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Role of CARM1 (PRMT4) in High Grade Serous Ovarian Cancer metabolism and function of PRMT5 in ARID1A-deficient Endometrial Cancer invasion

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Abstract

The arginine methyltransferase CARM1 (PRMT4) is amplified and overexpressed in ~20% of high-grade serous ovarian cancer (HGSOC) and correlates with a poor survival. Therapeutic approaches based on CARM1 expression remain to be an unmet need. Here we show that fatty acid metabolism represents a metabolic vulnerability for HGSOC in a CARM1 expression status dependent manner. CARM1 promotes the *de novo* synthesis of fatty acids and monounsaturated fatty acids (MUFAs). The disruption of MUFAs synthesis by inhibition of SCD1 results in excessive accumulation of cytotoxic saturated fatty acids and it is synthetic lethal with CARM1 expression. Collectively, our data show that the pharmacological inhibition of MUFAs synthesis via SCD1 inhibition represents a therapeutic strategy for CARM1-high HGSOC.

Another arginine methyltransferase, PRMT5, has been identified by our CRISPR screening analysis as a promising candidate for invasive ARID1A-deficient endometrial cancer. Endometrial Cancer frequently harbor somatic inactivating mutation of ARID1A that can promote an invasive phenotype. Our *in vitro* approach validated the CRISPR screening showing that both PRTM5 knock down and its pharmaceutical inhibition specifically hamper the invasion of ARID1A inactivated cells. Mechanistically, PRMT5 directly regulates the epithelia to mesenchymal transition pathway genes interacting with the SWI/SNF complexes. Moreover, *in vivo* experiments showed that PRMT5 inhibition contrasted the myometrium invasion highlighting PRMT5 inhibition as promising therapeutic strategy for ARID1A-inactivated aggressive endometrial cancer.

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1. Introduction

1.1 Female gynecological cancers

Cancer represents one of the main causes of death in the world every year motivating the scientific community to keep investigating these diseases and to develop new strategies and therapies. The estimation of GLOBOCAN based on cancer mortality, produced by the International Agency for Research on Cancer, resulted in ten million cancer related deaths worldwide during 2020 (Sung et al. 2021).

The female gynecological cancers are classified in ovarian, uterine, cervical, vaginal, vulvar, and fallopian tube cancers. The most frequent types of gynecological cancers among women worldwide according to GLOBOCAN 2020 were: cervical cancer with 341,831 cases, ovarian cancer with 207,252 and uterine cancer with 97,370 cases. The American Cancer Society estimated six hundred thousand cancers deaths in 2023 only in the United States, divided in 322,080 for male and 287,740 for female (Siegel et al. 2023).

Estimated cancer related death number in female population in 2023

Location	Number	Percentage
Lung & Bronchus	59,910	21%
Breast	43,170	15%
Colon & Rectum	24,080	8%
Pancreas	23,930	8%
Ovary	13,270	5%
Uterine corpus	13,030	5%
Liver & Intrahepatic bile duct	10,380	4%
Leukemia	9,810	3%
Non Hodgkin Lymphoma	8,400	3%
Brain and other nervus system	7,970	3%
all sites	**287,740**	

Figure 1. Leading sites of cancer deaths: estimation for 2023. Data from the reference American Cancer Society Cancer Facts & Figures 2023.

Approximately, as shows in figure 1, female gynecological cancer are estimated to be responsible of about 10% of female cancer related death during 2023 counting 13,270 cases of ovarian cancers (5%) and 13,030 cases of uterine cancers (5%) (Cancer Facts & Figures 2023).

1.2 Ovarian cancer

Ovarian cancer is often described as a silent killer since it is diagnosed only at an advantage stage due to generally vague symptoms, making the advanced cancer hard to treat on a curative basis (Stewart, 2019). According to the Ovarian Cancer Research Alliance (OCRA), unfortunately, only 17% of patient with FIGO (Federation of Gynecology and Obstetrics) stage I ovarian cancer are early detected when the primary tumor is localized in the ovary or in the fallopian tubes. Even though the survival rate is influenced by the type of tumor and the genetic mutations, overall, the women diagnosed with stage I tumor have a good prognosis with a possibility of 5-year survival rate of 93%. Stage II tumors, that are found in ovary or fallopian tubes and are spread to other points of the pelvis, are diagnosed in 19% of the cases, with a general 5-year survival rate of 74%. All the remaining cases are diagnosed at the advanced stages III and IV. Stage III tumors, that have already spread outside pelvis in the abdomen or in the nearby lymph nodes, have a 5-year survival rate of 41%. Last, stage IV tumors, characterized by metastasis in liver and lungs or other distant sites, have the lowest 5-years survival rate of 31% (Ovarian Cancer Stages, Survival Rate and Prognosis, *OCRA*).

1.3 Ovarian cancer classification and genetic features

Ovarian cancers are subdivided in type I and type II and this model takes in consideration the histopathologic features integrating them with the molecular alterations and the FIGO stage for the definition of the grade (Fig. 2-3). Type I cancers are typically characterized by a large and unilateral cystic neoplasm with a usual low-

grade behavior. Therefore, type I tumors comprise only 10% of the ovarian cancer deaths. On the other hand, type II cancers are universally high grade with rapid development and aggressive phenotype, and frequently accompanied by ascites. More than 75% of type II ovarian cancer are present in advanced stage and they are responsible for 90% of the deaths (Cho and Shih 2009; Magaña-Pérez 2020).

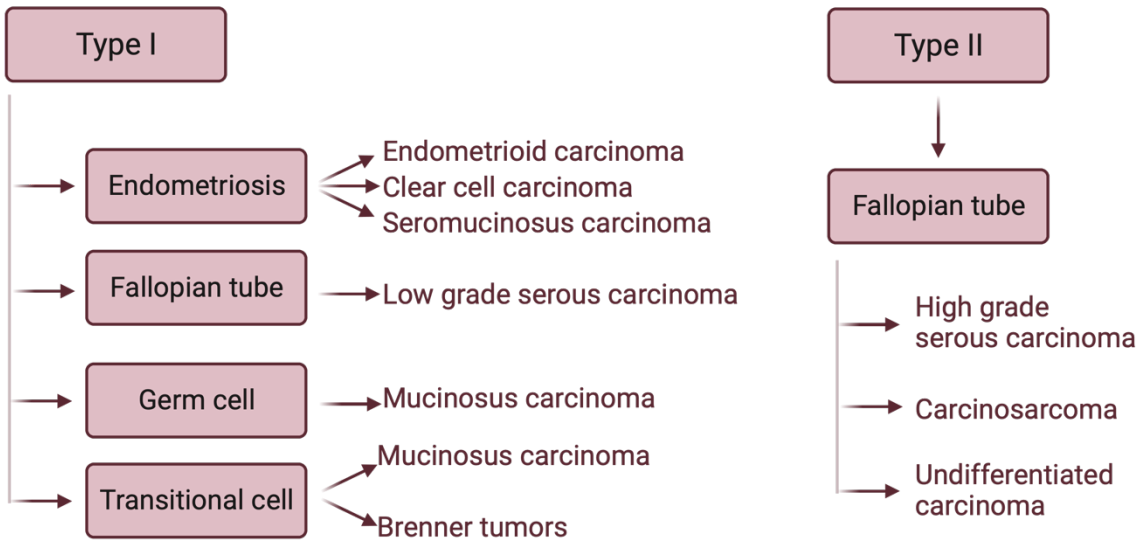


Figure 2. Classification of type I and type II ovarian cancers. Image created with BioRender.com.

1.3.a Type I ovarian cancer

Type I ovarian cancers comprehend ovarian endometrioid, clear cell and seromucinous carcinomas that may be originate from endometriosis; low-grade serous carcinoma that derive from fallopian tube; Brenner tumors that originates from transitional cells and mucinous carcinoma that may derive from both germ cells and transitional cells (Kurman and Shih 2016). Ovarian endometrioid carcinoma is generally well differentiated and ovarian clear cell carcinoma are not graded but usually regarded as high-grade, in contrast with others type I. The inactivating

mutation of the tumor suppressor gene ARID1A could be detected in 50% of clear cell ovarian cancer cases and 30% of the endometrioid ones. Low-grade serous ovarian cancers evolved from the atypical proliferative serous tumors (APST) and they present mutation in the KRAS, ERBB2 and BRAF oncogenes. These mutations are mutually exclusive and appear in the early development of the tumor. Seromucinous carcinomas present a mixture of epithelial cells such as squamous, endometrioid and endocervical-type mucinous cells. One third of these tumors have inactivation of ARID1A. Ovarian mucinous carcinoma are well differentiated and quite heterogeneous with areas of atypical proliferative tumors and cystadenoma mixed with areas of carcinoma. In 65% of these tumors, it has been reported KRAS-activation mutation and KRAS, BRAF and ERBB2 amplification are present in more than 90% of the cases, denoting recurrent activation of the RAS/MEK pathways in these carcinomas. Last, Brenner tumors are mostly benign and comprehensive molecular analysis of these tumors has not been performed (Cho and Shih 2009; Matulonis et al. 2016; Stewart, Ralyea, and Lockwood 2019).

1.3.b Type II ovarian cancer

Type II ovarian cancer mainly originated from fallopian tubes and they are subclassified in high grade serous carcinoma, carcinosarcoma and undifferentiated carcinoma. Undifferentiated carcinoma are uncommon tumors and currently, there is uncertainty regarding whether they represent separate tumors or variations of poorly differentiated HGSCs, or high-grade endometrioid carcinomas. Ovarian carcinomas, also called Malignant Mixed Müller tumors, are a biphasic tumor composed of sarcoma and carcinoma, and they present different genetic alteration such as TP53 mutation and CDKN2A overexpression (Kurman and Shih 2016).

The typical form HGSOC present compact cell masses that have narrow slit-like spaces and display glandular, cribriform and papillary cribriform patterns that are often accompanied by necrosis. Another variant consists solid cell masses that closely resemble endometrioid and transitional cell carcinomas and tumors in this group are defined as SET (solid eudoendometrioid transitional) variant to distinguish them from the typical HGSOC. The SET tumors have a bigger number of tumor-infiltrating lymphocytes and a higher mitotic index compared with the usual type of HGSC.

Considering the genetic profile, TP53 mutation characterized almost all the tumors, copy number alteration are widespread as well and other common threads involved CCNE1 amplification, somatic and germline mutation of BRCA1 and BRCA2 and other alteration in the pathways that regulate the homologous recombination (HR) DNA damage repair pathways. In this regard, the SET variants are more frequently associated to BRCA1 mutation then the typical HGSOC. It is important to notice that the so called *BRCAness* signature is characterized by deficiencies in the double strand DNA break repair pathway (involving BRCA1/2 mutations or epigenetic silencing), and can sensitize tumors to PARP inhibitor therapy. This carries significant implication since analysis of TGCA discovered that a considering portion of HGSOC harbor mutation in BRCA1/2 (Cho and Shih 2009; Matulonis et al. 2016; Stewart, Ralyea, and Lockwood 2019; Arnaoutoglou et al. 2023).

Type	Histological subtype	Mutations
Type I	Endometrioid	MMR deficiency
	Clear cell	ARID1A inactivation; PI3K pathway activation; PTEN inactivation
	Mucinous	ERRB2/KRAS/MEK pathway activation
	Low grade serous	ERRB2/KRAS/MEK pathway activation
Type II	High grade serous	P53 pathway inactivation; HR DDR deficiency; CCNE1, NOTCH activation; Rb, NF1 inactivation.

Figure 3. Main mutations in type I and type II ovarian cancers. Mutations are sorted based on the histological subtypes.

1.4 Ovarian cancer metabolism

The main theme in the field of cancer metabolism is that the restructured activities improve the overall cellular fitness granting a selective advantage during the formation of the tumor. When the enhanced metabolic functions are suppressed, the growth of the tumors is impaired (M. Wang, Zhang, and Wu 2023). The modified metabolic behaver supports anabolic growth during nutrition replete condition, promotes catabolism when nutrients are scarce supporting cell survival and reinforces the equilibrium of the redox homeostasis to offset the metabolic repercussions deriving from loss of tumor suppressor, activation of oncogenes and other stress (Roopak Murali et al. 2023). The rapid proliferation of cancer cells demands a large quantity of energy supply to reconstruct biomasses, consequentially, the reprogramming of the main metabolic pathways such as lipid, glucose, and amino acids, stands out as a well-recognized tumor hallmark (Hanahan 2022).

One main feature of metabolic alterations in cancer is the distinct glucose metabolic mode from the healthy cells. The best described metabolic alteration of the glucose pathway is the well-known Warburg effect. The Warburg effect described a phenomenon by which there is shift in the metabolism of glucose, and due to this the cancer cells use carbon from glucose for the construction of various molecules instead complete the fully oxidation of the glucose. In normal condition, when cells have abundant amount of oxygen, cells use glycolysis to use glucose within the cytoplasm for the production of pyruvate. Subsequently, pyruvate enters the mitochondria, and it is transformed into acetyl-CoA, that is an important player for the Krebs cycle for the oxidative phosphorylation. Normal differentiated cells mainly chose the oxidative phosphorylation due to its efficiency in producing more energy (36 ATP molecules) from one molecule of glucose. However, under conditions of limited oxygen, the fermentation is chosen to use the surplus of pyruvate and as result of fermentation the lactate is produced within the cytoplasm. The adoption of fermentation even when oxygen is accessible is well known as Warburg effect. This process is characterized by a remarkable increase in glucose intake and utilization and a decreased oxidative phosphorylation, and the following production of the end product lactate (Warburg 1956; Liberti and Locasale 2016). As example, in ovarian clear cell carcinoma, the

overexpression of HNF1β promotes glucose uptake. Moreover, the key component of glycolysis Glucose Transporter 1 (GLUT1) and the Hexokinase 2 (HK2), important enzyme in initial steps of glycolysis, are upregulated in ovarian cancer and they are regulated by the transcription factor FOXM1. FOXM1 knock down significantly reduce their expression downregulating the aerobic glycolysis and the cell proliferation (Macheda, Rogers, and Best 2005). Additionally, the Lactate Dehydrogenase A (LDHA), that is a key player in the maintenance of glycolysis converting the pyruvate into lactate, is upregulated in ovarian cancer respect to the normal ovary. It has been reported that targeting LDHA reduce the tumor proliferation by suppressing the glycolysis (Qiu et al. 2015).

Another important metabolic assignment for the cell is the regulation of amino acid metabolism to support the protein. For example, in ovarian cancer cells some amino acid transporters from the ABC and SLC families are upregulated or downregulated to promote the uptake of the amino acid required to satisfied the metabolic need of the cancers (Wang, Zhang, and Wu 2023).

Additionally, cancer cells display alterations in various aspects of lipid metabolism that can influence, for example, the availability of structural lipids and the abundance of lipid with signaling functions. Lipids constitute a diverse group of water-insoluble molecules composed by triglycerides, phosphoglycerides, sterols, and sphingolipids. Alterations in lipid metabolism can impact a multitude of cellular processes, including cell growth, motility, proliferation, and differentiation. The fatty acid are important component of lipids and alteration on fatty acid metabolism in ovarian cancer will be detailed in the next paragraphs.

In adult mammalian cells, lipids are obtained from dietary sources and therefore they are present in the bloodstream either bound to proteins like low-density lipoproteins or in the form of free fatty acids. Additionally, many lipids are synthesized either in the liver or within adipocytes producing carbohydrate-derived fatty acids that may be amassed and stored within cellular component called lipid droplets in adipocytes (C. R. Santos and Schulze 2012).

An important lipid related pathway is the mevalonate pathway that is required for the synthesis of diverse isoprenoids, such as cholesterol group. It has been published that inhibitors of different enzymes of the mevalonate pathway can suppress growth of ovarian cancer (Nieman et al. 2011; Abdullah, Abed, and Richardson 2017).

Many enzymes involved in fatty acid and cholesterol biosynthesis are regulated by the sterol regulatory element-binding proteins (SREBPs) (Horton 2002; Eberlé et al. 2004). In particular, the transcription factor SREBP1 was found overexpressed in metastatic ovarian cancer (Mukherjee et al. 2012; Yueying Liu et al. 2015). Moreover, SREBP1 knockdown experiments showed inhibition of ovarian cancer cell growth (Nie et al. 2013)

1.4.a Fatty acid metabolism alteration in ovarian cancer

Fatty acids represent as significant energy reservoirs and foundational building constituents for cellular component across various species. Indeed, the fatty acid oxidation not only is a crucial aspect of lipid metabolism but it is also a vital process for generating adenosine triphosphate (ATP) and producing novel lipid metabolites (Currie et al. 2013). In a healthy scenario, normal cells typically depend on external sources for fatty acids. On the other side, tumor cells may intensify the uptake of fatty acid or have the ability to synthetize new ones. In term of up taking fatty acids, cells are required to exhibit specialized transporters on the plasma membrane. Among these, some well-studied transporters are CD36 (fatty acid translocase), the FABPpm (plasma membrane fatty acid-binding proteins), and FATPs (fatty acid transport protein family). Notably, high RNA and protein levels of these transporters are detected within cancers cells. As example, high expression level of CD36 has been detected in various tumor types, including prostate, breast and ovarian cancers, and these enhanced expression correlated with a poor prognosis (Su and Abumrad 2009; Ladanyi et al. 2018).

Fatty acids have a central role as building blocks in biological membranes and also represent key constituents of diverse lipids type like membrane phospholipids, triacylglycerols, signaling molecules and others (Beloribi-Djefaflia, Vasseur, and Guillaumond 2016).

The general structure of a fatty acid consists of a hydrocarbon chain and a carboxylic acid group, where the hydrocarbon chain may vary in lengths and degrees of unsaturation (Los and Murata 1998). In normal organism, the de novo lipogenesis primarily occurs in adipocytes and hepatocytes while tumor cells, to sustain their rapid growth, can reactivate this pathway (Currie et al. 2013). The figure 4 summarize the

step of fatty acid synthesis. The cytoplasmic acetyl-CoA is the primary substrate for fatty acid synthesis. The acetyl-CoA undergoes carboxylation by the enzyme ACC (acetyl-CoA carboxylases) forming malonyl-CoA. Next, the enzyme FASN (fatty acid synthase) facilitates the condensation of 1 acetyl-CoA molecule and 7 malonyl-CoA molecules, producing the palmitic acid, a saturated 16-carbon fatty acid. The enzyme SCD1(stearoyl-CoA desaturase 1) can further modified the palmitic acid producing monounsaturated fatty acids (MUFA). Therefore, the palmitic acid is primary product of *de novo* fatty acid synthesis, and it is the precursor for other fatty acid species like stearate and oleate. Those fatty acid afterward serve for the generation of more intricate lipids. It is well known the significance of the activity of SCD1 for cancer cells. In recent publication is highlighted how the cell proliferation is impaired by the inhibition of SCD1 that disrupts the balance between unsaturated and saturated fatty acids. In addition, the blocking of SCD1 led to the accumulation of saturated fatty acids that triggers cell death (Röhrig and Schulze 2016; Lien et al. 2021).

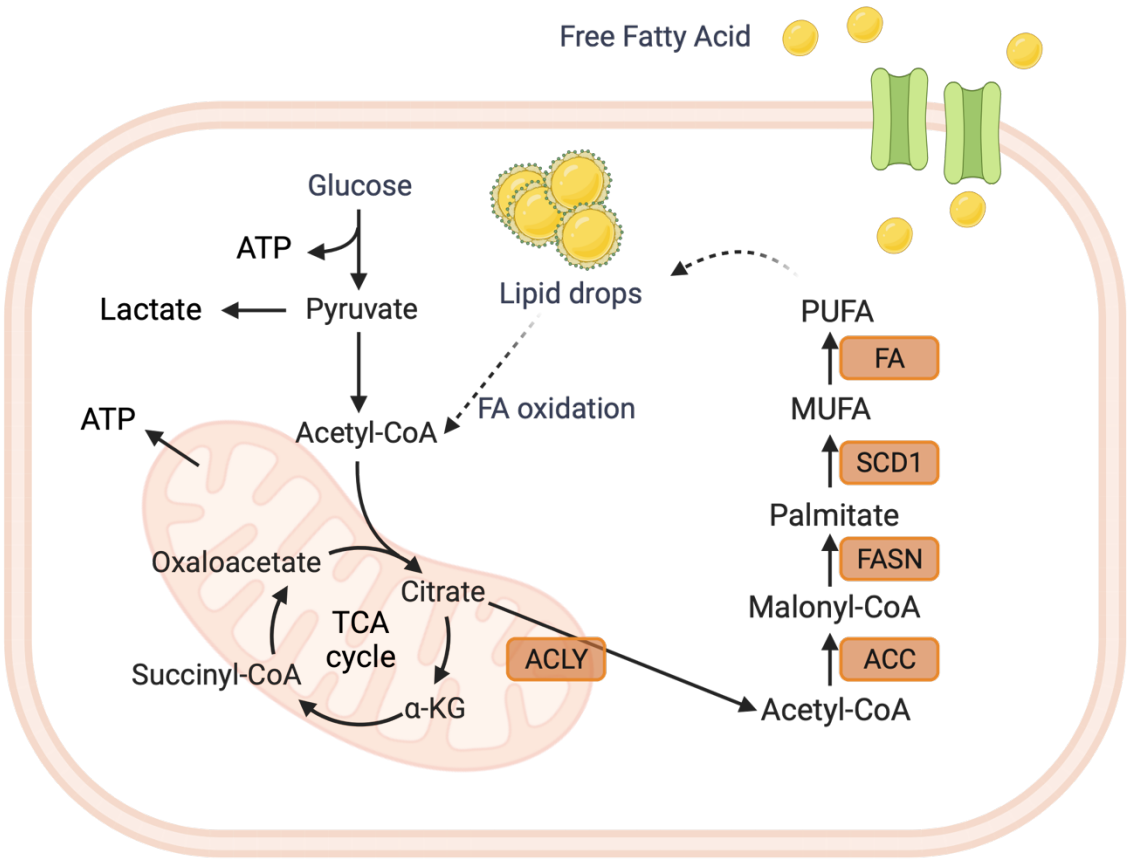


Figure 4. Schematic representation of fatty acid synthesis pathway. Image crated with BioRender.com.

Emerging studies indicated the possibility to use the plasmatic fatty acids composition as potential biomarkers gynecological cancers including ovarian cancers. Moreover, many research groups are showing that changes in fatty acid metabolism could influence the development and aggressiveness of ovarian cancer (Xu et al. 1998; Shen et al. 2001). As mentioned above, the increased uptake of external fatty acid is frequent in cancer cell and the transporter present in the cell membrane surface offer a potential avenue for cancer treatment. For example, the inhibition of FABP4 with the small molecule BMS309403 competes with the fatty acid in the binding of the pocket in the transporter and this FABP4 inhibition in ovarian cancer amplifying the responsiveness of cancer cells to carboplatin and suppresses cell proliferation (Furuhashi et al. 2007; Nieman et al. 2011; Mukherjee et al. 2020). Another example, in mouse xenografts of ovarian cancer, the administration of monoclonal antibodies against CD36 led to a reduction of the tumor size within (Pascual et al. 2017; Ladanyi et al. 2018).

But cancer cells can also activate the *de novo* fatty acid synthesis. Indeed, FASN is frequently upregulated in ovarian cancer tissues and this alteration has been linked to a decreased survival rate and unfavorable prognosis. It has been reported how FASN interact with ErbB2 (HER2/neu), and this interaction triggers the activation of PI3K-mTOR pathway, that in turn promote cell proliferation and survival of ovarian cancer cells. The elevated expression of FASN in ovarian cancer cell lines result in an elevated *de novo* fatty acid synthesis, that lead to increased cell growth and viability, and enhanced the resistance to cisplatin, a chemotherapy drug (Grunt et al. 2009; Y. Cai et al. 2014; Bauerschlag et al. 2015; Papaevangelou et al. 2018).

FASN is indeed emerging as a promising target for cancer therapies. The used of cerulenin, a specific FASN inhibitor, considerably reduced the fatty acid synthesis in a tumor xenograft model of ovarian cancer and increased the survival rates (Pizer et al. 1996; Veigel et al. 2015; Lu et al. 2018; R. R. Chen et al. 2019)

Another well studied and targetable enzyme of the fatty acid synthesis pathway is SCD1 that is emerging a potential therapeutic focus for ovarian cancer cure. SCD1 is situated within the endoplasmic reticulum and convert saturated fatty acids (like palmitoyl-CoA and stearoyl-CoA) into unsaturated fatty acids (like palmitoleate and oleate). SCD1 has been frequently observed to be upregulate in ovarian cancer stem cells and the administration of SCD1 inhibitors in mouse model effectively shortens the proliferation of ovarian cancer stem cells. In this contest, investigation of the

mechanism reveled that SCD1 may be regulated by NF-kB (Roongta et al. 2011; Igal 2016; J. Li et al. 2017).

In has been showed how the small-molecule and specific SCD1 inhibitor CAY10566 or A939572, can suppresses the proliferation and trigger apoptosis in diverse cancer cell types, including liver, kidney, colon, bladder cancer cells. In ovarian cancer it has been reported that the use of these SCD1 inhibitors notably potentiated the growth-inhibitory effected obtained by the ferroptosis-inducer agents RSL3 in both ovarian cancer cells and within xenograft models in vivo (Tesfay et al. 2019).

1.5 Endometrial cancer

The prediction of having approximately 66,200 cases of uterine cancers has been anticipated in the United States for the year 2023, with a prevision of 13,030 correlated deaths. The uterine cancer is often denoted as endometrial cancer, since in more than 90% of the cases, this malignancy is manifested in the endometrium, the inner layer of the uterus. Luckily, a notable majority of 69% of cases are diagnosed during early stages (“Cancer Facts & Figures 2023”). Localize tumors that do not show sign of cancer spreading outside the uterus, have a 5-years survival rate of 96%. Defined regional tumors, in which the cancer has spread from the uterus to the nearby structures of lymph nodes, the 5-year survival rate is 72%. Lastly, distant cancer, where the cancer masses have already spread in distant location of the body such as liver, lungs and bones, present the lowest rate that account only 20% of survival in 5 year (“Survival Rates for Endometrial Cancer” n.d.).

1.6 Endometrial cancer classification and genetic feature

Traditionally, the classification of endometrial carcinomas defined type I and type II based on Bokhman's taxonomy, that rely on clinical, endocrine, and epidemiological observations. The histology classification, as specified below, consider the World Health Organization (WHO) categories.

Over the time, it become gradually evident that endometrial cancer is composed of a heterogeneous range of tumors with biological, clinical, morphological, and genetic differences. The traditional classifications resulted inadequate to address such diversity especially with the increase in the related knowledge. To address this issue, it is emerging a genomic classification of endometrial carcinoma with the aim to identify potential targetable markers among the various subsets (Rajmohan Murali, Soslow, and Weigelt 2014).

1.6.a Bokhman classification

Bohhman introduced the concept of classifying endometrial cancers in two types based on clinical, endocrine, and metabolic features (Fig. 5) (Bokhman 1983). According to this classification, type I tumors were influenced by estrogen, associated to obesity, and linked to endometrial hyperplasia. Type I tumors overall exhibit a moderate or high rate of differentiation and clinically have a positive prognosis. On the other hand, type II tumors are associated with endometrial atrophy, and they are not estrogen dependent. Type II tumors could originate without metabolic and endocrine anomalies, and they are mainly diagnosed in non-obese individuals. In addition, type II tumors show poor differentiation and correlate with an atrophic endometrium, and overall have less favorable prognoses (Bokhman 1983; Stefanick 2005; Setiawan et al. 2013).

Bokhman classification	type I	type II
Distribution	60 - 70%	30 - 40%
Onset of menopause	after age 50	before age 50
Background endometrium	hyperplasia	atrophy
Tumor grade	grade 1-2	grade 3
Myometrial invasion	superficial	deep
Potential for lymphogenic metastatic spread	low	high
Prognosis	favorable	unfavorable
Estrogen associated	yes	no
Prototypical histological type	Endometrioid	Serous

Figure 5. Common features of type I and type II endometrial tumors.

The model defined by Bokhman placed the foundation for the principle by which type I tumors involved low-grade endometrioid carcinomas that are associated to unobstructed estrogen exposure, and they received a better prognosis; while type II tumors are mainly composed of serous and clear-cell carcinomas that ended with unfavorable prognosis (Cancer Genome Atlas Research Network et al. 2013).

1.6.b Histological classification of endometrial cancer

Considering the histology of endometrial cancer, this neoplasm originates in the uterine corpus and comprehend a variety of histological types that the WHO categorizes as epithelial carcinomas that includes endometrioid, serous, clear cell, mucinous, squamous cell, transitional cell, small cell, and undifferentiated), mixed epithelial and mesenchymal tumors (like carcinosarcomas), or mesenchymal tumors (including endometrial stromal and smooth-muscle tumors) (Böcker 2002; Helga B. Salvesen, Haldorsen, and Trovik 2012).

The epithelia endometrial tumors are the most common with a frequency of 75% of endometrioid, 5-10% of serous and 1-5% of clear cell carcinomas. Serous and clear-cell carcinomas are fundamentally classified as high grade, but Endometrioid adenocarcinomas embrace a range of cancers that vary from well-differentiated to poorly differentiated tumors varying from low to high grade. The low-grade endometrioid carcinomas are often linked to endometrial hyperplasia, clinically display less aggressive features. Low grade endometrioid is frequently diagnosed in premenopausal women and, in contrast, serous carcinomas occur mainly in postmenopausal women in association with atrophic endometrium and clinically display an aggressive progression (Creasman et al. 2006; Abu-Rustum et al. 2010).

1.6.c Genetic alteration in endometrial cancer

The conceptual sense of the histological and Bokhman classifications are indubitably valuable. Nevertheless, further information has been gathered by numerous investigations that have examined the genetic changes in endometrial cancer. Early

studies were predominantly focused on the identification of pathways or single candied genes, whereas more recent next-generation sequencing studies allowed scientist to have a better sense of genome-wide genetic modifications within these tumors (Creasman et al. 2006; Rajmohan Murali, Soslow, and Weigelt 2014).

The extensive investigation of the Endometrioid subtype revealed a significant mutational burden. One of the most mutated pathways is the PI3K/AKT/mTOR signaling pathway. Additionally, mutations in the tumor suppressor gene PTEN, that is a regulator of the PI3K/AKT/mTOR pathway, are proposed to represent an early event in the development of endometrioid cancer since it is disrupted in up to 80% of the cases (Mutter et al. 2000; Oda et al. 2005; Hayes et al. 2006; McConechy et al. 2012)m. Meanwhile, researcher have identified other frequent alteration such as PIK3CA mutations that are observed to occur in about 52%, and KRAS mutations that are identified in about 43% of endometrial cancers and in the endometrioid subtype these mutations frequently coexist. In serous endometrial cancer subtype, PIK3CA amplification and mutations are identified with a rate of 35% and 46% respectively and other less common mutation involved genes such as PTEN (11%), PIK3R1(12%), and KRAS (8%). Additionally, in up to 45% of serous cancers, the PI3K/AKT/mTOR pathway may be activated by amplification in the HER2 gene (Lax et al. 2000; Oda et al. 2005; Cheung et al. 2011; Rudd et al. 2011; Urick et al. 2011).

Another well studied pathway in endometrial cancer is the Wnt/β-catenin signaling pathway. About half of endometrioid and around 80% of serous endometrial cancers present loss of E-cadherin. Moreover, gain of function mutation of CTNNB1 (β-catenin) are identified in about 25% of endometrioid cancers (Schlosshauer, Ellenson, and Soslow 2002; Moreno-Bueno et al. 2003; Weigelt and Banerjee 2012; Matias-Guiu and Prat 2013).

Moreover, up to one-third of sporadic endometrioid carcinomas are characterized by microsatellite instability, that is the alteration of repetitive nucleotide sequence lengths. The microsatellite instability on the other hand is rare in serous carcinomas (Simpkins et al. 1999; H. B. Salvesen et al. 2000).

Another widely mutated gene is the TP53 tumor suppressor gene which mutations are present in up to 90% of serous carcinomas, 30% and 10% of high and low grade endometrioid carcinoma respectively (Tashiro et al. 1997; Lax et al. 2000; Jia et al. 2008; Urick et al. 2011).

Mutations in the suppressor gene ARID1A or loss of its protein expression are identified in various endometrioid cancer, and they are largely studied (see paragraph 1.11) (Wiegand et al. 2011; Mao et al. 2013; Werner et al. 2013). Other frequent detected mutations regard PPP2R1A gene that are observed in 40% of serous and 5% of endometrioid carcinomas (McConechy et al. 2011).

The Carcinosarcomas subtype presents recurrent mutations in TP53 (44–64%), in PTEN (11–33%) and PIK3CA (22–29%), and present other common alteration in PIK3R1 (6%), ARID1A (24%), KRAS (17%), PPP2R1A (21%), and CTNNB1 (up to 5%) (Cheung et al. 2011; McConechy et al. 2012).

Genetic alteration data regarding other non-endometrioid endometrial cancers are scarce. The mutational landscape of endometrial cancers is heterogeneous and intricate. Although the prevalence of specific somatic genetic changes varies between endometrioid and serous carcinomas, no mutations among the studied genes have been exclusively associated with either tumor type (Fig. 6) (Weigelt and Banerjee 2012).

Common genetic alteration	type I	type II
PTEN mutation	52 - 78%	1 - 11%
PIK3CA mutation	36 - 52%	24 - 42%
PIK3R1 mutation	21 - 43%	0 - 12%
KRAS mutation	15- 43%	2 - 8%
ARID1A mutation	25 - 48%	6 - 11%
CTNNB1 mutation	23 - 24%	0 - 3%
TP53 mutation	9 - 12%	60 - 91%
PPP2R1A mutation	5 - 7%	15 - 43%
HER2 amplification	0	27 - 44%
Microsatellite instability	28 - 40%	0 - 2%

Figure 6. Common alterations of type I and type II endometrial tumors.

1.6.d Genetic classification of endometrial cancer

The Cancer Genome Atlas Research Network (TCGA) has presented a comprehensive transcriptomic and genomic analysis of endometrial cancers. For this study, a large collection of endometrial cancers was used assessing advanced next-generation sequencing technologies, together with reverse-phase protein array, analyses of microsatellite instability and DNA methylation (Cancer Genome Atlas

Research Network et al. 2013). The samples used represented the main histological types, and in particular were utilized 307 endometrioid, 53 serous, and 13 mixed endometrioid and serous carcinomas. This comprehensive analysis allowed sorted the endometrial cancer in four distinct genomic categories: POLE ultra-mutated tumors, copy number low and copy number high groups and microsatellite instable. More in details, the POLE ultra-mutated group are tumors that present mutation in the DNA Polymerase ε. This polymerase is involved in the DNA replication and, in this group of tumors, present hotspot mutation in its proofreading domain and overall display an elevated mutation rate. Next, the copy number low group mainly gather grade 1 or 2 endometrioid tumors. Those tumors in general have low mutation rates and the characteristic to be microsatellite stable. Additionally, they present frequently mutations in CTNNB1 (Church et al. 2013). Then, the copy number high collect serous-like tumors that share the feature of having low mutations rates and a wide rete of copy number alterations. These tumors present poor outcome and recurring mutations in PPP2R1A and TP53, and less commonly mutations in KRAS and PTEN. Lately, the endometrioid subtype of endometrial cancers are the main histological type involved in the microsatellite-instable group. Those tumors present high mutation rates and often have MLH1 promoter methylation that is the cause of the microsatellite instability. In addition, other recurrent alterations responsible of the microsatellite-instability identified by the analysis are PTEN and KRAS mutations and frameshift deletion in RPL22 (Cancer Genome Atlas Research Network et al. 2013; Gilks, Oliva, and Soslow 2013; Soslow 2013).

1.6.e Moving toward a unified classification for endometrial cancer

Due to the noticeable diversity of endometrial cancers in histology, morphology, genetic attributes, and molecular features the current classification may appear confuse and individually can’t properly define endometrial cancers. Therefore, the classification system needs a refinement. The scientific community is moving toward an integration of the current classification systems with the aim of unify them in a unique classification method that consider altogether the clinicopathological, histological, genetic, and molecular alteration. The ultimate goal achieved by fusing all

these features is to provide a more potent and precise prognostic tool. Nonetheless, the establishment of these connections requires further exploration and additional validation (Rajmohan Murali, Soslow, and Weigelt 2014).

1.7 Endometrial cancer metastasis

A crucial and well recognized prognostic factor used to diagnose the progression of the endometrial cancer invasion is the degree of myometrial invasion. In endometrial cancer patients, the myometrium is analyzed to identify the thickness infiltrated by the carcinoma. Based on the severity of the infiltration, the myometrial invasion is classified in three groups: none, when the myometrium results clean; less than 50% and equal or more than 50% based on the dimension and spreading of the cancer from the endometrium layer. (Lindauer et al. 2003; Schwab et al. 2009; Chattopadhyay et al. 2012). Moreover, the FIGO subdivide the endometrial cancer in the following four stages: stage I when the tumor is confined in the uterine corpus; stage II when the tumor invades the cervical stroma without extrauterine extension or with substantial lymph vascular space involvement or present aggressive histological types with myometrial invasion; stage III when there a local or regional spreading is present in any histological subtypes; stage IV when the tumor spread to the bladder or intestinal mucosa or present distant metastasis (Berek et al. 2023).

The metastatization cascade involves several distinct steps: early loss of cellular adhesion, intensified motility and invasiveness, entrance and survival in circulation, exit into new tissue, and final establishment at a distant site (Berek et al. 2023).

An increasing number of publications characterized metastatic cancer molecular profiles and revealing candidate genes whose expression in the primary tumor potentially correlate with high probability of offset of metastasis (Weigelt, Peterse, and van't Veer 2005).

As first step of the metastatic process, the cancer cells reduced the intercellular adhesion (Cavallaro and Christofori 2004). Frequently this step is mediated by E-cadherin which expression may be suppressed during the cancer reprogramming process defined as epithelial-to-mesenchymal transition (EMT). During the EMT, the cancer cells can activate the expression of some transcription factors such as Twist,

Snail and Slug. Interestingly, many of these transcription factor have important functions in EMT of the embryonic development (Guo and Giancotti 2004).

In endometrial cancer cells it has been noticed that the upregulation of BMI-1 contributes to the development of the invasive behavior derived by the EMT and the following downregulation of BMI-1 reduce the cancer invasion by reverting the EMT (Dong et al. 2011).

Moreover, another well factor involved in the EMT of many cancers is TGFb and in particular for endometrial cancer, studies that compared the risk of recurrence of endometrial cancer highlighted the predominant role of TGFb and its signaling pathway in the determination of an aggressive behavior. Moreover, additional publications validated and confirmed the promotion of the EMT by TGFb is an early and key event in the initialization of the invasive phenotype (Massagué 2008; Lei et al. 2009; Muinelo-Romay et al. 2011).

As part of the initial step of the metastatic cascade, the cancer cell gains the ability of proteolytically digest the basement membranes, that are mainly composed of proteoglycans and glycoproteins such us laminin and type IV collagen. To fulfill this action the cancer cells specifically use and regulate extracellular matrix proteases (Liotta and Kohn 2001; Egeblad and Werb 2002).

In endometrial cancer, some of those extracellular matrix proteinases, are found to be overexpressed, in particular, MMP11, 23, 24 and 28. And the expression of that category of proteinase has been linked to the progression of the endometrial cancer (Schröpfer et al. 2010).

Moreover, it has been reported that in endometrial cancer the expression of another protease, MMP2, correlated with high risk of development of local and distant metastasis and therefore MMP2 could be a potential marker (Graesslin et al. 2006). As further confirmation, high concentration of MMP2 as well as high concentration of MMP9 were detected in lymph node invaded by endometrial cancer metastasis. (Honkavuori et al. 2008).

During the progression of the metastatic process the cancer cells face some challenges since they require new vascularization and enter in contact with the immune system cells that may infiltrate the tumor mass itself (Liotta and Kohn 2001). In patient diagnosed with endometrial cancer, the vascular epithelia growth factor A (VEGF-A) has been found upregulated in many patients and that overexpression correlates with poor prognosis (Doldi et al. 1996). Additional studies reported that, to

better identify high risk endometrial cancer patient a prognostic marker for the myometrial invasion could be the expression of VEGF-C or VEGF-D (Hirai et al. 2001; Yokoyama et al. 2003). Regarding the relationship with the immune cell infiltrating the tumors, those cells my collaborate or hamper the tumors mass progression. The myometrial invasion has been seen to be associate to the T cell suppressor indoleamine 2,3-dioxygenase (IDO) as well as the involvement of the lymph vascular space and the presence of metastasis in lymph node (Ino et al. 2008). Additionally, high expression of IDO correlated with a reduction of natural killer cell and $CD8^+$ TIL and was associated with poor survival (de Jong et al. 2012).

Following the metastatic cascade, the cancer cells must infiltrate the blood vessel in order to be able to invade distant locations of the organism via the circulatory system, and this process is named intravasation. Next, via a process called extravasation, the cancer cells complete the action to escape from the blood vessel and to infiltrate into the target tissue (Alitalo, Tammela, and Petrova 2005). The challenge the tumors cells must face one they get in the distant location is to establish a premetastatic niche in the new organ. As following step the newly metastatic cells have to exhibit ability to successfully growth in the new location (Al-Mehdi et al. 2000). In endometrial cancer it has been observed that the CXCR4-CXCL12 axis performs an important function during the promotion and the process of extravasation. Moreover, the CXCR4-CXCL12 axis plays a significant role in the establishment of metastasis. Interestingly, using monoclonal antibody against CXCR4 to neutralize its function dramatically reduced the number and the size of metastases in *in vivo* models (Gelmini et al. 2009).

1.8 Epigenetic alteration in ovarian and endometrial cancers

Genetic alterations have been considered the main cause of cancer for a long time, but the epigenetics changes are gaining and increasingly acknowledged in the context of the development and progression of tumors. The epigenetics can be defined as the study that investigate and explore the heritable changes in gene function, changes that occur without the involvement of modification in the DNA sequence. There are

different epigenetic mechanisms, such as nucleosome repositioning, comprehend DNA methylation, post-translational modifications of histones, and the post-transcriptional regulation of genes through miRNAs (Berger et al. 2009).

In the past years, a set of cancer-associated genes are identified as influenced by epigenetic modifications, and the resulting regulation emerged to have a pivotal contributors during the initiation and progression of malignant tumors, including ovarian and endometrial cancers (Natanzon, Goode, and Cunningham 2018; Inoue et al. 2021).

Most of the epigenetic changes involved the chromatin. The chromatin in composed of nucleosomes, that are formed by an octameric core wrapped by a 145-146 base pair peace of DNA. The octamer by two copies of the histone proteins H2A, H2B, H3, and H4, and the histone H1 linked together two nucleosomes. This link serves to organize the nucleosomes and ultimately the chromatin in a more complex chromatin structures (Becker and Workman 2013). The histones are characterized by the present of amino-terminal histone tails that have the flexibility to be editable, and the resulting modification directly influence the structure of the chromatin and the gene expression. The epigenetic changes are mediated by a group of protein that collaborate with each other and they are defined as writers, erasers, and readers. The writers are enzyme with the ability to add chemical groups to both histone tails and DNA sequence. On the other hand, the erasers have the enzymatic function to remove chemicals groups from DNA and histone tails. Lastly, the readers are a group of protein that present various motifs that enable them to recognize specific modifications in histones and DNA and next recruiting and interacting with additional remodeling factors and chromatin modifiers. The sum of these processes, known as chromatin remodeling, result in the modification of the structure of the chromatin. Ultimately, the chromatin can transit between two states: euchromatin, that is an open and accessible state of the chromatin; heterochromatin, that define a clone and compact version of the chromatin. This way, the euchromatin promote a better accessibility of DNA to other chromatin regulators and transcription factor (Kanwal and Gupta 2012; Dawson and Kouzarides 2012; Cheng et al. 2019).

1.8.a DNA methylation

One of the most extensively investigated epigenetic mechanism is represented by the DNA methylation. This mechanism required the action of a group of enzymes known as DNA methyltransferases (DNMTs), which include five members: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. The methylation events mainly occur within DNA regions called CpG islands. The CpG islands have the characteristic to show a cytosine followed by a guanine. It is interesting to notice that roughly 70% of CpG islands are located in the promoter of genes in the human genome. The methylation of the CpG sites result in the suppression of gene transcription and therefore this epigenetic mechanism is a main actor in the context of cancer development and progression (Klutstein et al. 2016; Pan et al. 2018).

In the past years, in ovarian cancer, a considerable list of genes has be found to be subject to hypermethylation, that ends in silencing of their expression. Whitin that list, BRCA is one of the most extensively studied gene. The loss of BRCA1 in epithelial ovarian cancers may be caused by the hypermethylation of BRCA1. Moreover, as further confirmation of the fundamental role of BRCA1 in ovarian cancer, its silencing is associated with high-grade tumors (Baldwin et al. 2000; Wilcox et al. 2005; Wiley et al. 2006).

DNA methylation is also an epigenetic modification widely explored in endometrial cancer. DNMT1 and DNMT3B are often upregulated in type I endometrial cancer. Thus, the hypermethylation of gene promoters, such as PTEN and MLH1, mainly occur in this type of endometrial cancer (X. C. Zhou et al. 2007). On the other side, in type II endometrial there is an increased genomic instability and a global hypomethylation, that may be partially responsible to the clinical and histological difference between these two types of endometrial tumors (Stampoliou, Arapantoni-Dadioti, and Pavlakis 2016). For example, MLH1, that has an important role in abnormal DNA mismatch repair and microsatellite instability, shows promoter hypermethylation and this leads to gene silencing. That promoter hypermethylation is frequently detected in endometrial cancer and present a strong correlation with microsatellite instability (MSI) high group of endometrial cancer. More in details, around 83–98% of MSI-high endometrial cancer exhibit MLH1 promoter hypermethylation (Peterson et al. 2012; Goodfellow et al. 2015).

1.8.b Histon modifications

There are at least eight distinct histone modifications recognized: methylation, acetylation, ubiquitination, glycosylation, phosphorylation, SUMOylating, ADP-ribosylation and carbonylation (Bannister and Kouzarides 2011).

One main histone modification is the acetylation of lysine residues. The regulation of this histone modification is a dynamic process, and it is mainly ruled by two opposing enzymes: histone acetyltransferases (HATs), responsible for adding acetyl groups abolishing the positive charge of lysine and therefore disrupting the electrostatic bond between DNA and histones unfolding the local chromatin in a more accessible status; and histone deacetylases (HDACs), responsible for removing acetyl groups and therefore restoring the positive charge in the lysine residue (Shahbazian and Grunstein 2007). Consequently, in a general context, the HATs are associate to a relaxed chromatin state that facilitate the gene transcription and the HDACs are associated to a condense chromatin status and gene repression. The imbalance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) plays a role in the development of cancers, including ovarian and endometrial cancers. For example, in epithelia ovarian cancer the histone acetylase MOF is frequently downregulated, decreasing the overall level of H4K16 acetylation causing gene instability and it has been reported to be a potential tumor suppressor gene for endometrial cancer (N. Liu et al. 2013; M. Cai et al. 2015; Y. Wu et al. 2019). Moreover, HDAC1,2 and 3 are over-expressed in both ovarian and endometrial cancers tissues and play a critical role in carcinogenesis and are associated with a poor prognosis (Khabele et al. 2007; Weichert et al. 2008).

Another well studied histone modification is the methylation that occurs through the transfer of methyl groups to the arginine or lysine residues in histone tails by histone methyltransferases (HMTs) and the removal by the histone demethylases (HDMs). In particular, the HMT are divided in histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs). The PRMT family is better describe in a next paragraph. Lysine residues can be mono-, di-, or tri-methylated and arginine residues can be mono-, symmetrically or asymmetrically di-methylated. The effect on gene transcription depends on the state of the methylation and may regulate both gene transcription and repression (Dawson and Kouzarides 2012; Cheng et al. 2019)]. For examples, the methyltransferase EZH2, responsible for the H3K27me3 is

frequently upregulated in ovarian cancer and positively correlate with advanced tumor status, as well it is also overexpressed in endometrial cancer in which correlated with poor prognosis (Q. Li et al. 2017; B. A. Jones, Varambally, and Arend 2018; Krill et al. 2020).

1.8.c Chromatin remodelers

Chromatin remodelers are essential for the dynamic reorganization of nucleosomes, enabling the movement, ejection, or restructuring of nucleosome compositions and they are crucial for a wide range of cellular processes, including transcription, DNA repair, DNA replication, and chromosome segregation. There are four distinct families of chromatin remodeling complexes: SWI/SNF (sucrose non fermenting, that will be described in a following paragraph), ISWI, CHD and INO80 families. All these four families of chromatin remodeler use the hydrolysis of ATP to modify interactions between histones and DNA and possess a common ATPase domain and their dysregulation can lead to various diseases, including cancer (Clapier and Cairns 2009). For examples, mutations in the ATPase SNF2L, component of ISWI complex, have been linked with ovarian clear cell carcinoma tumorigenesis (Itamochi et al. 2017). CHD5 is one of the most studied CHD family proteins associated to cancer and its mRNA expression has been frequently found downregulated in invasive epithelia invasive ovarian carcinomas correlating with shorter disease-free survival time (Bagchi et al. 2007; Wong et al. 2011). Moreover, in endometrial cancer CHD4 mutations have been found to promote the stemness by activating the TGF-beta signaling pathway (Y. Li et al. 2018). The ACT6LA, component of INO80, is often significantly amplified in ovarian cancer and its amplification status is linked to platinum resistance (Xiao, Lin, and Lin 2021).

1.9 PRMT family

The PRMT proteins, as mentioned above, are a family of protein arginine methyltransferases. Collectively, the PRMT proteins are involved in a wide array of

cellular process including gene expression, chromosome organization, protein folding RNA processing, and others. The PRMT proteins have been connected to different diseases including cancers, and since it represents a targetable modification, a lot of effort is being employed in investigating therapeutic potential. There are nine different PRMT proteins that can mono- or di-methylate substrate by using the cofactor SAM, and they are dived in three types (Fig. 5). Type I enzymes can asymmetrically di-methylate the substrates, and this group includes PRMT1, PRMT2, PRMT3, PRMT4 (also called CARM1), PRMT6 and PRMT8. Type II includes PRMT5 and PRMT9 that have symmetrically di-methylase activity. And group III contains PRMT7 that can monomethylate the substrates. Functionally, the PRMT enzymatic activity require SAM as donor and preferentially recognize the RG/RGG motif in the targets (Wolf 2009; Yang and Bedford 2013; Q. Wu et al. 2021).

type	members	methyltransferase activity product
type I	PRMT1	ADMA
	PRMT2	ADMA
	PRMT3	ADMA
	PRMT4 (CARM1)	ADMA
	PRMT6	ADMA
	PRMT8	ADMA
type II	PRMT5	SDMA
	PRMT9	SDMA
type III	PRMT7	MMA

Figure 7. PRMT family members and enzymatic activities. Abbreviations: ADMA = asymmetric di-methylation; SDMA = symmetric di-methylation; MMA = mono methylation.

1.9.a PRMT4 (CARM1)

Coactivator-associated arginine methyltransferase (CARM1) was the first PRMT to be functionally associated to the regulation of gene transcription (D. Chen et al. 1999).

CARM1 can regulate a various number of cellular processes, such as cell cycle progression, DNA damage response and mRNA splicing. CARM1 carries out its functions by methylating different category of targets: transcriptional factors, histones, coregulators, RNA polymerase II and splicing factors. CARM1 can participate to tumorigenesis in different tumors, including ovarian cancer (M. Santos, Hwang, and Bedford 2023). For example, the inhibition of EZH2 has been shown to suppress the growth on ovarian tumor cells that express high level of CARM1, and this is due to the induction of apoptosis and the reactivation of EZH2 target tumors suppressor genes, which expression depend on CARM1 (Karakashev et al. 2018). A following study showed how the inhibition of EZH2 can sensitize epithelial ovarian cancer cells that express high level of CARM1 and that carry proficient homologous recombination, to PARP inhibitors (Hatchi and Livingston 2020).

In addition to the enzymatic activity, CARM1 can also works as co-activator. It has been published a study that report that ovarian cancer cells expressing high level of CARM1 show sensitivity to the inhibition of the IRE1α/XBP1s pathway. IRE1α RNase processes mRNA encoding the transcription factor XBP1, as response to endoplasmic reticulum stress, leading to the translation of its spliced form, XBP1s. XBP1s translocates to the nucleus promoting the transcription of genes involved in the reduction of the endoplasmic reticulum stress (Urra et al. 2016). In this context, CARM1 acts as a coactivator of XBP1s. The inhibition of the IRE1α/XBP1s pathway was effective against ovarian cancer models in CARM1 depend manner (Lin, Liu, et al. 2021). These studies together underling the role of CARM1 as biomarker for ovarian cancer.

1.9.b PRMT5

The protein arginine methyltransferase 5 has complex function in oncogenesis. PRMT5 is known to both control cancer promotion and suppression being oncogene or tumor suppressor. Due to its cytoplasmatic and nuclear localization, PRMT5 has a wide range of targets involved in different cellular processes. PRMT5 is well known to regulate the RNA splicing, participating in the DNA damage response, modulating

transcription factors activity and the gene transcription via methylating arginine residues in histones (Kim and Ronai 2020). In particular, PRMT5 can catalyze methylation of the following arginine residues: H3R8, H4R3, H2AR3 and H3R2 (Branscombe et al. 2001). There is evidence that those modification are either associated with gene expression activation or repression. Moreover, PRMT5 has been also find associated with protein complexes. For examples, PRMT5 can interact with BRG1, a subunit of SWI/SNF complexes (described in paragraph 1.10) and it has been reported that this association contribute to the regulation of gene expression (Pal et al. 2003; 2004; Tae et al. 2011). More in details, the modifications H3R8me2s and H4R3me2s are largely considered to be associated with gene repression but they can also participate to gene transcription activation as it has been shown in the regulation of androgen receptor in prostate cancer. In contrast, H3R2s2me is commonly linked to the activation of the transcription (Zhao et al. 2009; Migliori et al. 2012; Yuan et al. 2012; Deng et al. 2017; R. Liu et al. 2018).

PRMT5 activity is related to different phenotype connected to tumorigenesis, including metastatization. For examples, PRMT5 can regulate can regulate the tumor invasion an migration via regulating E2F pathway (Barczak et al. 2020). Moreover, the PRMT5-MEP50 complex can both activate and repress the gene transcription during cancer invasion in response of TGFb stimuli (H. Chen et al. 2017).

1.10 SWI/SNF complexes

The switch deficient sucrose nonfermenting (SWI/SNF) class of chromatin remodeling complexes utilize ATP hydrolysis to obtain energy for remodeling the chromatin status in to make the genomic DNA accessible for the important cellular processes of gene transcription, DNA replication and repair. The SWI/SNF complexes have the ability to both slide the histone octamer or eject it from the genomic DNA. This ability of change the status of the chromatin make the SWI/SNF complexes crucial components in the cell fate decision and reprogramming in response to external stimuli. Moreover, according with this picture, there are plenty of evidence that mutations in their components are connected to several diseases, including cancers (Bieluszewski et al. 2023).

The SWI/SNF complex is evolutionally conserved across animals, and it is composed of at least 9 subunits that include a core complex, a DNA binding core and variable other subunits required to bind and recruit other factors for the activation or repression of the gene expression (Tang, Nogales, and Ciferri 2010).

There are three types of human SWI/SNF complex defined as canonical BRG1/BRM associated factor complex (cBAF), polybromo containing complex (pBAF) and no-canonical BAF. The core of all these complexes contains the ATPase subunit, responsible for the hydrolysis of the ATP, that can be either BRG1 (SMARCA4) or BRM (SMARCA2). SNF5 and BAF155 represent others common component of the core. Other subunits are present in different configuration such as SMARCC1/2 and SMARCD1/2/3. Additionally, one important difference is the DNA binding subunits. The cBAF complex present two mutually exclusive AT-rich interactive domain proteins called ARID1A (BAF250a) and ARID1B (BAF250b), whereas the pBAF comprises ARID2 (BAF200) (Kadoch and Crabtree 2015).

The scientific community has largely studied the multiple alterations of the SWI/SNF complexes subunits in cancer development highlighting possibility for the establishment of therapeutical interventions. Alterations that occur in multiple SWI/SNF component mutation have been largely reported in gynecological cancers. In particular, mutations in ARID1A are predominant and widely study in both ovarian and endometrial cancers (Roberts and Orkin 2004; Reisman, Glaros, and Thompson 2009).

1.11 ARID1A mutation in ovarian and endometrial cancers

ARID1A gene encodes for the cBAF subunit BAF250a. ARID1A inactivating mutations are frequent in ovarian and endometrial cancer, in particular are mainly detected in endometriosis-associated ovarian cancer such as clear cell carcinoma and endometrioid ovarian cancers, and endometrioid endometrial cancer. Plenty of evidence shown that ARID1A inactivation is an early event during cancer initiation that alone is not sufficient to drive tumorigenesis, additionally mutations like PTEN or PIK3CA alteration collaborate to drive the tumor development (Y. Wang et al. 2020).

Somatic mutation of ARID1A, that result in a complete loss of the protein, are detected in around half of the case of clear cell ovarian cancers and around 30% of endometrioid ovarian cancers. As mentioned above, the mevalonate pathway is a fundamental cellular metabolic process, and a recent publication shows that the inactivation of ARID1A in OCCC create a dependency on the mevalonate pathway for the tumor survival. The authors showed that the inhibition of the mevalonate pathway not only suppress the tumor growth but also boosted antitumor immunity promoting pyroptosis in ARID1A deficient cells but not in the wildtype counterpart (S. Jones et al. 2010; Wiegand et al. 2010; Ayhan et al. 2012; Yamamoto et al. 2012; Anglesio et al. 2017; W. Zhou et al. 2023).

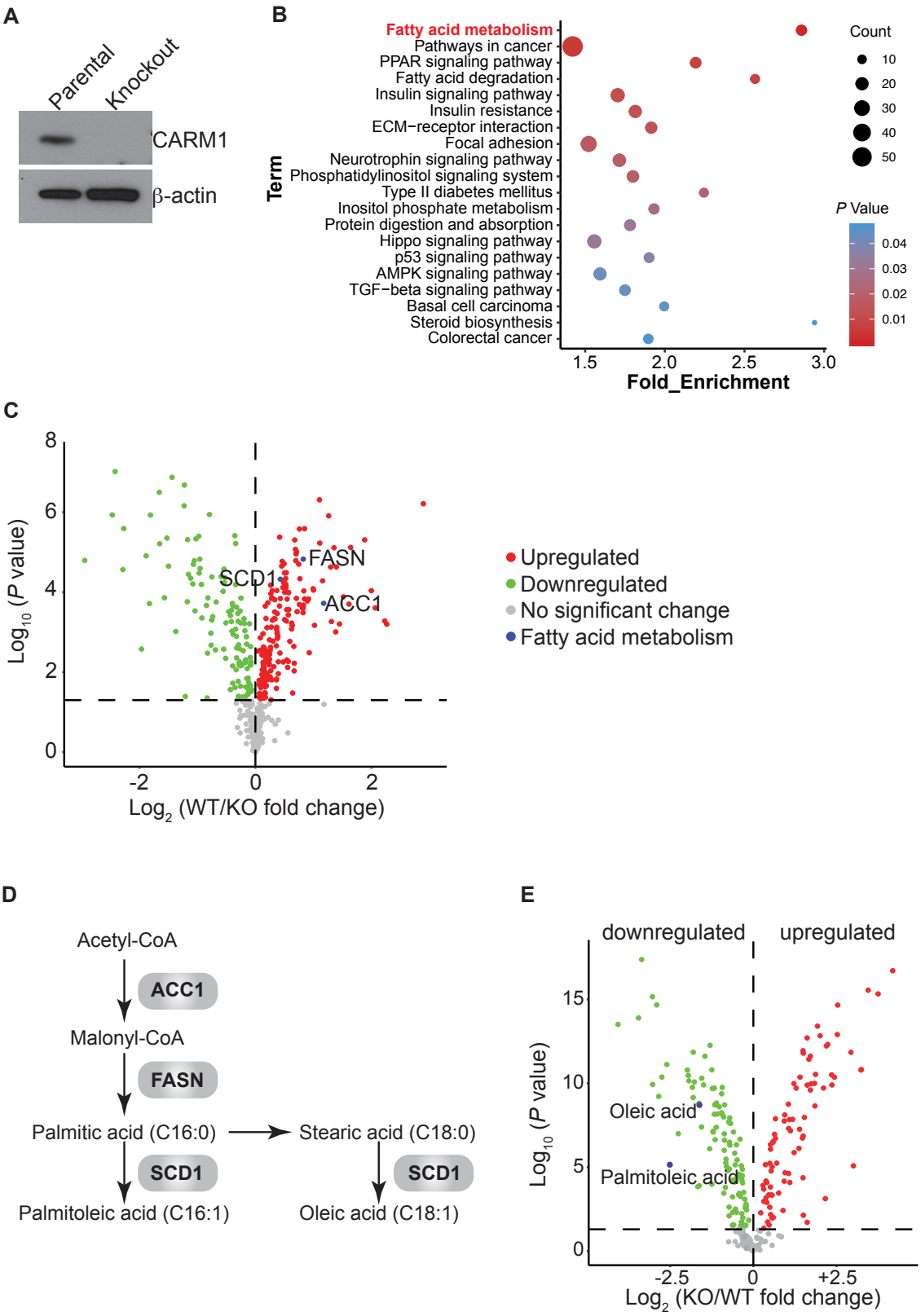
Focusing on endometrial cancer, inactivating mutation of ARID1A are detected in all types of endometrial cancer with the high frequency in low and high grade endometrioid endometrial cancer that account alteration in 29% and 39% of the case respectively. Co-occurrence of PTEN or PIK3CA mutation are observed in endometrial cancer that carry ARID1A inactivating mutations. It has been recently published by two different groups that the inactivation of ARID1A can drive the endometrial cancer invasion when associated with PTEN mutation or PIK3CA mutation (Wilson et al. 2019; Suryo Rahmanto et al. 2020). More in details, Shih and collaborators identify that ARID1A deficiency led to loss of the tumor suppressing function of TGFb and this inactivation of the ARID1A/TGFb axis favor the promotion of migrative and invasive phenotype in endometrial cancer cell carrying PTEN deletion. Moreover, Chandler and collaborators, shown that ARID1A loss increase the chromatin accessibility in gene belonging to the epithelia to mesenchymal transition pathway and therefore promoting their expression. The authors shows that the PIK3 activation partially rescue this phenotype driven by ARID1A and the co-occurrence of PIK3 inactivation collectively promote the EMT transition promoting the tumor invasion (Wiegand et al. 2011; Guan et al. 2011; Fadare, Renshaw, and Liang 2012; Mao et al. 2013; Werner et al. 2013; DeLair et al. 2017; Yen et al. 2018).

2. Results part I

2.1 Preliminary data and aim of the project

CARM1 is an arginine methyltransferase that is involved in different pathway but its role in Ovarian Cancer tumorigenesis has not been fully understood and clinical data show that CARM1 high expression is a frequent alteration in the High-Grade Serous subtype of Ovarian Cancer (Karakashev et al. 2018). To systematically explore the role of CARM1 in HGSOC, we analyzed previously published RNA-seq data (Karakashev et al. 2018) from CARM1 wt and CAMR1 knock out A1847 cells (Fig. 8A). The KEGG analysis conducted from the RNA-seq data shows that the Fatty Acid metabolism is the top one pathway regulated by CARM1 in this cellular model (Fig. 8B). To further confirm this result, we investigated the expression change at the protein level. For this aim, we performed the reverse phase protein array (RPPA), in A1847 wt and CARM1 KO cells. Consistently with the RNA seq data, the RPPA showed a downregulation of the protein levels of the key enzyme of the fatty acid metabolic pathway SCD1, FASN and ACC1 by CARM1 KO (Fig. 8C,D). Similar result has been obtained with another HGSOC cell model PEO4 wt and CARM1 KO (data not shown, reference Lombardi et al. 2023).

Additionally, to explore the change at a metabolic by CARM1 KO, the global metabolites analysis has been performed in A1847 wt and CARM1 KO. Interestingly, monounsaturated fatty acid such as oleic and palmitoleic acid, products of SCD1, are downregulated by CARM1KO (Fig. 8E). These preliminary results suggested that CARM1 promotes the production of the MUFA first promoting the *de novo* FA synthesis via ACC1 and FASN and the next conversion in MUFA through SCD1. Then, a more detailed fatty acid profiles have been performed in A1847 revealed that monounsaturated palmitoleic and oleic acid are significantly downregulated both upon SCD1 inhibition, consistently with the function of SCD1, in CARM1 KO (Fig. 8F). The FA are the building components of the lipid, and we investigated the lipid species composition performing a lipid profile. The lipid profile in A1847 cells showed that the ratio between monounsaturated and saturated fatty acid 18:1/18:0 decreased in CARM1 KO cells (Fig. 8G), consistent with the upregulation of MUFA by CARM1, their


A
Parental
Knockout
CARM1
β-actin
B
Fatty acid metabolism
Pathways in cancer
PPAR signaling pathway
Fatty acid degradation
Insulin signaling pathway
Insulin resistance
ECM−receptor interaction
Focal adhesion
Neurotrophin signaling pathway
Phosphatidylinositol signaling system
Type II diabetes mellitus
Inositol phosphate metabolism
Protein digestion and absorption
Hippo signaling pathway
p53 signaling pathway
AMPK signaling pathway
TGF−beta signaling pathway
Basal cell carcinoma
Steroid biosynthesis
Colorectal cancer
Term
1.5
2.0
2.5
3.0
Fold_Enrichment
Count
10
20
30
40
50
P Value
0.04
0.03
0.02
0.01
C
Log10 (P value)
Log2 (WT/KO fold change)
FASN
SCD1
ACC1
Upregulated
Downregulated
No significant change
Fatty acid metabolism
D
Acetyl-CoA
ACC1
Malonyl-CoA
FASN
Palmitic acid (C16:0)
Stearic acid (C18:0)
SCD1
SCD1
Palmitoleic acid (C16:1)
Oleic acid (C18:1)
E
downregulated
upregulated
Log10 (P value)
Log2 (KO/WT fold change)
Oleic acid
Palmitoleic acid

F

Compound Name	Relative nomalized peak area: WT 1	WT 2	WT 3	WT+SCDi 1	WT+SCDi 2	WT+SCDi 3	CARM1 KO 1	CARM1 KO 2	CARM1 KO 3	(WT+SCDi) / WT FC	(WT+SCDi) / WT *P*	(CARM1 KO) / WT FC	(CARM1 KO) / WT *P*
Palmitoleic Acid-16:1										-1.71	0.0025	-1.69	0.0009
Oleic Acid-18:1										-1.59	0.0093	-1.46	0.0104
Nervonic Acid-24:1										-1.44	0.0139	-2.18	0.0005
Adrenic Acid-22:4										-1.36	0.0057	-1.24	0.0025
Lauric Acid-12:0										-1.32	0.0100	-1.66	0.0015
Palmitic Acid-16:0										-1.29	0.0519	-1.31	0.0175
Myristic Acid-14:0										-1.24	0.0672	-2.23	0.0001
Arachidonic Acid-20:4										-1.17	0.1152	-1.50	0.0019
Docosapentaenoic Acid-22:5										-1.16	0.1724	-2.08	0.0001
Docosahexaenoic Acid-22:6										-1.14	0.1836	-1.15	0.0543
Lignoceric Acid-24:0										-1.12	0.5102	1.10	0.5703
Dihomo-gamma-Linolenic Acid-20:3										-1.07	0.5450	-1.54	0.0005
Eicosapentaenoic Acid-20:5										-1.01	0.9647	-1.97	0.0036
Linoleic Acid-18:2										1.00	0.9852	-1.55	0.0010
Stearic Acid-18:0										1.20	0.1259	-1.19	0.1271
Arachidic Acid-20:0										1.27	0.1178	1.27	0.0450
Linolenic Acid-18:3-first_peak										1.84	0.0072	-1.14	0.2676
Stearidonic Acid-18:4										2.01	0.0042	-3.67	0.0000

G

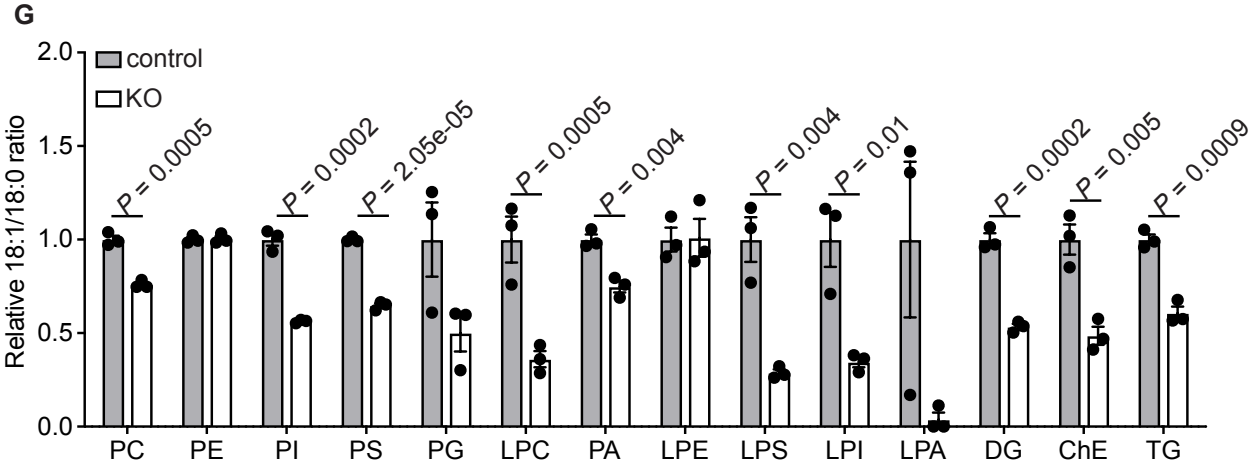


Fig 8. CARM1 may promotes fatty acid metabolism. A Immunoblot that shows the expression of CARM1 and a loading control β-actin in control and CARM1 knockout A1847 cells. **B** Scatter plot generated to visualize the KEGG pathway analysis results for genes that were upregulated in wildtype (WT) compared to CARM1 knockout (KO) A1847 cells, fold change greater than 2 and a false discovery rate (FDR) lower than 0.05. The dot size in the scatter plot represents the number of genes, and the color of the dots represents the corresponding P value. The P values were calculated through KEGG analysis. **C** Volcano plot generated to visualize the protein expression detected by Reverse Phase Protein Array (RPPA) in wildtype and CARM1 knockout (KO) A1847 cell lysates. Red dots

represent proteins that were significantly upregulated in wildtype cells, while green dots represent proteins that were significantly upregulated in knockout cells. The proteins SCD1, FASN, and ACC1 were highlighted in blue. The statistical significance was determined using a two-tailed Student t-test, and the corresponding P values were used for the plot. **D** Schematic representation of the monounsaturated fatty acid synthesis pathway. **E** Volcano plot showing changes of metabolite levels in wildtype or CARM1 KO A1847 cells detected by LC/MS. Significantly upregulated metabolites are indicated in red dots and downregulated in green. In blue were highlighted the free oleic acid, palmitoleic acid, palmitic acid, and stearic acid (P value calculated using a two-tailed Student t test). **F** Fatty acid profile that shows modification in fatty acid composition upon SCD1 inhibitor treatment or vehicle or CARM1 KO in A1847 cells. **G** Ratio between total oleic acid (18:1) and stearic acid (18:0) in various lipid species determined by lipid profiling in wildtype and CARM1 KO A1847 cells (lipid names see reference Lombardi et al. 2023). Data represent mean ± SEM, n = 3 biologically independent experiments (P value calculated using a two-tailed Student t test)

incorporation in lipid is decreased in CARM1 KO. A similar trend has been obtained for 16:1/16:0 ratio (data not shown, reference Lombardi et al. 2023).

These preliminary data show that CARM1 may regulate the fatty acid metabolism in HGSOC and this pathway could represent a new vulnerability for CARM1 high HGSOC. The aim of this project is to target the fatty acid pathway in order to selectively kill the CARM1-high expressing cells.

2.2 CARM1 regulates fatty acid metabolic pathway

On the base of the preliminary data, we want to further validate if CARM1 may regulate the Fatty Acid metabolic pathway. We employed the same cellular system as in the preliminary analysis, the HGSOC isogenic A1847 gctrl or CARM1 KO cell lines, and we analyzed the expression of the key enzymes in the FA pathway. Briefly, the fatty acids are produced from the Acetyl-CoA that is converted in Malonyl-CoA by the ACC1 enzyme. Seven Malonyl-Coa are condensed by the FASN enzyme in palmitic acid that can be extended in stearic acid. Those saturated fatty acid are ultimately converted in monounsaturated fatty acid by the SCD1 enzyme. We validated the downregulation detected by the RNAseq and RPPA of these enzymes at both RNA and protein levels. (Fig. 9A-D). Similar results have been obtained with the isogenic PEO4 gctrl and CARM1 KO cell line (data not shown, reference Lombardi et al, 2023). Consistently, the overexpression of CARM1 in the CARM1-low CAOV3 cell line, increased the expression of ACC1, FASN and SCD1 (Fig. 9E). Additionally, from clinal samples in the HGSOC TGCA dataset there is a positive correlation between the CARM1 expression and SCD1, FASN and ACC1 expressions (data not shown, reference Lombardi et al, 2023). Hence, CARM1 promotes the expression of the key enzymes SCD1, FASN and ACC1 regulating the FA metabolism pathway.

To mechanistically comprehend in what way CARM1 regulates the FA metabolism genes, we performed ChIP seq analysis in A1847 gctrl and CARM1 KO cells. Interestingly, CARM1 was associated with the promoter of SCD1 (Fig. 10A), FASN and ACC1 (data not shown, reference Lombardi et al, 2023) suggesting a direct regulation. CARM1 is an arginine methyltransferase that can asymmetrically di-methylates the arginine residue 17 in histone 3 and therefore modulate the gene expression. Consistently with the CARM1 ChiP-seq track, the H3R17me2a ChIP-seq shows an overlapping distribution of that histone modification and CARM1 association on SCD1, FASN and ACC1 promoters (Fig. 10A and other data not shown, reference Lombardi et al). We validated these results with ChIP qPCR, and consistently with the ChIP seq, CARM1 KO significantly decreases the association of CARM1 and

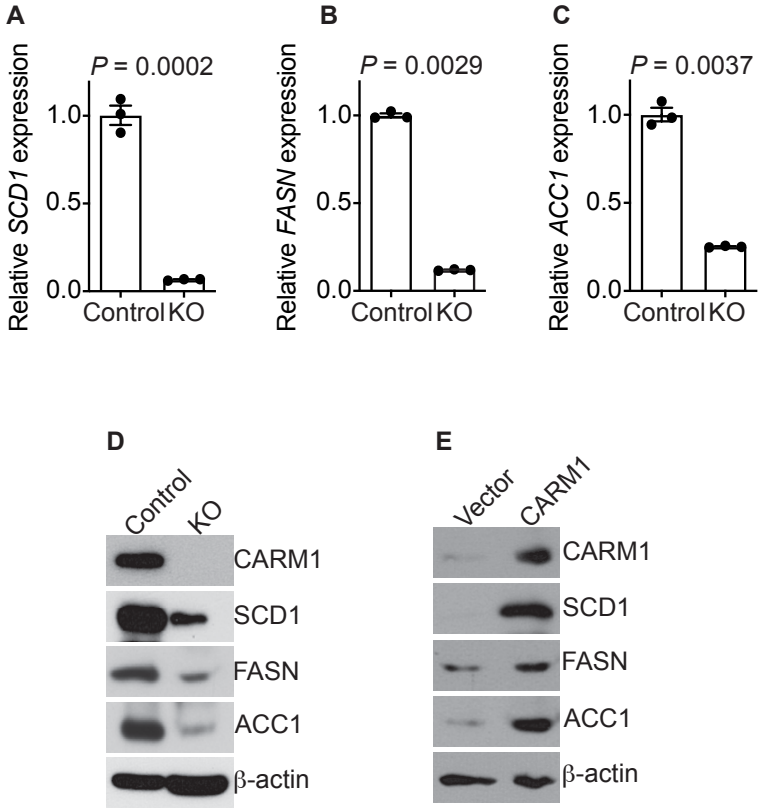


Figure 9. CARM1 regulates FA metabolism gene pathway. A-C qRT-PCR analysis showing mRNA levels of *ACC1* **(A)**, *FASN* **(B)**, and *SCD1* **(C)** in control and CARM1 KO A1847 (*P* value was calculated using a two-tailed Student *t* test, data represent mean ± SEM, *n* = 3). **D** Immunoblot that shows protein expression of SCD1, FASN, ACC1 and the loading control β-actin in control and CARM1 KO A1847 cells. **E** same as **D** but related to CAOV3 wt and CARM1-overexpression CAOV3.

H3R17me2s in SCD1, FASN and ACC1 promoters (Fig. 10B,C and other data not shown, reference Lombardi et al).

We therefore tested the hypothesis that CARM1 may regulate the gene expression through its enzymatic activity. We treated CARM1 expressing A1847 cell with EZM2303, a specific CARM1 inhibitor that blocks its enzymatic activity. Interestingly, we did not observe any changes in the SCD1, FASN and ACC1 expression level in cells treated with the CARM1 inhibitor (data not shown, reference Lombardi et al

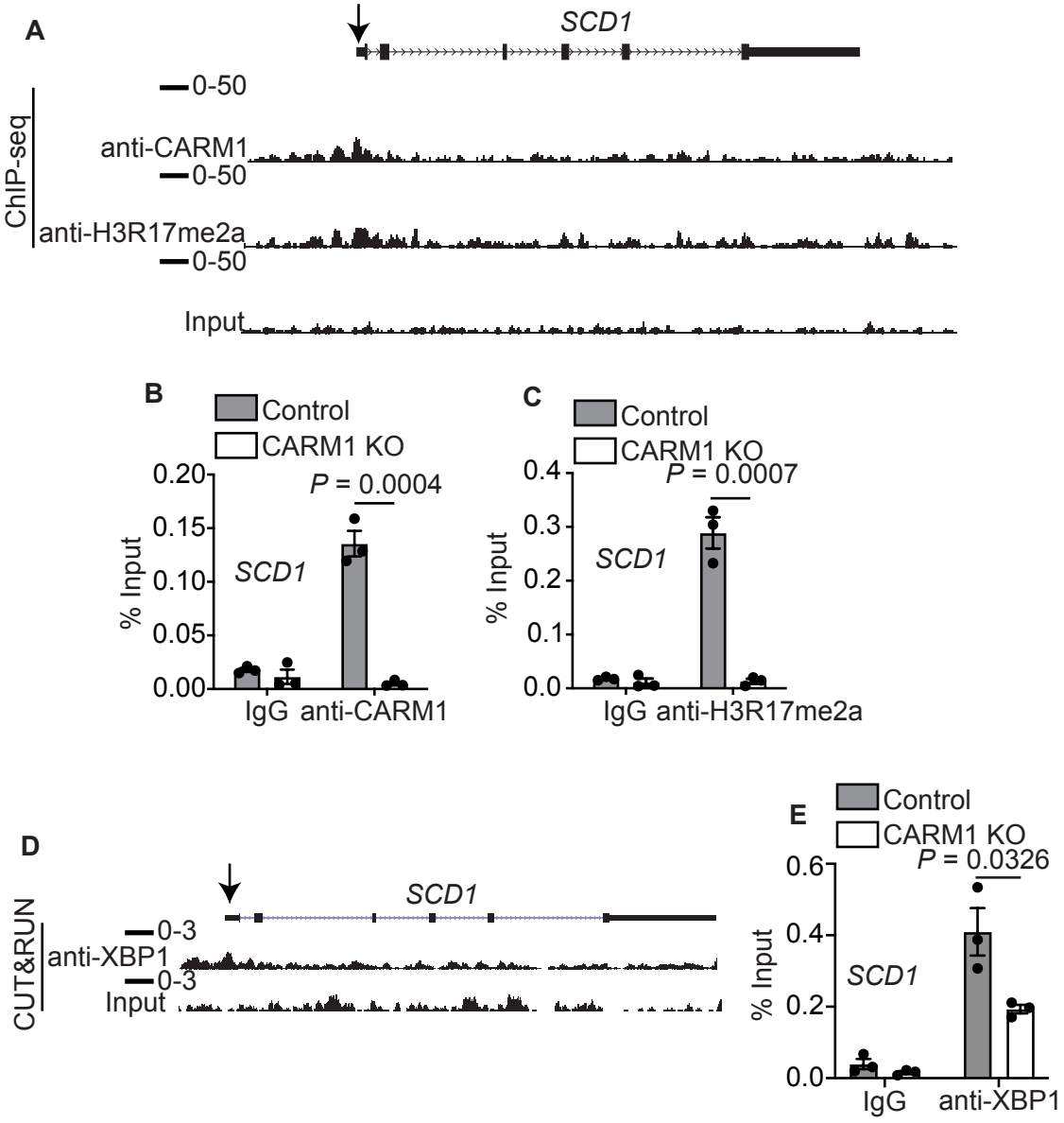


Figure 10. CARM1 enhances SCD1 expression independently of its enzymatic activity and by recruiting XBP1s to its promoter. **A** ChIP-seq track of CARM1 and H3R17me2a in the *SCD1* promoter, the CARM1 and H3R17me2a peaks are indicated by the arrow. **B** ChIP-qPCR analysis showing the binding of CARM1 and H3r17me2a or negative control IgG at the *SCD1* promoter in control and CARM1 KO A1847 cells. **C** CUT&RUN track of XBP1s in the *SCD1* promoter, the arrow indicates the XBP1s peak. **D,** ChIP-qPCR analysis showing the binding of XBP1s or negative control IgG at the *SCD1* promoter in control and CARM1 KO A1847 cells (*P* value calculated using a two-tailed Student *t* test, data represent mean ± SEM, *n* = 3 biologically independent experiments).

2023). Since the inhibitor fail to modify the expression of those key enzyme, we excluded the involvement of CARM1enzymatic activity in their regulation.

It is known that CARM1 can function as cofactor, and it has been published by Lin in 2021 that CARM1 can recruit the transcription factor XBP1s (Lin, Liu, et al. 2021). From this reference, the XBP1s Cut&Run seq shows that XBP1s directly binds the SCD1, FASN and ACC1 promoters (Fig. 10D and other data not shown, reference Lombardi 2023 and Lin). We validated this result with ChIP-qPCR analysis (Fig. 10E and other data not shown, reference Lombardi et al). The data support the hypothesis that CARM1 works as coactivator that recruit XBP1s to regulates the fatty acid metabolism gene.

2.3 The pharmaceutical inhibition of SCD1 is selective against CARM1 expression

CARM1 first regulates the *de novo* saturated FA synthesis and then the further conversion in MUFA by upregulating SCD1. This ultimate step mediated by SCD1 avoid the accumulation of the saturated FA in the cells that it is known to be lipotoxic. To exploit this phenomenon, we treat CARM1-expressing and CARM1 KO A1847 cells with the CAY10566, a selective SCD1 inhibitor to test whether blocking SCD1 may block the proliferation of CARM1 expressing cells. As expected, CARM1-expressing cells are more sensitive to the inhibitor (Fig. 11A,C), consistent with the upregulated production of saturated FA observed in the Fatty Acid Profile (Fig. 8F) and supported by the enhanced regulation of the ACC1 and FASN enzymes by CARM1. Similar results have been obtained with the PEO4 gctrl and CARM1 KO cell line (data not shown, reference Lombardi et al, 2023). Inversely, the overexpression of CARM1 in CAOV3 cells, sensitize these cells to SCD1 inhibitor (Fig. 11B, D). Consistently, using a panel of ovarian cancer cell line which known CARM1 expression level, the SCD1 inhibitor sensitivity was lower in CARM1-high cells then

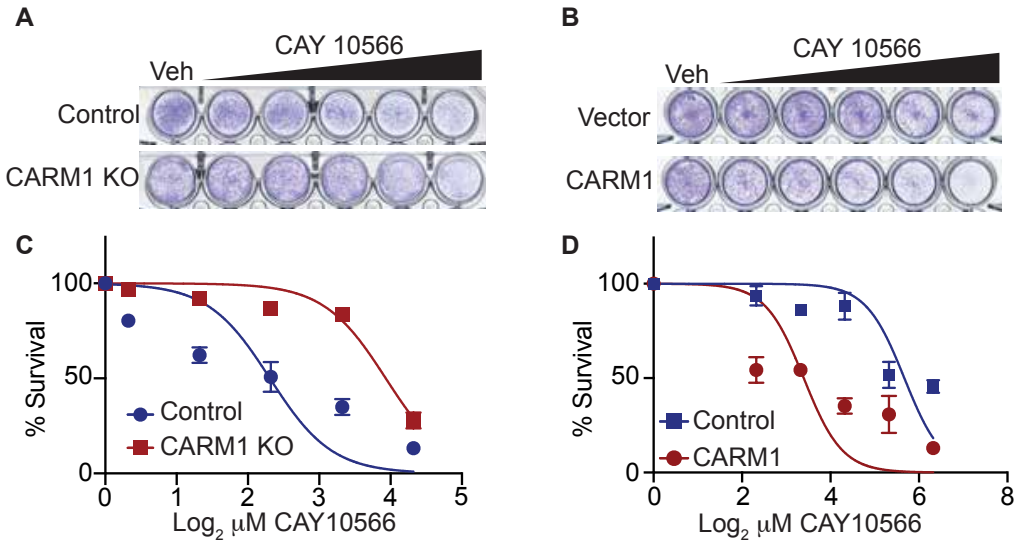


Figure 11. CARM1 expression sensitizes cells to SCD1 inhibition. **A** Sensitivity to SCD1 inhibitor CAY10566 of control and CARM1 KO A1847 cells determined by colony formation assay. **B** same as **A** but related to control and CARM1-overexpressing cells. **C,D** Quantified dose response curves of **A** and **B** respectively.

the CARM1-low cells (data not shown, Lombardi 2023). Therefore, these data indicated a correlation between CARM1 expression status and the sensitivity to SCD1 inhibitor.

Based on recent study, the accumulation of saturated fatty acid contributes to the effect of SCD1 inhibitor in suppressive the tumor growth. The accumulation of saturated fatty acid is lipotoxic for the cells. We therefore tested whether administer the saturated palmitic acid could affect the cell proliferation. As expected, CARM1 KO decreases the sensitivity to palmitic acid consistent with the downregulation of SCD1 by CARM1 KO (Fig. 12 A,C). Similar results have been obtained with the isogenic cell line PEO4 gctrl and PEO4 CARM1 KO (data not shown, reference Lombardi et al, 2023). Contrarywise, the overexpression of CARM1 in CAOV3 cells, increases the tolerance of the administration of palmitic acid (fig B,D), consistent with the increased expression of SCD1 by the overexpression of CARM1 (Fig. 12B,D).

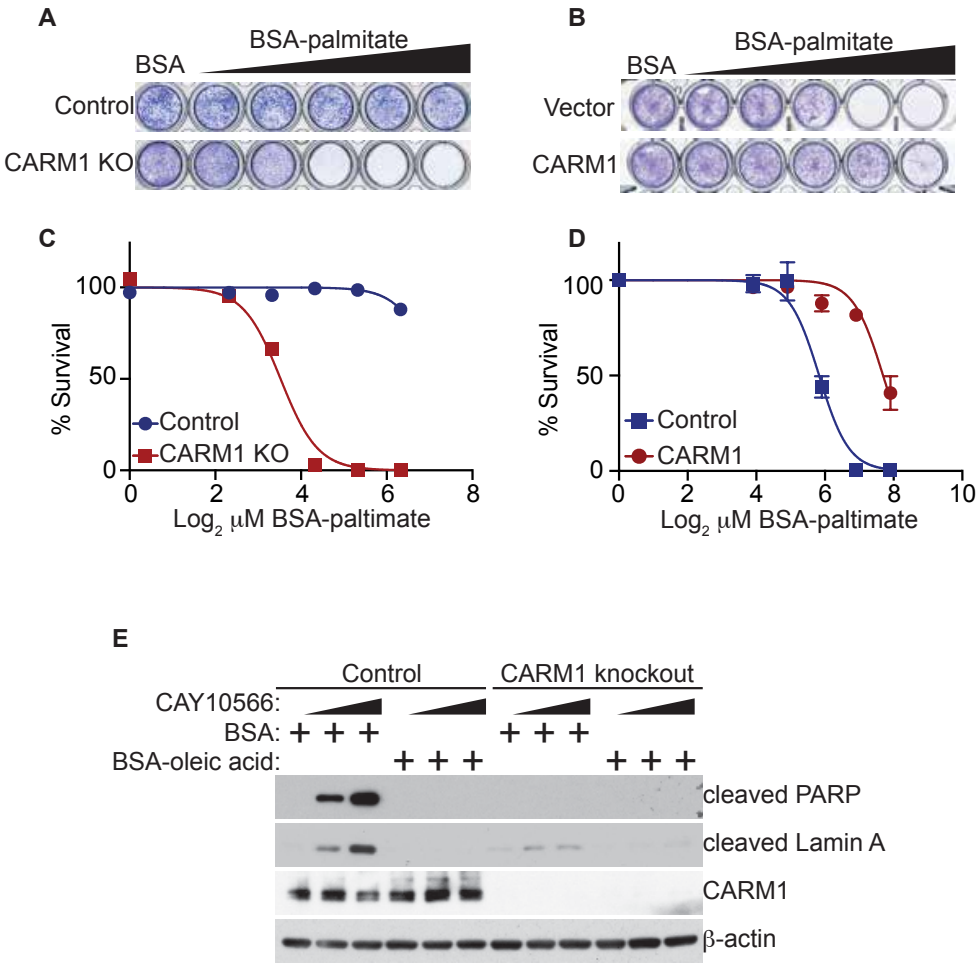


Figure 12. CARM1 KO sensitizes cells to saturated FA palmitate. **A** Sensitivity to BSA conjugated palmitate FA of control and CARM1 KO A1847 cells determined by colony formation assay which was quantified as dose–response curves. **B** Same as **A** but related to control and CARM1-overexpressing CAVO3 cells. **C,D** Quantified dose response curves of **A** and **B** respectively. **E** Immunoblot showing the protein expression of cleaved PARP, cleaved Lamin A, CARM1 and a loading control β-actin in control and CARM1 KO A1847 cells treated with or without SCD1 inhibitor CAY10566 and supplemented with BSA conjugated oleic acid (1 mg/mL) or BSA control (data represent mean ± SEM, *n* = 3 biologically independent experiments).

We then treated A1847 gctrl and CARM1 KO cells with the MUFA oleic acid, in combination with or without increasing concentration of SCD1 inhibitor CAY10566. As expected, the SCD1 inhibitor promotes cells death via apoptosis in CARM1 expressing cells and increasing dose of SCD1 inhibitor correlates with increasing protein level of the apoptotic markers cleaved PARP and cleaved lamina A. The supplements of BSA-oleic acid rescue the apoptosis induced by SCD1 inhibition (Fig. 12E). This indicates that the observed effects are due to the effect of SCD1 inhibition activity and the production of MUFA and the balance between saturated and unsaturated FA in the cells.

These data together support the role of CARM1 in promoting the de novo FA synthesis and the MUFA synthesis, first promoting the production of the saturated fatty acid and then the MUFA synthesis by the key enzyme SCD1. The inhibition of SCD1 disrupt this chain production bringing to the accumulation of the intermediate saturated products that cause lipotoxicity and cell death via apoptosis. We consequently concluded that SCD1 inhibition is selective against CARM1 expression status in HGSOC.

2.4 SCD1 inhibition suppresses CARM1-expressing HGSOC *in vivo*

To validate *in vivo* the previous results, we pursued to test whether SCD1 inhibition suppresses the proliferation in vivo in ovarian tumors based on CARM1-status depent manner. For this purpose, we employed an orthotopic xenograft model using the A1847 CARM1 expressing cells that were unilaterally injected in the *bursa sac* of the ovary of female mice. A schematic representation of the experiment design is shown in Fig. 13A. CARM1 expressing wildtype cells were injected in the *bursa sac* and once the tumors were well established after ten days, we administrated either vehicle or SCD1 CAY10566 inhibitor via oral gavage in randomized groups. The CAY10566 treatment significantly decreases the tumor burden in wildtype tumors (Fig. 13B,C) according with the decreased cell proliferation observed in vitro. Moreover, similar results have been obtained with another syngeneic xenograft model in which we used the murine cell lines UPK10 CARM1 expressing cells. Consistently, the SCD1

treatment reduces the tumors burden versus the vehicle treated group in the UPK10 syngeneic model (Fig D,E). In parallel, the same experiment design has been applied in orthotopic or syngeneic xenograft model with A1847 CARM1 KO or UPK10 CARM1 KO cells respectively and the randomized group have been treated accordingly. Consistent with the previous *in vitro* findings, the SCD1 inhibitor does not affect the tumor growth in CARM1 deficient tumors (data not shown, Lombardi 2023).

Examination of internal organs such as liver, kidney and pancreas, did not show alterations in their morphology indicating that the treatment is well tolerated by the organism (data not shown, Lombardi 2023).

We have observed that SCD1 inhibition drive apoptosis in CARM1 expressing cells. We next examined whether the decreased in tumors burden in CARM1 wildtype tumors treated with SCD1 inhibitor is due to apoptosis. Consistently, in consecutive tumor slides a significantly decreased expression of the cell proliferative marker Ki67 and an increased in the apoptotic marker cleaved Caspase 3 are detected upon SCD1 inhibition in CARM1 wildtype tumors (Fig. 13F,G).

As further validation of the in vitro findings, we examined the expression of the FA synthesis pathway ACC1, FASN and SCD1 in CARM1 wildtype and CARM1 KO tumors. Staining in consecutive tumors slide shows a significant lower level of those enzyme in CARM1 KO tumors respect the wildtype tumors and those levels are not affected by SCD1 treatment (data not shown, Lombardi 2023).

Collectively, these data demonstrate that *in vivo*, the inhibition of SCD1 suppresses the growth of high grade serous ovarian cancer with its efficacy being dependent on CARM1 status. Moreover, these *in vivo* observations support that SCD1 inhibition is effective in suppressing the tumor growth of HGSOC trough inhibiting the proliferation and trigging the apoptosis accordingly with the mechanism with defined *in vitro*.

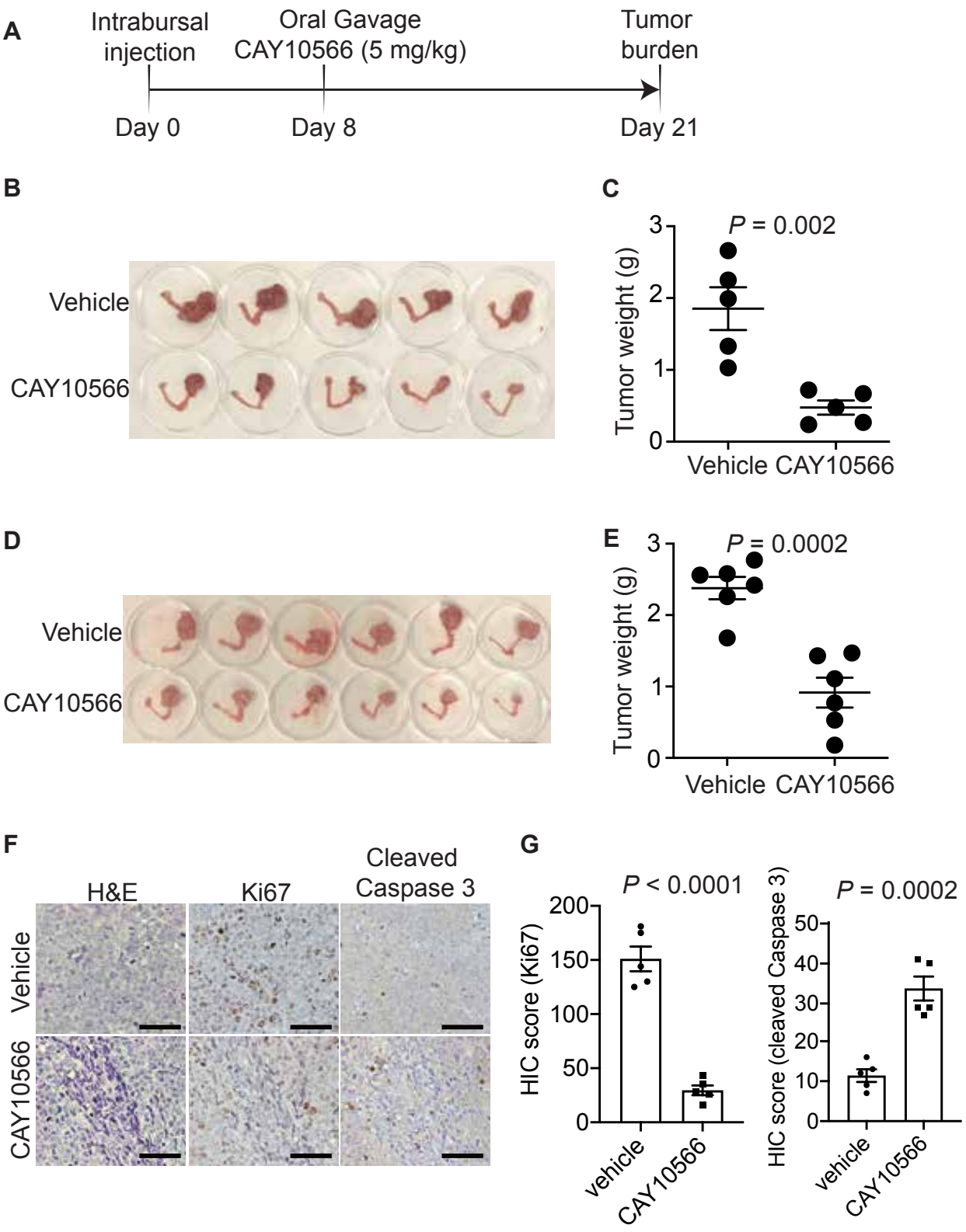

A
Intrabursal injection
Oral Gavage CAY10566 (5 mg/kg)
Tumor burden
Day 0
Day 8
Day 21
B
Vehicle
CAY10566
C
P = 0.002
Tumor weight (g)
Vehicle CAY10566
D
Vehicle
CAY10566
E
P = 0.0002
Tumor weight (g)
Vehicle CAY10566
F
H&E
Ki67
Cleaved Caspase 3
Vehicle
CAY10566
G
P < 0.0001
HIC score (Ki67)
vehicle
CAY10566
P = 0.0002
HIC score (cleaved Caspase 3)
vehicle
CAY10566

Figure 13. SCD1 inhibition suppresses CARM1-expressing ovarian cancer *in vivo*. A, Schematic representation of experimental design for ovarian cancer mouse model. **B** Reproductive tracts with tumors from the A1847 xenograft model indicated treated groups that were dissected at the end of treatment (*n* = 5 mice per group). **C** Weights of tumors dissected in **B** measured as a surrogate for tumor burden. **D,E** Same as **B,C**, but for syngeneic ovarian cancer mouse model using CARM1-expressing UPK10 cells (*n* = 5 mice per group). **F** IHC staining for the cell proliferative marker Ki67 and apoptotic marker cleaved caspase 3 of slides from tumors formed by A1847 cells treated with vehicle control or SCD1 inhibitor CAY10566 (*N* = 5 mice per group. Scale bars = 100 μm). **G** Quantification of F base on the H score (data represent mean ± SEM. *P* values were calculated using two-tailed *t* test).

2.5 Discussion

The methyltransferase CARM1 is involved in cancer tumorigenesis regulating different pathways and its role in Ovarian Cancer has not been fully comprehended yet. Given the high frequency of CARM1 overexpression in HGSOC we found that CARM1 enhances the production of monounsaturated fatty acid, that are important cellular components for the cellular structure and proliferation. Briefly, as summarized in Fig, CARM1 promotes the expression of ACC1 and FASN that are responsible of the *de novo* production of saturated fatty acid such as palmitic and stearic acid, then, CARM1 also promotes SCD1 that converts the saturated FA in mono-unsaturated FA such as palmitoleic and oleic acid.

As methyltransferase, CARM1 can asymmetrically di-methylates the arginine 17 residue in histone 3. Therefore, CARM1 can modulate the gene expression through its enzymatic activity. Nevertheless, CARM1 also function as co-factor and it has been published that it can recruit the transcription factor XBP1s (Lin, Liu, et al. 2021) regulating the gene expression independently by its enzymatic activity. Here we demonstrated that CARM1 regulates the key enzymes of the FA pathways through recruiting the co-factor XBP1s.

Consistent with our finding, it has been reported that CARM1 can enhance ACC1 promoting the *de novo* fatty acid production in the neurodegenerative disease,

supporting that CARM1 has a role in the FA metabolism in different diseases (Yang Liu et al. 2018).

A limitation of this study is that CARM1 can potentially regulate other pathway beyond the fatty acid pathway. Nevertheless, we showed that the FA pathway represents a vulnerability for high grade serous ovarian cancer in CARM1 status dependent manner and the inhibition of the key enzyme SCD1 is crucial to selectively target the CARM1-high cells. We showed that SCD1 inhibition suppresses the proliferation in CARM1 status dependent manner both in vitro and in vivo causing the accumulation of saturated FA (Fig. 14). Hence, our results align with recent research indicating that the accumulation of saturated fatty acids contributes to the tumor suppressive effects of SCD1 inhibition (Lien et al. 2021).

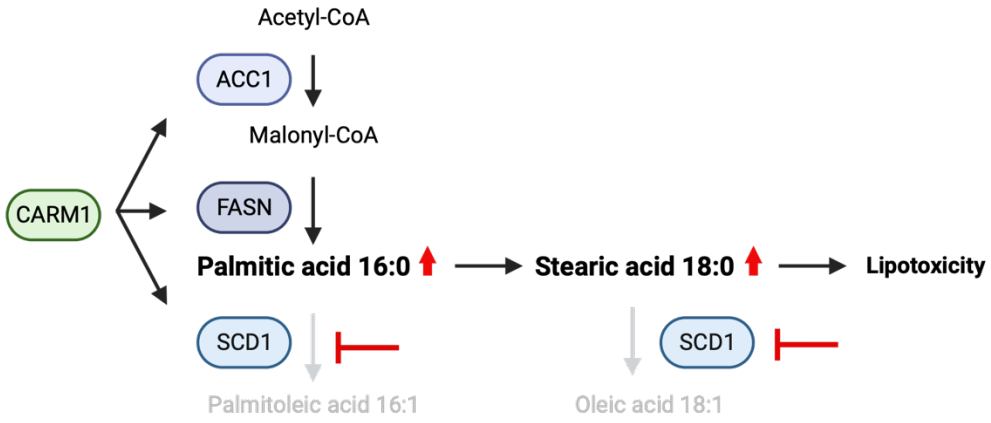


Figure 14. Current model by which the inhibition of SCD1 is induce toxicity in CARM1-high cells.

3. Results part II

3.1 Preliminary data and aim of the project

The SWI/SNF component ARID1A is frequently lost in many cancers. Analysis of the TGCA Endometrial Cancer dataset shows that ARID1A is mutated in 48% of the cases and the majority of these aberrations consist of truncating mutations that, as well as the deep deletions, cause the loosing of the protein (Fig. 15). It has been reported that the inactivation of ARID1A is responsible of tumorigenesis in different tumors including endometrial cancer (Chatterjee, Rodger, and Eccles 2018).

Additionally, two independent publications demonstrated that the *in vivo* inactivation of ARID1A not only collaborates to drive the tumorigenesis but also promotes the invasion of the tumors toward the surrounding myometrium regulating the epithelia to mesenchymal transition (Wilson et al. 2019; Suryo Rahmanto et al. 2020). This let the primary tumor escape from the endometrium developing into a high-grade tumor with the ability to form metastasis.

There is an unmet need to identify a therapeutic strategy for metastatic endometrial cancers. The regulatory impact of the epigenetic factors, as well as for ARID1A, is widely recognized in the regulation of cancer invasion (Chatterjee, Rodger, and Eccles 2018). We therefore decide to adopt the CRISPR screening technique to identify an epigenetic factor which inhibition can, synthetically with ARID1A inactivation, contrast the invasion of ARID1A deficient endometrial cancer.

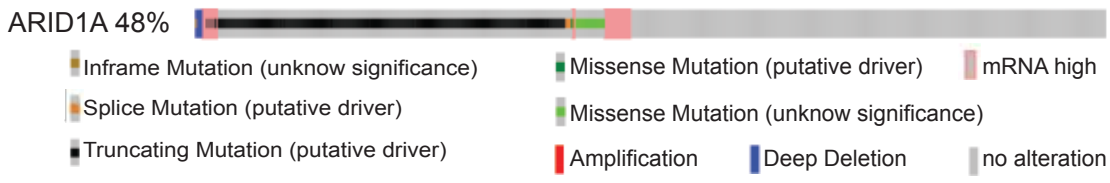


Figure 15. ARID1A alteration profile in 529 samples from the TGCA Uterine Corpus Endometrial Cancer dataset.

3.2 CRISPR screening identify PRMT5 as promising target for decreasing invasion in ARID1A KO cells

It has been reported that ARID1A inactivation in endometrium collaborates to drive the invasion of the tumors in the surrounding myometrium (Wilson et al. 2019; Suryo Rahmanto et al. 2020). Moreover, it is well known that the epigenetic factors are implicated in tumor invasion (Chatterjee, Rodger, and Eccles 2018). In order to identify an epigenetic factor which can be targeted to diminish the invasiveness of ARID1A deficient cells, we applied the CRISPR screening technique combined with the invasion assay.

We first generated the isogenic cell lines 12Z gctrl and 12Z ARID1A KO using the CRISPR Cas9 system and we validate the efficiency of the knockout analyzing the protein level of ARID1A (Fig. 16A). The vectors used in the system also allowed the integration of the Cas9 coding sequence in the cell genome, therefore both 12Z gtrl and 12Z ARID1A KO cell lines expressed similar amount of Cas9 (Fig. 16B). The stable expression of Cas9 allowed the isogenic cell 12Z gtrl and 12Z ARID1A KO to be suitable for the following CRISPR screening.

More in details, the experimental workflow of the screening is summarized in Fig. 16C. To conduct the CRISPR screening we used a plasmid gRNA library that target 1200 epigenetic factors. The plasmid backbone of the library only contains the gRNA sequence merged with the tracrRNA without carrying the Cas9 coding sequence. For this reason, we previously ensured that the cell model express stable level of Cas9 (Fig. 16B). Then, each plasmid containing the specific gRNA has been packaged into lentivirus particles, generating a lentivirus library, according with the maintenance of the library coverage. Both 12Z gtrl and 12ZARID1A KO were infected with the lentivirus library with a multiplicity of infection equal to 0.3 ensuring a single infection for each cell. This resulted in the generation of a specific gene knockout in each cell by the Cas9 previously introduced and the gRNA brought by the lentivirus, collectively forming a pool of cells according to the conservation of the initially library coverage, for both 12Zgtrl and 12Z ARID1A KO. Each pool has been subject to antibiotic selection for 3 days to eliminate the cells that were not infected. Next, the selected pools were expanded to ensure enough library coverage for the next applications. The cell libraries were then assayed for invasion or collected as input cells. The invasion

assay has been conducted *in vitro* for 36 hours and at the end point the invading cells have been collected for both 12Zgtrl pool and 12Z ARID1A KO pool. The genomic DNA have been purified from each bunch of input cells and invading cells and have been used as template for following PCR amplification that amplify the gRNA region present in the insert integrated in the genomic DNA upon lentivirus infection. Next, the samples have been then sequenced. The detection of the gRNA sequences (form the insertion in the genome of each cell) proved information regarding which gene has been knocked out in the respective cells and groups. The ultimate bioinformatic analysis reveals the distribution of the gRNA sequences in each sample that match to the distribution of respective gene knockouts in the sample. For our propose, we seek for the gene KOs that were less represented in the ARID1A KO cells meaning that those gene KOs may potentially diminish the invasion of these cells.

The screening analysis calculated the distribution of the gRNA depleted in the invading cells samples, resulting in the identification of 79 KOs that are depleted in wildtype invading cells and 133 KOs in ARID1A invading cells (Fig. 16D). These genes may therefore represent potential targets that could decrease the invasion of ARID1A deficient cells. From the analysis we also calculate the gene KOs that may be synthetic lethal with ARID1A inactivation, resulting from the comparison of the 12Z gtrl pool and 12Z ARID1A KO pool inputs, and that have been quantify as 185 potential synthetic targets (Fig. 16D). In order to only explore the invasive phenotype, we have to exclude a potential inhibition of the proliferation. Those KOs were excluded to eliminate false results resulting in 68 gene whose downregulation may potentially reduce the invasion of ARID1A inactivated cells (Fig. 16D. Interestingly there are 5 common gene KOs that may affect the invasion independently by ARID1A status as they are common between the two cell lines (Fig. 16D).

Notably, the top ten hits of the analysis (Fig. 16E) are reported to promote the invasion in different cancers, giving the confidence that the screening was successfully conducted. More importantly, it has been previously published that PELP1 can promote the invasion in Endometrial Cancer confirm the accuracy of the screening (Wan and Li 2012). Across the potential targets, PRMT5 is a well know arginine methyltransferase that is involved in several cancers' aspects, but its role in endometrial cancer it has not been fully explored. Moreover, some PRMT5 inhibitor are in clinal trials standing out PRMT5 as a potential clinical target for invasive endometrial cancer.

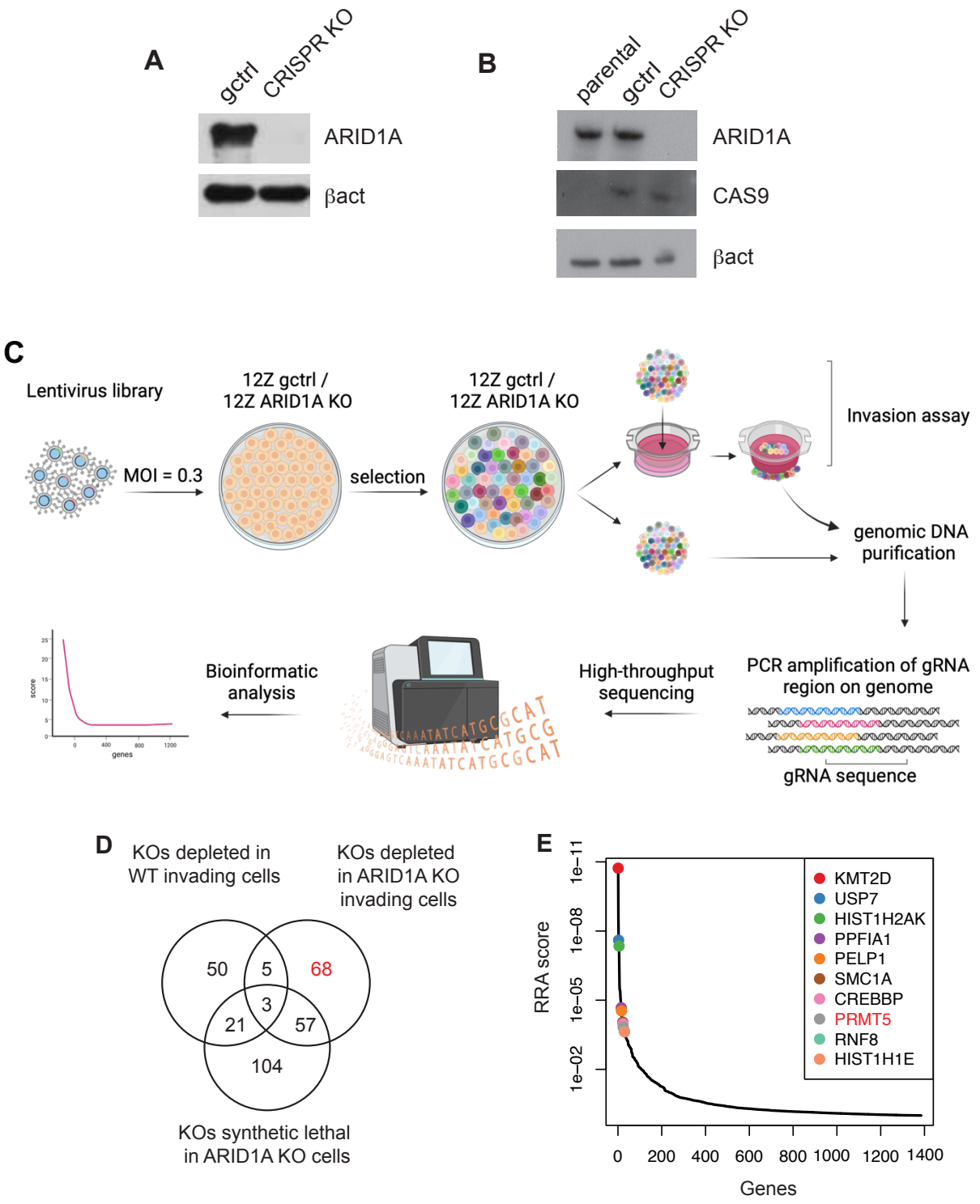


Figure 16. Identification of PRMT5 as promising target to reduce invasion in ARID1A deficient cells. A Immunoblot that shows ARID1A knock out with CRISPR cas9 system in 12Z cell line. **B** Immunoblot that shows CAS9 protein level in the 12Z gctrl and 12Z ARID1A knock out cells. **C** Schematic representation of the CRISPR screening combined with the invasion assay. **D** Output and analysis

of the CRISPR screening. **E** Robust rank aggregation (RRA) of the top ten hits from the screen whose knockout specifically decrease invasion in ARID1A knock out cells.

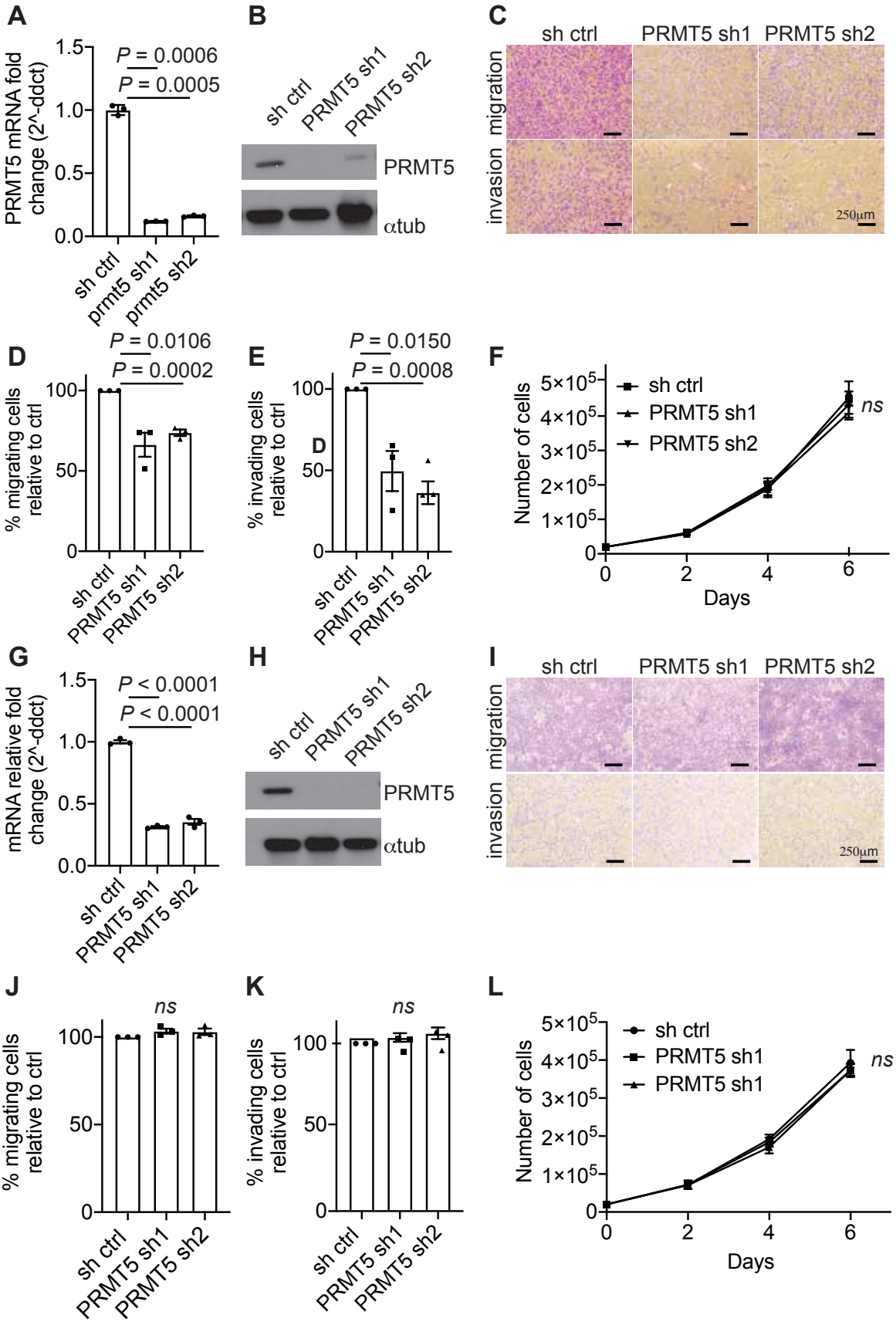
In conclusion, the combination of the CRISPR screening with the invasion assay identify PRMT5 as a promising target which inhibition may decrease the invasion of ARID1A deficient endometrial cancer cells.

3.3 PRMT5 genetic knock down decrease invasion of ARID1A inactivated cells

To systematically validate the screening results, PRMT5 has been knockdown with two different shRNA in 12Z ARID1A KO and the efficiency of the knockdown has been evaluated at both RNA and protein levels compared to the control short hairpin (Fig. 17A,B). To establish the effect of PRMT5 knockdown, the invasion assay has been performed accordingly to the condition previously set for the screening. Notably, both the PRMT5 short hairpins 1 and 2 can significantly decrease the invasion of 12Z ARID1AKO cells compared to the control, and consistently the migration is reduced as well (Fig. 17C-E). Interestingly, the growth of the 12Z ARID1A KO cells is not affected by PRMT5 knockdown confirming that the diminishing of the invasive or migrating phenotype detected in the previous assay is not correlated to cell proliferation (Fig 17F).

The initial hypothesis was that PRMT5 depletion contrasts the invasion synthetically to ARID1A inactivation, as predicted by the screening analysis. To further validated this hypothesis, PRMT5 has been successfully knocked down in 12Z gtrl cells (Fig. 17G,H). Consistently, any significant changes are detected in the invasion or migration ability of the 12Z gtrl cells upon PRMT5 knockdown with the two different short hairpins respect the control one (Fig. 17I-K), as well as in the proliferation (Fig. 17L).

Additionally, to further validate these results we utilized the SNG-M cell line which presents two deletions (p.D1633fs and p.F1924fs) that cause the loss of the ARID1A protein. The absence of the ARID1A protein in SNG-M cells has been confirmed by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PRMT5 mRNA fold change (2^-ddct)
P = 0.0006
P = 0.0005
sh ctrl
prmt5 sh1
prmt5 sh2
B
sh ctrl
PRMT5 sh1
PRMT5 sh2
PRMT5
αtub
C
sh ctrl
PRMT5 sh1
PRMT5 sh2
migration
invasion
250μm
D
% migrating cells relative to ctrl
P = 0.0106
P = 0.0002
E
% invading cells relative to ctrl
P = 0.0150
P = 0.0008
F
Number of cells
sh ctrl
PRMT5 sh1
PRMT5 sh2
ns
Days
G
mRNA relative fold change (2^-ddct)
P < 0.0001
P < 0.0001
H
I
J
% migrating cells relative to ctrl
ns
K
% invading cells relative to ctrl
ns
L
Number of cells
sh ctrl
PRMT5 sh1
PRMT5 sh1
ns
Days

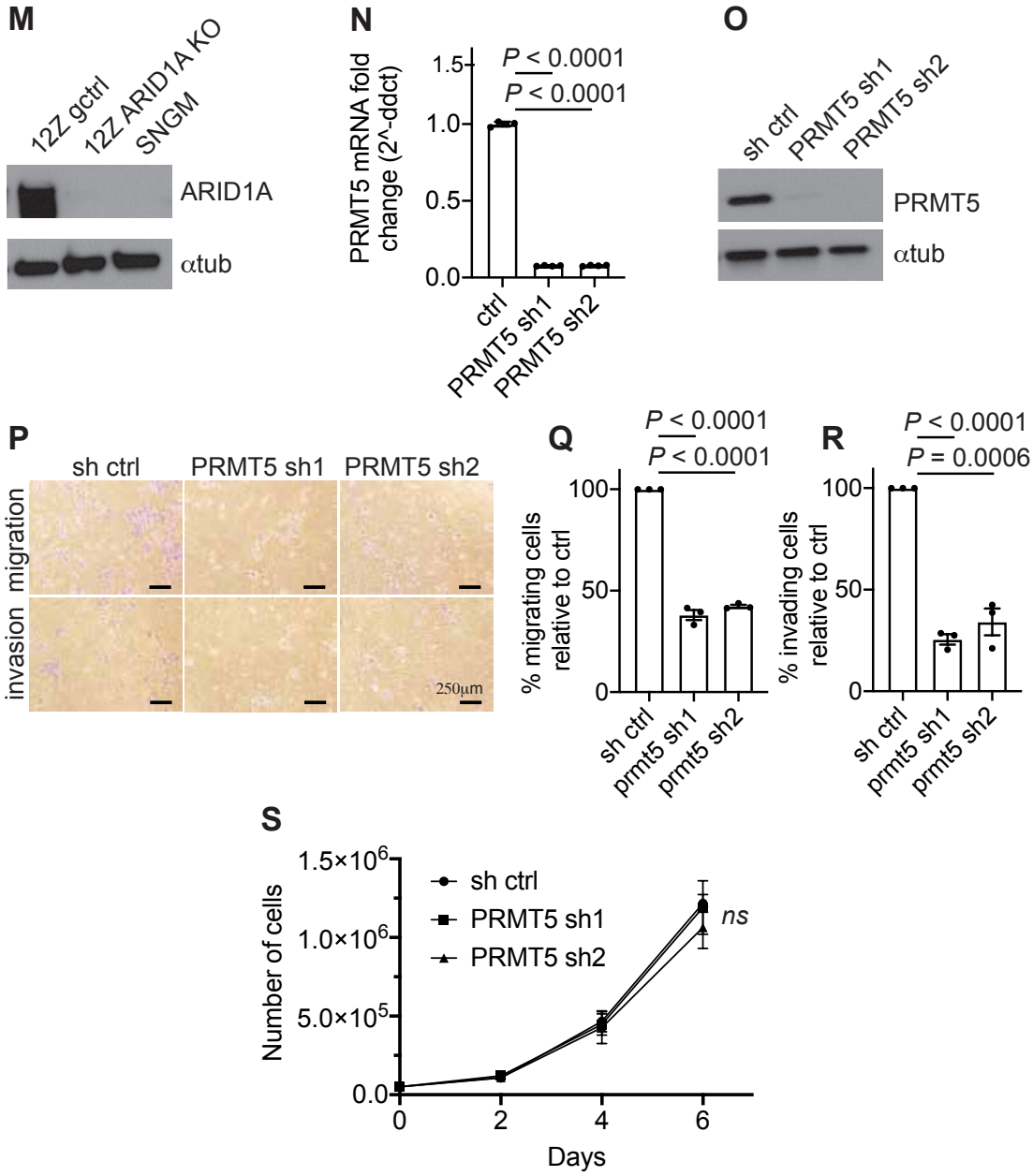


Figure 17. PRMT5 genetic Knock down decrease migration and invasion specifically in ARID1A deficient cells. A,B RNA expression (**A**) and protein level (**B**) of PRMT5 upon genetic knock down of PRMT5 with two different shRNA in 12Z ARID1A knock out cells. **C** Representative images of stained membrane from transwell assay of 12Z ARID1A knock cells with PRMT5 knock down or control vector. First Upper images show migration and lower images show invasion. Data represent three biological replicates. **D,E** Quantification of migration (**D**) and invasion (**E**) of the transwell assay showed in panel **C**. Data represent three biological replicates, mean +/- SEM. **F** Growth curve of 12Z ARID1A knock out with PRMT5 genetic knock down or control vector (n= 3 biological replicates, mean +/- SEM). **G-L** same as **A-F** but related to 12Z gctrl. **M** Immunoblot that shows ARID1A protein level in SNGM cells compared to 12Z cells. **N-S** same as **A-F** but related to SNGM cells.

the analysis of the protein level (Fig. 17M). Next, PRMT5 has been knockdown in SNG-M cells and the efficiency of the knockdown has been validated analyzing both the RNA and the protein expressions (Fig. 17N,O). Accordingly with the previous results, the invasion and the migration are significantly decreased by PRMT5 KD versus control in SNG-M cells (Fig. 17P-R). Remarkably, the PRMT5 knockdown does not affect the cell growth validating that the observed phenotype is not related to the proliferation of the cells (Fig. 17S).

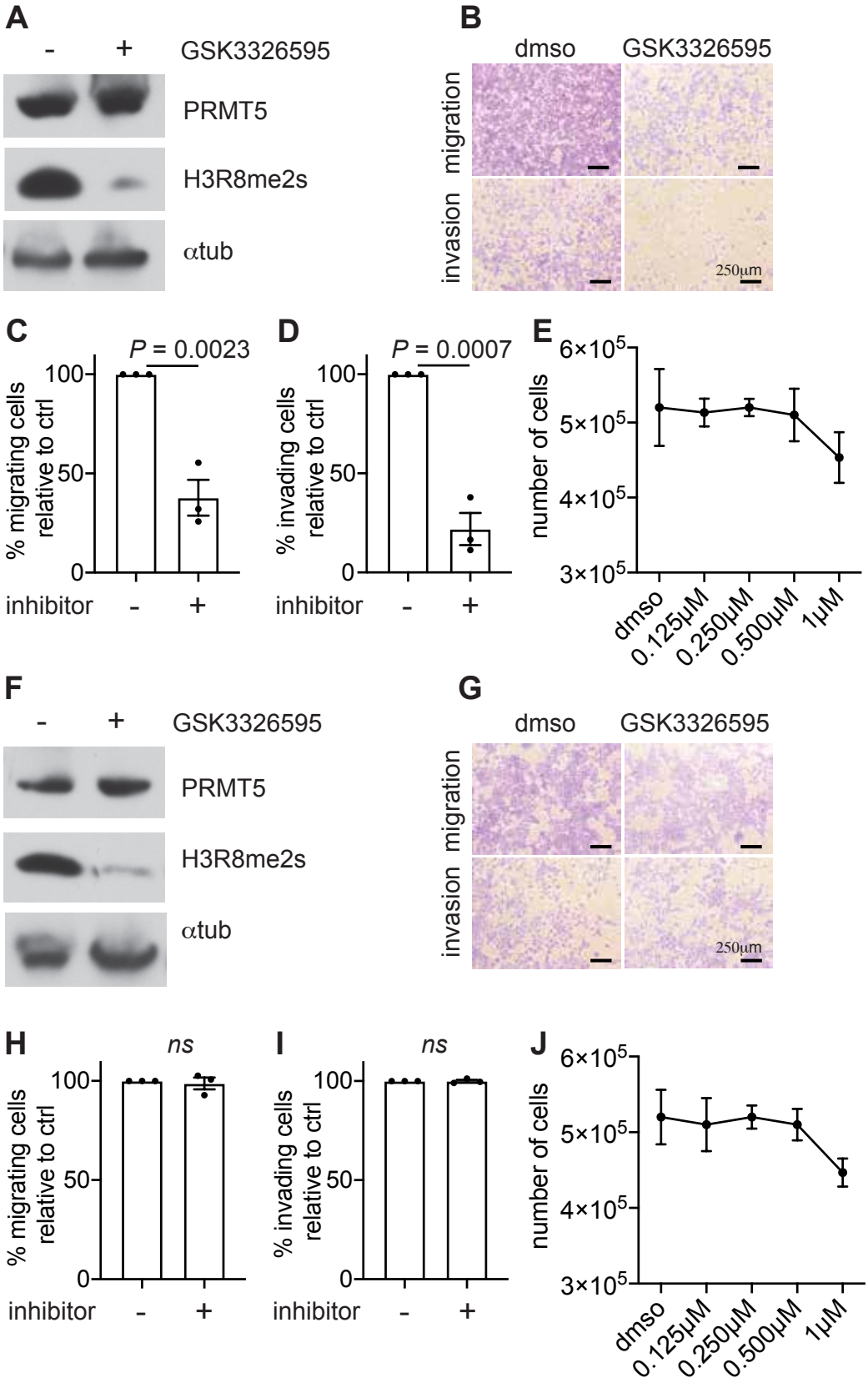
Together, these results validated the screening prevision confirming that, in a background of ARID1A inactivation, PRMT5 represents a target for invasive endometrial cancer.

3.4 PRMT5 pharmaceutical inhibition reduces invasion of ARID1A inactivated cells

PRMT5 is an arginine methyltransferase that symmetrically demethylates arginine residues in histone and no-histone proteins (Kim and Ronai 2020). To further validate the role of PRMT5 in the regulation of the invasion we test whether its enzymatic activity is involved in the regulation of the phenotype. For this aim, we used the specific PRMT5 inhibitor GSK3326595 that is currently used in clinical trial (NCT02783300).

The 12Z ARID1A KO have been treated with GSK3326595 for a total of 72 hours and the efficiency of the treatment have been determinate by analyzing the decreasing of protein level of H3R8 symmetric de-methylation that is one product of PRMT5 enzymatic activity (Fig. 18A). Next, 12Z ARID1A KO cells were pre-treated for 36 hour and then assayed for 36 hours invasion assay, with the supplementation of the drug, according to the conditions previously set. Notably, the GSK3326595 treatment significantly diminishes the invasiveness and migration of 12Z ARID1A KO cells compared to the vehicle (Fig. 18B-D). Additionally, the GSK3326595 treatment does not affect the cell proliferation at the used dose (Fig. 18E).

To further confirm the specificity of PMRT5 inhibition in the regulation of the invasion


A
- +
GSK3326595
PRMT5
H3R8me2s
αtub
B
dmso
GSK3326595
migration
invasion
250μm
C
P = 0.0023
% migrating cells relative to ctrl
100
50
0
inhibitor - +
D
P = 0.0007
% invading cells relative to ctrl
100
50
0
inhibitor - +
E
number of cells
6×10^5
5×10^5
4×10^5
3×10^5
dmso
0.125μM
0.250μM
0.500μM
1μM
F
- +
GSK3326595
PRMT5
H3R8me2s
αtub
G
dmso
GSK3326595
migration
invasion
250μm
H
ns
% migrating cells relative to ctrl
100
50
0
inhibitor - +
I
ns
% invading cells relative to ctrl
100
50
0
inhibitor - +
J
number of cells
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5×10^5
4×10^5
3×10^5
dmso
0.125μM
0.250μM
0.500μM
1μM

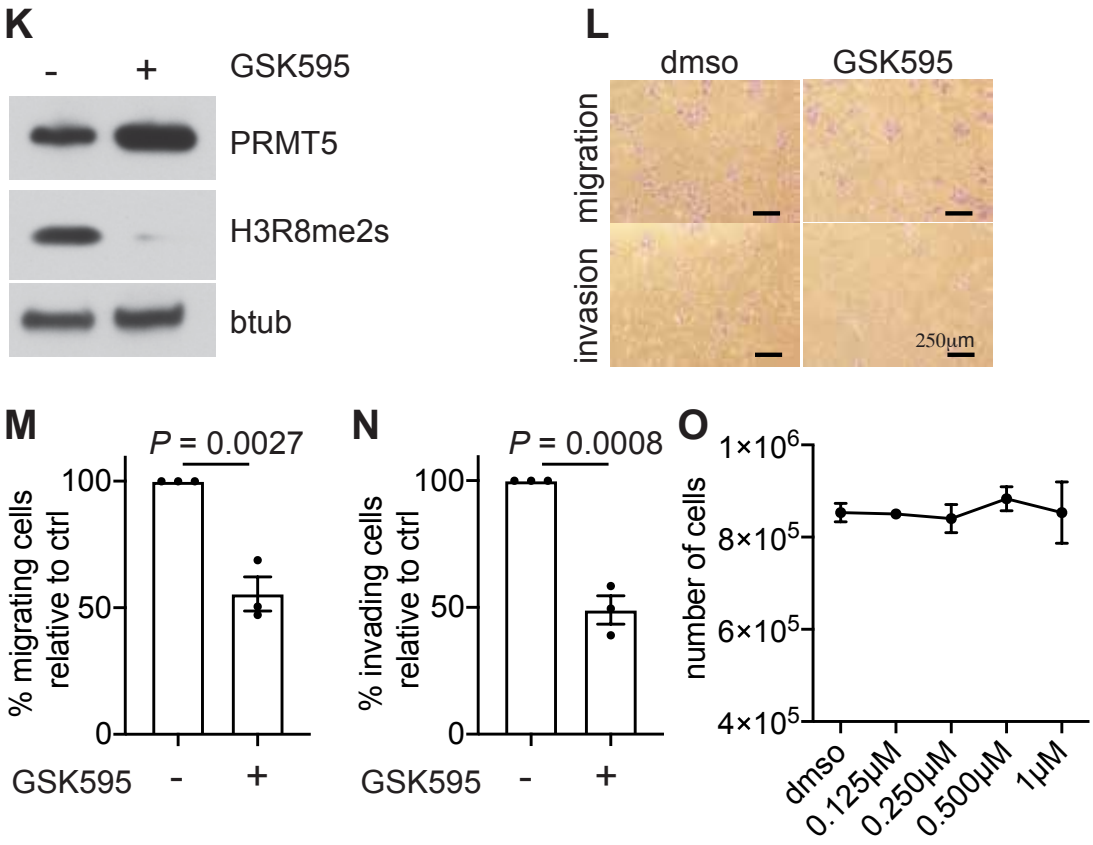


Figure 18. PRTM5 pharmaceutical inhibition decrease migration and invasion specifically in ARID1A deficient cells. A Arginine symmetric dimethylation level upon PRMT5 pharmaceutical inhibition treatment or vehicle showed as immunoblot in 12Z ARID1A cells. **B** Representative images of stained membrane from transwell assay of 12Z ARID1A KO cells upon PRMT5 inhibition vehicle. Migration showed in upper line and invasion in lower layer. Data represent three biological replicates. **C,D** Quantification on migration (**C**) and invasion (**D**) showed in panel **B**. **E** Cell growth base on 3 days total dose curve treatment (n= 3 biological replicates, mean +/- SEM). **F-J** same experiments showed in panel **A-E** but related to 12Z wt cells. **K-O** same experiments showed in panel **A-E** but related to SNGM cells.

of ARID1A deficient cells, we test the drug in the ARID1A wildtype 12Z gctrl. As expected, the 72 hours GSK3326595 treatment successfully reduces the product of PRMT5 enzymatic activity H3R8me2s (Fig. 18F). Then, the invasion assay has been conducted with the same condition of drug concentration and duration as for 12Z ARID1A KO. Notably, the GSK3326595 inhibitor does not affect either the invasion and migration of 12Z gtrl cells or the cell proliferation (Fig. 18G-J).

Moreover, we confirmed the results with the other SNG-M ARID1A deficient cell line. SNG-M cells have been treated with GSK3326595 for 72 hours and the treatment successfully decreases the symmetric methylation of the PRMT5 target H3R8 (Fig. 18K). Additionally, SNG-M cells have been pre-treated for 36 hours then the invasion assay has been performed for 36 hours time with GSK3326595 as supplement. Consistently with the previous results, the GSK3326595 treatment reduces the invasion and the migration of SNG-M versus the vehicle (Fig. 18L-N) without affecting the proliferation at that concentration (Fig. 18O).

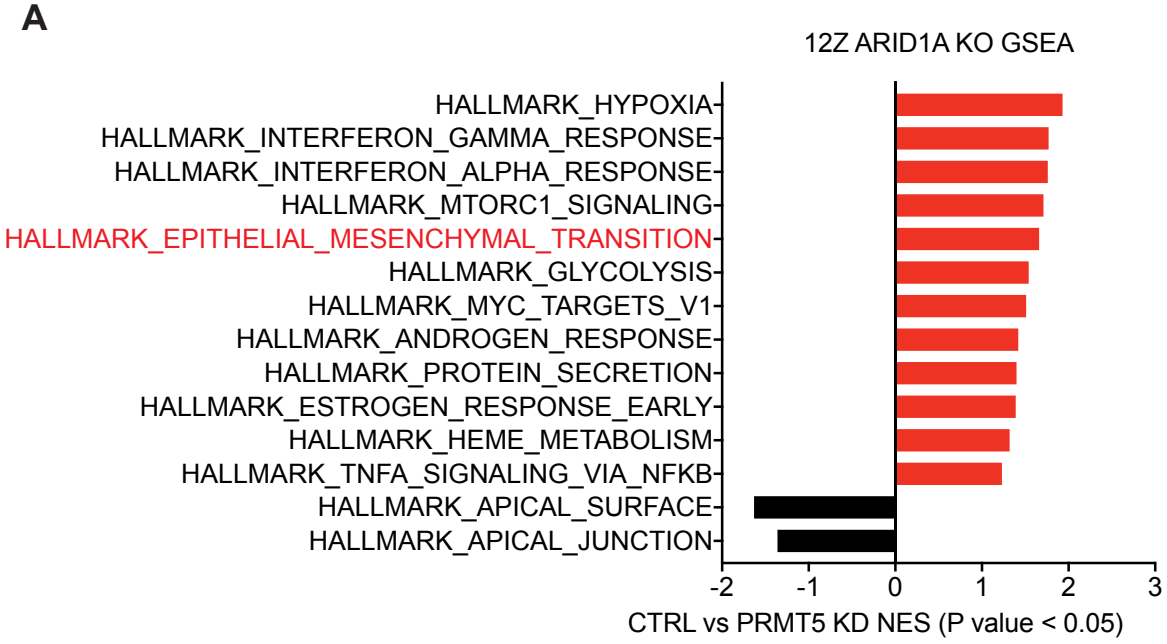
Collectively, these results show that the arginine methyltransferase activity of PMRT5 is responsible for the phenotype and targeting the enzymatic activity of PRMT5 is a promising strategy to reduce the invasiveness of ARID1A deficient endometrial cancer cells.

3.5 PRMT5 inhibition reduce the Epithelia to Mesenchymal transition in ARID1A KO cells

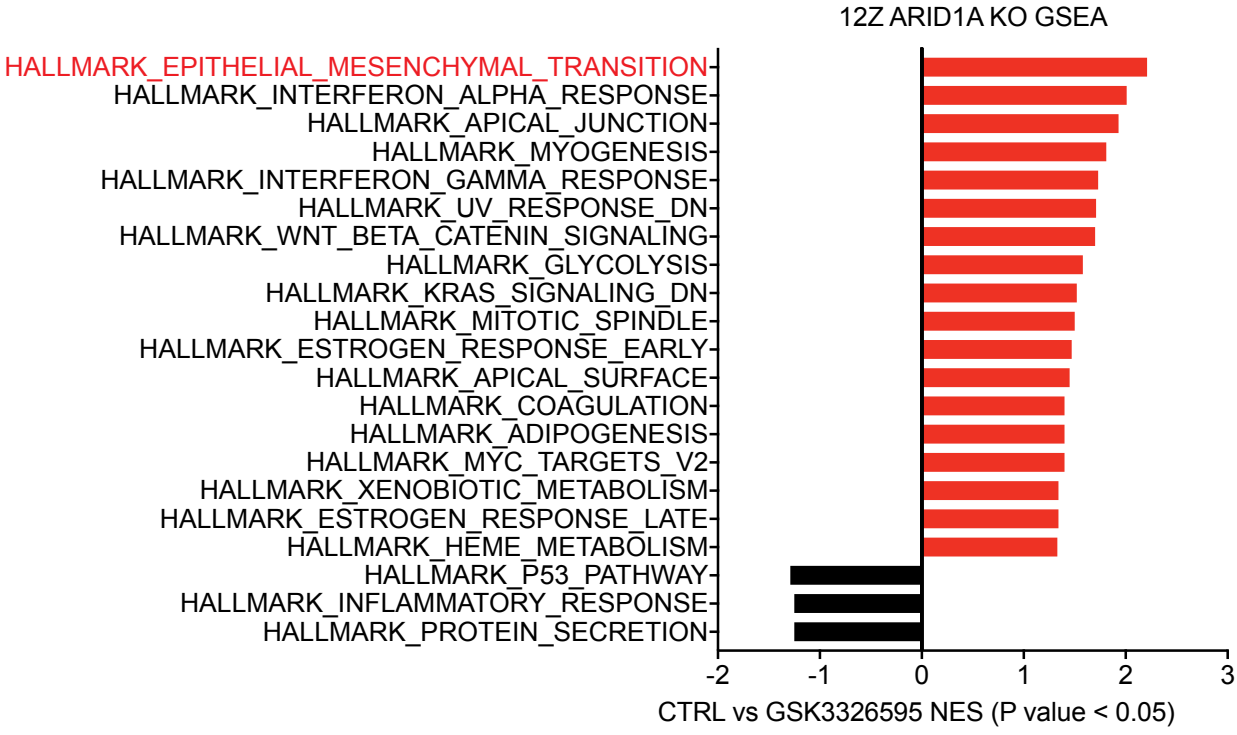
To systematically explore in which way PRMT5 inhibition regulates the invasion of ARID1A deficient cells we investigate the change in gene expression with RNA seq analysis upon PRMT5 inhibition. We therefore performed RNA seq analysis in both 12Z gtrl and 12Z AKO upon PRMT5 KD or treatment with GSK3326595 inhibitor. For this last condition, both 12Z gtrl and 12Z ARID1A KO have been treated with GSK3326595 for a total of three days, then total RNA has been purified as well as from PRMT5 KD samples and controls.

Remarkably, the gene set enrichment analysis (GSEA) shows the Epithelia to Mesenchymal transition (EMT) as one of the pathways downregulated by PRMT5 KD

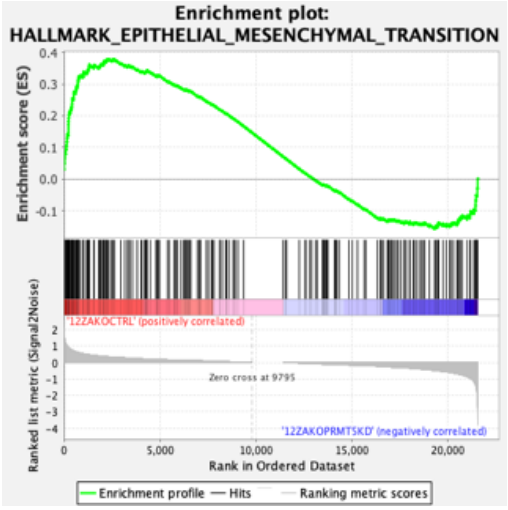
A


12Z ARID1A KO GSEA
HALLMARK_HYPOXIA
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_MTORC1_SIGNALING
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_GLYCOLYSIS
HALLMARK_MYC_TARGETS_V1
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_PROTEIN_SECRETION
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_HEME_METABOLISM
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_APICAL_SURFACE
HALLMARK_APICAL_JUNCTION
-2
-1
0
1
2
3
CTRL vs PRMT5 KD NES (P value < 0.05)

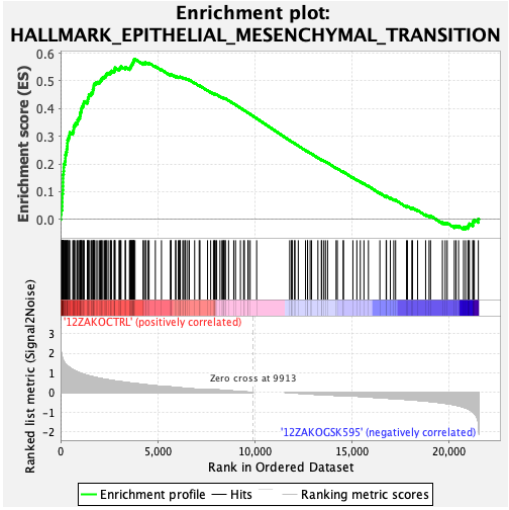
B


12Z ARID1A KO GSEA
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_APICAL_JUNCTION
HALLMARK_MYOGENESIS
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_UV_RESPONSE_DN
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_GLYCOLYSIS
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_MITOTIC_SPINDLE
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_APICAL_SURFACE
HALLMARK_COAGULATION
HALLMARK_ADIPOGENESIS
HALLMARK_MYC_TARGETS_V2
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_HEME_METABOLISM
HALLMARK_P53_PATHWAY
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_PROTEIN_SECRETION
-2
-1
0
1
2
3
CTRL vs GSK3326595 NES (P value < 0.05)

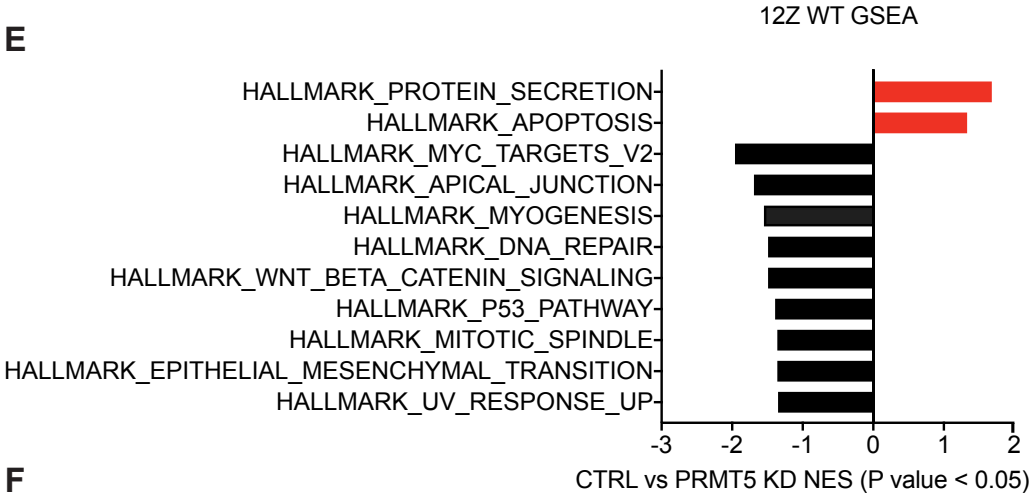
C


Enrichment plot:
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
Enrichment score (ES)
'12ZAKOCTRL' (positively correlated)
Zero cross at 9795
'12ZAKOPRMT5KD' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

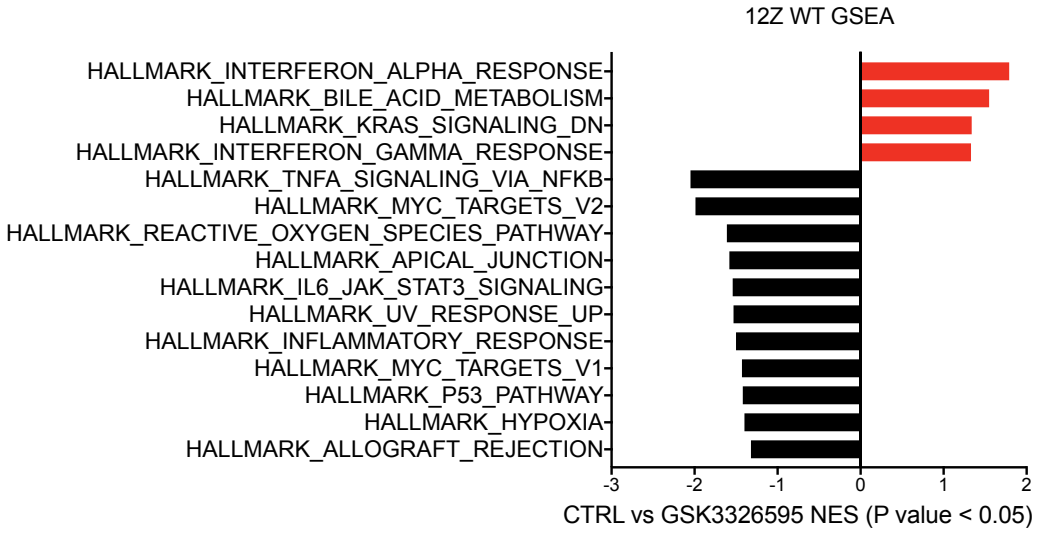
D


Enrichment plot:
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
Enrichment score (ES)
'12ZAKOCTRL' (positively correlated)
Zero cross at 9913
'12ZAKOGSK595' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

E


12Z WT GSEA
HALLMARK_PROTEIN_SECRETION
HALLMARK_APOPTOSIS
HALLMARK_MYC_TARGETS_V2
HALLMARK_APICAL_JUNCTION
HALLMARK_MYOGENESIS
HALLMARK_DNA_REPAIR
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_P53_PATHWAY
HALLMARK_MITOTIC_SPINDLE
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_UV_RESPONSE_UP
-3
-2
-1
0
1
2
CTRL vs PRMT5 KD NES (P value < 0.05)

F


12Z WT GSEA
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_MYC_TARGETS_V2
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY
HALLMARK_APICAL_JUNCTION
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_UV_RESPONSE_UP
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_MYC_TARGETS_V1
HALLMARK_P53_PATHWAY
HALLMARK_HYPOXIA
HALLMARK_ALLOGRAFT_REJECTION
-3
-2
-1
0
1
2
CTRL vs GSK3326595 NES (P value < 0.05)

G

Gene Name	12 WT		12 ARID1A KO		12 WT		12 ARID1A KO		12 WT		12 ARID1A KO	
	prmt KD/ctrl	Inh/ctrl	prmt KD/ctrl	Inh/ctrl	prmt KD/ctrl	Inh/ctrl	prmt KD/ctrl	Inh/ctrl	prmt KD/ctrl	Inh/ctrl	prmt KD/ctrl	Inh/ctrl
	FC	FC	FC	FC	p	p	p	p	fdr	fdr	fdr	fdr
MEST	0	0	-3.09	-1.27	0.069481	0.573322	3.4E-118	3.6E-08	39%	100%	0%	0%
CXCL12	1.94	0	-1.94	-2.36	6.83E-27	0.435587	2.3E-144	6.2E-234	0%	100%	0%	0%
TGFBI	1.10	0	-1.78	-1.50	0.001145	0.292871	2.3E-122	3.65E-63	3%	94%	0%	0%
IGFBP3	0	-1.28	-1.72	-2.13	0.005972	1.42E-07	4.8E-92	4.7E-177	8%	0%	0%	0%
COL6A3	2.32	0	-1.54	-1.59	1.04E-09	0.232658	3.38E-18	4.27E-20	0%	86%	0%	0%
ITGA5	0	0	-1.45	-1.20	0.571284	0.184655	1.05E-23	4.94E-07	100%	79%	0%	0%
SPOCK1	-1.32	0	-1.45	-1.53	3.29E-08	0.010213	3.58E-46	9.6E-62	0%	15%	0%	0%
ADAM12	0	0	-1.44	-2.03	0.4333	0.055724	1.09E-24	3.91E-81	100%	44%	0%	0%
LOX	1.13	1.22	-1.44	-1.10	0.002315	1.37E-05	4.23E-39	0.00031	5%	0%	0%	0%
MMP2	1.47	1.28	-1.42	-1.45	9.35E-07	0.001638	1.01E-15	1.37E-17	0%	4%	0%	0%
SERPINE1	1.42	0	-1.39	-1.31	4.31E-07	0.321501	3.36E-16	1.05E-11	0%	97%	0%	0%
LOXL1	0	0	-1.30	-1.38	0.032241	0.657678	0.002272	0.00026	24%	100%	4%	0%
HTRA1	-1.33	0	-1.29	-1.24	4.68E-06	0.026133	0.000383	0.002307	0%	28%	1%	2%
RHOB	0	0	-1.28	-1.75	0.734144	0.029323	8.29E-06	5.21E-23	100%	30%	0%	0%
THBS2	0	0	-1.24	-1.71	0.446096	0.773954	9.68E-08	4.34E-41	100%	100%	0%	0%
ENO2	0	-1.20	-1.21	-1.39	0.384905	0.000118	6.28E-10	1.8E-26	98%	1%	0%	0%
MYLK	0	0	-1.18	-1.27	0.091366	0.042535	4.52E-07	1.13E-13	46%	37%	0%	0%
TPM1	0	0	-1.16	-1.41	0.659737	0.539185	1.73E-07	1.55E-32	100%	100%	0%	0%
WNT5A	0	0	-1.15	-1.16	0.991248	0.897625	3.66E-05	7.44E-06	100%	100%	0%	0%
ITGB1	-1.23	0	-1.14	-1.21	0.000944	0.159884	6.45E-05	2.32E-09	3%	74%	0%	0%

Figure 19. Epithelia to mesenchymal transition is downregulated by PRMT5 inhibition in ARID1A deficient cells. **A,B** GSEA hallmark enrichment analysis of Differentially Expressed Genes between control and PRMT5 knockout (**A**) or vehicle versus PRMT5 pharmaceutical inhibition (**B**) in 12Z ARID1A KO cells. Data showed as normalized enrichment score (NES). **C,D** Enrichment plots showing the epithelia to mesenchymal transition hallmark for 12Z ARID1A KO upon PRMT5 KD (**C**) or inhibition (**D**). **E,F** same as **A,B** but related to 12Z wt cells. **G** List epithelia to mesenchymal transition pathway genes commonly downregulated by PRMT5 knock down or PRMT5 pharmaceutical inhibition based on RNA seq data.

in 12Z ARID1A KO cells respect the control. Notably, the EMT is the top one pathway downregulated by the PRMT5 inhibitor GSK3326595 in 12Z ARID1A KO cells versus the control (Fig. 191-D).

Contrarywise, neither PRMT5 KD nor GSK3326595 inhibitor treatment in 12Z gtrl reduce the EMT. Additionally, the EMT is enhanced by the PRMT5 KD in 12Z gtrl but not upon GSK3326595 inhibitor treatment (Fig. 19E-F).

In conclusion, the transcriptome analysis reviled that the effect of PRMT5 genetic knock down or the inhibition of its enzymatic activity corroborate with the phenotype previously observed. Both the PRMT5 knockdown and more specifically the inhibition of its arginine methyltransferase activity specifically downregulates the expression of the EMT genes in 12Z ARID1A KO cells (Fig. 19G).

3.6 PRMT5 inhibition reduce the myometrial invasion *in vivo*

To explore the clinical impact of this finding, we employed the previously established endometrial cancer transgenic mouse model ARID1A$^{flox/flox}$/PTEN$^{flox/flox}$. Briefly, in this transgenic mouse model, ARID1A and PTEN are selectively inactivated in the uterus via a doxycycline induced Pax8-Cre system in which the Cre is under the specific uterine PAX8 promoter, resulting in the inactivation of ARID1A and PTEN in uterine epithelia cells upon doxycycline administration.

The Figure 20A shows the workflow of the animal design. After the induction of the system with the administration of doxycycline, primary tumors develop within ten days. Then the mice were randomized and subdivided in two bunches for two weeks and three weeks treatment with GSK3326595 inhibitor or vehicle. At the end point the mice were scarified and the uteruses were harvested for the pathologic evaluation of the myometrium. Remarkably, in the control group the tumor progressed into the late stage 1b (more than 50% of myometrial invasion) in 50% of the case after two weeks treatment and 75% after three weeks (Fig 20B-D).

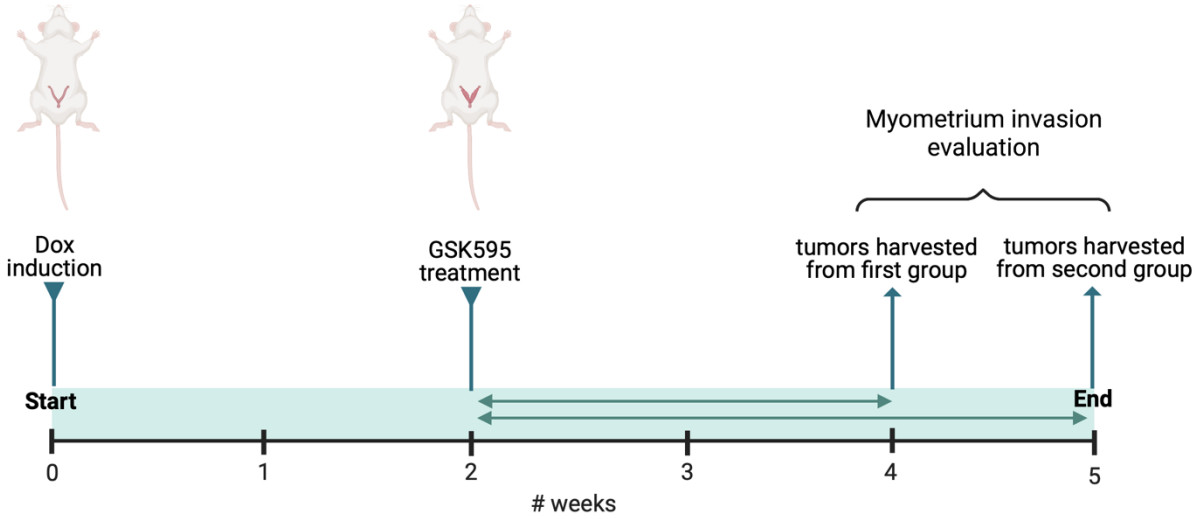
Notably, two and three weeks GSK3326595 treatment slows down the progression maintained 100% of tumors in stage 1a (less than 50% of myometrial invasion).

Overall, the GSK3326595 inhibitor significantly contrasts the evasion of the primary tumor from the endometrium reducing the myometrial invasion respect the vehicle (Fig 20B-D).

The limitation of this result is that we had not test the toxicity of the GSK3326595 inhibitor treatment in the internal organs but the usage of this drug in clinical trial suggests a good tolerability by the organism (Fig 20B-D).

These *in vivo* data strongly suggest that the inhibition of PRMT5 reduces the myometrial invasion and may represent a new clinical strategy for ARID1A inactivated endometrial cancer.

A



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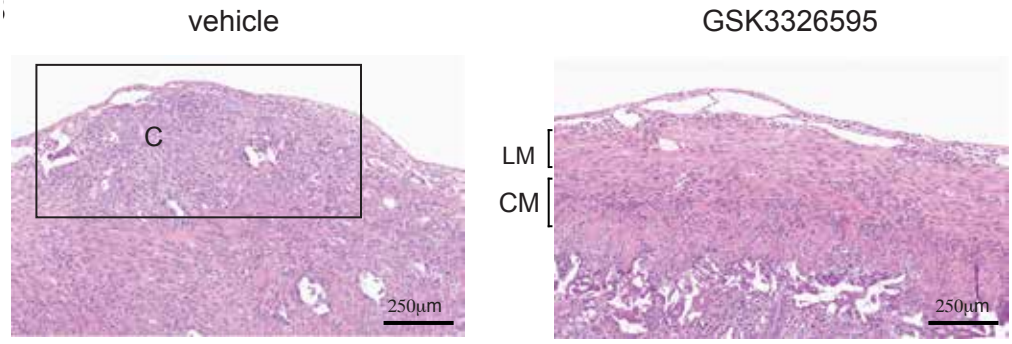


C

group	number	weeks	stage of invasion
control	4	two	50% 1a; 50% 1b
treatment	4	two	100% 1a
control	4	three	75% 1b, 25% 0
treatment	3	three	100% 1a

D

	early stage	late stage	total
control	3	5	8
treatment	7	0	17
total	10	5	15
Fisher's exact test			0.0256

Figure 20. PRMT5 inhibition treatment contrast tumor invasion in myometrium. A Schematic representation of the *in vivo* experiment design. **B** H&E staining of longitudinal sections of murine uterus of "strain" treated with PRMT5 inhibitor or vehicle for three weeks, C = carcinoma, LM = longitudinal myometrium, CM = circular myometrium; scale bar = 200um. **C,D** Output and statistic significance of the in vivo experiment described in **A** and **B**.

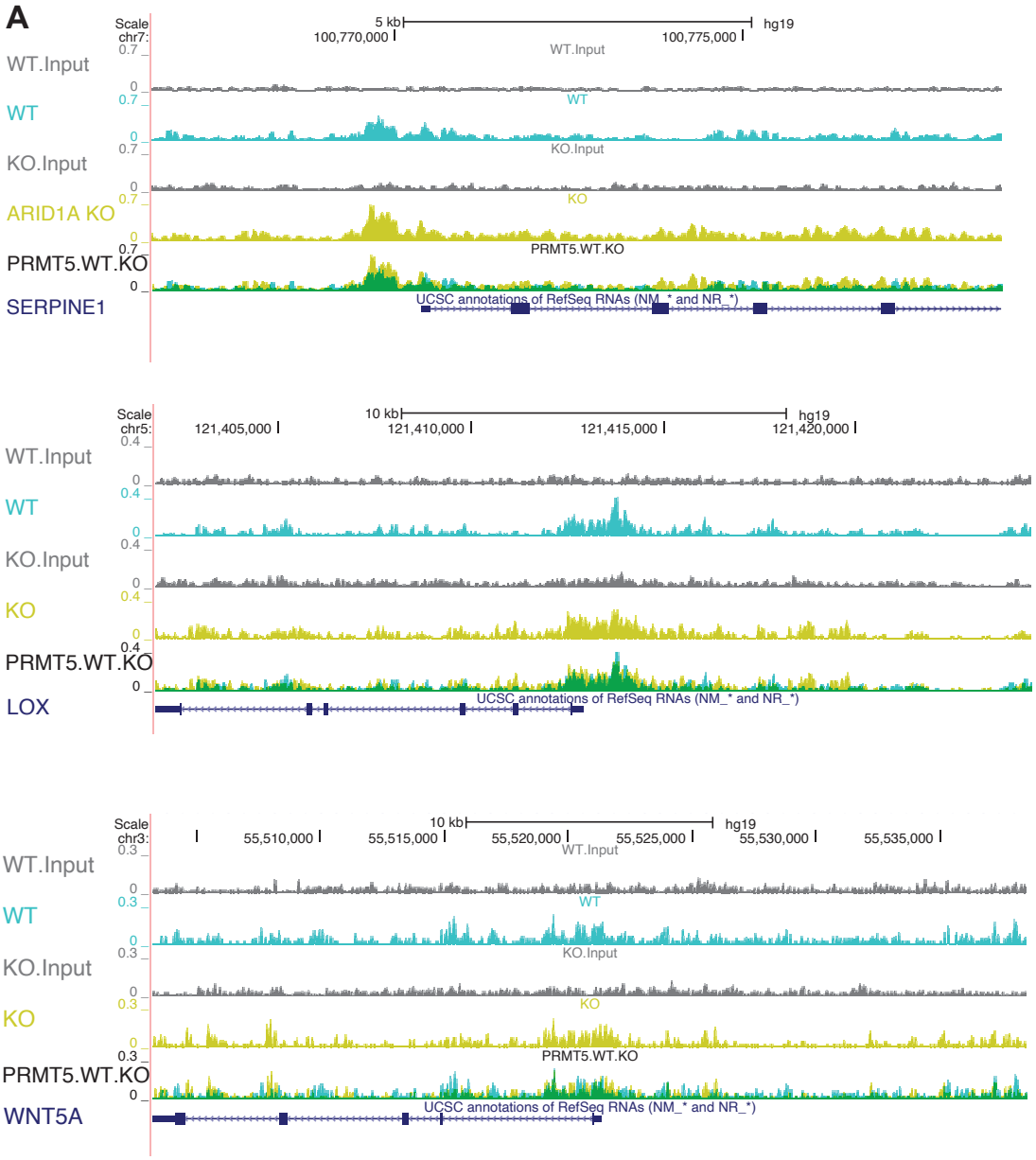
3.7 PRMT5 directly regulates Epithelia to Mesenchymal transition pathway genes

To mechanistically apprehend the mechanism by which PRMT5 regulates the EMT genes with tested both the hypothesis of direct and indirect regulation. PRMT5 enzymatic activity can symmetrically methylate arginine in histone and non-histone proteins such as transcriptional factors. Based on the RNA seq data we performed the Ingenuity Pathway Analysis (IPA) to predict upstream regulators. Among the top hits of the IPA analysis there were not PRMT5 targets accordingly to PRMT5 methylome profiles previously published (data not shown, (Musiani et al. 2019; Radzisheuskaya et al. 2019), allowing the exclusion of an indirect regulation.

Then, we test the hypothesis of a direct regulation and for this aim we performed the ChIP seq with a specific PRMT5 antibody. Interestingly the ChIP seq tracks show PRMT5 association with the promoters of some EMT pathway genes such as SERPINE1, LOX and WNT5A in both 12Z gtrl and 12Z ARID1A KO, suggesting that the PRMT5 localization on those genes is not affect by the inactivation of ARID1A (Fig. 21A).

Intriguing, PRMT5 has been reported to interacted with BRG1 that is the catalytic subunit of the SWI/SNF complexes (Pal et al. 2003). There are different SWI/SNF complexes containing BRG1 that bind the genome through the AT rich interacting domain such as ARID1A, ARID1B and ARID2. On the base of these knowledge, we analyzed the interaction between PRMT5 and BRG1 upon inactivation of ARID1A. The co-immunoprecipitation performed in both 12Z gtrl and 12Z ARID1A KO cells shows that PRMT5 binds BRG1 independently on the ARID1A status (Fig. 21B). In support to these results, we know from a published ARID1A ChIP seq performed in 12Z wildtype cell, that ARID1A binding on the above-mentioned gene promoters overlaps with PRMT5 binding site (data not shown ChIP seq from reference Wilson et al. 2019). Collectively, these results raised the hypothesis that PRTM5 directly regulates the gene expression based on the association with different SWI/SNF complexes.

Consequently, we performed ChIP-qPCR analysis to validate the association of PRMT5 on the SERPINE1, LOX and WNT5A promoter regions observed in the ChIP-seq. Differentially from the ChIP-seq, the PRMT5 association decrease in ARID1A KO cells and accordingly the H3R8me2s binding is also reduced (Fig. 21C-E).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hg19
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WT
KO.Input
ARID1A KO
PRMT5.WT.KO
SERPINE1
UCSC annotations of RefSeq RNAs (NM_* and NR_*)
Scale
chr5:
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121,415,000
121,420,000
hg19
WT.Input
WT
KO.Input
KO
PRMT5.WT.KO
LOX
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chr3:
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55,515,000
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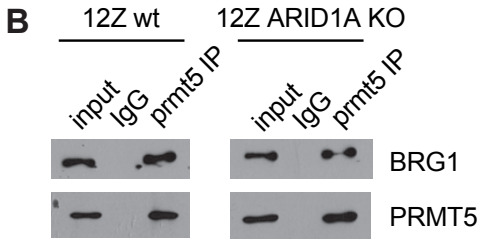
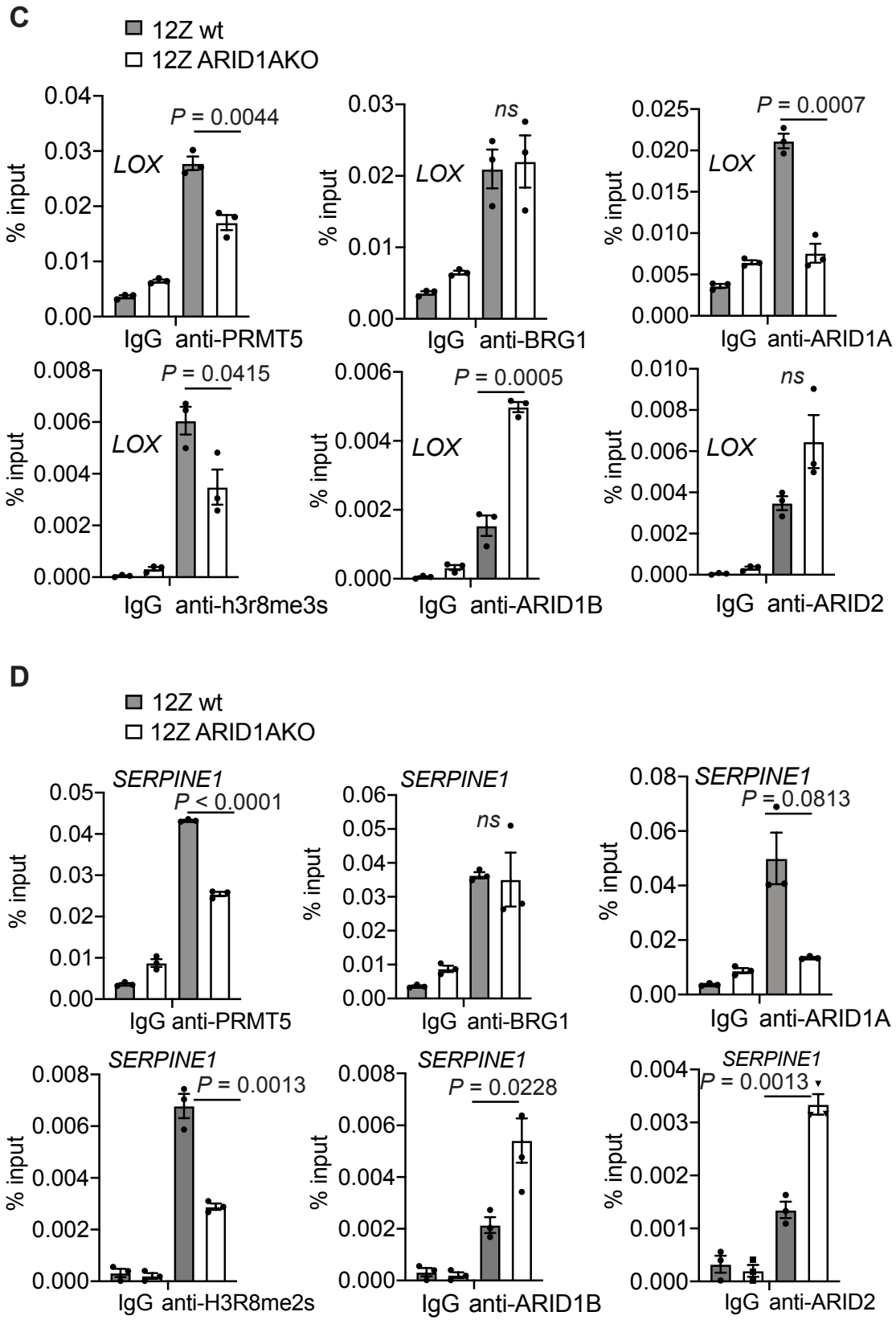
WNT5A
UCSC annotations of RefSeq RNAs (NM_* and NR_*)


B
12Z wt
12Z ARID1A KO
input
IgG
prmt5 IP
input
IgG
prmt5 IP
BRG1
PRMT5


C
12Z wt
12Z ARID1AKO
LOX
% input
P = 0.0044
IgG anti-PRMT5
ns
IgG anti-BRG1
P = 0.0007
IgG anti-ARID1A
P = 0.0415
IgG anti-h3r8me3s
P = 0.0005
IgG anti-ARID1B
ns
IgG anti-ARID2
D
12Z wt
12Z ARID1AKO
SERPINE1
P < 0.0001
IgG anti-PRMT5
ns
IgG anti-BRG1
P = 0.0813
IgG anti-ARID1A
P = 0.0013
IgG anti-H3R8me2s
P = 0.0228
IgG anti-ARID1B
P = 0.0013
IgG anti-ARID2

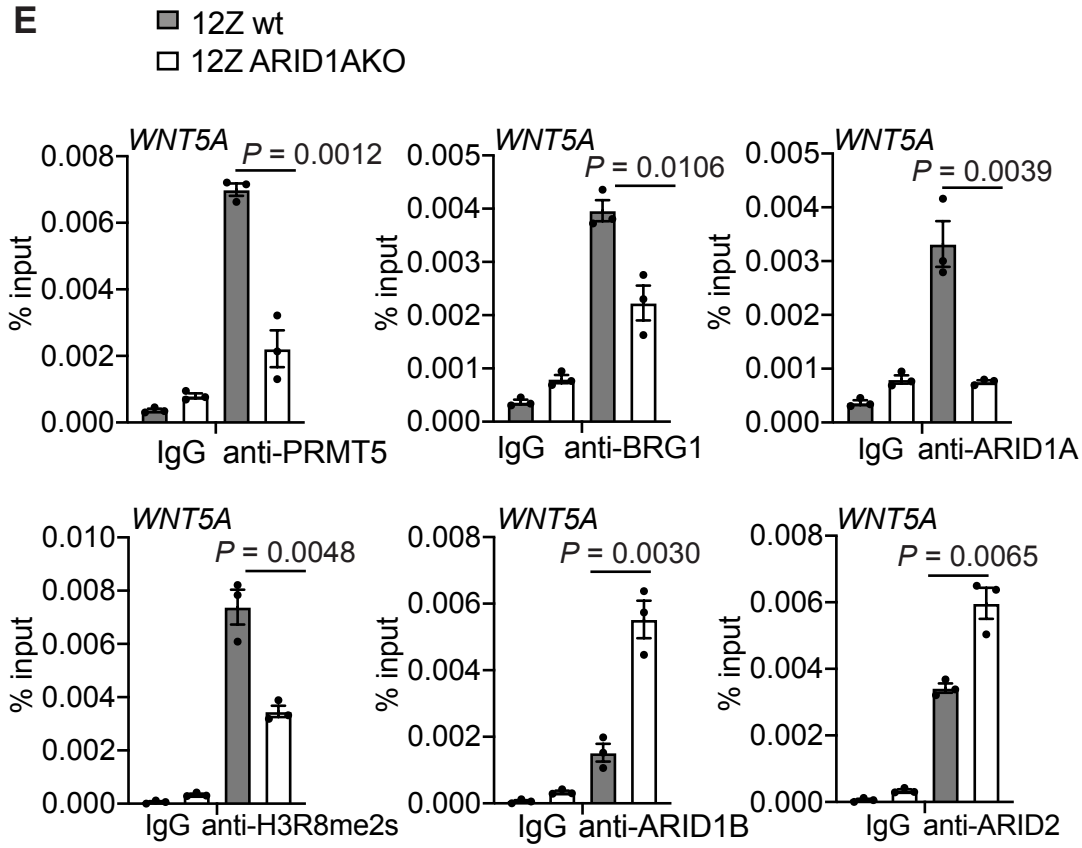


Figure 21. PRMT5 may directly regulates the EMT genes. A ChIP-seq tracks showing the PRMT5 picks in *serpine1*, *lox* and *wnt5a* promoters (from top to bottom) in both 12Z wt and 12Z ARID1A KO normalized to the respective inputs. **B** Immunoblot that show co-binding of PRMT5 and BRG1 in both 12z wt and 12Z ARID1A KO. **C** ChIP-qPCR showing binding of (in order) PRMT5, BRG1, ARID1A, H3R9me2s, ARID1B and ARID2 on *serpine1* promoter in both 12Z wt and 12Z ARID1A KO cells. **D,E** same as **C** but related to *lox* and *wnt5a* promoter respectively.

To test the model by which PRMT5 interact with the SWI/SNF complexes we performed ChIP-qPCR analysis testing other components such as BRG1, ARID1A, ARID1B and ARID2. BRG1 association does not change in ARID1A KO cells, consistent with the notion that BRG1 is a common subunit across different SWI/SNF complexes, except for the WNT5A promoter where there is a slight reduction of its binding (Fig. 21C-E). Additionally, as expected, ARID1A association in those promoters is strongly reduced in ARID1A KO cells (Fig. 21C-E)

Remarkably, both ARID1B and ARID2 promoter association are enhanced in ARID1A KO cells, apart from ARID2 binding in LOX promoter where there is an increased but not statistically significant (Fig. 21C-E). These final finding is consistent with the literature from which we have the knowledge that the SWI/SNF complexes have overlapping genome wide distribution and the ARID1B- or ARID2-SWI/SNF complexes can regulates a similar set of gene as ARID1A-SWI/SNF in ARID1A inactivated contest.

Notably, ARID1B and ARID2 are frequently related to gene repression but there are emerging pieces of evidence that they can also promote the gene transcription, for examples when they recruit transcription factors such as BRCA and MAX (Raab, Resnick, and Magnuson 2015).

PRMT5 related arginine symmetric di-methylation of the histones may also promote or repress the gene expression. The genetic knockdown and the pharmacological inhibition of PRMT5 specifically downregulates the expression of the EMT genes (Fig. 20G) and mechanistically PRMT5 and the SWI/SNF complexes components binds some of those genes (Fig. 21A) suggesting that different SWI/SNF complexes may require the PRMT5 enzymatic activity in order to regulate the gene expression of the EMT genes.

In conclusion, PMRT5 interact with the SWI/SNF complexes via the binding of BRG1 and those factors are directly associated to the EMT gene promoters, supporting a model by which the ultimate regulation of the EMT gene expression may be dependent on the combinatorial effect of PRMT5 and SWI/SNF complexes activities.

3.8 Discussion

The SWI/SNF subunit ARID1A is frequently altered in endometrial cancer with a predominancy of truncated mutations and out of frame deletions that are translated in loss of the protein (Reisman, Glaros, and Thompson 2009). The inactivation of ARID1A in primary endometrial cancer collaborates to drive the invasion of the surrounding myometrium (Wilson et al. 2019; Suryo Rahmanto et al. 2020). Moreover, it is well known that epigenetic factor could regulate tumor invasion and metastatic pathways (Chatterjee, Rodger, and Eccles 2018). There is an unmet need to establish a therapy for high grade endometrial cancer. We therefore studied the invasive

endometrial cancer that specifically carries inactivating mutations of ARID1A in order to find another targetable epigenetic factor whose inhibition can hamper the myometrial invasion synthetically to ARID1A inactivation.

Using the isogenic cell line 12Z gtrl and 12Z ARID1A KO, with the combination of the CRISPR screening and the *in vitro* invasion assay we identified PRMT5 as novel therapeutic target for reducing the spreading of ARID1A deficient endometrial cancer cells. Supporting to this initial screening finding, PRMT5 has been previously reported that can promote the EMT, for example via regulating E2F pathway in colon cancer, or via activating the AKT pathway in high-risk neuroblastoma (Barczak et al. 2020; Huang et al. 2022). Moreover it has been shown that the Snail/PRMT5/NuRD(MTA1) complex promotes the invasion and metastasis of cervical cancer (Gao et al. 2021).

With the *in vitro* invasion assay, we validated the screening result in both 12Z ARID1A KO cells and another ARID1A-deficient SNGM cell line confirming that PRMT5 inhibition decreases the invasion of these cells. As counter evidence, PRMT5 inhibition does not affect the invasion of 12Z gtrl cells. Additionally, the data show that the enzymatic activity of PRMT5 is involved in the observed phenotype. The PRMT5 inhibitor used in this study is under clinical trial further highlighting PRMT5 as a promising clinical target for endometrial cancer. To validate the results in vivo we employed the transgenic murine endometrial cancer model ARID1A/PTEN double conditioned knock out (Suryo Rahmanto et al. 2020). The mice carrying the primary tumor were treated with GSK3326959. The treatment was tested for a period of two and three weeks at the same concentration and the final pathologic evaluation showed the treatment better reduced the invasion of the myometrium holding the tumors in stage 1a without developing into a more aggressive stage. We then explore the transcriptome changing upon PRMT5 inhibition. PRMT5 symmetric demethylation of the arginine residues of histones can both result in repression and activation of the transcription (H. Chen et al. 2017). Intriguing, the RNA seq analysis, reviled that both the PRMT5 knockdown and the PRMT5 pharmaceutical inhibition with GSK3326959 downregulate Epithelia to Mesenchymal transition pathway genes specifically in AIRD1A knock out cells. Mechanistically, our ChIP-seq analysis reviled that PRMT5 is associated with the promoter of some EMT genes such as SERPINE1, LOX and WNT5A in both ARID1A wildtype and ARID1A KO cells, even though PRMT5 inhibition can downregulate those EMT gene expression only in ARID1A KO cells. PRMT5 can interact with the SWI/SNF complexes via binding the subunit BRG1 (H. Chen et al.

2017), suggesting that SWI/SNF complexes may require PRMT5 activity to regulates the gene expression. To explore this possibility, we analyzed the association of the different SWI/SNF complexes with the PRMT5 targets. Furthers ChIP-qPCR analysis show that both ARID1B and ARID2 association to the PMRT5 targets increase in ARID1A KO cells, These results together with the transcriptome analysis suggest a model by which, in ARID1A deficient cells, the ARID1B- or ARID2- SWI/SNF complexes may require the PRMT5 activity to promote the expression of the EMT genes, and in a contest of ARID1A wildtype cell the ARID1A- SWI/SNF complex function is not affected by PRMT5 activity. Therefore, PRMT5 inhibition is effective in the reduction of the invasion of endometrial cancer cells only synthetically to ARID1A inactivation. This hypothesized model is consistent with the evidence that ARID1A, ARID1B, and ARID2 have a highly overlapping role in transcriptional regulation that is expressed by the existence of both competitive and cooperative interaction among distinct SWI/SNF complexes that recruit and interact with other factor in order to regulate the transcription (Raab, Resnick, and Magnuson 2015). Collectively the model suggests that the ARID1B- and ARID2- SWI/SNF complexes may also require the PRMT5 to promote that EMT gene in a contest of ARID1A inactivation, but more pieces of evidence are needed to further validate this model. In conclusion, the data strongly shows that PRTM5 represents an intriguing therapeutic target for ARID1A inactivated endometrial cancer. The pharmacological inhibition of PRMT5 enzymatic activity may be a new therapeutic strategy to hamper the myometrial invasion in ARID1A deficient endometrial cancer.

3.9 Future directions

Limitations of this study is that we have not tested other ARID1A wildtype high grade endometrial cancer cell lines and that we have not rescued the expression of ARID1A in SNG-M cells to further confirm the results. Additionally, the mechanism by which PRMT5 promotes the EMT interacting with the SWI/SNF complexes in a contest of ARID1A inactivation, require additional investigation, for examples exploring the effect of ARID1B or ARID2 knock down to confirm the phenotype observed by PRMT5 inactivation. On a big picture, PRMT5 inhibition may become part of other endometrial cancer therapies as combinational strategy to improve the tumor arrest.

4. Materials and Methods

4.1 Cell culture, transfection and reagents

12Z endometrial epithelia immortalized cells (RRID: CVCL_0Q73), gift from Lawrenson lab, were cultured in DMEM/F12 (Corning, #10-092 CM); SNGM (RRID: CVCL_1707), A1847 (RRID:CVCL_9724) and CAOV3 (RRID:CVCL_0201) were cultured in RMPI (Corning, #10-040 CM), HEK293T cells were cultured in DMEM (Corning, #10-013 CM); all the media were supplemented in 10%FBS (R&D system, #S11510), penicillin/streptomycin (1%) with 5% CO2 at 37°C. Before used for experiments, the cell lines were first retrieved from liquid nitrogen and subsequentially cultured for at least two passages. The cells were authenticated by STR (short tandem repeat) analysis performed by the The Wistar Institute Genomics Facility based on the available STR profiles. *Mycoplasma* testing was perfomed monthly using LookOut *Mycoplasma* PCR detection (Sigma). The reagent Lipofectamine 2000 (Life Technologies) was used to transfect the cells following the manufacturer instruction. The following inhibitor have been purchased from MCE: SCD1 inhibitor CAY10566 (#HY- 15823), CARM1 inhibitor EZM2302 (#HY-111109), PRMT5 inhibitor GSK595 (#HY-101563). BSA-palmitate was purchased from Cayman (#29558) and BSA-oleic acid from Sigma (#O3008).

4.2 Animal experiment

For the first part of this thesis, the in vivo protocol for animal experiments was subject to review and approval by the Institutional Animal Care and Use Committee of The Wistar Institute. Female C57BL/6 mice were purchased from Charles River Laboratories, and female NSG mice were purchased from The Wistar Institute Animal Facility. For the Intra-bursal injection, 0.5*10^6 wildtype or CARM1 knockout UPK10 or A1847 cells were unliterally injected into the bursa sac of the ovary in 6-8 weeks old C57BL/6 or NSG mice respectively (n= 5 mice/group, except n=3 for CARM1 knockout UPK10 IN C57BL/6) Once tumors had formed, the mice were randomized into groups that received either the SCD1 inhibitor at a dosage of 5 mg per kg in 0.5% methyl

cellulose, or the vehicle, administered once a day by oral gavage for a duration of two weeks. After treatment, the tumors were surgically excised, and the tumors weight was measured as an indicator of the tumor burden.

For the second part of this thesis, the animal experiment has been conducted by our collaborators in Wang Lab and Shih Lab at the Johns Hopkins University according with their institutional procedure. Briefly, female transgenic mice, previously generated (Suryo Rahmanto et al. 2020) have been used for this experiment. 15 mice have been feed with doxycycline, after 10 days the mice were randomized in 4 groups administrated either with PRMT5 inhibitor (100mg/kg) or vehicle, daily for two weeks (first bunch, 4 mice per group) and three weeks (second bunch, four mice for control and three mice per treatment). After the treatment, the uterus has been collected and a pathologic evaluation has been performed by Wang Lab and Shih Lab.

4.3 Protein extraction and immunoblot

Cells were harvested and cell pellets were collected. The pellets were washed two times with cold PBS and lyseted with RIPA lysis buffer (150 mmol/L NaCl, 50mmol/L Tris (pH 8.0), 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, protease inhibitor) on ice for 30 minutes to extracted protein. Protein concentration was detected by the BCA assay (Pierce, #23225). Samples were separated by SDS-PAGE and transferred to PVDF (polyvinylidene fluoride membrane, Millipore). Membranes were then blocked with 5% BSA/TBS-T, incubated with primary antibodies and then secondary antibodies. The primary and secondary antibodies used for immunoblotting are listed in table 1.

4.4 Co-immunoprecipitation

12Z and 12ZARID1A KO Cells were harvest and wash twice with cold PBS. The cell pellet have been lysated with No Denaturing buffer (137mM NaCl, 20mM TrisHCl pH8,2mM EDTA, 1% NP40). 1mg of protein has been incubated with 2ug of PRMT5 or IgG antibodies (see table 1), overnight at 4C. Then, the lysates have been incubated

with protein A/G magnetic beads for 2 hours, rotation at 4C. The protein-beads complexes have been isolated with a magnetic rack and washed twice with the lysis buffer. The immunoprecipitated complexes have been eluted from the beads with loading buffer 2X. The eluted proteins have been used for immunoblotting (see above) in 8% polyacrylamide gel.

4.5 Quantitative reverse transcriptase PCR

First the total RNA has been purified from cells using Triazol according to manufacturing instructions. 2mg of purified RNA has been used for RT-PCR (reverse-transcriptase PCR) using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, #4374967). The cDNA has been then utilized for qPCR (quantitative PCR) using iTaq Universal SYBR Green Supermix (Bio-Rad, #1725121) running the reaction on QuantStudio 3 Real-Time PCR System. The gene expression fold change was calculated using the 2^{DDCT} analysis. The primers used for the experiments are listed in table 2.

4.6 CRISPR mediated knock out

The pLentiCRISPR v2-blast plasmid (Addgene #83480, RRID: Addgene_83480) or pLentiCRISPR v2-puro plasmid (Addgene #98290, RRID: Addgene_9829) was digested with BsmBI enzyme (NEB, #R0739) at 55°C for 1 hour and subsequently analyzed on a 1% agarose gel. The digested plasmid band was excised from the gel and purified using the QIAquick gel extraction kit (QIAGEN, #166047244). Each pair of oligos (listed in table 2) were phosphorylated using T4 PNK enzyme (M0201S) in T4 ligation buffer (New England Biolabs, #B0202S) and then annealed in a thermocycler at 37°C for 30 minutes, followed by 95°C for 5 minutes, and gradually cooled to 25°C at a rate of 5°C per minute. The annealed oligonucleotides were diluted 1:200 in RNase/DNase-free water. Ligation of the annealed oligonucleotides and the digested pLentiCRISPR v2 plasmid was performed using Quick Ligase (New England

Biolabs, #M2200). The resulting plasmids were utilized for lentivirus infection (details below) to generate CARM1 knockout (KO) cells in A1847 and UPK10 cell lines, as well as ARID1A KO cells in the 12Z cell line. In brief, the respective lentivirus was used to infect the cells, and single clones were selected in 96-well plates. Subsequently, the knockout efficiency of the selected single clones was evaluated using western blot analysis.

4.7 Lentiviral infection

HEK293FT cells were transfected with the designated target plasmids (mentioned above), along with the packaging plasmids psPAX2 (Addgene #12260, RRID: Addgene_12260) and pMD2G (Addgene #12259, RRID: Addgene_12259), using Lipofectamine 2000. The transfection was performed at a ratio of 1 mL Lipofectamine per 1 mg DNA, and the cells were incubated with the transfection mixture for 6 hours before replacing the medium. After 48 hours of transfection, lentivirus was harvested and filtered through a 0.45μm filter. The filtered medium was then used to infect the target cells for an additional 48 hours. Following infection, the cells were selected by culturing them in medium containing either 1 mg/mL puromycin or blasticidin for a period of 3 days. The following target-specific short hairpin RNA (shRNA)-expressing plasmids, obtained from the Molecular Screening Facility of the Wistar Institute, were utilized: pLKO.1-shPRMT5 TRCN0000107086 and TRCN0000107088.

4.8 CRISPR screening

The CRISPR screening in this study was conducted following a previously published protocol (Lin, Guo, et al. 2021). The library used consisted of single-guide RNAs (sgRNA) targeting 1,218 genes involved in chromatin regulation, obtained from Kristian Helin Lab (Müller et al. 2021). A total of 6 * 10^6 12Z wildtype and 12Z ARID1A knockout cells were transduced with a filtered DMEM/F12 containing a virus expressing the human epigenetic gDNA library. The multiplicity of infection (MOI) was set at 0.3 to ensure a library representation of 1,000. Subsequently, the established cell library was either used for invasion assays or harvested as input cells.

For the invasion assay, approximately 12 million cells were seeded in multiple 6-well inserts placed in 6-well plates, with each well containing 0.3 mg/mL Matrigel. After 36 hours, the invading cells were collected from the lower surface of the trans-well inserts. Genomic DNA was extracted from both the input cells and the invading cells using a salt precipitation method previously described by Lin et al. 2021. In summary, cell pellets were suspended in 6 mL of gDNA lysis buffer (50 mmol/L EDTA, 1% SDS, 50 mmol/L Tris pH 8.0), and then 30 µL of 20 mg/mL proteinase K was added. The mixture was incubated at 55°C overnight, followed by the addition of 30 µL of 10 mg/mL RNase A and incubation at 37°C for 30 minutes. After cooling on ice, protein precipitation was achieved by adding 2 mL of a 7.5 mol/L stock solution of Ammonium Acetate and centrifuging at > 4,000 g for 10 minutes. The supernatant containing genomic DNA was collected and precipitated by adding 6 mL of 100% isopropanol and centrifuging at > 4,000 g for 10 minutes. The resulting DNA pellet was washed with 70% ethanol, air-dried, and dissolved in H2O for PCR amplification. The eluted DNA was then used to construct a library for sequencing, following previously published methods. The specific primers used for the library construction can be found in the table 3.

The library construction involved a two-step PCR amplification process. In the first PCR step, eight PCR reactions were performed, each containing 5 µg of genomic DNA, 1.5 µL of 10 mmol/L forward and reverse primers, and 50 µL of NEBNext Q5 HotStart HiFi PCR Mastermix. The amplification was carried out for 15 cycles using a Bio-Rad T100 thermal cycler PCR machine, with cycling conditions of 98°C for 20 seconds, 60°C for 30 seconds, and 65°C for 45 seconds. The first PCR products from the eight reactions for each sample were pooled together and purified using AMPure XP beads, following the manufacturer's instructions. The purified products were then suspended in 800 µL of H2O.

To barcode the samples, a second PCR step was performed. For each sample, 10 µL of the first PCR product was used in separate PCR reactions containing 1 µL of 10 mmol/L forward and reverse Illumina primers, and 50 µL of NEBNext Q5 HotStart HiFi PCR Mastermix. This amplification was carried out for 20 cycles using the same cycling conditions as described above. The final PCR product was purified using AMPure XP beads, following the manufacturer's instructions. Subsequently, the purified products were subjected to 75 bp single-end sequencing on the NextSeq 500 platform (Illumina) at the Wistar Genomic facility. The screening analysis has been

performed by the former post doc of the lab J. Lin PhD in collaboration with the Wistar Bioinformatic facility.

4.9 Invasion assay

A total of 5 * 10^4 cells were suspended in a serum-free medium and seeded into the chamber of a trans-well insert placed in a 24-well plate. For invasion assays, a layer of 100 µl of Matrigel was applied on top of the insert membrane, resulting in a final concentration of 0.3 mg/mL. In the case of migration assays, the membrane was left uncovered. After 36 hours of incubation, the upper chamber was carefully cleaned using a cotton swab, and the lower surface of the membrane was subjected to staining using the following steps: methanol fixation (10 minutes), crystal violet staining (10 minutes), and two washes with PBS. Subsequently, images of the stained cells were captured using an inverted microscope, and the invading cells were quantified.

4.10 Colony formation

First, 3,000 cells were plated into individual well of 24-well tissue culture plate, followed by treatment with the designated compounds at the specific concentrations. The cell medium was replenished every three days, maintaining the prescribed compound doses over a10 days period. Following the 10 days incubation, colonies were subjected to staining using a solution of 0.05% crystal violet/10% methanol. Quantification of the colonies was performed using the NIH ImageJ software (RRID: SCR_003070).

4.11 Reverse Phase Protein Array (RPPA)

For each replicate, approximately 0.5 × 106/3 mL of wildtype or CARM1 knockout A1847 or PEO4 cells were seeded into 6-well plates and allowed to incubate for 24 hours. Then, the cells were washed with PBS and lysed using 100 µL of lysis buffer containing the following components: 50 mmol/L HEPES pH 7.4, 1% Triton X-100, 1.5 mmol/L MgCl2, 150 mmol/L NaCl, 100 mmol/L NaF, 1 mmol/L ethylene glycol-bis(β-

aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mmol/L sodium pyrophosphate, 10% glycerol, and 1 mmol/L Na3VO4. The lysis buffer also contained phosphatase and protease inhibitors (Roche Applied Science, catalog nos. 04906837001 and 05056489001, respectively) and was incubated with occasional shaking on ice for 20 minutes. Subsequently, the cells were scraped off the wells and the lysates were centrifuged (14,000 rpm, at 4°C for 10 minutes). The resulting supernatants were collected, and protein concentrations were determined using the BCA assay and adjusted to 1.5 μg/μL. The lysates were then mixed with 4x SDS sample buffer (comprising 8% SDS, 10% beta-mercaptoethanol, 40% glycerol, and 0.25 mol/L Tris-HCL pH 6.8) and boiled at 95°C for 5 minutes. Finally, the prepared samples were submitted to the reverse phase protein array (RPPA) core facility at MD Anderson Cancer Center for further analysis.

4.12 Metabolomic analysis

Metabolomics analysis was conducted using a previously described method (13). To summarize, polar metabolites were extracted using 80% methanol. Subsequently, LC/MS analysis was performed using the Thermo Fisher Scientific Q Exactive HF-X mass spectrometer with HESI II probe. This instrument was coupled with the Thermo Fisher Scientific Vanquish Horizon Ultra-High-Performance Liquid Chromatography (UHPLC) system. Chromatographic separation was carried out via hydrophilic interaction liquid chromatography (HILIC) at 0.2 mL/minute and 45°C, employing a ZIC-pHILIC column (2.1-mm i.d. × 150-mm; EMD Millipore). The solvents used for the gradient were solvent A (0.1% ammonium hydroxide pH 9.2, 20 mmol/L ammonium carbonate) and solvent B (acetonitrile) with the following gradient profile: 0 minute-85% B; 2 minutes-85% B; 17 minutes-20% B; 17.1 minutes-85% B; and 26 minutes-85% B.

For quantification, the samples were randomized and analyzed using full mass spectrometry (MS) scans with polarity switching, scanning a range of 65 to 975 m/z, with an automated gain control (AGC) target of 1E6, 120,000 resolution, and a maximum injection time of 100 ms. Additionally, a sample pool (QC) was created by combining equal volumes of each sample and periodically analyzed throughout the

run sequence with full MS scans. MS-MS analysis was also performed for the QC samples using different runs for negative and positive mode analysis. For MS-MS, a full MS scan was obtained, followed by MS-MS of the 10 most abundant ions, with an AGC target of 5E4, 15,000 resolution, an isolation width of 1.0 m/z, max IT of 50 ms, and a stepped collision energy of 20, 40, and 60.

Metabolite identification (MS-MS data) and quantitation (MS only data) were conducted using Compound Discoverer 3.0 (Thermo Fisher Scientific). Metabolites were identified based on accurate mass and either retention time using a mass list generated from standards or by comparing MS-MS spectra with the mzCloud database. Putative annotations were made with a matched score of at least 50. Metabolite levels were then normalized to the protein amount for each sample.

4.13 Global lipidomics and fatty acid saturation analysis

Control or CARM1 knockout A1847 cells were washed with cold PBS, collected in cold methanol, and then supplemented with EquiSPLASH mix (Avanti Polar Lipids). Lipids were extracted using a modified Folch extraction method (2:1:1 chloroform:methanol:0.88% sodium chloride) and subjected to LC/MS-MS analysis. LipidSearch 4.2 software (Thermo Fisher Scientific) was employed to detect lipid species based on MS-MS spectra, with product ion mass tolerances and 5 ppm precursor.

Identification of lipid species was filtered based on the expected identification quality and main adduct. Peak areas were utilized for quantification and were adjusted using EquiSPLASH lipids to represent their respective classes. Furthermore, the values were normalized to the protein content in each sample. The quantification of lipid classes was achieved by summing the peak areas of all species within the same class. For saturation analysis, the fatty acids (FAs) incorporated into lipids with high-confidence identifications (grades A and B from LipidSearch) were categorized according to the number of carbon double-bonds as saturated (0), monounsaturated (1), or polyunsaturated (>1). The quantification of each lipid species with identified FA levels was weighted by the number of FAs of each type present in the respective species.

4.14 Total fatty acid quantification

The lipids underwent saponification with methanolic KOH, and fatty acids (FAs) were subsequently extracted using hexane. The total FA extracts were then analyzed using LC/MS on a Thermo Fisher Scientific Q-Exactive HF-X coupled with a Vanquish Horizon UHPLC system. For reversed-phase chromatography, an Accucore C18 column with a size of 2.1 mm × 150 mm (Thermo Fisher Scientific) was used, employing water and acetonitrile solvents (both containing 0.1% acetic acid).
In negative mode, full MS scans were acquired ranging from 180 to 650 m/z with a resolution of 120,000. To identify the FAs, TraceFinder 4.1 (Thermo Fisher Scientific) was utilized, using accurate retention time and mass information derived from standards. Peak areas were quantified, and the FA levels of each sample were corrected using the level of deuterated FA 18:d7, which originated from saponified EquiSPLASH lipids. The data were further normalized based on the protein content in each sample.

4.15 IHC staining

For the serial dissection of tumors, which were fixed in PBS containing 10% formalin (Thermo Fisher Scientific, #SF100-4) and embedded in paraffin, immunohistochemical (IHC) staining was conducted using the Dako EnVision+ system. In brief, the sections were deparaffinized, rehydrated, and treated with a solution of 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed using sodium citrate buffer (Thermo Fisher Scientific, #005000). Subsequently, each section was blocked with PBS containing 1% BSA. The primary antibodies used for overnight incubation at 4°C are listed in table 1. Counterstaining was performed using Mayer's Hematoxylin (Dako, #3309S). The expression of the target was assessed using the histologic score (H score) method.

4.16 RNA sequencing

The total RNA from the control, PRMT5 sh1, and 3-day GSK595-treated samples of both 12Z wildtype and 12Z ARID1A knockout cells was isolated using the RNeasy mini Kit (Qiagen, #74106), following the instructions provided by the manufacturer. Subsequently, the extracted RNA was treated with DNase I (Qiagen, #79254) to remove any remaining DNA. The RNA sequencing libraries were prepared by the Wistar Genomics facility using the ScriptSeq complete Gold kit (Epicentre, #SCL24EP). The libraries were then subjected to a 75 bp paired-end sequencing run on the NextSeq 500 platform, utilizing Illumina's NextSeq 500 high output sequencing kit (#20024906), following the manufacturer's guidelines. The bioinformatic analysis has been performed by the Bioinformatic facility at Wistar.

4.17 CHIP sequencing and CHIP-qPCR

Chromatin immunoprecipitation (ChIP) was performed following previously established protocols (Lin, Liu, et al. 2021). Briefly, the cells were fixed using 1% formaldehyde/PBS for 10 minutes at room temperature and then quenched with 0.125 mol/L glycine. Following fixation, the cells were lysed in ChIP lysis buffer 1 on ice for 30 minutes, followed by lysis buffer 2 at room temperature for 10 minutes. Chromatin fragmentation was achieved by sonication. After centrifugation, the digested chromatin from each sample was collected and incubated overnight at 4°C with the specific antibodies listed in the table. The following day, the antibody and target protein/DNA complexes were captured using Protein A/G Dynabeads with 1.5 hours of incubation at 4°C. Post-incubation, the Dynabeads were washed, and the chromatin was eluted using TES buffer [1 mmol/L EDTA, 1% SDS, 10 mmol/L Tris-Cl (pH 8.0)]. The eluted DNA/protein complexes underwent proteinase K digestion and de-crosslinking. The resulting target DNA was purified using the Zymo ChIP DNA Clean and Concentrator Kit (Zymo Research, catalog no. D5205). The ChIP-qPCR primer sequences can be found in the table.

For chromatin immunoprecipitation sequencing (ChIP-seq), the NEBNext Ultra DNA Library Prep Kit (NEB; #E7645) was utilized to construct libraries using the purified

DNA, following the manufacturer's instructions. The libraries were then subjected to a 75-bp single-end sequencing run on the NextSeq 500 platform (Illumina) at the Wistar Genomic facility. The bioinformatic analysis has been performed by the Bioinformatic facility at Wistar.

4.18 GSEA (Gene set enrichment analysis)

RNA seq data produced for this thesis were utilized for the pathway enrichment analysis. GSEA was performed following the guidelines on the GSEA website of Broad Institute (http://www.broadinstitute.org/gsea/index.jsp) using hallmark gene set. The GSEA analysis has been performed by the PhD candidate in collaboration with the Wistar Bioinformatic facility.

4.19 Data availability

The ChIP-seq data generated for the first part of this thesis have been deposited in the GEO (RRID: SCR_005012) under accession no. GSE202259 (Lombardi et al. 2023). XBP1s CUT&RUN sequencing citied was previously deposited in the GEO database under accession GSE157118 (Lin, et al. 2021). ChIP-seq and RNA seq data generated for the second part of this thesis have not been published yet. TCGA high-grade serous ovarian cancer or endometrial cancer RNA-seq datasets were downloaded from cBioPortal.

5. Tables

Table 1. List of antibodies used in this thesis.

protein name	company	catalog	dilution/ amount	application	RR:ID
ACC1	Cell Signaling Technology	#4190	1:1000	immunoblot	RRID: AB_10547752
anti-β-actin	Sigma	#A5316	1:5000	immunoblot	RRID:AB_476743
ARID1A	Cell Signaling Technology	#12354	1:1000	immunoblot	RRID:AB_2637010
CARM1	Cell Signaling Technology	#3379S	1:1000	immunoblot	RRID:AB_2068433
cleaved LAMIN A/C	Cell Signaling Technology	#2032S	1:1000	immunoblot	RRID:AB_2136278
cleaved PARP	Cell Signaling Technology	#5625S	1:1000	immunoblot	RRID:AB_10699459
FASN	Cell Signaling Technology	#3189	1:1000	immunoblot	RRID: AB_2100798
H3R8me2s	abcam	ab130740	1:1000	immunoblot	RRID:AB_2801510
PRMT5	Cell Signaling Technology	#79998	1:1000	immunoblot	RRID:AB_2799945
SCD1	abcam	#ab236868	1:1000	immunoblot	RRID: AB_2928123
ACC1	Cell Signaling Technology	#4190	1:100	IHC staining	RRID: AB_10547752
CARM1	Cell Signaling Technology	#12495	1:100	IHC staining	RRID: AB_2797935
cleaved caspase 3	Cell Signaling Technology	#9661	1:200)	IHC staining	RRID: AB_2341188
FASN	Cell Signaling Technology	#3189	1:100	IHC staining	RRID: AB_2100798
Ki67	Cell Signaling Technology	#9449	1:1,000	IHC staining	RRID: AB_2797703
SCD1	Proteintech	#23393-1-AP	1:100	IHC staining	RRID: AB_2744674
PRMT5	active motif	61001	2ug	Co-IP	RRID: AB_2615010
ARID1A	abcam	ab182560	10ug	ChIP-seq, ChIP qPCR	RRID:AB_2889973
CARM1	Cell Signaling Technology	#12495S	10ug	ChIP-seq, ChIP qPCR	RRID: AB_2797935
PRMT5	active motif	61001	10ug	ChIP-seq, ChIP qPCR	RRID: AB_2615010
ARID1B	abcam	ab57461	10ug	ChIP qPCR	RRID:AB_2243092
BRG1	abcam	ab110641	10ug	ChIP qPCR	RRID:AB_10861578
H3R17me2a	abcam	ab8284	4ug	ChIP qPCR	RRID:AB_306434
H3R8me2s	abcam	ab130740	10ug	ChIP qPCR	RRID:AB_2801510
XBP1s	Novus Biological	#NBP1-77681	4ug	ChIP qPCR	RRID: AB_11010815

Table 2. List of oligos used in this thesis.

target	sequence 5' -> 3'	application	species
arid1a forward	caccGTGCCTTCATTTCCCCGCGCT	CRISPR KO	Homo sapiens
arid1a reverse	aaacAGCGCGGGGAAATGAAGGCAC	CRISPR KO	Homo sapiens
carm1 forward	caccAGCACGGAAAATCTACGCGG	CRISPR KO	Homo sapiens
carm1 reverse	aaacCCGCGTAGATTTTCCGTGCTc	CRISPR KO	Homo sapiens
carm1 forward	caccTCGCGTCGCCGATAGTGAGG	CRISPR KO	Mus musculus
carm1 reverse	aaacCCTCACTATCGGCGACGCGAc	CRISPR KO	Mus musculus
prmt5 forward	TCCCCACTAGCATTTTCCTGA	RT-qPCR	Homo sapiens
prmt5 reverse	ATGAACTGCACCTCCAACTTGA	RT-qPCR	Homo sapiens
scd1 forward	CTTGCGATATGCTGTGGTGC	RT-qPCR	Homo sapiens
scd1 reverse	CCGGGGGCTAATGTTCTTGT	RT-qPCR	Homo sapiens
fasn forward	CCTGGCTGCCTACTACATCG	RT-qPCR	Homo sapiens
fans reverse	CACATTTCAAAGGCCACGCA	RT-qPCR	Homo sapiens
acc1 forward	CATCTCCCTTGGCCCAACC	RT-qPCR	Homo sapiens
acc1 reverse	TCTGAGCCAACAGAAGCAGG	RT-qPCR	Homo sapiens
scd1 forward	GGAAGTGCTGGGAGGTGTCATT	RT-qPCR	Mus musculus
scd1 reverse	CTGCCTTCGTCCTTCTTCTTCA	RT-qPCR	Mus musculus
fasn forward	TGCACCTCACAGGCATCAAT	RT-qPCR	Mus musculus
fans reverse	GTCCCACTTGATGTGAGGGG	RT-qPCR	Mus musculus
acc1 forward	GAGAGTTCACCCAGCAGAATAA	RT-qPCR	Mus musculus
acc1 reverse	CTGATCCACCTCACAGTTGAC	RT-qPCR	Mus musculus
fasn forward	CGGGGAAAGCCACCAACA	Chip-qPCR	Homo sapiens
fans reverse	GCTCCTCCAGGCCCTTCA	Chip-qPCR	Homo sapiens
acc1 forward	GTGAACGGCCTGGAGTAACC	Chip-qPCR	Homo sapiens
acc1 reverse	CCCCTGTCTCCCACCTCAG	Chip-qPCR	Homo sapiens
scd1 forward	AGAGGGAACAGCAGATTGCG	Chip-qPCR	Homo sapiens
scd1 reverse	CTGTAAACTCCGGCTCGTCA	Chip-qPCR	Homo sapiens
lox forward	CGAAGCGCATCACTCCTTTT	Chip-qPCR	Homo sapiens
lox reverse	CGTGATTTGAGCCCCGTTTT	Chip-qPCR	Homo sapiens
serpine1 forward	ATCAAAAGGACGGAGTGGGG	Chip-qPCR	Homo sapiens
serpine1 reverse	CCCCTTGCATTTCTGCTCCT	Chip-qPCR	Homo sapiens
wnt5a forward	GAAAACGCACAAGTCGCCAT	Chip-qPCR	Homo sapiens
wnt5a reverse	CCCGTTTTTGCCGAACCCTA	Chip-qPCR	Homo sapiens

Table 3. Primers used for library construction for CRISPR screening.

	Name	Sequence 5'-3'
First PCR primers	lentiCRISPR v2Adaptor_F	AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG
	lentiCRISPR v2Adaptor_R	TCTACTATTCTTTCCCCTGCACTGTtgtgggcgatgtgcgctctg
Second PCR primers	Control-P5 #1_mGeCKO	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTtAAGTAGAGtcttgtggaaaggacgaaacaccg
	Control-P5 #2_mGeCKO	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTatACACGATCtcttgtggaaaggacgaaacaccg
	Control-P5 #3_mGeCKO	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTgatCGCGCGGTtcttgtggaaaggacgaaacaccg
	Control-P5 #4_mGeCKO	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTcgatCATGATCGtcttgtggaaaggacgaaacaccg
	Control-P7_mGeCKO	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTG CTCTTCCGATCTTCCTTGGTTCTACTATTCTTTCCCCTGCACTGT

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