between the expression of *MEN1* and 2 inhibitory factors in the *MYC* pathway, *MYCB2* and *MYCT1*, in ER+ BC from the TCGA dataset. **d** Proliferation curve of MCF7 cells transfected with either SiMEN1 hs1 or SiMYC or both (left panel), and transfected with SiMEN1 hs1 or treated with 10058 F4 (*MYC/MAX* inhibitor) or both (middle panel). Western blot showing *MYC* protein expression in MCF7 cells treated with DMSO or 20 and 50 μ M of 10058 F4 inhibitor (right panel).

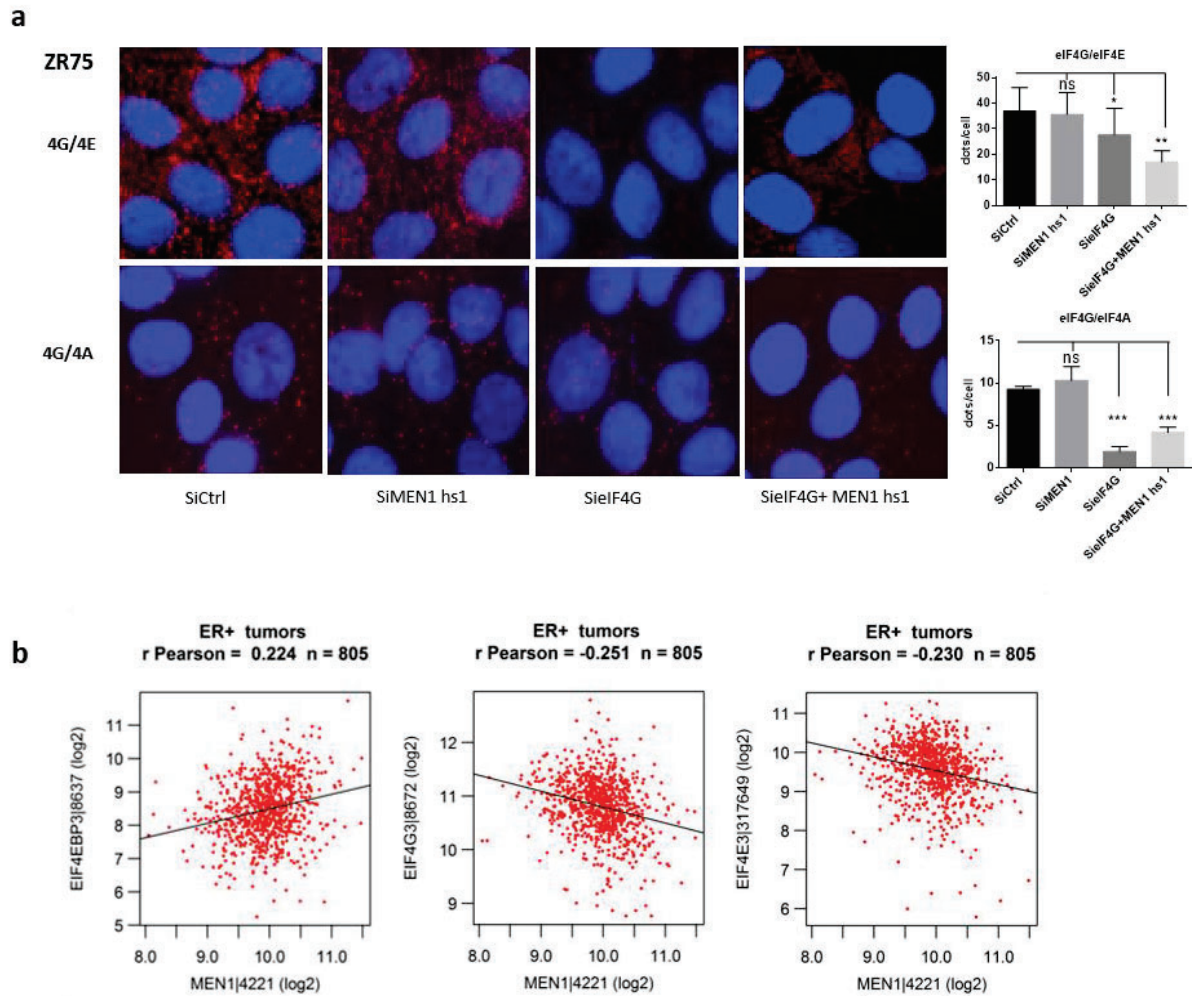
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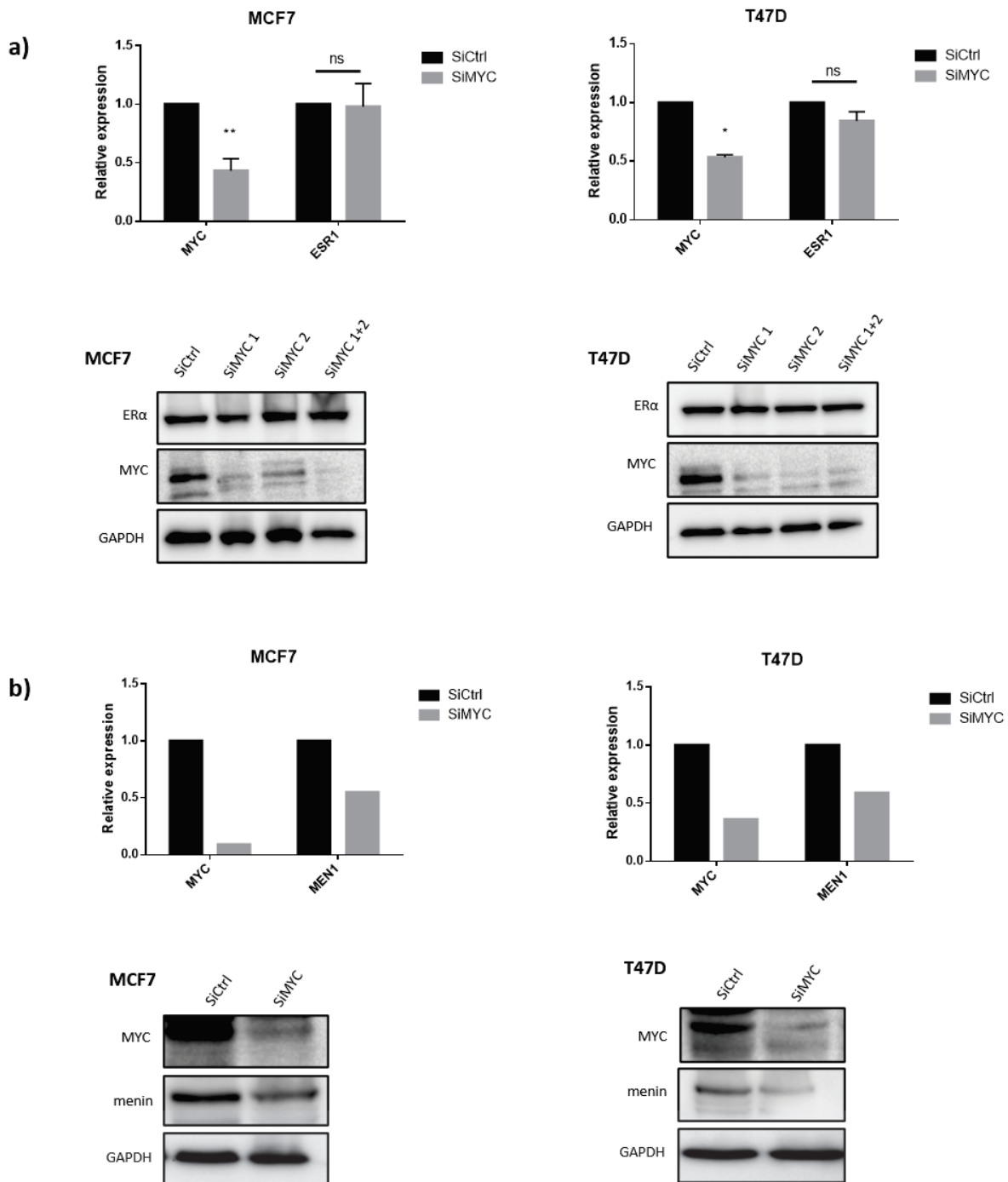
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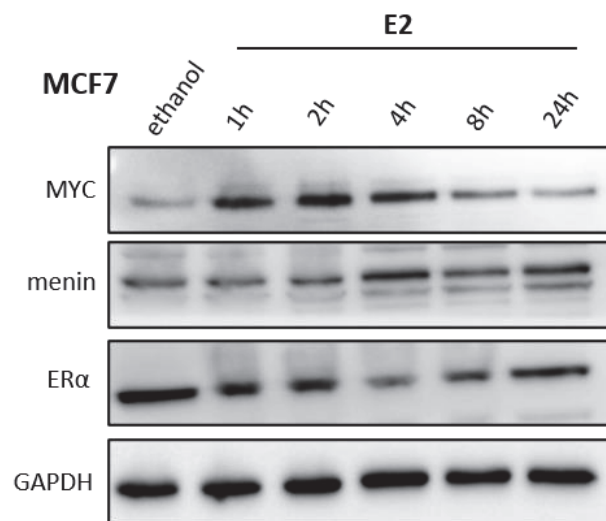
Supplementary Fig 1. Effects of reduced menin expression on mTORC1 activation: **a** Western blot analysis showing the protein expression of the different phosphorylated S6K1 in MCF7 cells transfected with SiCtrl or SiMEN1 and treated with insulin and/or essential amino acids (EAA), stimulators of AKT. **b** Western blot analysis showing the protein expression of the different phosphorylated S6K1 and S6RP in MCF7 cells transfected with SiCtrl or SiMEN1 and treated with insulin and/or AKT inhibitor MK2206.



Supplementary Fig 2. Menin is involved in the regulation of translation initiation complex in ER+ BC. **a** Proximity ligation assay (PLA) showing the interaction between either eIF4G/eIF4E or eIF4G/eIF4A in ZR75 cell line transfected with SiCtrl, SiMEN1, SiEIF4G and SiEIF4G+MEN1 hs1, respectively. Quantification of PLA signals, represented by dots/cell, for each condition is shown on the right panels. **b** Clinical correlation from TCGA datasets between *MEN1* and 4EBP3 (positive correlation) and eIF4G2, eIF4G3, and eIF4E3 (negative correlation) in ER+ BC.

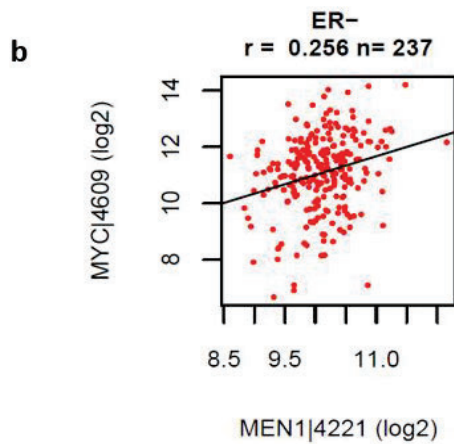
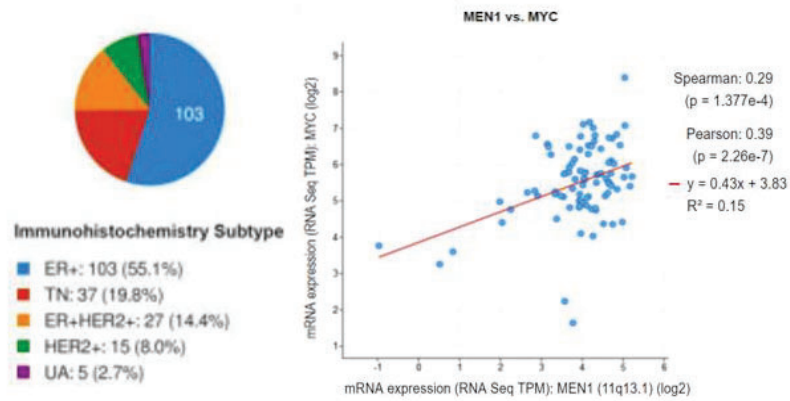


Supplementary Fig 3. MYC KD affects menin but not ER α expression: **a** Quantitative RT-PCR (upper panel) and Western blot (lower panel) showing the mRNA and protein expression of *ESR1* and ER α respectively in MCF7 cells treated with SiCtrl or 2 different SiMYC. **b** Quantitative RT-PCR (upper panel) and Western blot (lower panel) showing the mRNA and protein expression of menin in MCF7 cells treated with SiCtrl or SiMYC1.



Supplementary Fig 4. MYC, menin and ER α expression under estrogen stimulation. Western blot analysis showing the protein expression of MYC, menin and ER α in MCF7 cells treated with E2 for 1, 2, 4, 8, or 24h.

a Breast Cancer (SMC 2018)
User-defined Patient List (103 patients/samples)



Supplementary Fig 5. Significant positive clinical correlation between *MEN1* and *MYC* mRNA expression in ER+ and ER- BC. a Positive correlation of mRNA expression from SMC 2018 dataset between *MEN1* and *MYC* in ER+ BC. **b** Positive correlation of mRNA expression between *MEN1* and *MYC* observed in ER- tumors from TCGA dataset.

Discussion

The first clue of menin's involvement in breast cancer came from our observations in female aged heterozygous *Men1* mutant mice that developed, in addition to endocrine tumors, mammary gland carcinomas at low but noticeable frequency (*Bertolino et al., 2003a*). Few years later, we generated a mammary specific *Men1* knock-out mouse model, showing that ER α expression and the number of ER α -positive cells were clearly reduced in mammary intraepithelial neoplasia (MIN), precursor lesions of mammary cancer lesions of mutant mice, compared with normal mammary glands (*Seigne et al., 2013*). More recently, female MEN1 patients found to be at a higher risk to develop breast cancer (*Dreijerink et al., 2014*) and *Men1* mutations were also detected (though at very low frequency) in sporadic breast cancer (*Nik-Zainal et al., 2016*). Interestingly, an international clinical panel on MEN1 disease researched a consensus last year, proposing to the community that breast cancer is from now on considered as among the tumor panel developed in female MEN1 patients.

However, we are still far from knowing exactly the role of menin in BC development, since both the data from basic research and clinical observations are divers and even contradictory. Nevertheless, the recent works carried out in different laboratories in the field, including in our lab, afford some new clues into better understanding of the complex and multifaceted role played by menin in BC.

I. What we know now about the role of menin in breast cancer

Consistent to the above-mentioned works, our clinical studies carried out during my thesis work showed that lower menin expression was significantly associated with ER- and luminal B subtype breast cancers displaying either no, for the former, or weaker ER α expression for the latter, whereas high menin expression was associated with luminal A ER+ breast cancers. These in vivo analyses in total consistency with the regulatory role of menin in *ESR1* transcription revealed previously and more detailed in this study is. These data also reinforced what we reported previously based on our observations obtained in mammary-specific *Men1*-deficient mouse model, suggesting the tumor suppressor role of menin during the initiation

procedure of tumorigenesis of mammary cells, since both ER- and luminal B subtype breast cancers are more aggressive than luminal A subtype breast cancers.

On the contrary, in ER+ luminal A subtype breast cancer, menin expression may promote cell proliferation by activating ER α expression and ER α target genes (Dreijerink et al., 2006). In the matter of fact, Imachi et al found that menin binds and coactivates ER α in a hormone (estrogen) dependent manner and that menin could be a predictive factor of tamoxifen resistance (Imachi et al., 2010). These data highlight a complex and even opposite role played by the *Men1* gene in the carcinogenesis of mammary cells. A hypothesis of “dual for menin” proposed by dual role Dreijerink et al., in 2017 may help to solve these discrepancies and the eventual confusions thus elicited. They proposed that menin could play as a tumor suppressor during the procedure of the initiation of tumorigenesis of normal luminal mammary epithelial cells, but as an oncogenic role in sporadic ER+ breast cancer initiated by other factors, the key point being the essential role of menin in the regulation of the *ESR1* gene mediated by MLL/menin complex via H3K4me3 sites. The data obtained from my thesis works support fully such a hypothesis. Therefore, when the *MEN1* gene is mutated or inactivated in normal mammary cells, it results in decreased ER α expression, leading to aberrant cell proliferation and differentiation, and tumor development, whereas in ER+ breast cancer triggered by other factors, menin could act as a coactivator of ER α , playing a crucial role in promoting the cell proliferation by the latter.

II. Menin regulates *ESR1* transcription in ER+ BCs

We, as well as others, have showed that menin positively regulates ER α expression as *MEN1* knockdown leads to decreased ER α expression on both the protein and transcriptional level. After the fine analyses carried out by Dreijerink et al mainly on distal regulatory sequences of the *ESR1* gene, our current works has been focalized on the proximal *ESR1* promoter. We have demonstrated by ChIP analysis and luciferase assay that menin binds to a large area of the proximal promoter of *ESR1* and regulates its activity, the area extending from -2500bp to +2000bp of *ESR1* transcription start sites (TSS). Dreijerink et al showed that menin regulates ER α through binding to *ESR1* enhancer region through a loop-structure bound by a complex involving MLL1/2, GATA3 and FOXA1. Intriguingly, our results showed that, though

treatment with menin/MLL inhibitor MI503 decreased the expression of ER α and menin binding to the *ESR1* proximal promoter, no changes were detected in the H3K4me3 marks. Moreover, the combined *MLL1* and *MLL2* knockdown had no effect on ER α expression in MCF7 cells, thus raising the questions about the exact role of MLL in the regulation of *ESR1* transcription by menin. Taking Dreijerink et al's observations into account, we also investigated the relation between menin, GATA3 and FOXA1. We found that menin does physically interact with GATA3 and FOXA1 in our cellular model, but only regulate their expression at the protein not the transcriptional level. All these results suggest that the complex described by Dreijerink may not be enough to fully explain the molecular mechanisms underlying the regulation of *ESR1* transcription by menin.

To better understand how menin regulate the *ESR1* proximal promoter and why MI503 treatment decreased ER α expression and menin binding but not H3K4me3, we investigated the eventual participation of JunD, menin's first identified partners (*Agarwal et al., 1999*), by using MI503, which inhibits MLL1/2 binding to menin's pocket, the same pocket where JunD binds to menin. Our results, however, showed no effect of JunD knockdown on ER α expression or cell proliferation (data not shown). We also checked other epigenetic factors like HDAC1 and found no effects of its knockdown on ER α expression (data not shown). Bearing in mind that menin can interact with a large number of epigenetic factors (like HMTs and HATs), and transcription factors that could bind to the *ESR1* promoter and due to the limitation of our study, we expect that other factors could be involved in this regulation, and further detailed studies like ChIP-seq or RHIME techniques could be more suitable to identify the factors involved in the regulation of *ESR1* transcription.

III. How can *MEN1*-KD activate mTOR pathway and yet cause cell death?

As we showed in our first part of the work, menin regulates, as a cofactor, *ESR1* transcription in ER+ breast cancer cells. Bearing in mind the importance of ER α for mammary cell growth and survival, we thought that it's important to study the biological effect of menin in these types of cells. We found that *MEN1*-KD decreased cell proliferation in both ER+ cell lines, whereas its effects in ER- cell lines were rather heterogenous. The latter can be explained by

the fact that ER- breast cancer is heterogenous and can be subdivided into at least 7 further subtypes (*Lehmann et al., 2011*) and nothing is known about menin's role in these types of cancers. It was, therefore, not surprising for us to observe different outcomes of *MEN1*-KD, due to possible different roles played by in the different subtypes of ER- breast cancers.

We focused on ER+ breast cancer cell lines and sought to know how menin *MEN1*-KD decreased cell proliferation in these cells and the molecular mechanisms behind it. Since AKT/mTOR pathway is highly activated in breast cancer (*Castaneda et al., 2010*), and that menin has been shown to interact with partners of this pathway in other types of cancer (*Wang et al., 2011*) (*Wuescher et al., 2011*), we studied the relation between menin and AKT/mTOR pathway in our breast cancer cells. Surprisingly, our results showed that menin inactivation activates the mTOR pathway and activates the downstream translation initiation complex. We also investigated other mTOR downstream targets like those involved in lipid metabolism, aerobic glycolysis, and pentose phosphate pathway (data not shown) but did not find any obvious change after *MEN1*-KD. Interestingly, these results may explain why breast cancer patients with low menin expression had better survival rates when treated with tamoxifen plus the mTOR inhibitor everolimus (RAD001) compared with those treated with tamoxifen only. We noticed that, in 2016, Dermit et al observed that PI3K inhibitor (PI3Ki)-resistant cells developed proliferative defects under the actions of PI3K/mTORC1 in an AKT independent manner, due to accumulated reactive oxygen species (ROS), and hypoxia-inducible factor-1 α (HIF1 α)- produced lactate (*Dermit et al., 2017*). This metabolic phenotype made resistant cells more sensitive to hydrogen peroxide and nutrient starvation thus hindering their division. Unfortunately, because of the lack of time, it was not possible for me to carry out further investigation to know whether this is also the case in my cell model.

IV. Menin and MYC, another major partnership underlying menin's oncogenic effect?

We looked at other menin partners that play important role in cell proliferation and death in BC cells, in particular MYC. MYC, an estrogen-regulated oncogene involved in cell proliferation, apoptosis, differentiation, metabolism, is overexpressed in 20%–30% of breast

cancers and interacts with ER α in ER+ positive cell lines (Bièche et al., 1999) (Cheng et al., 2006). Menin was reported to interact with MYC in different tissues to regulate MYC target genes, whereas its regulatory role on MYC transcription has not been so far reported. In our lab, a preliminary menin ChIP-seq analysis in MCF7 cells has showed that menin binds to the MYC promoter (Data not shown). Our further analyses in the current study confirmed that menin positively regulates MYC transcription and its target genes especially those involved in cell cycle and apoptosis. We also detailed, for the first time, that menin not only binds to several areas of the MYC proximal promoter but also to an enhancer previously identified (Wang et al., 2011). We checked if menin and MYC could regulate together ER α expression, but MYC knockdown had no effect on ER α expression, whereas menin knockdown decreased both ER α and MYC expression. Furthermore, our data demonstrated that menin is crucial for the activation of MYC expression, both on transcription and protein levels, under estrogen stimulation. The data highlighted the importance of menin, as an oncogenic factor, in the tumorigenesis procedure mediated by the ER α pathway in ER+ BC cells.

Interestingly, we also found that MYC knockdown led to decreased menin expression at both protein and transcriptional level. The data suggest that menin and MYC may form a positive feedback regulation, playing a crucial role in promoting the proliferation of ER+ BC cells. This part of the project would be interesting to follow, since very limited studies focus on the regulation of the *MEN1* gene and nothing is known so far about on the regulation of menin expression by MYC. It would be interesting to investigate more deeply on the crosstalk between menin/ER α /MYC in ER+ BC cells.

In this study, we were also able to reveal some clinical potential related to the importance of menin as a regulator of MYC expression, one of the most important proliferative genes. Although menin inactivation alone decreases cell proliferation of ER+ BC cells, combined menin and MYC inactivation gives better results in cultured cell works. The data may provide relevant clue into new therapeutic strategy, since menin expression in ER+ BC cells reported to be more resistant to hormone-therapy. Also, data mining analyses demonstrated certain positive correlations between *MEN1* and *MYC* mRNA expression in breast cancer,

supporting that the interplay between menin and MYC could be existing in a substantial proportion of BC.

The current work allowed us to uncover an important partnership between menin and MYC in ER+ BC cells. It'll be relevant to know, in the future, what would be the role of menin/MYC in ER- BC cells, and whether menin could also be involved, as an oncogenic factor, in other cancers where MYC plays a decisive role.

Conclusion and Perspectives:

Menin, a multifunctional scaffold protein, can have different roles in a tissue and molecular specific manner. In cancers, menin have been first identified as a tumor suppressor in endocrine tumors, and later on as an oncogenic factor in several types of leukemia. However, its involvement in hormone-dependent cancers like prostate and breast seems to be more complex. Menin, as a cofactor and regulator of ER α and *ESR1* respectively, is now considered playing a dual role depending on the stage and type of the tumor, meaning that in the early stages or initiation of breast cancer, menin plays a tumor suppressive role, while in already developed ER+ breast cancer, menin plays an oncogenic role. Both molecular and clinical data acquired during my thesis works provide relevant clues supporting this notion.

I noticed, though Dreijerink et al showed that menin recruits MLL/compass-like complex to the distal (enhancer) *ESR1* promoter and regulate its expression, we didn't find any changes in H3K4me3 on the proximal promoter after treatment with menin/MLL inhibitor. Thus, this area of the promoter remains to be an interesting field of research to study. How menin exactly regulates *ESR1* on this site? What other factors could be involved? We have tried the candidate gene approaches (already tested few factors like HDAC1 and JunD). But given the fact that menin interacts with a wide number of epigenetic and transcription factors, this approach could be long and inefficient. For this reason, applying techniques like RIME (Rapid Immunoprecipitation Mass spectrometry of Endogenous proteins) on this area of the promoter could give later on better, faster, and more detailed description of all the factors and mechanisms involved in this regulation.

We have also shown that menin inhibition activates the mTORC1 pathway and its downstream translation initiation complex. We have also checked if menin had an influence on other downstream effectors of mTORC1 but didn't go deep into these investigations. Consequently, it could be interesting to investigate if menin regulates pathways like lipid metabolism, glycolysis, pentose phosphate, and autophagy all being controlled by the mTOR pathway.

Our works also revealed that menin physically interacts and regulates *MYC* transcription in ER+ BC cells, through binding to its proximal and enhancer promoter. Menin not only regulate *MYC* protein and transcriptional expression, but also regulates several *MYC* target genes mainly those implicated in cell cycle and apoptosis. For this reason and taking into consideration that menin knockdown inhibits cell proliferation in ER+ BC cell lines, it would be interesting to investigate more thoroughly whether menin regulates *MYC* target genes directly or through *MYC* itself, and if menin's role on the proximal and enhancer could be different and/or effected by estrogen. In addition, it would be also relevant to dissect the positive feedback regulation between *MYC* and menin observed in our work, which could be relevant to investigate more deeply the crosstalk between menin, ER α and *MYC* in ER+ BC cells. We also found that menin could regulate *MYC* differently in ER+ and ER- BC cells, however we didn't investigate more deeply how menin regulate *MYC* expression in ER- BC cells, making this an interesting field for other people to explore. Lastly, taking into consideration the clinical complexity and aggressiveness of TNBCs, and that many ER+ BCs eventually acquire resistance to endocrine therapy, menin/*myc* relation could serve as a potential strategy for novel targeted therapy.

List of publications:

During my 3-years PhD, I had the opportunity to contribute to other work done by my colleague Yakun Luo, who works on menin's role in prostate cancer. I helped with the results analysis and discussion as well as provided technical help and support.

Article 1: *Men1 disruption in Nkx3.1-deficient mice results in AR low/CD44 + microinvasive carcinoma development with the dysregulated AR pathway (published in the journal Oncogene)*

Article 2: *Menin is essential for activating the MYC locus and MYC-mediated androgen receptor transcription in AR-dependent prostate cancer cells (submitted to the journal Oncogene)*

I also had the chance to read and integrate different studies done in our field, mainly menin. Together with my colleague and Dr. Chang, we were able to summarize this work in three review articles:

Article 3: *Involvement of the MEN1 Gene in Hormone-Related Cancers: Clues from Molecular Studies, Mouse Models, and Patient Investigations (published in the journal Endocrines)*

Article 4: *Research progress of MEN1 gene mutation mouse model: exploring the molecular mechanism of MEN1 inactivation in tumor development (published in in Journal of Pancreatology)*

Article 5: *Generation and characterization of Men1 mutant mouse models for studying MEN1 disease (published in Chinese Journal of Digestion)*



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.

These authors contributed equally: Romain Teinturier, Yakun Luo

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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*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 injection (Fig. 1a). Immunohistochemical (IHC) analysis confirmed *Men1* disruption in prostate luminal cells exclusively in *Men1*NT 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*NT mice would allow us to determine whether additional *Men1* disruption rescues or exacerbates the phenotype of *Men1*N mice. We observed mPIN lesions in dorsal, ventral, lateral, and anterior lobes in all male *Men1*NT mice ($n = 7$) 1.5 months after induction of the Cre recombinase, with high-grade mPIN being observed in six of the seven *Men1*NT mice and low-grade mPIN in the remaining *Men1*NT mouse, whereas only one mouse displayed low-grade mPIN among the untreated age-matched *Men1*N mice ($n = 5$, Figs. 1d and S1 and Table 1). Having shown that a majority of prostate cells within lesions in *Men1*NT 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*N 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*NT 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*NT mice by analyzing several major cell

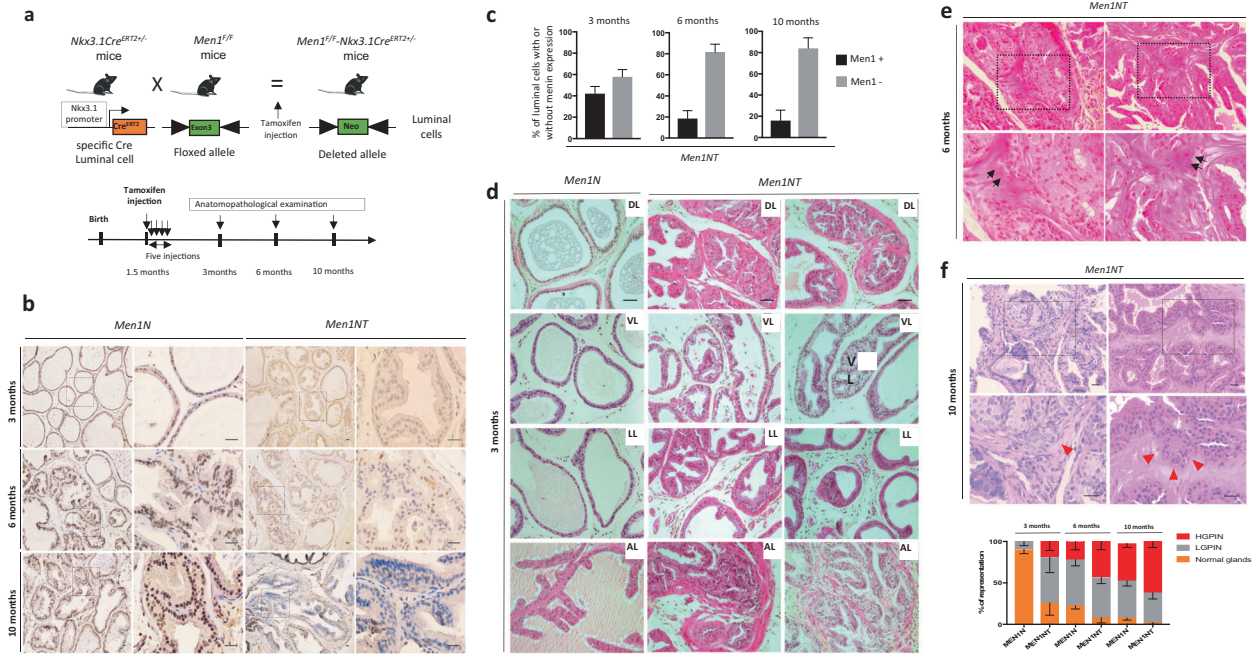


Fig. 1 Characterization of the *Men1^{F/F}-Nkx3.1Cre^{ERT2+/-}* mouse phenotype. **a** Schematic overview of the approach used for the generation of *Men1^{F/F}-Nkx3.1Cre^{ERT2+/-}* mice. **b** Depletion of menin in prostate glands and development of prostatic intraepithelial neoplasia (PIN) lesions 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 μ m. **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 μ m. **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 μ m. **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 μ m. 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>MEN1NT</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.

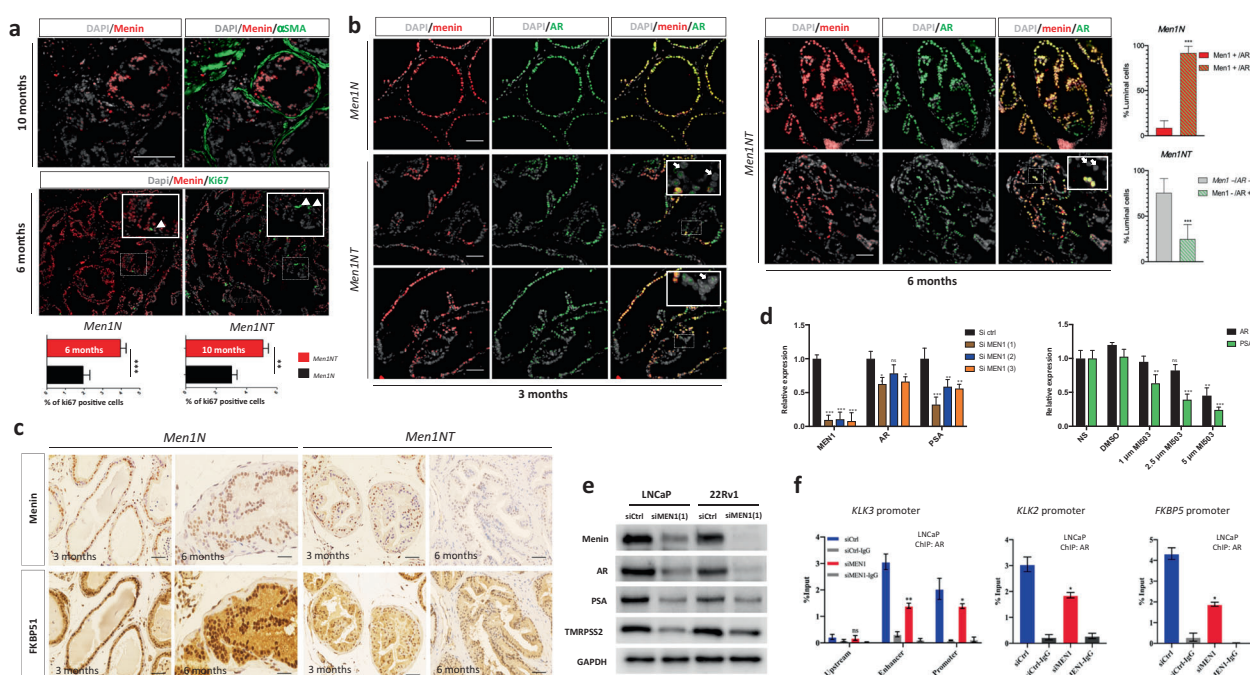


Fig. 2 *Men1* disruption in *Nkx3.1*-deficient prostate cells results in deregulated AR expression and the AR pathway. **a** Upper: α SMA immunofluorescence (IF) performed in prostate specimens of *Men1NT* mice at 10 months of age, arrows indicate discontinuous α SMA staining in surrounding stromal compartment adjacent to menin-deficient luminal cells. Scale bar = 50 μ m. Middle: IF staining against Ki67 on prostate sections from *Men1N* and *Men1NT* 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 (*Men1NT*) 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 *Men1NT* mice (n = 5). Scale bar = 25 μ m. **c** Immunohistochemical (IHC) detection of

FKBP5 was performed in 6-month-old *Men1N* and *Men1NT* 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 TMPRSS2 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

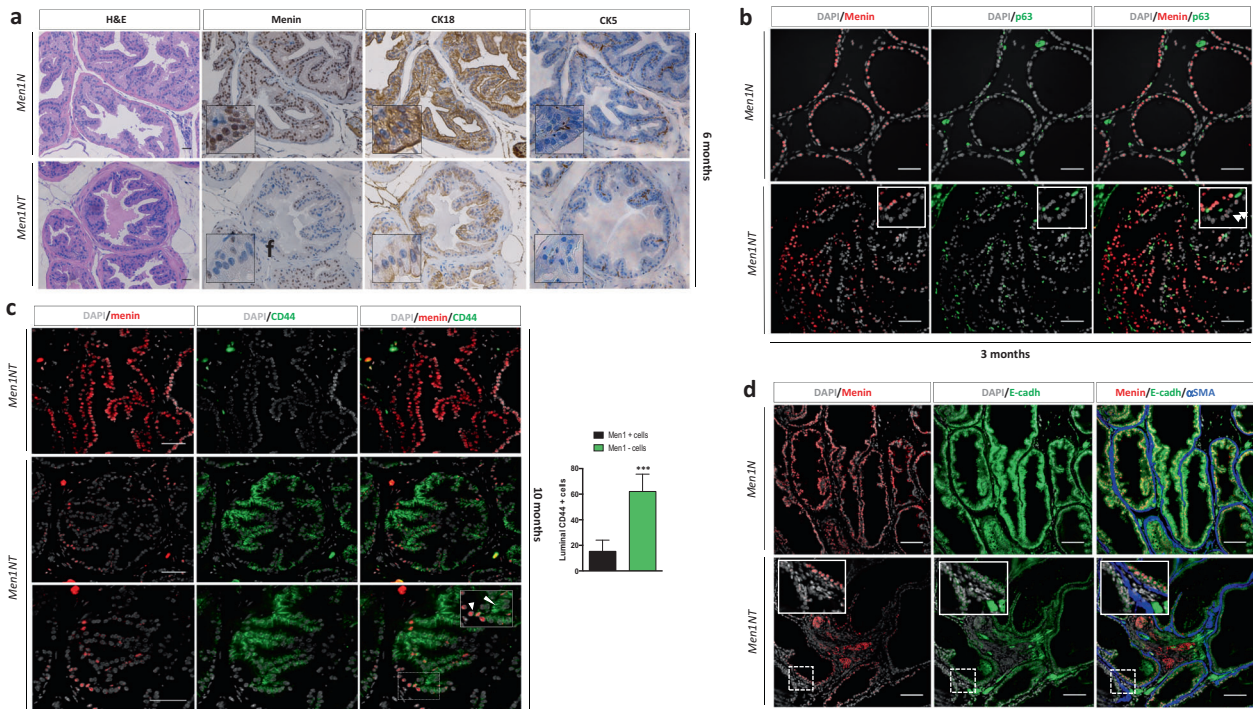


Fig. 3 *Men1* inactivation in *Nkx3.1*-deficient prostate cells led to cell dedifferentiation. **a** H&E staining (left column) and IHC against menin, CK18, and CK5 on *Men1N* and *Men1NT* mouse prostate sections at 6 months of age. **b** Double IF staining against menin and p63 with DAPI counterstaining on prostate sections from *Men1N* (upper panel) and *Men1NT* (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 *Men1N* (upper

panel) and *Men1NT* (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 *Men1N* and *Men1NT* 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 *Men1NT* 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 *Men1NT* 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

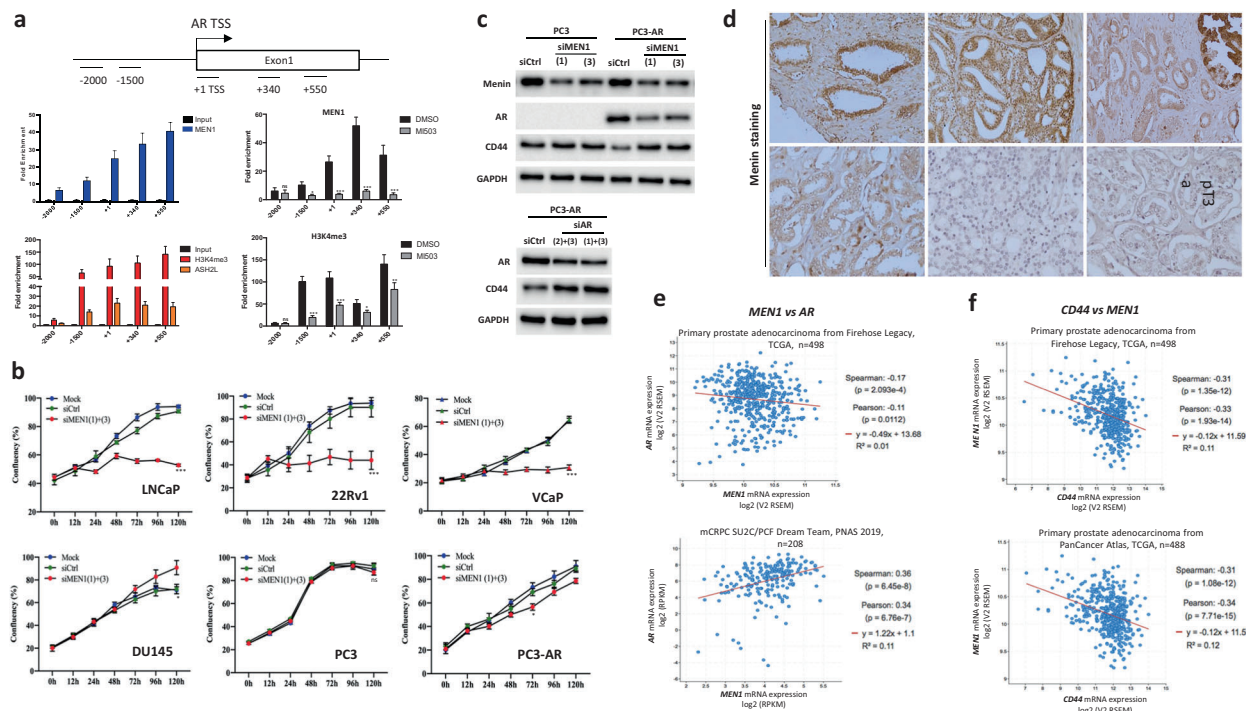


Fig. 4 Menin binds to the proximal AR promoter and is crucial to cell differentiation and proliferation of AR-dependent cells. **a** Upper graph: ChIP analysis was used to define the binding of menin and menin–MLL complex to the AR promoter. Quantitative PCR analyses of menin and H3K4me3 binding on the -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 $5 \mu\text{M}$ (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 StepOne 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].

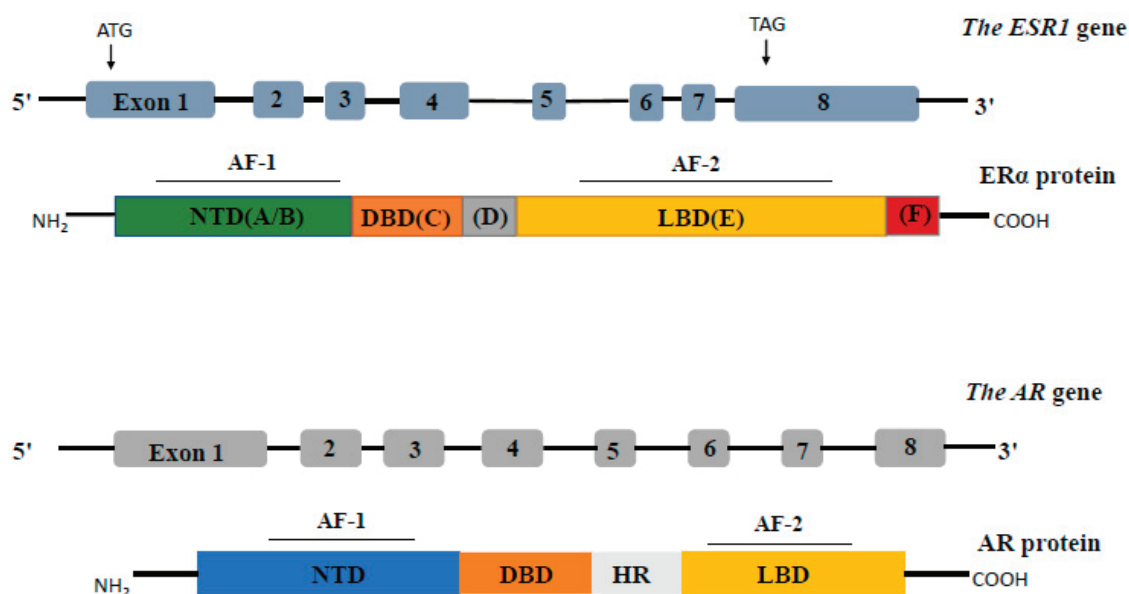


Figure 1. Schematic representation of the structure of the *ESR1* gene and the *AR* gene and their proteins. Different protein domains are indicated, including the N-terminal domain (NTD), the DNA binding domain (DBD), the hinge domain (D for ER α protein and HR for AR protein), and the ligand binding 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 msn3A [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 $\text{pur-}\alpha$ 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).

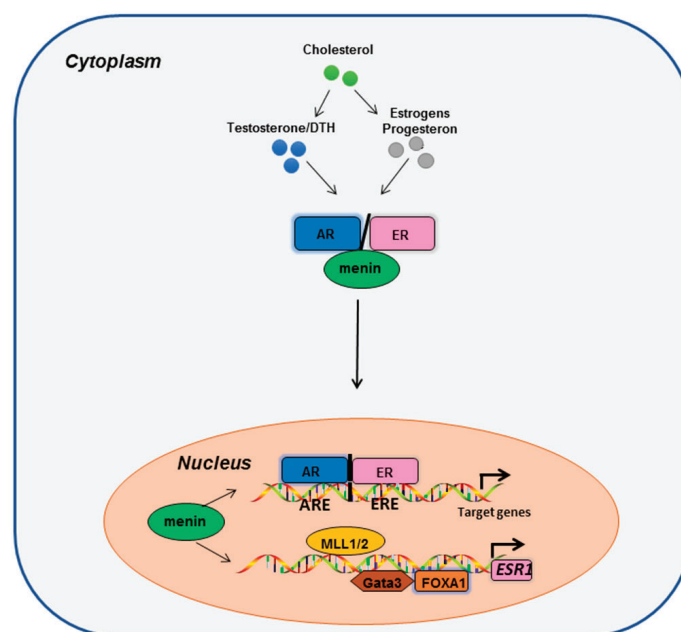


Figure 2. The menin protein interacts physically and functionally with ER α and AR, and is involved in the regulation of ESR1 transcription and the transactivation of the target genes of both ER α and AR.

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).

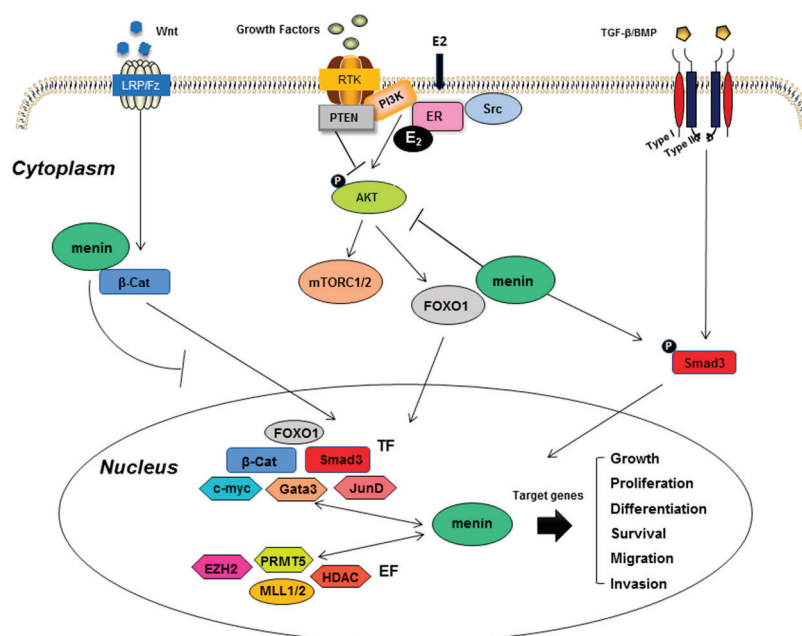


Figure 3. Mechanistic clues underlying the involvement of menin in mammary and prostate cell tumorigenesis. Menin interacts with numerous menin-interacting factors, consequently participating in the regulation of many target genes and interfering with different signaling pathways strongly implicated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

Schnepp 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^{F/F}-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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· 胃肠胰神经内分泌肿瘤专家笔谈 ·

MEN1 基因突变小鼠模型的研究进展:探究 MEN1 失活在肿瘤发展过程中的分子作用机制

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张昌贤,法国国家医学科学院研究员,长期从事肿瘤领域基础研究。毕业于复旦大学上海医学院和法国里昂第一大学,现任职于法国里昂肿瘤研究中心,负责内分泌肿瘤发生机理的研究,主要以基因敲除小鼠为研究模型,研究多发性内分泌肿瘤 1 型(MEN1)基因失活相关的肿瘤发生机制。基于团队建立的 MEN1 敲除小鼠模型研究,证明 MEN1 基因在胰腺内分泌细胞的发育,细胞增殖和分化,以及成人胰腺内分泌细胞可塑性的控制中起至关重要作用,已在该研究领域发表论文 50 余篇。

【摘要】 MEN1 突变会影响多种内分泌和非内分泌细胞,从而使患者具有了对多发性内分泌肿瘤 1 型(MEN1)的易感性。使用基因打靶方法建立动物模型尤其有助于观察 MEN1 失活后发生的细胞和分子水平的变化,由此揭示该疾病发展的详细机制。本文重点关注通过 MEN1 突变小鼠模型的研究所揭示的 MEN1 基因分子功能。

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根据美国的统计数据,胃肠胰神经内分泌肿瘤(neuroendocrine tumors, NET)的发病率在不断地升高。由于其相对缓慢的肿瘤进展,患病率相对较高。NET 包括由基因突变引起的家族遗传性综合征。多发性内分泌肿瘤 1 型(multiple endocrine neoplasia type 1, MEN1)即为遗传性 NET 之一,系常染色体显性遗传。MEN1 基因异常的患者会发生多种内分泌肿瘤,最常见为甲状旁腺、胰腺、垂体前叶、胃肠道乃至胸腺和肾上腺的 NEN,以及脑膜瘤、血管纤维瘤和脂肪瘤^[1-2],这些肿瘤除了家族遗传外也可以是散发的。MEN1 基因突变是内分泌肿瘤发生过程中已知的一种基因异常,这使得该基因成为研究内分泌细胞肿瘤发生的关键。在患者的肿瘤发生过程中,该基因常发生杂合性缺失,进一步提示 MEN1 基因在内分泌肿瘤中发挥着抑癌基因的作用。

1997 年 MEN1 基因被发现后^[3],世界各地的不同实验室(包括笔者实验室)纷纷采用不同的方法和模型对 MEN1 进行研究,同时构建和研究 MEN1 突变小鼠模型。此后,更多实验室参与到应用这些

模型来剖析 MEN1 基因功能。正如所期,对 MEN1 突变小鼠模型的大量研究,不仅获得了 MEN1 相关的病理学研究进展,而且也促进了由于 MEN1 失活所导致的细胞和分子机制的探讨。本文综述目前 MEN1 基因突变小鼠的研究进展,重点阐述对肿瘤发展中因 MEN1 基因缺失所造成下游分子生物学机制的认知现状。

一、MEN1 基因突变小鼠模型

随着当前小鼠的基因打靶的技术进展,既可以在胚系水平上破坏特定的基因(常规突变小鼠),也可以在特定细胞类型中使基因失活(条件突变小鼠)。常规 MEN1 突变模型概括了 MEN1 患者中发生的遗传事件,因此有助于研究 MEN1 疾病的自然史。多篇文献指出纯合 MEN1 突变体胚胎在妊娠中期死亡,而杂合 MEN1 突变体小鼠从出生后 12 月龄开始出现多发性内分泌肿瘤,累及胰岛、甲状旁腺、垂体和肾上腺,并伴有相关的高胰岛素血症。通过将携带 floxed MEN1 等位基因(MEN1^{F/F})的小鼠与特定细胞类型或组织中起作用的 Cre 小鼠(包括

Title

Lessons learnt from *Men1* mutant mouse models: molecular mechanisms underlying the role played by *MEN1* inactivation in tumorigenesis

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Key words

The *MEN1* gene; mouse models; tumorigenesis

Abstract

MEN1 mutation results in the predisposition to multiple endocrine neoplasia type 1 (MEN1) syndrome affecting various endocrine and non-endocrine cells. The generation of murine models using gene targeting approach has afforded remarkable tools for studying the *MEN1* gene and its related diseases. In particular, these studies led to unravelling the cellular and molecular events occurred after *MEN1* inactivation, revealing, therefore, detailed mechanisms underlying the development of the disease. In this review, we focus on the currently available data concerning the molecular functions of the *MEN1* gene uncovered using *Men1* mutant mouse models in connection with MEN1 syndrome.

Lessons learnt from *Men1* mutant mouse models: molecular mechanisms underlying the role played by the *MEN1* gene in tumorigenesis

I. Introduction:

Neuroendocrine tumours form a rare group whose incidence is constantly increasing and the prevalence is relatively high because of its relative slow tumour progression, according to the statistics collected in the States.¹ They may occur as familial syndromes that are caused by genetic mutations. Multiple endocrine neoplasia syndrome type 1 (MEN1) is one of these hereditary neuroendocrine tumour syndromes, transmitted with an autosomal dominant trait². Patients affected by the disease develop multiple endocrine tumors, including the parathyroid glands, endocrine pancreas and anterior pituitary gland. *MEN1* mutation is among the genetic abnormalities clearly involved, making this gene a crucial key to address studies of tumorigenesis of endocrine cells. In tumors developed by patients, there is frequently a loss of heterozygosity at the locus of this gene, consistent with the notion that the *MEN1* gene is a tumor suppressor. It is important to indicate that *MEN1* inactivation is also involved in sporadic neuroendocrine tumours affecting endocrine pancreas, parathyroid, anterior pituitary and adrenal glands, as well as in angiofibromas and lipomas³.

The *MEN1* gene was identified gene in 1997^{4,5}. Since then, along with MEN1 studies using diverse approaches and models, different laboratories around the world, including our own, have engaged in the generation and characterisation of *Men1* mutant mouse models. Later on, more laboratories were involved in the use of these models to dissect *MEN1* functions. As expected, numerous studies carried out in using *Men1* mutant mouse models have allowed not only studying the events related to MEN pathology, but also, dissecting cellular and molecular mechanisms triggered by *MEN1* ablation. In this review, we present the currently available data on the downstream molecular events due to *MEN1* disruption revealed by studies exploring *Men1* mutant mice.

II. *Men1* mutant mouse models

Gene targeting in the mouse allows currently to either disrupting a given gene on germline level (conventional mutant mice) or inactivating the gene in a specific cell type (conditional mutant mice). Conventional *Men1* mutant models recapitulate genetic events happened in MEN1 patients, hence facilitating the study of the natural history of MEN1 disease. Several published works unanimously pointed out that homozygous *Men1* mutant embryos died at mid-gestation stage, whereas heterozygous *Men1* mutant mice developed multiple endocrine tumors starting from around 12-month-old of age, affecting the islet, parathyroid, pituitary and adrenal glands and associated with hyperinsulinemia. Conditional *Men1* mutant models were generated by crossing the mice carrying the floxed *Men1* allele, *Men1^{F/F}*, with selected Cre mice functioning in given cell types or tissues, including different islet cells, parathyroid, pituitary and mammary cells. In addition, inducible *Men1* mutant models were also generated and provided interesting tools for fine analyses of temporal molecular and physio-pathological consequences of *Men1* inactivation. All these mouse models played a vital role in dissecting cellular and molecular mechanisms involved in MEN1 syndrome development (Table 1).

Different models	Temporo-spacial spectrum of <i>Men1</i> disruption	References
Conventional	germline mutation (<i>Men1</i> ^{-/-} mice died at embryonic mid-gestation stage; <i>Men1</i> ^{+/-} mice mimics genetic and disease events in MEN1 patients)	<i>Crabtree et al., 2001</i> ⁶ <i>Bertolino et al., 2003</i> ⁷ <i>Loffler KA et al., 2007</i> ⁸ <i>Harding B et al., 2009</i> ⁹
Conditional	β -Cell <i>Men1</i> mutant mice (<i>Men1</i> ^{F/F} - <i>RipCre</i> ⁺)	<i>Bertolino P et al., 2003</i> ¹⁰ ; <i>Crabtree et al., 2003</i> ¹¹ ; <i>Biondi CA et al., 2004</i> ¹²
	α -Cell <i>Men1</i> mutant mice (<i>Men1</i> ^{F/F} - <i>GluCre</i> ⁺)	<i>Lu J et al., 2010</i> ¹³ ; <i>Shen HC et al., 2010</i> ¹⁴
	Pan-pancreatic <i>Men1</i> mutant mice (<i>Men1</i> ^{F/F} - <i>Pdx1Cre</i> or - <i>pTF1Cre</i>)	<i>Shen HC et al., 2009</i> ¹⁵ ; <i>Bonnaïon R et al., 2015</i> ¹⁶
	Parathyroid <i>Men1</i> mutant mice (<i>Men1</i> ^{F/F} - <i>PthCre</i>)	<i>Libutti SK et al., 2003</i> ¹⁷
	Mammary gland <i>Men1</i> mutant mice (<i>Men1</i> ^{F/F} - <i>WapCre</i> ⁺)	<i>Seigne C et al., 2013</i> ¹⁸
	Craniofacial osteogenic cell and osteoblast <i>Men1</i> mutant mice (<i>Men1</i> ^{F/F} - <i>osteocalcin-Cre</i> and - <i>Runx2Cre</i>)	<i>Kanazawa I et al., 2015</i> ¹⁹ <i>Liu P et al., 2017</i> ²⁰
Inducible	Crossing <i>Men1</i> ^{F/F} mice with transgenic mice carrying tamoxifen-inducible CreER	<i>Schnepp RW et al., 2006</i> ²¹
	inducible β -cell-specific <i>PdxCreER</i>	<i>Yang Y et al., 2010</i> ²²
	Inducible CreER driven by the rat insulin promoter (<i>Men1</i> ^{F/F} - <i>RIP2CreER</i>)	<i>Lines KE et al., 2017</i> ²³

III. Molecular mechanisms triggered by *Men1* inactivation

The *MEN1* gene codes for a protein named menin²⁴ that interacts physically with many protein partners, including in particular those involved in transcriptional regulation²⁵. It is believed, therefore, that menin may exert various biological functions through its partners to influence different cell signaling pathways in a cell context dependent manner. Different *Men1* mutant mouse models and derived *Men1*-null cells provide relevant tools for dissecting complex events triggered by *MEN1* inactivation under distinct cellular and molecular contexts.

III.1 Synergistic effects of *Men1* disruption with other cancer genes and pathways

Heterozygous *Men1* mutant mice have been used to investigate eventual synergistic effects between the *Men1* gene and other cancer genes involved in endocrine tumour development. The double heterozygotes *Men1* and *pRb* mutant mice displayed a combined tumour spectrum observed in each of the individual heterozygote's mutant mice, without decrease in age of onset²⁶. Similar approach was used by the same lab to determine whether complete or partial *Trp53* inactivation could alter the tumour phenotype seen in *Men1* mutant mice. Their data demonstrated that simultaneous heterozygous deletion of *Men1* in mice with either heterozygous or homozygous *Trp53* disruption did not result in formation of tumours at any new sites. The works implies that there are independent

rather than synergistic effects of these pathways on tumorigenesis²⁷. Pei et al. reported that p18, but not p27, functionally collaborates with *Men1* in suppressing lung tumorigenesis, likely due to loss of the control on stem cell expansion²⁸.

Agarwal et al. has reported that JunD, the first identified menin partner²⁴, activates proliferation of menin-deficient MEFs²⁹. It appears that, in the absence of menin, c-Jun N-terminal kinase (JNK) phosphorylates JunD, leading the activation of JunD-induced pro-proliferative gene expression^{27,30}. The activation of JunD after menin inactivation could also result from the loss of binding to the co-repressor mammalian sin3 protein (mSin3A) and its associated HDAC³¹. However, the role played by JunD in *Men1*-related tumours remains so far elusive.

The analyses carried out in our laboratory revealed the physical interaction between menin and the Wnt pathway effector β -Catenin, and the nuclear translocation of the latter in menin-deficient islet cells^{10,32}. Furthermore, Cao et al have shown that menin plays an essential role in controlling β -Catenin cyto-nuclear shuttling. Importantly, Jiang et al have provided genetic evidence showing that β -Catenin ablation could restore *Men1* insulinoma phenotype³³, highlighting the importance of β -Catenin in *Men1* insulinoma development. Finally, β -Catenin inhibitor was shown to successfully suppress insulinoma development in *Men1*^{F/F}-*RipCre*⁺ mice³³.

Wang, Y. et al investigated whether menin can regulate the AKT signaling using immortalized menin-null MEFs stably infected or not with menin-expressing retroviruses³⁴. They found that menin inhibited AKT1-mediated cell proliferation likely by reducing the translocation of Akt1 from the cytoplasm to the plasma membrane. They also demonstrated that p-AKT (S473) expression was detected in *Men1* mouse insulinomas. Interestingly, it is reported that menin deficiency in CD8 T cells affected the mTOR complex 1 (mTORC1) signaling, resulting in increased glycolysis and glutaminolysis, highlighting its role in regulating the AKT/mTOR pathway in these cells³⁵.

By using *Men1*-deficient mouse embryonic fibroblasts (MEF) and primary islets derived from *Men1* mutant mice, Gurung et al have provided compelling data showing that menin plays a crucial role in inhibiting the Hedgehog pathway through its physical interaction with PRMT5 and its binding to both the *Gli* and *Gas* promoters³⁶. The data suggest that menin inactivation may lead to the activation of the Hedgehog pathway known to be enhanced in human PanNET.

Mammary precancerous lesions were found in more than half of *WapCre-Men1*^{F/F} female mice where the *Men1* gene was disrupted in luminal cells¹⁸. Further analyses demonstrated that ER α expression was reduced in menin deficient mammary cells. Similarly, the quantitation of epithelial cell nuclei displaying either no or very low staining of AR expression showed a significant increase in the number of cells with altered AR expression in prostate carcinomas from heterozygous *Men1* mutant mice, compared with prostate glands from *Men1*^{+/+}³⁷. These observations are in consistence with the molecular analyses showing the physical and functional interaction of menin with ER α and AR^{38,39}.

III.2. Cell cycle control & Growth factors

Several studies of the altered gene expression in mouse *Men1* insulinomas demonstrated early deregulated expression of the genes involved in cell cycle control, such as cyclin A2, B2, D2, CDK2, CDK4, p18 and p27, and growth factors of the insulin pathway, such as Igf1, Igf2, Igfbp3, and Igfbp6^{40,41, 42,43, 44}. Moreover, the analysis of the methylation state of the DMR2 regulatory regions of the *Igf2*

gene revealed a hyper-methylation of the four CpG sites whose methylation leads to the increased Igf2 expression^{40,45,46,47}. Interestingly, Zhuang *et al* recently showed that neuron-specific *Men1* ablation in the mouse caused decreased CDK5 expression and synaptic dysfunction. Their detailed investigation demonstrated that menin binds the *p35* promoter, the main activator of CDK5, and activates its expression via the MLL complex⁴⁸.

III.3 Cell adhesion and angiogenesis

The alteration of several cell adhesion markers were documented in mouse *Men1* insulinomas⁴⁵. Interestingly, we were also able to demonstrate an evident decrease in membrane β -Catenin and E-cadherin expression in the mammary lesions developed in *Men1^{F/F}-WapCre⁺* model¹⁸. Early appearance of increase in angiogenesis was observed in different *Men1* insulinoma models, using respectively CD31¹⁰, lectin, VEGF¹⁵, reminiscent of what observed in MEN1 PanNET.

III.4 Pancreatic endocrine transcription factors

Our works revealed that the expression of Maf family members, in particular MafA and MafB both having important functions in islet cells, is dysregulated in islet tumors in murine models. More precisely, we observed an ectopic expression of MafB, which is normally expressed only in mouse α -cells, in insulinomas. Our analyses demonstrated that MafB expression is negatively regulated by menin and its overexpression can promote the ability of β -cells to form foci on soft agar and the level of cyclin D2 expression⁴⁹. On the contrary, MafA expression is decreased in mouse and human MEN1 insulinomas⁵⁰. The analyses demonstrated that menin binds to the *MafA* promoter and activates its transcription, which is essential for keeping intact β -cell program. Therefore, a finely controlled expression of MafA and MafB seems crucial for normal β -cell proliferation and differentiation.

The expression of another islet transcriptional factor Hlxb9 was found increased in mouse islet tumors, the former being a menin-interacting factor⁵¹. The authors also reported that combined menin knockdown and Hlxb9 overexpression partially rescued the apoptosis induced by Hlxb9 overexpression alone.

We were able to demonstrate the existence of physical and functional interaction between menin and Foxa2. In addition, Foxa2 is overexpressed very early in menin deficient α -cells in *Men1^{F/F}-GluCre⁺* mice and in a substantial proportion of human glucagonomas. This is consistent with our observation that the overexpression of Foxa2 in cultured α -cells (α TC1-9) led to an increase in the proliferation of these cells⁵².

III.5 Epigenetic regulation

The decreased expression of P18 and P27, as well as Mll was observed in mouse and human MEN1 insulinomas⁵³. The analyses showed that menin positively regulated the transcription of these genes by recruitment of Mll to the *p27Kip1* and *p18Ink4c* promoters and coding regions. Thus, loss of menin resulted in reduced expression of p27Kip1 and p18Ink4c, and deregulated cell growth.

Several studies have demonstrated that menin stably associates with the N-terminus of MLL and is essential for initiation and maintenance of leukemogenic transformation by MLL fusions^{54,55,56}. Thiel *et al* showed that menin recruits both wild-type MLL and oncogenic MLL-AF9 fusion protein to the loci of HOX genes to activate their transcription through both H3K4 and H3K79 methylation. They also

showed that that menin binds the *Ezh2* promoter, a polycomb component, and activates its expression, leading to the repression of C/EBP α target genes and of cell differentiation⁵⁷. In parallel, Yokoyama et al. showed that conditional *Men1* disruption in the mouse hematopoietic system impaired the self-renewal capacity of hematopoietic stem cells, associated with altered MLL and Ldgf-mediated Hoxa9 expression⁵⁶.

Menin loss resulted in increased ActivinB expression in β -cell-specific *Men1* mutant mice, which occurs along with the loss of insulin expression⁵⁸. The work further revealed that ActivinB expression was mediated through a direct modulation of H3K27me3 marks on the *Inhbb* locus in *Men1*-deficient cell lines⁵⁹. More importantly, the data demonstrated that menin binds to the *Inhbb* promoter where it favours the recruitment of Ezh2 via an indirect mechanism involving Akt-phosphorylation. The data may provide insights onto the molecular mechanisms underlying the occurrence of less differentiated PanNET, likely including non-functional PanNETs.

Feng, Z.J. reported that menin could bind to the *PTN* promoter together with polycomb repressive complex (PRC)2, increase the repressive chromatin mark H3K27me3, thus repress the expression of the *PTN* gene. The latter encodes a pro-cell migration receptor, pleotro-phin, in lung cells⁶⁰.

Cao et al reported that menin expression is reduced in the liver of aging mice and that hepatocyte-specific *Men1* ablation induced liver steatosis in aging mice, due to loss of menin-mediated SIRT1 recruitment to control hepatic CD36 expression and triglyceride accumulation through histone deacetylation⁶¹.

IV. Summary

The generation and characterisation of *Men1* mutant mouse models have greatly accelerated studies to pinpoint cellular and molecular mechanisms underlying MEN1 disease development, from cells of origin of tumorigenesis related to MEN1 syndrome to different factors and /or cell signaling pathways involved in the procedure due to menin inactivation. These advances would help us to better understand the tumour spectrum and tumour progression seen in MEN1 patients. Needless to say, acquired knowledge should also be valuable for finding prognostic biomarkers and potential therapeutic targets for MEN1 disease.

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