Role of JARID1 histone demethylases in modulating steroid receptor function

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1. Gutachter	**Prof. Dr. Petra Knaus** Institut für Biochemie Freie Universität Berlin
2. Gutachter	**Prof. Dr. Peter Donner** Proteinchemie Bayer Schering Pharma AG

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1 INTRODUCTION

1.1 Epigenetic control of gene expression

Almost all somatic cells of an organism contain the same genetic information. During development, cells start in a pluripotent state before differentiating into many cell types with different morphologies and functions. The regulatory mechanisms that determine which genes will be transcribed at which time point during differentiation are very precisely controlled and recent studies have shown the importance of epigenetic modifications in this process (Alberts et al. 2002; Jones and Baylin 2007; Reik 2007).

Many definitions of epigenetics have been proposed and it is now generally accepted that it refers to changes in gene expression that are not directly caused by the DNA sequence itself. A precise epigenetic program is crucial during development and its stability is essential to maintain all functions of each specific cell type during the lifetime of an organism. Gene expression can be epigenetically regulated through two main mechanisms: DNA methylation and histone modifications. The complex interplay between these two processes will determine whether a particular gene is transcriptionally active or repressed. It should also be mentioned that in the broader sense, regulation of expression levels by miRNAs also represents an epigenetic mechanism (Allis et al. 2007; Ellis et al. 2009).

The DNA molecule carries the genetic information and is about 2 meters long in each higher eukaryote cell. In order to fit into the tiny eukaryotic nucleus it therefore needs to be condensed about 10.000-fold. On the other hand, the DNA needs to be accessible for replication, transcription and repair. The solution to these challenges is the compaction of the eukaryotic genome into chromatin, a complex combination of DNA and basic proteins. Besides solving the packaging problem, chromatin forms a dynamic platform which controls all DNA-mediated processes within the nucleus (Peterson and Laniel 2004; Allis et al. 2007).

Nucleosomes represent the basic units of eukaryotic chromatin (Fig. 1). Each nucleosome contains 147 bp of DNA wrapped about twice around an octamer of the core histones. The histone octamer consists of a central heterotetramer of histones H3 and H4, and two heterodimers of histones H2A and H2B. Each nucleosome core particle is separated by a linker DNA, which can vary in length from a few up to 80 nucleotide pairs. Nucleosomes provide the first level of DNA packing and convert a DNA molecule into a chromatin fiber of 10 nm in diameter and about one-third of its initial length. The second level is the folding of this so-called “beads-on-a-string” arrangement in 30 nm thick fibers

that are stabilised by binding of linker histones called histone H1 to both sides of the nucleosome. Finally, the chromosome structures are formed by condensation of the 30 nm fibers and this represents the highest level of condensation, visible during mitosis.

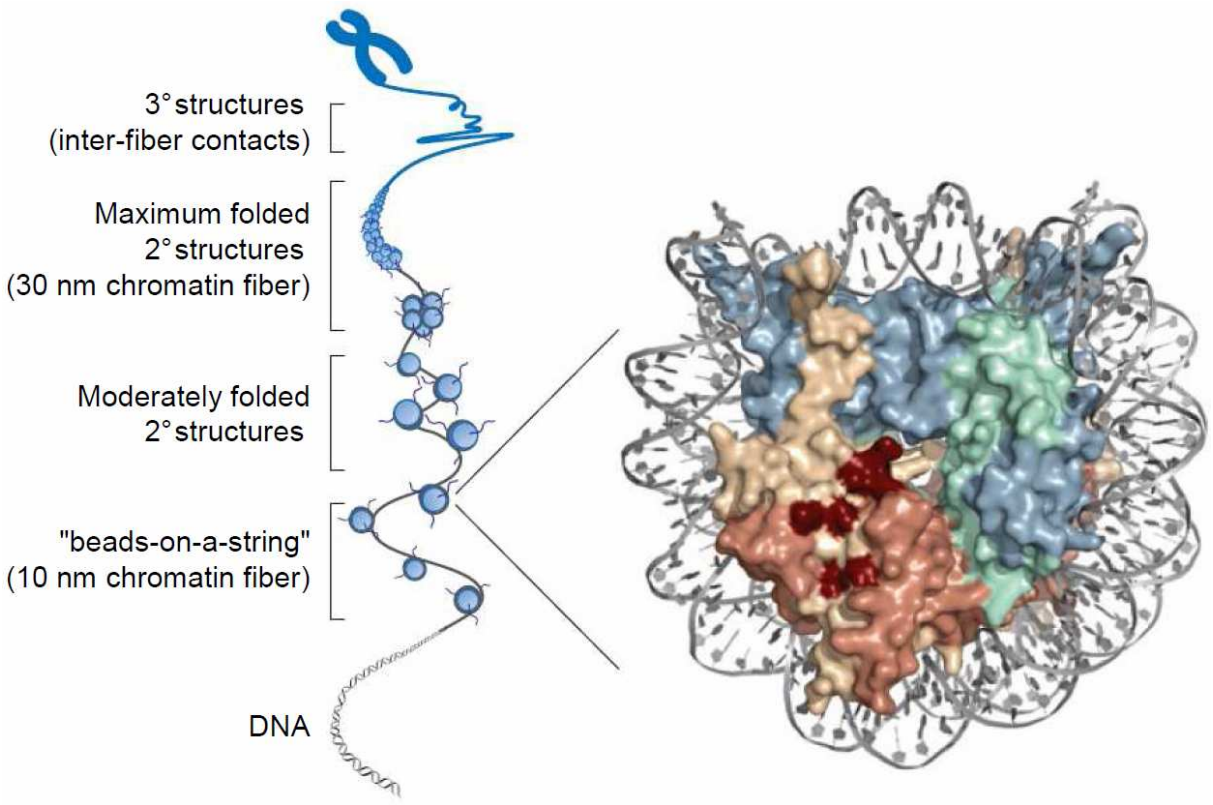


Figure 1

Levels of chromatin structure. Left, schematic representation of chromatin compaction levels, from the "beads-on-a-string" formation to the folding into secondary and tertiary structures. Right, details of the nucleosome surface showing histones H2A (yellow), H2B (light red), H3 (blue) and H4 (green) (modified from (Caterino and Hayes 2007)).

Chromatin exists in two main conformation states: (1) heterochromatin, which is densely compacted and transcriptionally inactive and (2) euchromatin, which is decondensed and transcriptionally active. Changes in chromatin conformation are mediated through post-translational modifications of the core histones. Each of the core histones has a globular domain and is rich in basic amino acids, lysine and arginine. Their positive charge allows interaction with the negatively charged DNA backbone. All four core histones have an extended N-terminal acid tail, which protrudes out of the nucleosome. These histone ends are subject to several types of post-translational modifications, including methylation, acetylation, phosphorylation and ubiquitylation (Alberts et al. 2002; Peterson and Laniel 2004).

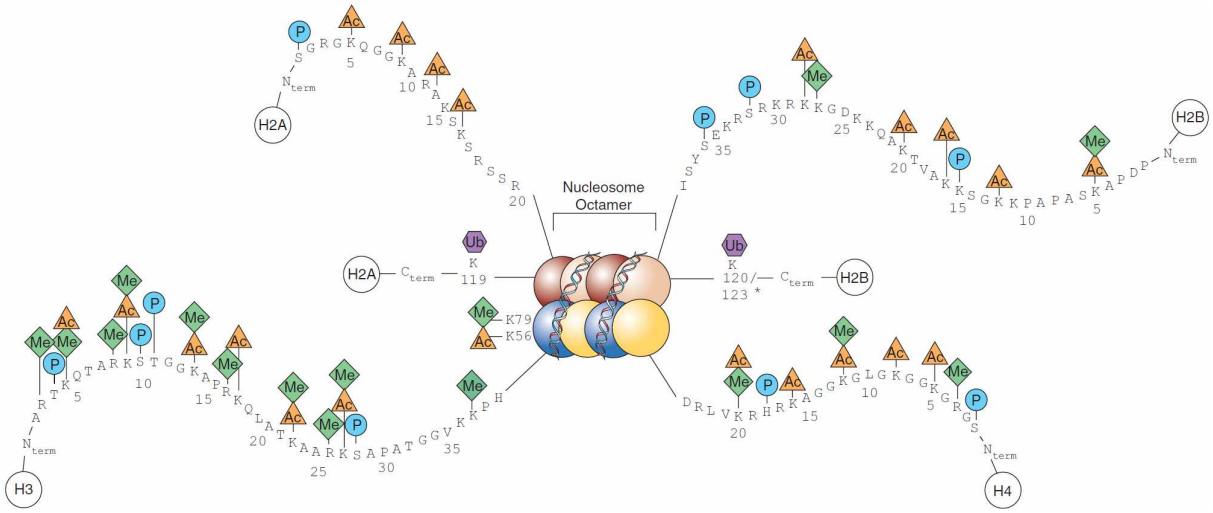


Figure 2

Core histone modifications in mammals. N- and C-terminal tails of histones H2A, H2B, H3 and H4 can be post-translationally modified by covalent additions such as methylation (green diamonds), acetylation (orange triangles), phosphorylation (blue circles) and ubiquitylation (purple hexagons). Human histone tail amino acid sequences are shown. Corresponding modified residue positions within yeast histones are indicated by asterisks. Lysine positions 56 and 79 on histone H3 are located within the globular domain (Keppler and Archer 2008).

Methylation and acetylation take place at lysines (K) and arginines (R), ubiquitylation is restricted to lysines and phosphorylation occurs at serines (S) and threonines (T). In general, acetylation is found in transcriptionally active regions, whereas methylation can be either an activating or a repressive mark. It was found that combinations of histone modifications correlate with particular biological functions. For instance, the combination of H4K8 acetylation, H3K14 acetylation and H3S10 phosphorylation often correlates with gene expression whereas trimethylation of H3K9 and the lack of H3 and H4 acetylation are associated with transcriptional repression in higher eukaryotes. This observation led to the concept of the “histone code” which suggests that a histone “language” is encoded by the modifications found on these tail domains and read by specific recognition modules found on many regulatory nuclear proteins (Strahl and Allis 2000). For instance the bromodomain binds to acetylated lysines and the chromodomain to methylated lysines.

1.1.1 Histone lysine methyltransferases and demethylases

Already in the sixties it was known that histone proteins can be post-translationally modified by methylation and acetylation (Allfrey et al. 1964). The first histone acetyltransferases and deacetylases were identified in 1996 (Hassig and Schreiber 1997). But it was not until 2000 that the molecular identity of the first histone

methyltransferase (HMT) was discovered (Rea et al. 2000; Shi and Whetstine 2007). It took then another four years until the first true histone demethylase (HDM), the lysine-specific demethylase 1 (LSD1), was discovered. This finding showed that the prevailing dogma that methylation is an irreversible process was not correct. In the meantime about 22 histone methyltransferases and 19 histone demethylases have been identified in humans.

Histone lysine methylation is catalysed by two families of proteins: the SET-domain-containing family and the non-SET-domain proteins DOT1/DOT1L (Martin and Zhang 2005). The SET domain (named after Su(var)3-9, Enhancer of Zeste, Trithorax) is an evolutionarily conserved sequence motif that is found in over 200 proteins ranging from mammals to bacteria and viruses (Zhang and Reinberg 2001). So far, one lysine residue in H1 isoforms and H4 and five lysine residues in H3 have been reported to be sites of methylation (Agger et al. 2008; Lu et al. 2009). Typically, methylation at H3K4, H3K36 and H3K79 is associated with transcriptional activation, whereas H3K9, H3K27 and H4K20 methylation is associated with transcriptional repression (Fig. 3). Adding to the complexity, each lysine can be either mono-, di- or trimethylated by specific HMTs. The histone modifications H3K9 and H4K20 methylation are highly relevant for the regulation of heterochromatin. On the other hand H3K9 and H4K20 methylation can also be found in euchromatin. The difference consist in the methylation status: heterochromatin is enriched in trimethyl-H3K9 and H4K20, whereas mono- and dimethyl-H3K9 and H4K20 seem to be restricted to euchromatic regions (Peters et al. 2003; Rice et al. 2003; Schotta et al. 2004). Also the HMTs involved are different. For instance, SUV39H is responsible for heterochromatin H3K9 methylation whereas G9a together with the highly related G9a-related protein (GLP) take over this function in euchromatin. It should however be stressed that this is not an absolute rule. In addition to heterochromatin formation, histone methyltransferases are also involved in transcriptional regulation. Important histone methyltransferases implicated in the silencing of genes required for development and in female X-chromosome inactivation are SET-domain-containing proteins of the polycomb group (PcG) of proteins such as enhancer of zeste homologue 2 (EZH2), which catalyses H3K27 methylation. Several studies report that EZH2 is involved in cancer progression (Varambally et al. 2002; Simon and Lange 2008). The methylation of all three lysine sites associated with gene activity, including K4, K36 and K79 of histone 3, seems to be directly coupled to the transcriptional process. For instance, the H3K4 methylase SET1 associates with RNA polymerase II during elongation, resulting in histone trimethylation in the coding region and providing a memory of recent transcriptional activity (Ng et al. 2003).

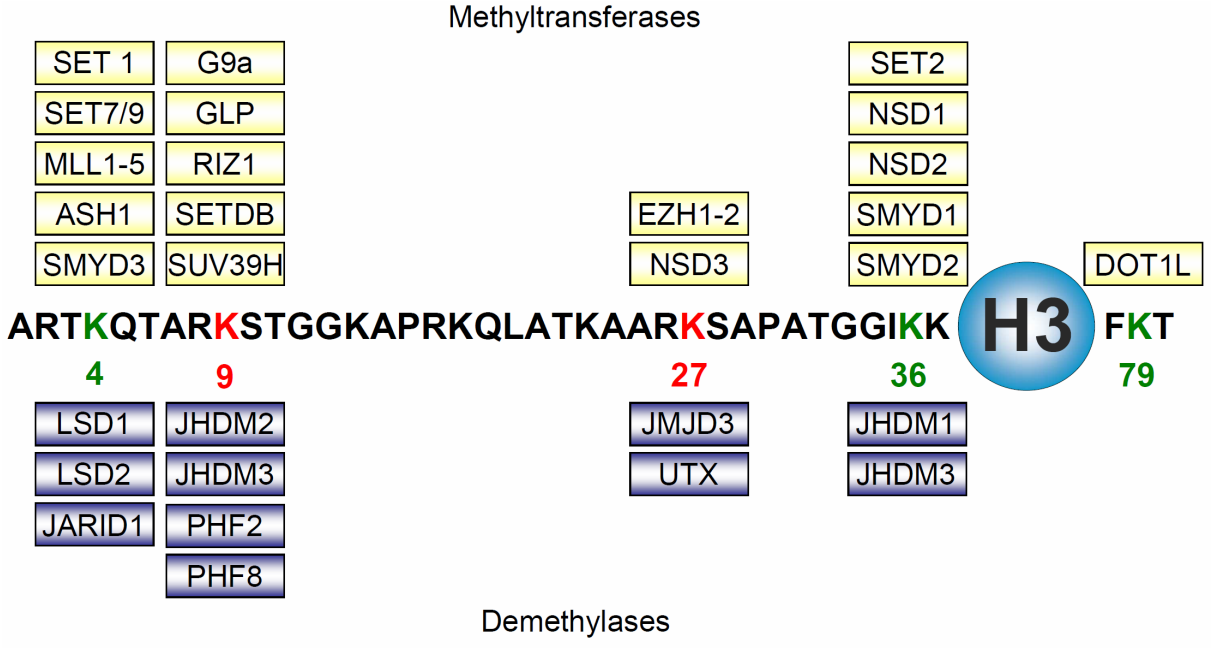


Figure 3

Regulation of histone methylation. The first 37 amino acid of the histone H3 tail and lysine residue 79 plus neighbouring amino acids are shown. Lysine residues that undergo methylation are highlighted in green to indicate a function in active transcription, or in red to indicate involvement in transcriptional repression. The histone methyltransferases targeting various lysine residues on histones are shown at the top and the corresponding demethylases are shown at the bottom. ASH (absent, small or homeotic)-like (Drosophila); DOT1L, disruptor of telomeric silencing 1-like; EZH, Enhancer of zeste homologue 2; G9a, also known as EHMT2 (euchromatic histone-lysine N-methyltransferase 2); GLP, G9a-related protein; JHDM, JmjC-domain-containing histone demethylase 1; JMJD, Jumonji-domain-containing protein; LSD, lysine-specific histone demethylase 1; MLL, myeloid/lymphoid or mixed-lineage leukaemia-associated protein; NSD, nuclear receptor-binding; PHF, PHD finger protein; RIZ, retinoblastoma protein-interacting zinc-finger protein; SET, SET-domain-containing histone methyltransferase; SMYD, SET and MYND domain containing protein; SUV39H, suppressor of variegation 3-9 homologue; UTX, ubiquitously transcribed tetratricopeptide repeat, X chromosome.

Two classes of histone demethylases which are able to reverse lysine methylation have been identified (Klose et al. 2006). LSD1 and LSD2 belong to the first class. They directly reverse histone H3K4 methylation by an oxidative demethylation reaction using flavin as a cofactor. The LSD proteins can only remove mono- and dimethyl-lysine modifications. In complex with the androgen receptor (AR), LSD1 can additionally act as a transcriptional activator by removing the mono- and dimethylation of H3K9 (Metzger et al. 2005). The second and largest class of demethylase enzymes is characterised by the presence of a Jumonji C (JmjC) domain which catalyses lysine demethylation through an oxidative reaction that requires iron Fe(II) and α-ketoglutarate (αKG) as cofactors (Klose et al. 2006).

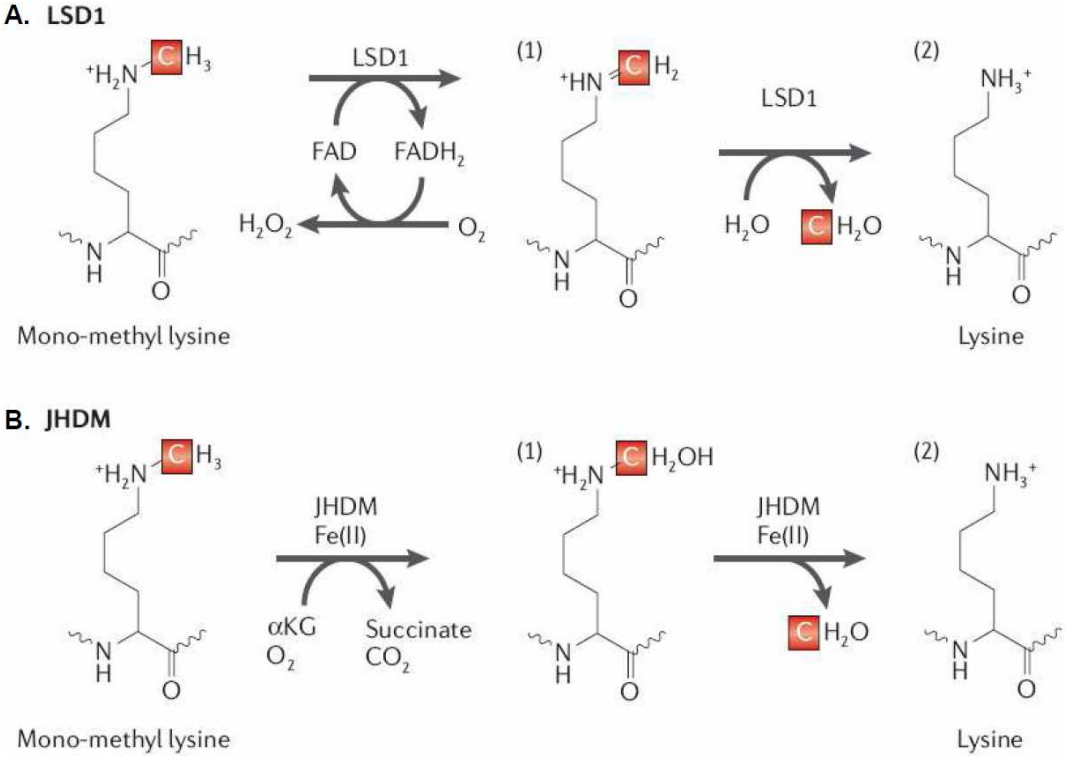


Figure 4

Distinct chemical mechanisms are used by the two classes of histone demethylases. **A.** LSD1 (lysine specific demethylase 1) uses flavin (FAD) as a cofactor to mediate demethylation of mono- and dimethylated lysine residues through an amine oxidation reaction. Loss of the methyl group from monomethyl lysine occurs through an imine intermediate (1), which is hydrolysed to form formaldehyde by a non-enzymatic process (2). **B.** JHDMs (JmjC-domain-containing histone demethylases) can demethylate mono-, di- and trimethylated lysine by an oxidative mechanism that requires Fe(II) and αKG as cofactors. Demethylation is thought to occur by direct hydroxylation of the methyl group (1), which results in an unstable hydroxymethyl product that is spontaneously released as formaldehyde (2) (modified from (Klose et al. 2006)).

The JmjC-domain-containing histone demethylases (JHDMs) can reverse all three histone lysine-methylation states. The first JmjC domain histone demethylase identified, JHDM1 (also known as Fbx11) was found by Yi Zhang and colleagues using a biochemical assay to isolate proteins with histone demethylase activity from human cells (Tsukada et al. 2006). They also demonstrated that the JmjC domain is the catalytic moiety involved in mediating the demethylation reaction (Tsukada et al. 2006). The JmjC domain is a highly conserved domain and more than 100 jumonji-domain-containing (JMJD) proteins have been found from bacteria to eukaryotes (Clissold and Ponting 2001). Human family members targeting H3K4, H3K9, H3K27 and H3K36 have been identified during the last years. They regulate a variety of cellular processes, have important roles in development and contribute to some of the cellular abnormalities observed in cancer (Klose et al. 2006).

JmjC-Group	members	Also known as	Substrate Specificity	Function
JHDM1	JHDM1A	FBXL11, KDM2A	H3K36me1/2	
	JHDM1B	FBXL10, KDM2B	H3K36me1/2	
JHDM2	JHDM2A	JMJD1A, KDM3A	H3K9me1/2	AR gene activation, spermatogenesis
	JHDM2B	JMJD1B, KDM3B	H3K9me	
	JHDM2C	JMJD1C		
JHDM3	JMJD2A	JHDM3A, KDM4A	H3K9/ H3K36me2/3	Transcription repression, genome integrity
	JMJD2B	KDM4B	H3K9/ H3K36me2/3	Heterochromatin formation
	JMJD2C	GASC1, KDM4C	H3K9/ H3K36me2/3	Putative oncogene
	JMJD2D	JMJD2D, KDM4D	H3K9me2/3	
JARID	JARID1A	RBP2, KDM5A	H3K4me2/3	Retinoblastoma-interacting protein
	JARID1B	PLU-1, KDM5B	H3K4me2/3	Transcription repression
	JARID1C	SMCX, KDM5C	H3K4me2/3	X-linked mental retardation
	JARID1D	SMCY, KDM5D	H3K4me2/3	Male-specific antigen
UTX		KDM6A	H3K27me2/3	Transcription activation
JMJD3		KDM6B	H3K27me2/3	Transcription activation
PHF2/ PHF8		JHDM1E/JHDM1F	H3K9me1/2	Transcription activation

Figure 5

The family of JmjC-domain-containing histone demethylases. Six groups of JmjC-domain-containing proteins with demethylating activity were identified in human. JHDMs reverse mono-, di- and trimethyl-marks from H3K4, H3K9, H3K27 and H3K36.

1.2 The JARID family

The JARID enzymes belong to the JmjC-containing class of demethylases. Two subgroups can be distinguished, JARID1 and JARID2. JARID2 is the founding member of the JMJD protein family and was originally identified in mouse as an important factor involved in neural tube formation. The name *Jumonji*, which is the Japanese word for cross, was given due to the abnormal cruciform shape that the neural groove formed in the homozygous null mice (Takeuchi et al. 1995). No histone demethylase activity was detected in JARID2, which is explained by the lack of conservation of two essential histidine residues in the metal-binding motif of the JmjC domain (Pasini et al.; Klose et al. 2006; Shirato et al. 2009). Very recently, three different groups found that JARID2

regulates the binding and activity of the Polycomb repressive complex 2 (PRC2) in stem cells (Pasini et al.; Peng et al. 2009; Shen et al. 2009). The PcG proteins are important regulators of the expression of genes that are essential for development, differentiation and maintenance of cell fates (Schuettengruber et al. 2007; Schwartz and Pirrotta 2007).

The JARID1 subgroup is formed by *bona fide* histone demethylases that specifically remove dimethyl and trimethyl marks from H3K4 (Christensen et al. 2007; Lee et al. 2007). Four members exist in mammals: JARID1A (KDM5A/RBP2), JARID1B (KDM5B/PLU1) and the two highly homologous proteins JARID1C (KDM5C/SMCX) and JARID1D (KDM5D/SMCY) (Klose et al. 2006). These four proteins contain the catalytic JmjC domain responsible for histone demethylation and the JmjN domain which is important for maintaining the structural integrity of the adjacent JmjC domain (Chen et al. 2006; Iwase et al. 2007). JARID1 members also contain an AT-rich-interacting domain (ARID) also known as the BRIGHT domain and a C5H2C zinc finger. Finally, the four JARID1s have a plant homeodomain-associated protein domain (PHD) which is a protein-protein interaction domain and a histone-methyl-lysine binding motif (Wysocka et al. 2006; Shi et al. 2007). The JARID1 family differs in the number of PHD domains; JARID1A and 1B contain three and JARID1C and 1D two PHDs.

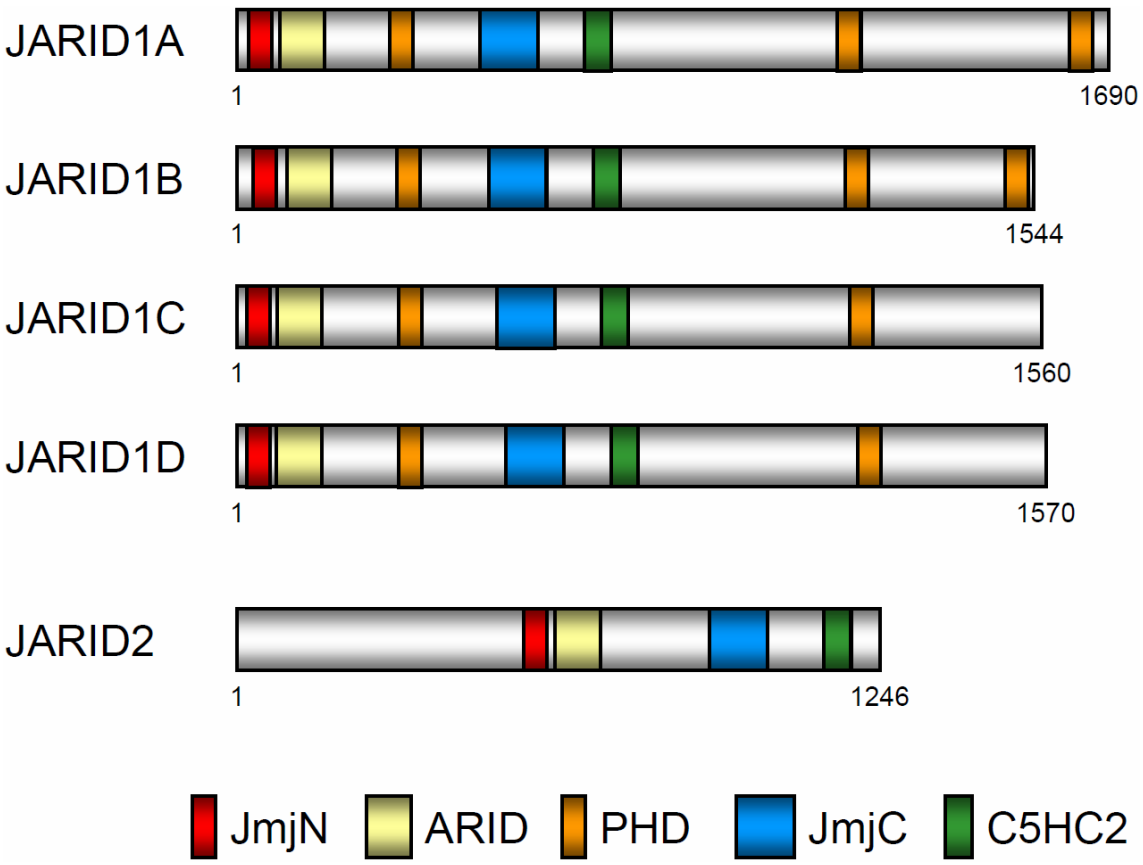


Figure 6

Structure of JARID1/2 proteins. There are two subgroups of JARID proteins in higher eukaryotes: JARID1 and JARID2. The JARID1 subgroup contains in addition to the catalytic JmjC (blue) domain, JmjN (red), AT-rich interactive (yellow), C5HC2-zinc-finger (green) and PHD-finger domains (orange). The JARID2 subgroup does not contain PHD domains and its JmjC domain lacks two essential histidine residues in the HXD/EX_nH metal-binding motif. Thus, only JARID1 members have a histone demethylase activity.

JARID1 proteins play important roles in cellular differentiation and development (Christensen et al. 2007). The best-described members are JARID1A and JARID1B. Less is known about JARID1C and JARID1D and their cellular function.

Human JARID1A is evolutionarily conserved, with homologs in mouse *(JARID1a)*, *Drosophila (Lid)*, *C. elegans (rbr-2)* and *S. pompe (Lid2)*. JARID1A was originally described as a nuclear protein that binds to the retinoblastoma protein (pRB), leading to promotion of cellular differentiation by preventing JARID1A from repressing genes required for this process (Defeo-Jones et al. 1991; Fattaey et al. 1993; Benevolenskaya et al. 2005). More recently its function as histone demethylating enzyme was evidence (Christensen et al. 2007; Klose et al. 2007). Since it demethylates H3K4me2/3, which is generally found in active gene regions, JARID1A has been suggested to function as a general transcriptional repressor. A genome-wide analysis showed two groups of genes to be controlled by JARID1A, some of which are involved in cellular differentiation, whereas others are implicated in mitochondrial function and RNA/DNA metabolism (Lopez-Bigas et al. 2008). Underlining its function in differentiation and development, it was found that mouse *JARID1a* is also associated to the PRC2 complex which represses developmental genes in embryonic stem cells (Pasini et al. 2008). *JARID1a-/-* mice appear to be grossly normal. Analysis of their global H3K4 trimethylation and comparison to wild-type animals did not reveal any significant differences (Klose et al. 2007). However, when comparing the global H3K4me3 status of the nematode *C. elegans* wild-type with that of a mutant strain lacking the entire JmjC domain of the unique worm ortholog rbr-2, a significant increase of H3K4me3 levels at all larval developmental stages in the mutant was observed (Christensen et al. 2007). This suggests compensatory mechanisms by the other JARID1 family members to have taken place in the *JARID1a-/-* mice.

JARID1B is up-regulated in 90 % of breast cancers, including breast cancer cell lines, primary and metastatic breast cancers (Lu et al. 1999; Barrett et al. 2002). In 2007, the demethylase activity of JARID1B was evidenced and it was shown to act as a transcriptional repressor in breast cancer, promoting tumour progression by repression of tumour suppressor genes, including BRCA1 (Yamane et al. 2007). Additionally, JARID1B

associates with the oncogene ZNF217 as part of a larger complex which also includes LSD1, and represses gene transcription (Banck et al. 2009). On the other hand, a protein described as a JARID1B isoform, RBP2-H1, is down-regulated in melanomas where it may have tumour suppressive effects (Roesch et al. 2008). Microarray studies showed about 100 genes to be controlled by JARID1B, several of which are involved in cell cycle and signal transduction (Scibetta et al. 2007).

JARID1C is encoded by the SMCX gene on chromosome X and escapes X inactivation (Iwase et al. 2007). It was suggested to play an important role in brain function and was found mutated in families with X-linked mental retardation (XLMR) (Jensen et al. 2005). Importantly, these mutations affect H3K4 demethylase activity, underlining the importance of this histone mark for brain development. JARID1C was also found to act as a Smad3 repressor, thus affecting transforming growth factor-β (TGF-β) signalling (Kim et al. 2008). The same group reported the existence of a shorter JARID1C isoform that predominantly resides in the cytoplasm, but still demethylates H3K4me3.

JARID1D is encoded by chromosome Y and contributes to epitopes of the male-specific histocompatibility (H-Y) antigen (Warren et al. 2000). It is associated with the polycomb-like protein Ring6a/MBLR and plays a role in transcriptional repression through H3K4 demethylation (Lee et al. 2007). Recent data show JARID1D to form a complex with a protein involved in meiosis during spermatogenesis (Akimoto et al. 2008).

1.3 Regulation of gene expression by steroid receptors and their cofactors

Steroid hormones have pleiotropic functions. They have important roles in reproduction, development, differentiation and metabolic homeostasis (Evans 1988; McEwan 2009). The effects of steroid hormones are mediated through specific receptors which belong to the nuclear receptor (NR) superfamily. In humans 48 members have been identified (Escriva et al. 2004; McEwan 2009). The steroid receptor subgroup is composed of the androgen receptor (AR), two estrogen receptors (ER) named ERα and ERβ, the progesterone receptor (PR), the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). All steroid receptors are structurally organised in four main domains: an N-terminal region of variable length with important transactivation functions, a DNA-binding domain (DBD) composed of two zinc fingers, a hinge region containing the nuclear localisation signal (NLS) and a ligand-binding domain (LBD) with additional transactivation functions (Faus and Haendler 2006).

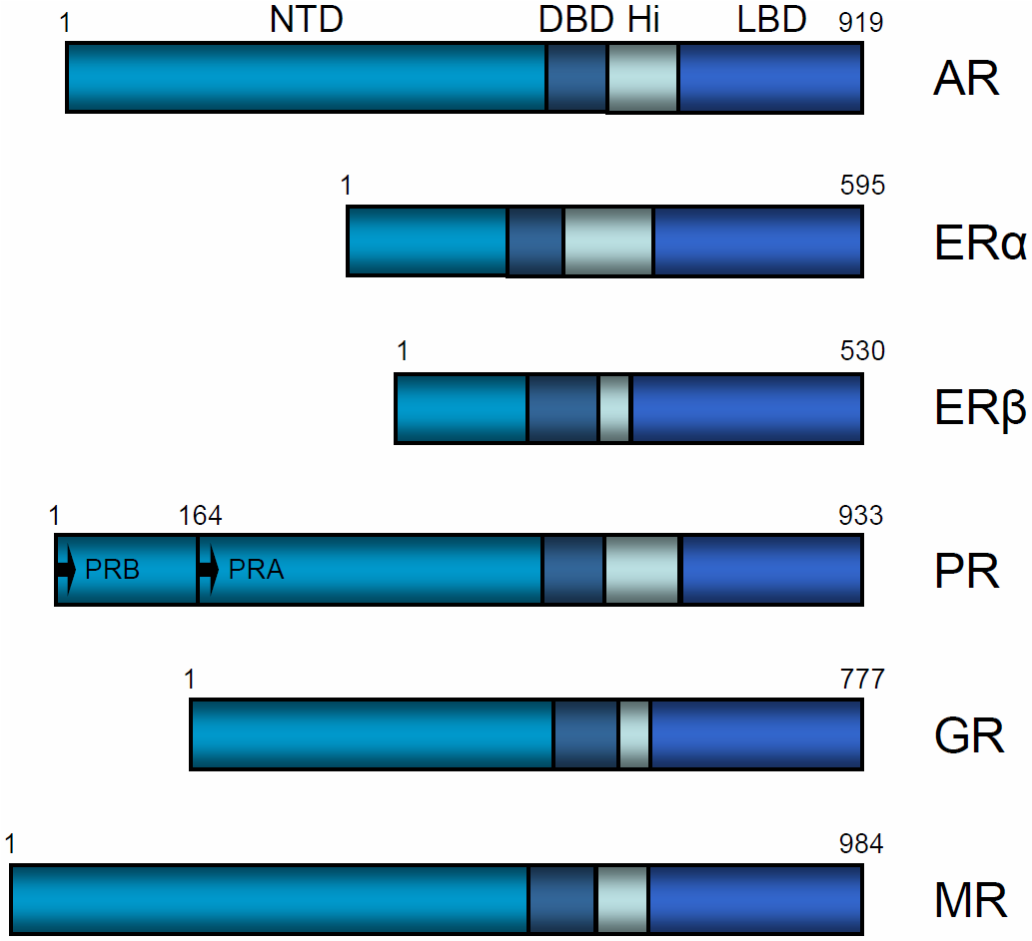


Figure 7

Steroid receptor protein structure. The steroid receptors contain four domains, the N-terminal domain (NTD), the DNA-binding domain (DBD), a hinge region and the ligand-binding domain (LBD).

Steroid receptors act as ligand-dependent transcription factors which activate or repress target gene expression. In the absence of hormone, the receptors are usually located in the cell cytoplasm and form a complex with heat-shock proteins. When the lipophilic ligand which diffuses passively through the plasma membrane binds to its cognate receptor, conformational changes leading to dissociation of heat-shock proteins take place. The NLS can then be recognised by import proteins and the steroid receptor is translocated into the nucleus. There, it binds as homodimer to target gene promoters at specific DNA elements called hormone-responsive elements (HRE). These elements usually comprise two half-sites of six nucleotides each and are arranged as inverted repeats. Direct repeat elements have more recently been identified as selective androgen-response elements (Kato et al. 2005; Faus and Haendler 2006; McEwan 2009).

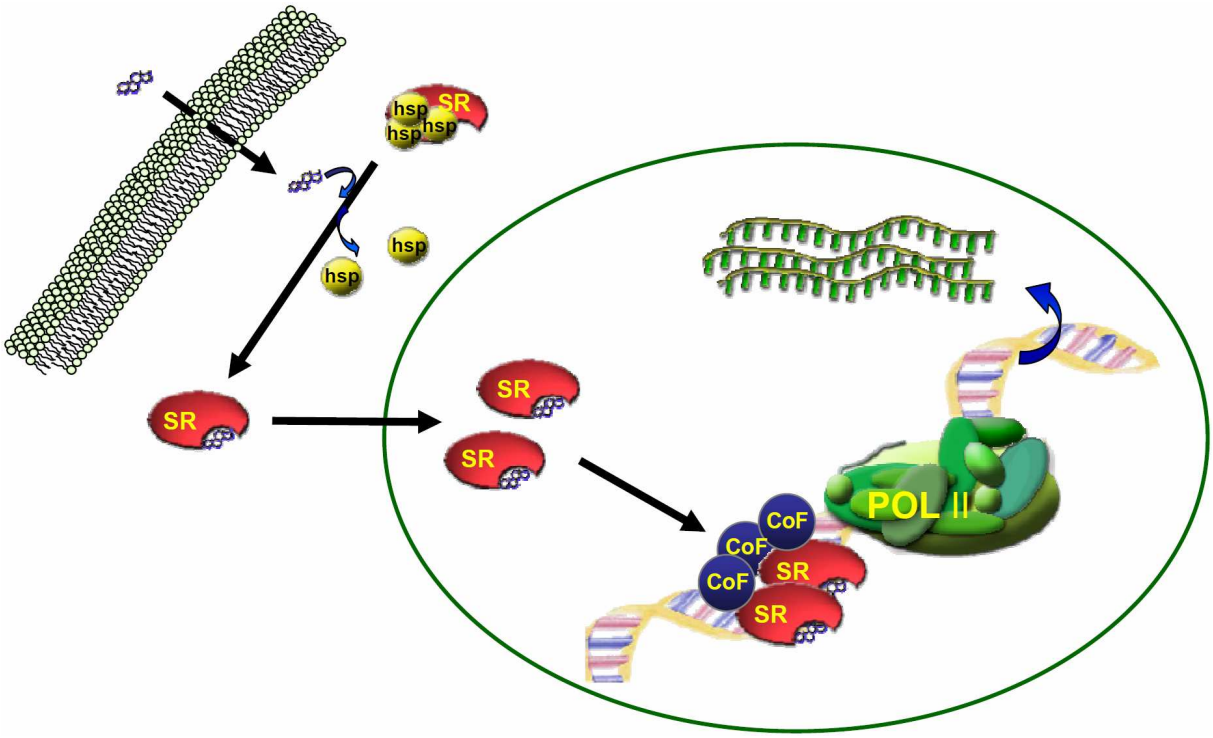


Figure 8

Steroid receptor mode of action. Steroid hormones diffuse through the cell membrane and bind to the steroid receptor (SR). Following hormone binding, the SR changes its conformation, dissociates from the heat-shock proteins (hsp) and translocates into the nucleus. There it binds to specific response elements as a homodimer. Cofactors (CoF) are then recruited and form an interaction platform with the general transcriptional machinery (Pol II complex), thus leading to transcriptional control of target genes (modified from Geserick).

For the recruitment of the transcription machinery and the activation or repression of target genes, steroid receptors need to interact with various coactivators or corepressors. These multi-subunit protein complexes interact with ATP-dependent chromatin remodelling complexes and form interaction platforms for the recruitment of a variety of histone-modifying enzymes. The movement of nucleosomes along the DNA and the post-translational modifications of histone tails will regulate in concert the access of the transcription machinery to DNA regulatory regions in target genes (Rosenfeld et al. 2006).

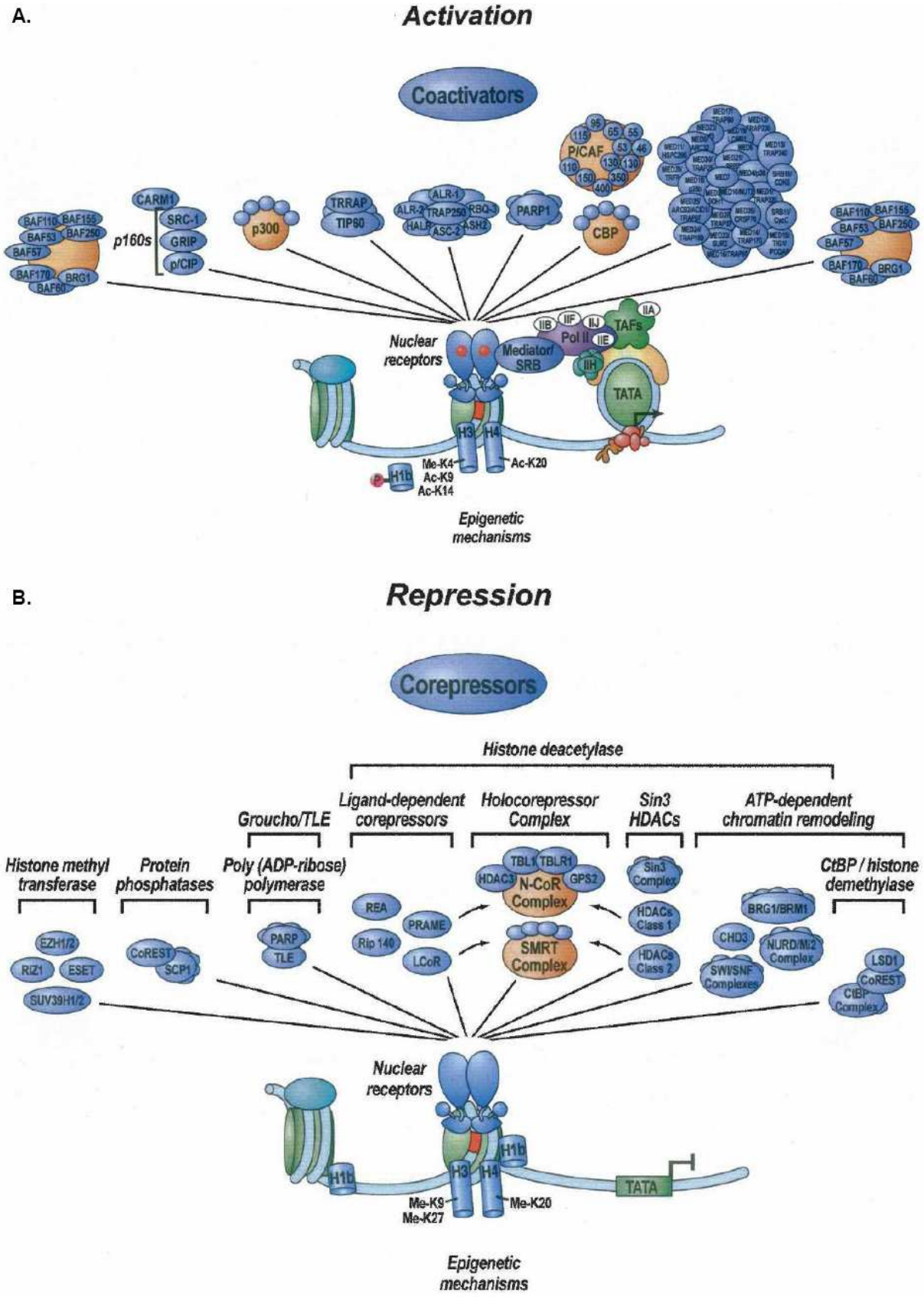


Figure 9

The coactivator and corepressor groups involved in nuclear receptor signalling pathways. These multisubunit cofactor complexes contain enzymes that change chromatin structure by covalent modifications of histone tails. **A.** NR coactivators overview. **B.** NR corepressors overview (Rosenfeld et al. 2006).

A small group of cofactors which plays an important role in modulating the activity of all steroid receptors is the p160 steroid receptor coactivator family (SRC). Three members have been identified: SRC-1, SRC-2/glucocorticoid receptor-interacting protein 1 (GRIP1)/transcriptional intermediary factor 2 (TIF2) and SRC-3/CBP-interacting protein (pCIP). These factors have weak intrinsic histone acetylase activity but can recruit the histone acetylase CBP/p300 (Leo and Chen 2000). They interact with the LBD of most steroid receptors via their LXXLL motifs (Bevan et al. 1999). Conversely, interaction of steroid receptors with histone deacetylases such as HDAC1 or sirtuin 1 in repressive complexes has also been described (Jepsen and Rosenfeld 2002).

Generally, the repressing activity of steroid receptor complexes is less well understood. Corepressors were originally identified with the help of receptor antagonists. They can be divided in three groups depending on their mode of interaction with NR. One can distinguish between corepressors (1) which dissociate from the receptor upon ligand binding, (2) which bind constitutively to the unliganded NR and (3) which bind only to liganded receptors (Moehren et al. 2004). The best-characterised corepressors are NCoR and SMRT, which belong to the first group and associate with HDAC3 (Yoon et al. 2003; Moehren et al. 2004).

Methylation of histone tails is also important for steroid receptor action. This can lead to activation or repression of target genes, depending on which lysine is modified. For instance, the coactivator-associated arginine methyltransferase 1 (CARM1) is an important coactivator for several NR. It facilitates transcriptional activation following association with p160 coactivators, due to its ability to methylate H3 at arginine 2, 17 and 26 (Chen et al. 1999). Another protein arginine methyltransferase, PRMT1, promotes nuclear hormone receptor transcriptional activation by specifically methylating arginine 3 of H4 (Wang et al. 2001). Concerning lysine methylation, the H3K9-methyltransferase G9a was identified as an NR coactivator which interacts with CARM1 and p300 (Lee et al. 2006). The H3K4 methylase SMYD3 is an important coactivator for ERα function.

Other steroid receptor cofactors are involved in ubiquitylation and sumoylation (Poukka et al. 1999; Conaway et al. 2002). Importantly crosstalk between these various modifications may take place, ultimately leading to activation or repression of downstream target genes.

1.3.1 The Estrogen Receptor

The steroid hormone estrogen is involved in the regulation of the reproductive, immune, cardiovascular, musculo/skeletal and central nervous systems. In mammals, the biological effects of estrogen are mediated by two different estrogen receptors, ERα and

ERβ, which arise from different genes. Both ERs show a 97 % identity in their DBD, but differ in their LBD, which has only 59 % identity (Williams et al. 2008). However, estrogen stimulates both receptors similarly and its action often occurs as a balancing act between ERα and ERβ, in a “yin-yang relationship”. ERα plays a proactive role whereas ERβ has the opposite effect and is antagonistic to ERα. In line with this, ERα drives proliferation whereas ERβ is antiproliferative (Gustafsson 2006). Also, ERα is expressed in 70 % to 80 % of all breast tumours, whereas in sharp contrast, ERβ is expressed at high concentration in normal human mammary gland tissue but at much lower levels in breast cancer (Palmieri et al. 2002; Esslimani-Sahla et al. 2005; Williams et al. 2008). Competitive ERα antagonists have been used successfully for years to treat ERα-positive breast cancer (Musgrove and Sutherland 2009). Mechanistically it was found that the anti-estrogen tamoxifen induces a conformational change in the LBD which prevents the interaction with p160 coactivators and favours recuitment of the corepressors NCoR and SMRT (Green and Carroll 2007).

ER transcriptional function can be mediated by two mechanisms, either direct binding to estrogen responsive elements (ERE) or interaction with other DNA-bound transcription factors such as Sp1, AP-1 or NF-κB. In the second case, the ER functions as a signal transducer which stabilises the binding to DNA and/or recruits coactivators to the complex (Klinge 2001; McDonnell and Norris 2002). An example for this is ERα interaction with the upstream transcription factors USF-1 and USF-2 mediating estrogen responsiveness of the cathepsin D promoter (Xing and Archer 1998). The first mechanism, direct binding of the ERα, takes place at regulatory regions of several downstream targets including the genes for *PGR* (Progesterone), *TFF1* (trefoil factor 1, also known as pS2) and *c-myc*, which all get induced by estrogen treatment (Brown et al. 1984; Dubik et al. 1987). Recent studies indicate the involvement of several histone-modifying enzymes in ERα transcriptional activation. For instance the histone methyltransferase SMYD3, which methylates H3K4, induces ERα target gene expression and enhances the proliferation of breast cancer cells (Hamamoto et al. 2006; Kim et al. 2009). Also the MLL2 methyltransferase complex was identified as a coactivator of ERα (Mo et al. 2006).

1.3.2 The Progesterone Receptor

Progesterone is a critical regulator of female reproductive function in the uterus, ovary, mammary gland and brain. In addition, it plays an important role in non-reproductive tissues such as the cardiovascular system, bone and the central nervous system (Scarpin et al. 2009). Progesterone effects are mediated in vertebrates and humans by

the two PR isoforms, PRA and PRB, which arise from a single gene by alternate initiation of transcription from two different promoters (Kastner et al. 1990). PRA and PRB are identical in sequence, except that PRB has an extension of 164 amino acids at the N-terminal domain which endows it with specific properties, possibley linked to differential recruitment of cofactors (Fig. 7). Indeed, it was found that PRA and PRB have unique roles as transcriptional regulators of progesterone-responsive genes, differentially regulating gene transcription and recognising distinct promoters (Brayman et al. 2006; Aupperlee and Haslam 2007). Knock-out studies in mice have revealed that PRA is essential for uterine development and reproductive function (Mulac-Jericevic et al. 2000), while PRB is required for normal mammary gland development (Mulac-Jericevic et al. 2003). However, in contrast to other species in which the predominant expression of one PR isoform is common, in humans the majority of PR-positive cells express both isoforms (Mote et al. 2000; Mote et al. 2002). In addition to its ligand-activated transcriptional effects in the nucleus, PR also regulates transcription through non-genomic pathways such as activation of second messenger signalling cascades (Lange 2008a; Lange 2008b). In contrast to ERα and AR, very few data on the interaction of PR with chromatin-modifying enzymes have been reported (Aoyagi and Archer 2007).

PR gene expression is regulated by estrogen and progesterone itself, whereby estrogen increases and progesterone decreases PR expression (Graham et al. 1995). Stimulation of PR expression by estrogen is solely due to the ERα isoform (Flototto et al. 2004). Motifs that mediate estrogen response have been identified in the human PR gene, including ERE half-sites, Sp1- and AP-1-binding sites. Several transcription factors involved in the regulation of PR gene expression have been identified, however nothing is known about cofactors that alter histone methylation. In breast cancer tissue, PR expression is up-regulated and the balanced expression of PRA and PRB isoforms is frequently changed, resulting in a predominant expression of the PRA isoform (Bamberger et al. 2000; Mote et al. 2002). Several studies show that breast cancer patients with PR-positive tumours have a better prognosis, due to the smaller size of the tumour and the better response to endocrine agents (Alghanem and Hussain 1986; Cordera and Jordan 2006). Knock-out and xenograft models additionally show the importance of PRs in breast cancer progression (Ismail et al. 2003; Sartorius et al. 2003).

1.3.3 The Androgen Receptor

The AR is bound and activated by the physiological androgens testosterone (T) and its more active metabolite dihydrotestosterone (DHT). The androgen-AR signalling pathway plays a key role in the development and function of the male reproductive system, such

as male sexual differentiation during embryogenesis, onset of sperm production at puberty and prostate development (Brinkmann and Trapman 2000). Besides, androgens have additional functions in the development of muscle, hair follicles and brain. As a transcription factor, the AR interacts with androgen response elements (ARE) in various androgen target genes and activates or represses transcription. Typical target genes up-regulated by the AR are coding for several proteases, including the prostate specific antigen (PSA, also known as kallikrein-related peptidase 3, KLK3), kallikrein-related peptidase 2 (KLK2), and transmembrane protease serine 2 (TMPRSS2). Genes that get significantly down-regulated by androgens are those for testosterone-repressed prostate message 2 (TRPM2, also known as clusterin) and the serpin peptidase inhibitor (SERPINI1) (DePrimo et al. 2002).

The AR gene is located on the X-chromosome so that in males gene mutations will cause direct phenotypic manifestations not compensated by a wild-type allele. Indeed abnormalities in the AR signalling pathway are linked to several pathologies, such as male infertility, androgen insensitivity syndrome (AIS), Kennedy's disease (also known as X-linked adult onset spinal/bulbar muscular atrophy (SBMA)) and prostate cancer (Lee and Chang 2003). A misregulation of the AR caused by mutations and resulting in a lower (in the case of AIS) or higher (in the case of prostate cancer) AR activity is the basis for most of these diseases, underlining the importance of a proper control of AR function. Regulation of AR activity can occur in several different ways: modulation of gene expression, androgen binding, nuclear translocation, protein stability and trans-activation (Lee and Chang 2003). During the last years, new AR cofactors involved in histone methylation have been identified. The histone methyltransferase G9a and the two histone demethylases LSD1 and JHDM2A interact with the AR and promote its activating function (Metzger et al. 2005; Lee et al. 2006; Yamane et al. 2006; Wissmann et al. 2007). Due to the implication of the AR in several diseases and the need to identify more potent and selective modulators acting longer than current anit-androgen therapies, efforts have recently converged towards the identification and analysis of coactivators and corepressors essential in the modulation of AR transcriptional activity.

1.4 Aim of this work

Nuclear receptor activity necessitates the interaction with various coactivators or corepressors. Among these cofactors, enzymes have been identified that locally modify histone tails, thereby controlling the access to DNA regulatory regions in target genes. Recently, several proteins that interact with steroid receptors have been shown to affect histone methylation. The lysine methyltransferase G9a methylates H3K9 and stimulates

AR and ERα function. More recently, members of the JmjC family of histone demethylases have also been found to regulate steroid receptor function. For instance, JHDM2A, which demethylates H3K9, acts as an AR activator. However, less is known about the role of H3K4 methylation and the enzymes responsible for adding or removing this histone mark in steroid receptor function. The objective of this work was therefore to study the potential role of the JARID1 family members, a subgroup of the JmjC family known to have a H3K4me2/3 demethylase activity, in ERα and AR function.

When this work was initiated, little was known about the biological function of these proteins. In view of the essential and general role of H3K4 methylation in gene activation, it was therefore decided to study the role of the JARID1 family members as possible cofactors in ERα and AR signalling pathways. Coactivators that are essential for ERα or AR function and harbour enzymatic activity may provide new suitable targets for the treatment of hormone-dependent cancers.

To test the hypothesis, siRNA knock-down studies of the JARID1 family members were carried out in breast and prostate cancer, using MCF7 and LNCaP cells as models for breast and prostate cancer, respectively. The outcome on ERα and AR signalling pathways was examined and the potential role of these demethylases was further characterised by overexpression, reporter gene assays, co-immunoprecipitation and chromatin-immunoprecipitation assays.

2 MATERIALS AND METHODS

2.1 Materials

Cell culture

RPMI 1640	BIOCHROM AG
RPMI 1640 w/o phenol red	BIOCHROM AG
PBS w/o Ca^{2+}, Mg^{2+}	BIOCHROM AG
Trypsin-EDTA	BIOCHROM AG
FBS	BIOCHROM AG
cFBS	BIOCHROM AG
L-Glutamine	GIBCO
Penicillin/Streptomycin	GIBCO
1x non-essential amino acids	BIOCHROM AG
Cell culture flasks	Corning
Test plates 96/24/6 wells	TPP
Centrifuge tubes	Corning
Stripettes	Costar

Cloning and mutagenesis

QUICK-Clone™ cDNA brain pool	BD Biosciences Clontech
QUICK-Clone™ cDNA testis pool	BD Biosciences Clontech
Human Genomic DNA	Clontech
pCMV-HA vector	BD Biosciences Clontech
pCR 2.1-TOPO® vector	Invitrogen
pGL4.26[luc2/minP/Hygro] vector	Promega
Primers	MWG
TOPO TA Cloning®	Invitrogen
HotStarTaq Master Mix	QIAGEN
Restriction enzymes and buffers	Roche
Shrimp alkaline phosphatase	Roche
PCR purification / Gel extraction kits	QIAGEN
Rapid DNA ligation kit	Roche
Ultra Pure™ Agarose	Invitrogen
DNA ladder	Invitrogen
QuikChange XL / II SDM kits	Stratagene
XL1-Blue supercompetent cells	Stratagene
XL10-Gold ultracompetent cells	Stratagene
S.O.C.-Medium	Invitrogen
Ampicillin, Kanamycin	Roche
Plasmid Purification Mini, Maxi kits	QIAGEN

Transfections

Stealth™ siRNA	Invitrogen
Stealth™ RNAi Negative Universal Control Medium	Invitrogen
Block-iT™ fluorescent oligo	Invitrogen
Lipofectamine™ 2000 reagent	Invitrogen

FuGENE® HD transfection reagent	Roche
Opti-MEM®	GIBCO
SteadyLite Plus Reagent	PerkinElmer Life Sciences
CulturPlate-96	PerkinElmer Life Sciences
TopSeal-A film	PerkinElmer Life Sciences
LumiCount	Packard

RNA extraction and reverse transcription

QIAshredder	QIAGEN
RNeasy Mini Kit	QIAGEN
RNase-Free DNase Set	QIAGEN
SuperScript™ III First Strand	Invitrogen

Quantitative PCR

QuantiFast™SYBR®Green PCR Kit	QIAGEN
Primers	MWG
TaqMan® Fast Universal PCR Mix	Applied Biosystems
TaqMan® Gene Expression Assays	Applied Biosystems
Fast-Optical 96-Well Reaction Plates	Applied Biosystems
Optical Adhesive Film	Applied Biosystems

Protein extraction, PAGE and western blot

M-Per	Pierce Biotechnology
Complete Mini EDTA-free tablets	Roche
Benzonase	Merck
Bradford protein assay	BIO-RAD
NuPAGE LDS Sample Buffer	Invitrogen
Precision Plus Protein Standards	BIO-RAD
NuPAGE 4-12 % Bis-Tris gels	Invitrogen
NuPAGE MOPS SDS Running Buffer	Invitrogen
XCell II SureLock Mini-Cell	Invitrogen
PVDF Membrane	Invitrogen
NuPAGE Transfer Buffer	Invitrogen
XCell II Blot Module	Invitrogen
Milk powder	ROTH
10x PBS	GIBCO
Western Lightning Reagent	PerkinElmer
Hyperfilm ECL	Amersham Biosciences
Hyperfilm cassette	Amersham Biosciences
Re-blot Plus Strong Solution	Millipore

Immunoprecipitation

Protein A/G-Agarose	Santa Cruz Biotechnology
Dynabeads® Protein A	Invitrogen
Dynabeads® Protein G	Invitrogen
DynaMag™-2	Invitrogen
RNase A	QIAGEN
Proteinase K	QIAGEN

DNeasy Blood and Tissue kit	QIAGEN
Blue Max™ Conical Tube, 15 ml	Falcon
Cell culture 150 mm dishes	Corning

Antibodies

JARID1A	rabbit	Bethyl Laboratories
JARID1B	rabbit	abcam
JARID1C	rabbit	Bethyl Laboratories
JARID1D	rabbit	Bethyl Laboratories
GAPDH	mouse	Advanced ImmunoChemical
Actin	rabbit	SIGMA
SMYD3	rabbit	Santa Cruz Biotechnology
ERα G-20	rabbit	Santa Cruz Biotechnology
ERα HC-20X	rabbit	Santa Cruz Biotechnology
RNA Pol II	rabbit	abcam
H3	rabbit	abcam
H3K4me1	rabbit	abcam
H3K4me2	rabbit	abcam
H3K4me3	rabbit	abcam
AR 441	mouse	Santa Cruz Biotechnology
AR C-19X	rabbit	Santa Cruz Biotechnology
IgG	rabbit	Millipore
Anti-mouse IgG - HRP[a]	sheep	GE Healthcare
Anti-rabbit IgG - HRP	donkey	GE Healthcare

[a] horseradish peroxidase

Chemicals

17β-estradiol	synthesised in-house at BSP[a]
R1881 (methyltrienolone)	Dupont NEN
sodium dodecyl sulfate (SDS)	BIO-RAD
Others	SIGMA and Merck

[a] Bayer Schering Pharma

Technical Devices

BBD 6220 incubator	Thermo Scientific
HERA safe cleanbench	Heraeus
Cell counter CASY®	Innovatis
Microscope Axiovert 40C	Zeiss
NanoDrop™ 2000	Thermo Scientific
7500 Fast Real-Time PCR System	Applied Biosystems
Sub-Cell GT Gel electrophoresis system	BIO-RAD
Power Pack P25	Biometra
Developing machine, CURIX 60	AGFA
Victor™ X3	PerkinElmer Life Sciences
Biorupter™	Diagenode
pH Meter 761 Calimatic	Knick
Centrifuge 5415R	Eppendorf
Heraeus Pico 21 Centrifuge	Thermo Scientific

Heraeus Multifuge 3S+	Thermo Scientific
Heraeus Multifuge 3SR+	Thermo Scientific
Speed vac, Concentrator 5301	Eppendorf
PCR Cycler Tetrad 2	BIO-RAD
Water bath	Julabo 5B
Shaker	Heidolph Titramax 100
Incubator	Heraeus
Incubator for shaking	INFORS

Buffers

1x Tris-Acetate-EDTA (TAE)	40 mM tris (pH 7.6), 20 mM acetic acid and 1 mM EDTA
IP-Lysis buffer	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Triton X-100, protease inhibitors and benzonase
Lysis buffer	5 mM PIPES pH 8, 85 mM KCl, 0.5 % Nonidet P40 and protease inhibitors
RIPA-Buffer	10 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1 % SDS, 0.1 % Na-deoxycholate, 140 mM NaCl and protease inhibitors
Wash Buffer I	20 mM Tris-HCl pH 8, 2 mM EDTA, 150 mM NaCl, 1 % Triton X-100 and 0.1% SDS
Wash Buffer II	20 mM Tris-HCl pH 8, 2 mM EDTA, 500 mM NaCl, 1 % Triton X-100 and 0.1 % SDS
Wash Buffer III	10 mM Tris-HCl pH 8, 1 mM EDTA, 250 mM LiCl, 1 % Nonidet P40 and 1 % Sodium deoxycholate
TE pH 8.0	10 mM Tris-HCl pH 8 and 1 mM EDTA
Elution-Buffer	0.1 M $NaHCO_3$ and 1 % SDS

2.2 Methods

2.2.1 Cell culture

Cells were grown at 37 ℃ in a 5 % CO_2 atmosphere in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. For MCF-7 cells phenol-red RPMI was used. The medium for MDA-MB-231 cells additionally contained 2 mM L-glutamine and 1x non-essential amino acids, the medium for LNCaP cells L-glutamine at the same concentration plus 10^{-10} M of the synthetic androgen analogue R1881 (methyltrienolone), which was dissolved in ethanol. For hormone stimulation experiments, cells were cultured in hormone-free medium consisting of RPMI 1640 media without phenol red supplemented with 2 % charcoal-stripped FBS (cFBS) and 2 mM L-glutamine for 3 days. Then 1 nM estrogen or androgen was added. 17β-estradiol (ZK5018) was synthesised in-house and dissolved in ethanol.

Table 1: Cell lines used in this work

Name	Description	Origin
MCF-7	Human breast cancer cell line derived from pleural effusion. ERα positive	ATCC[a]
MDA-MB-231	Human breast cancer cell line derived from pleural effusion. ERα negative	ATCC
LNCaP	Human prostate cancer cell line derived from left supraclavicular lymph node. AR positive (mutation at position T877A)	ATCC

[a]American Tissue Culture Collection

2.2.2 Cloning and site-directed mutagenesis

Cloning of JARID1A cDNA into pCMV-HA

For the generation of full-length human JARID1A cDNA (NM_001042603.1), pCMV-SPORT6-JARID1A, which includes the 5'-terminal 3257 bp, was purchased from the RZPD (Ressourcenzentrum für Genomforschung, Berlin, IRAKp961B10255Q). The remaining 3'-terminal 1868 bp were amplified from a cDNA brain pool using the primers JARID1A for and JARID1A rev (see table), and transferred into the pCR 2.1-TOPO® vector using TOPO TA Cloning® (Fig. 10). Digestion of pCMV-SPORT6-JARID1A-5'-terminal and partial digestion of TOPO-JARID1A-3'-terminal was performed with *Rsr*II. The resulting 5'-terminal fragment and the opened TOPO-JARID1A-3'-terminal plasmid were ligated for 10 min using the Rapid DNA Ligation Kit. For subcloning into the pCMV-HA expression vector, the full-length JARID1A insert was PCR-amplified with the JARID1A *Sal*I for and JARID1A *Not*I rev primers, which additionally contained the *Sal*I or *Not*I restriction sites. The resulting PCR product and the vector were appropriately digested and purified. The vector was dephosphorylated by incubation with the Shrimp alkaline phosphatase (SAP) for 1 h at 37 °C to prevent religation. The ligation reaction was performed as before. The product was then used to transform XL1-Blue supercompetent cells following the manufacturer's protocol. Bacterial colonies were allowed to grow on LB-Agar plates supplemented with 100 µg/ml ampicillin at 37 °C overnight. The colonies were tested for the presence of plasmids with the expected insert by PCR using JARID1A-specific primers. Positives clones were then amplified, the plasmid purified and the insert sequenced (AGOWA GmbH).

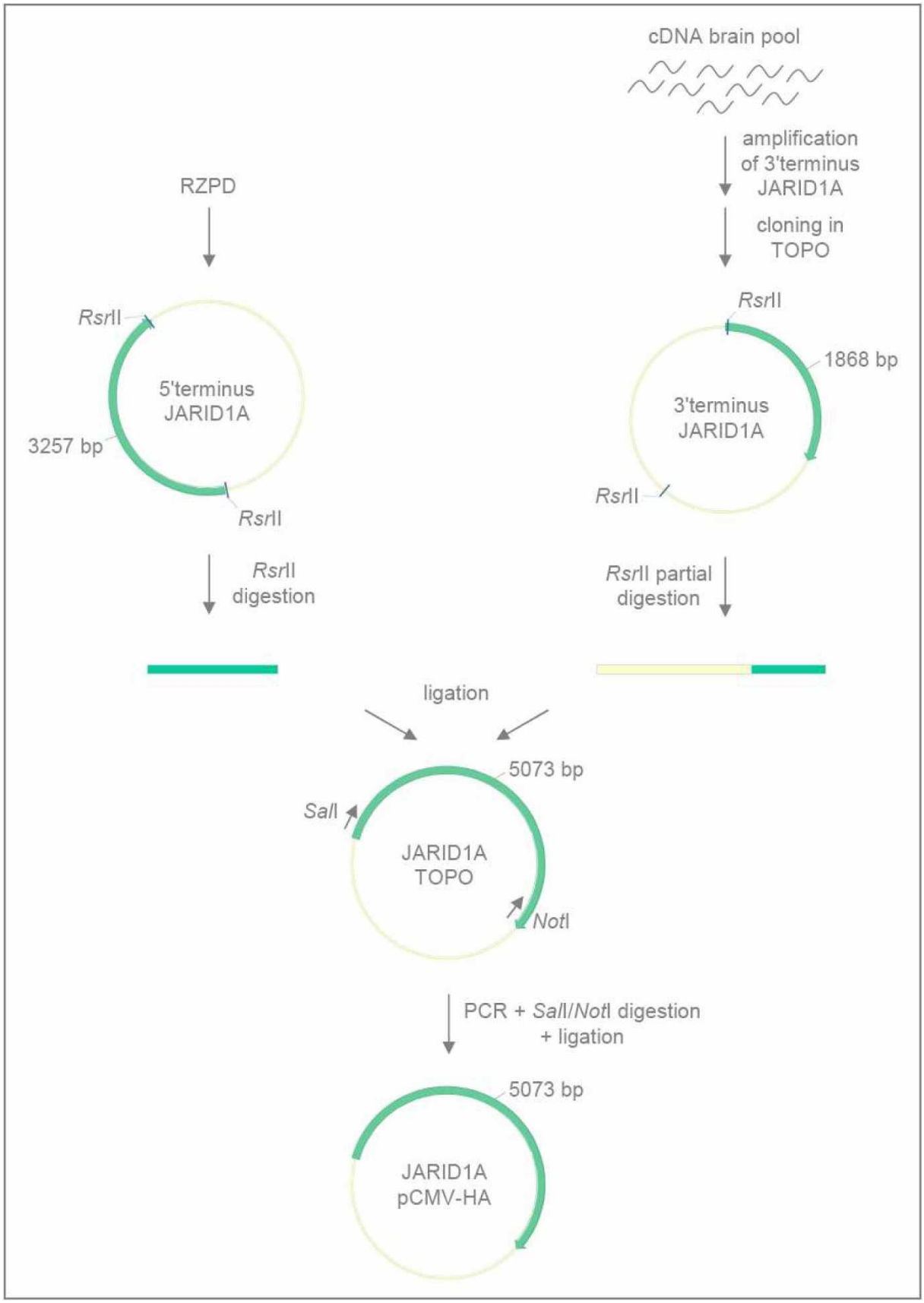


Figure 10

Schematic representation of the cloning procedure of full-length JARID1A into pCMV-HA.

PRB promoter in pGL4.26[luc2/minP/Hygro]

The PRB promoter luciferase (Luc) reporter construct was generated by amplifying the -711 to +29 bp region using human genomic DNA as template and the primers PRB for and PRB rev. The amplified fragment was then transferred into pCR 2.1-TOPO®. For subcloning into the pGL4.26[*luc2*/minP/Hygro] vector, TOPO-PRB promoter plasmid and vector were digested with *Kpn*I and *Xho*I, purified and ligated as before. The sequence of the resulting construct was verified by DNA sequencing.

SMYD3 in pCMV-HA

The SMYD3 coding sequence (NM_022743) was amplified from a cDNA testis pool using primers including *EcoR*I and *Xho*I restriction sites and transferred into pCR 2.1-TOPO®. For subcloning into the expression vector, pCR 2.1-TOPO-SMYD3 was digested with *EcoR*I and *Xho*I, the insert was purified and ligated into pCMV-HA, as before. In the resulting plasmid the SMYD3 sequence was downstream of and in-frame with the sequence of the HA tag.

The plasmids pSG5-ERα and pEGFP-N3 were available in the lab.

Table 2: Primers used for cloning

Name	Sequence (5' – 3')
JARID1A for	CATTGTTACAGGTGCTGAGCC
JARID1A rev	CTAACTGGTCTCTTTAAGATCCTC
JARID1A SalI for	ATATGTCGACAATGGCGGGCGTGGGG
JARID1A NotI rev	CGATGCGGCCGCCTAACTGGTCTCTTTAAGATCC
PRB for	GGATCCATTTTATAAGCTCAAAGATAATTAC
PRB rev	CTTACCCCGATTAGTGACAGCTGTGGAC
SMYD3 EcoRI for	CAGTGAATTCAAATGCGATGCTCTCAGTGCCGCG
SMYD3 XhoI rev	CGATCTCGAGTTAGGATGCTCTGATGTTGGCG

Site directed-mutagenesis was carried out with the Quik-Change® XL (JARID1A mutant) or II kit (SMYD3 mutants) following the recommended protocol. Briefly, the complete template plasmid was amplified using primers containing the desired change (see table). The extention time was 1 min per kb. The template plasmid was eliminated by *Dpn*I digestion and the product was used to transform XL1-Gold ultracompetent cells. Plasmids were prepared from selected colonies and the presence of the desired mutation was confirmed by sequencing.

To create the enzymatically inactive form of JARID1A, the codon CAC corresponding to the catalytic histidine at position 483 was substituted by GCC, coding for alanine (Klose et al. 2007). The two enzymatically inactive SMYD3 mutants were generated by deletion of 9 bp (ΔEEL) or 12 bp (ΔNHSC) region located in the SET domain (Hamamoto et al. 2004).

Table 3: Primers used for site-directed mutagenesis

Name	Sequence (5' – 3')
JARID1A H483 for	CTTCTCTTCTTTTTGCTGGGCCATTGAGGATCACTGGAG
JARID1A H483 rev	CTCCAGTGATCCTCAATGGCCCAGCAAAAAGAAGAGAAG
SMYD3 ΔEEL for	CCGAGACATCGAGGTGGGAACCATCTGCTACCTGGATATG
SMYD3 ΔEEL rev	CATATCCAGGTAGCAGATGGTTCCCACCTCGATGTCTCGG
SMYD3 ΔNHSC for	CCTATATCCCAGTATCTCTTTGCTCGACCCCAACTGTTCGATTGTG
SMYD3 ΔNHSC rev	CACAATCGAACAGTTGGGGTCGAGCAAAGAGATACTGGGATATAGG

2.2.3 Transfection

The transfer of foreign nucleic acids into eukaryotic cells is called transfection. The lipid reagents used here are able to form liposomes which have strong binding to DNA and RNA since they are positively charged. This complex merges with the cell membrane and is taken up inside the cell.

Transfections with small interfering RNAs (siRNAs) were performed with Lipofectamine™ 2000 following the recommended protocol. Stealth™ RNAi Negative Universal Control Medium was transfected in the control experiments. A transfection reaction with a fluorophore-linked, non-targeting RNA (Block-iT™) was included to control efficiency.

Plasmid transfections were generally carried out with FuGENE® HD. Cells were seeded in 96-, 24-, 6-well plates or 150 mm dishes and the transfection was performed the day after using 3 µl reagent per 1 µg DNA. As control empty pCMV-HA vector was transfected. GFP was routinely used in order to verify the transfection efficiency.

2.2.4 Luciferase gene reporter assay

Reporter gene assays rely on the activity measurement of certain reporter gene products, usually fluorescence and luminescence, which are expressed under the control of an inducible promoter. Here the ability of the enzyme luciferase to catalyse a reaction with luciferin to produce light was used.

For the transactivation assays, MCF-7 cells were seeded into 96-well plates at a concentration of 8,000 cells/per well. Transfection was carried out 44 h later using FuGENE® HD Transfection Reagent in Opti-MEM®. Expression plasmids for wild-type JARID1A or mutant form (30 ng/well) were cotransfected together with a Luc-based reporter vector harbouring the PRB promoter (40 ng/well) and a pSG5-based ERα-expressing plasmid (80 ng/well). Hormone induction was performed 4-5 h later by adding estrogen up to 100 nM. Measurement of Luc activity was carried out after 24 h in a Victor

multilabel reader, following the addition of 100 µl of SteadyLite Plus Reagent. For all points the average value of six wells treated in parallel was taken.

2.2.5 Gene knock-down

For the knock-down of the JARID1 family, siRNA technology was used. SiRNAs are short, usually 21-nucleotide long, RNA molecules, which bind to the target messenger RNA (mRNA) with the protein complex RISC (RNA-induced silencing complex), resulting in the degradation of the target mRNA and knock-down of expression.

Two Stealth™ siRNAs targeting different positions of JARID1A, 1B, 1C and 1D mRNA were purchased from Invitrogen. Cells seeded in 6-well plates or 150 mm dishes were transfected with siRNAs or a non-targeting siRNA control, at a final concentration of 40 nM. After 1, 2, 3, 4 or 5 days, the cells were harvested and the extent of the RNA knock-down was measured at the mRNA level by quantitative PCR and at the protein level by Western blot analysis (see below).

Table 2: siRNAs used for knock-down

Name	Sequence (5' – 3')
siJARID1A #1	ATACTAACCAGCCACCCTAGAGCTC
siJARID1A #2	CAGCCTCCATTTGCCTGTGAAGTAA
siJARID1B #1	CACGTATCCAGAGACTGAATGAATT
siJARID1B #2	GCCTTCTTGTTTGCCTGCATCATGT
siJARID1C #1	AGGCCCAGACGAGAGTGAAACTGAA
siJARID1C #2	CGGTTTCCCTGTCAGTGACAGTAAA
siJARID1D #1	TGCCAGCAACATTCCTGCTAGCATA
siJARID1D #2	GAGGAAGCCCAAGTGCGTACCTTTA

2.2.6 RNA extraction and reverse transcription

Cells were transfected or treated as indicated for each experiment and total RNA was then extracted using the RNeasy Mini Kit including the *DNase*I digestion on column. The RNA concentration was measured using the Nanodrop.

To transcribe RNA into DNA, first-strand cDNA synthesis was performed starting with 1 to 4 µg of total RNA with the random primers using SuperScript™ III reverse transcriptase.

2.2.7 Quantitative PCR

Quantitative real-time polymerase chain reaction (qPCR) is a technique used to amplify and simultaneously quantify a targeted DNA molecule. In contrast to the standard PCR, where the product of the reaction is detected at its end, the amplified DNA is detected as

the reaction processes in “real time”. It enables quantification as relative amount when normalised to DNA input or normalising gene (e.g. cyclophilin, GAPDH or 18S). Two common methods for detection in real-time PCR are: (1) sequence specific DNA-probes (here TaqMan® Gene Expression Assays) consisting of oligonucleotides that are labelled with a fluorophore, and (2) non-specific fluorescent dyes that intercalate with double-stranded DNA (SYBR-Green).

Real-time PCR quantification was performed on a Fast Real-Time PCR System using the standard cycling program (95 ℃ for 20 s, 40 cycles of 95 ℃ for 3 s followed by 60 ℃ for 30 s). The PCR Mix and the TaqMan gene expression assays used were purchased from Applied Biosystems (see table). As template 20 ng of cDNA per reaction was added. For the analysis of the results, the cycle threshhold (Ct) value of the gene of interest obtained for each sample was normalised to the Ct value of human cyclophilin A (ΔCt) and then to the control sample (ΔΔCt). Results are expressed as percentage ($2^{-\Delta\Delta Ct}$ x 100) in comparison to the control.

Table 3: TaqMan® Gene Expression Assays used for quantitative PCR

Name	Number
JARID1A	Hs00231908_m1
JARID1B	Hs00366783_m1
JARID1C	Hs00188277_m1
JARID1D	Hs00190491_m1
PGR	Hs00172183_m1
EGR1	Hs00174860_m1
pS2 (TFF1)	Hs00170216_m1
c-myc	Hs00153408_m1
SMYD3	Hs00224208_m1
PSA (KLK3)	Hs02576345_m1
KLK2	Hs00428383_m1
TMPRSS2	Hs00237175_m1
clusterin	Hs00156548_m1
AR	Hs00907244_m1
p21	Hs00355782_m1
hu Cyc	4326316E

For quantification of the DNA obtained by chromatin immunoprecipitation, the SYBR® Green (QIAGEN) method was performed following the recommended protocol (95 ℃ for 5 min, 40 cycles of 95 ℃ for 10 s followed by 60 ° C for 30 s). For each primer pair the generation of a unique population of amplicons was confirmed by running the dissociation protocol at 60 ℃. Serial dilutions (1 :10, 1:100 and 1:1000) of input cDNA were performed to find the linear range. For the analysis of the results, the Ct value of the region of interest obtained for each antibody approach was normalised to the input Ct

of this region. Results are expressed as percentage ($2^{-\Delta Ct}$ x 100) as compared to the input.

Table 4: Primers used for quantitative PCR

Name	Sequence (5' – 3')
PR half-ERE for	GGAGAACTCCCCGAGTTAGG
PR half-ERE rev	AGGGAGGAGAAAGTGGGTGT
PR enh 1 for	GCAGGACGACTTCTCAGACC
PR enh 1 rev	GCCTGACCTGTTGCTTCAAT
PR enh 2 for	GGGCTGGCTTTTATCATTCA
PR enh 2 rev	AACGTGTTTGCATCTTGCTG
Unrel. gene control for	CTTGAACCCAGGAAGCGTAG
Unrel. gene control rev	TGGAGTCTCACTGCGATGTC
PSA ARE I for	CCTAGATGAAGTCTCCATGAGCTACA
PSA ARE I rev	GGGAGGGAGAGCTAGCACTTG
PSA ARE III for	GCCTGGATCTGAGAGAGATATCATC
PSA ARE III rev	ACACCTTTTTTTTCTGGATTG
PSA middle for	CTGTGCTTGGAGTTTACCTGA
PSA middle rev	GCAGAGGTTGCAGTGAGCC

2.2.8 Protein extraction, SDS-PAGE and western blot

Protein extracts were prepared from transfected cells using the Mammalian Protein Extraction Reagent (M-Per) supplemented with a protease inhibitor mix and benzonase. Lysates were purified by 10 min at 13000 rpm and protein concentrations were measured using the Bradford assay and adjusted with lysis buffer.

For Western blot analysis the protein extracts were separated in NuPAGE gradient gels for 1 h at 200 V. NuPAGE gels are a neutral (pH 7.0), discontinuous SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis), which use an electric field to rapidly resolve proteins based roughly on their mass.

Then the separated proteins were transferred onto polyvinylidene fluoride membranes for at least 1 h at 40 V. The membranes were blocked in 5 % milk powder in PBS/0.1 % Tween and incubated overnight at 4 ℃ with the prim ary antibody using a 1:1000 dilution in 5 % milk solution for each antibody except for actin and GAPDH, where a 1:10000 dilution was used. AR western blot analysis was carried out with the AR-441 monoclonal antibody. Blots were washed with PBS/0.1 % Tween, incubated with the appropriate secondary antibodies in a 1:10000 dilution for 1 h at room temperature, washed again and developed using the Western Lightning chemiluminescence kit.

For re-blot experiments, the blots were incubated for 15 min in 1x Re-blot Plus Strong Solution at room temperature, washed with PBS/0.1 % Tween, incubated for 1 h in 5 % milk powder in PBS/0.1 % Tween and probed with the appropriate primary antibody at

4 ℃ overnight.

2.2.9 Co-immunoprecipitation

Co-immunoprecipitation allows to analyse protein-protein interactions occurring in the cell by isolation using an antibody against the protein of interest for purification of a protein complex.

Cells treated with hormone were washed with cold PBS, harvested and resuspended in cold lysis buffer. Following incubation on ice for 1 h, lysates were sonicated for 1 x 5 min with a 30 s interval at medium level, centrifuged at 13000 rpm for 10 min at 4 ℃ and supernatants were transferred to a new tube. The appropriate antibodies (4 µg) directed against JARID1A, JARID1D, ERα (G-20) and AR (C-19) (see material) were added and incubated overnight. Then, 40 µl of Protein A/G PLUS-Agarose were added and incubated for 3 h at 4 ℃. After washing four times with cold lysis buffer, proteins were eluted at 95 ℃ for 3 min in NuPAGE LDS Sample Buffer and analysed by Western blot.

2.2.10 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) extends the standard IP by allowing the analysis of protein-DNA interactions. First, proteins are cross-linked to DNA. These DNA/protein complexes are then lysed, sonicated and immunoprecipitated using an antibody against the protein of interest. After purification, the bound DNA is analysed by PCR.

Cells were grown in 150 mm dishes in hormone-free medium for 3 days. They were then treated with 1 nM hormone for 1 h and dual cross-linked at room temperature by a 45 min incubation in 2 mM disuccinimidyl glutarate, followed by treatment with 1 % formaldehyde for 10 min. The reaction was stopped by adding glycine to a final concentration of 125 nM. After 5 min, the cells were washed twice with cold PBS, detached by scraping into cold PBS and pelleted by centrifugation at 4 ℃ for 5 min at 1000 rpm. Chromatin was isolated after cell lysis in lysis buffer (see material). This was followed by nuclear lysis using RIPA-buffer. DNA was sheared in fragments of around 200 bp by sonicating for 2 x 10 min with a 30 s interval at high level using the Biorupter™. Sonication efficiency was controlled by agarose gel electrophoresis. The samples were then centrifuged at 13000 rpm for 10 min and at 4 ℃. In order to pre-clear the chromatin solution, 60 µl of Dynabeads® Protein A/G were added to the cell lysates and incubated for 1 h while rotating at 4 ℃. For immunoprecipitation, 3-4 µg of antibody specific for RNA Pol II, ERα (HC-20X), AR (C-19X), JARID1A, JARID1D, H3, H3K4me1, me2 or me3 were used. Rabbit IgG was used as control. The antibodies were first pre-bound to 40 µl Dynabeads® Protein A/G in PBS by incubating them for 2-3 h while

rotating at 4 °C. After removing an input sample, the cell lysate was added to the pre-bound antibodies and incubated overnight at 4 °C. Washing was performed with buffer I, buffer II and buffer III which contained rising salt concentrations. This was followed by two washes with TE. The immunocomplexes were eluted in 2 x 250 µl elution buffer at 65 °C, while shaking for 10 min. Then the cross-link was reverted by adding NaCl to a final concentration of 160 mM followed by incubation at 65 °C overnight. The eluates were sequentially treated for 1 h with RNase H and then with proteinase K. The DNA was then purified using the DNeasy Blood and Tissue kit. The immunoprecipitated DNA was analysed by quantitative PCR using QuantiFast™SYBR®Green PCR Kit. The reaction was performed using primers designed to specifically amplify sequences in the PR and PSA regulatory regions (see table). For the experiments in which JARID1A was immunoprecipitated, an expression plasmid containing the corresponding cDNA was transfected first.

3 RESULTS

3.1 Regulation of JARID1 gene expression by estrogen and androgen

The transcriptional expression of numerous genes is under the control of steroid hormones. The regulation of JARID1 gene expression by estrogen and androgen was therefore examined first. Estrogen regulation was tested in the ER-positive breast cancer cell line MCF-7 and androgen regulation in the AR-positive prostate cancer cell line LNCaP. The cells were cultured in hormone-free medium for three days prior to treatment with 1 nM of 17β-estradiol (MCF-7) or 1 nM of the synthetic androgen analogue R1881 (LNCaP). The mRNA levels of each JARID1 family member were determined 1, 6, 16 and 24 hours after hormone treatment by quantitative PCR (Fig. 11). Estrogen treatment of MCF-7 cells repressed JARID1A expression in a time-dependent manner with a maximum of 30-40 % reduction after 6 hours and at later time-points (Fig. 11A). A time-dependent decrease down to about 50 % was seen for JARID1B gene expression after 16 and 24 hours of estrogen treatment. JARID1C levels were initially reduced down to ~ 80 % by estrogen but went back to normal after 24 h. The JARID1D form is not expressed in MCF-7 cells as it is encoded by the chromosome Y, and was therefore not analysed. Concerning LNCaP cells, only a slight reduction of about 30 % was observed for JARID1A levels after androgen treatment (Fig. 11B). JARID1B gene expression was reduced around ~ 50 % after 16 and 24 hours of treatment. Virtually no effects were observed for JARID1C and 1D after androgen stimulation. These results show that estrogens and androgens regulate the expression of some JARID1 family members. Estrogen represses JARID1A and more so 1B expression in MCF-7 cells whereas androgen mainly affects JARID1B expression in LNCaP cells.

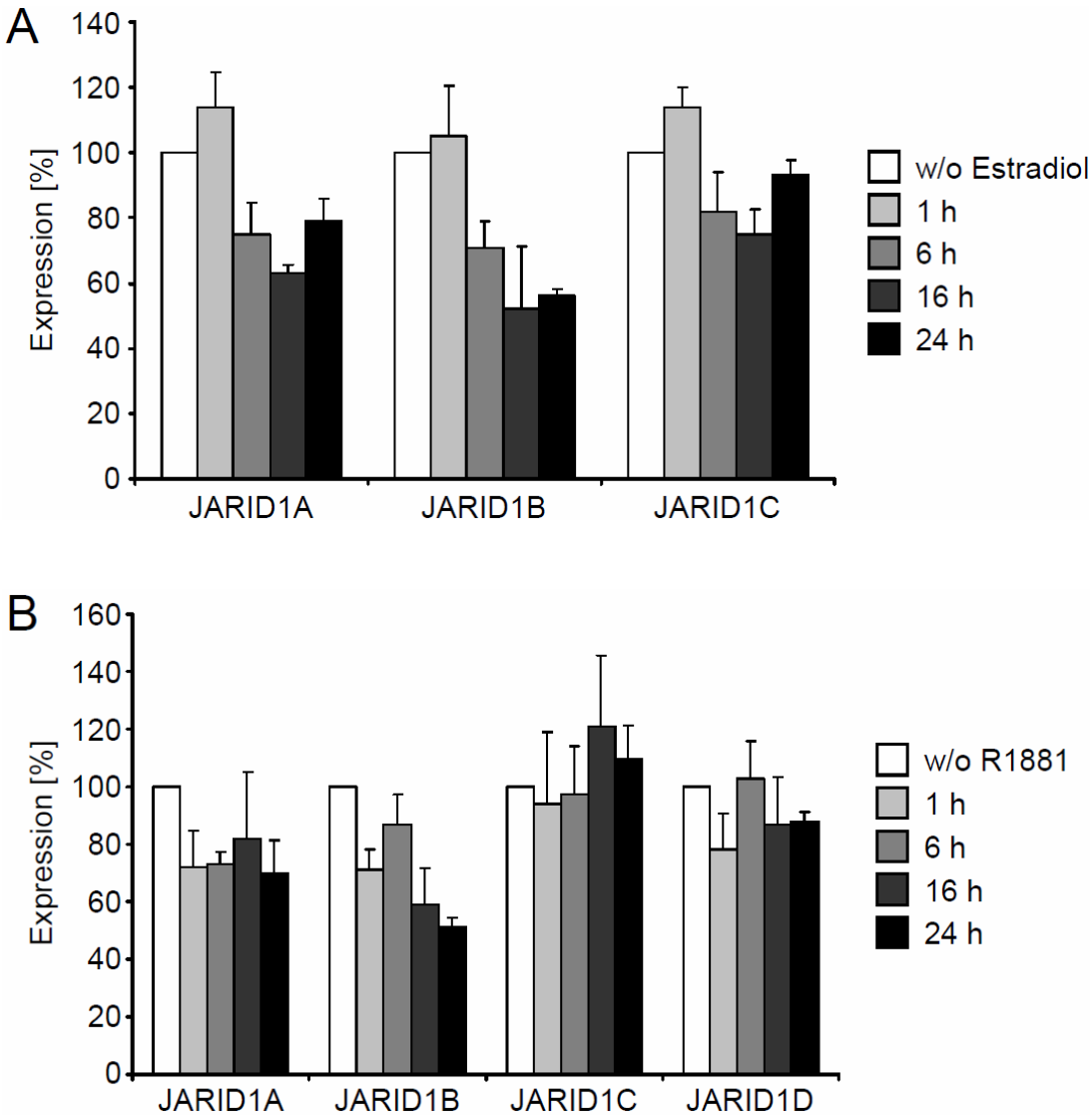


Figure 11

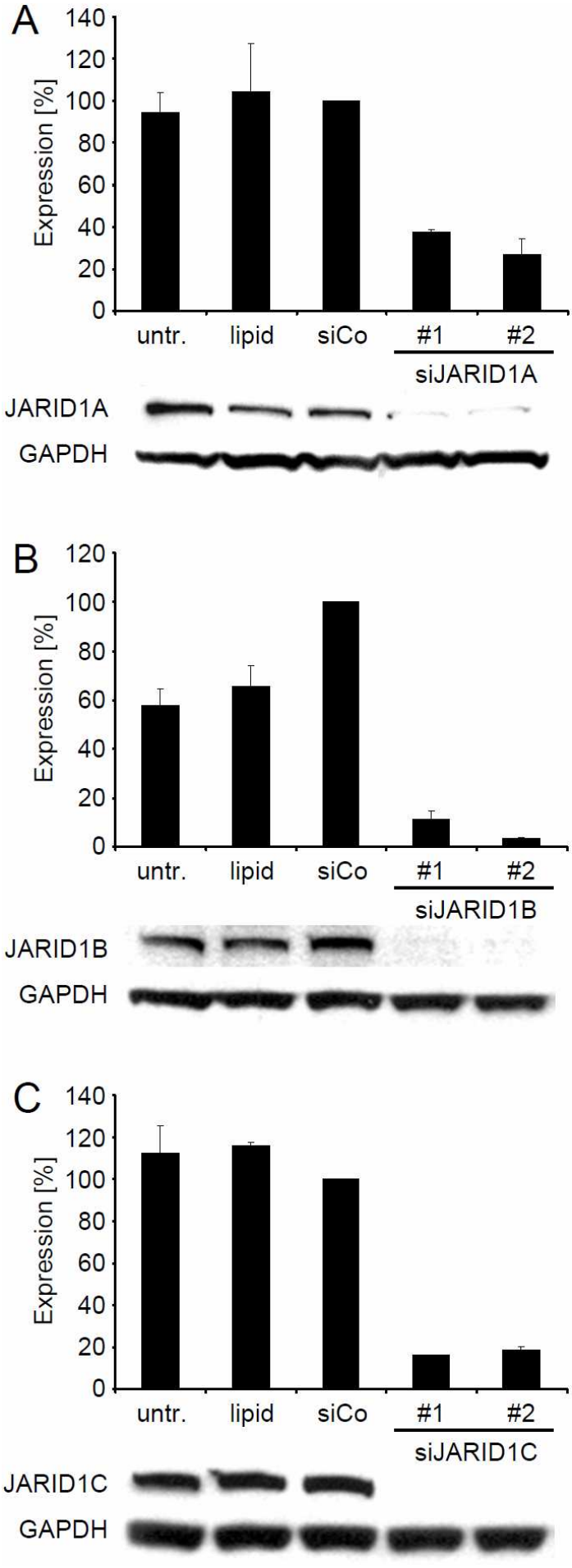
Regulation of JARID1 gene expression by steroid hormones. **A.** MCF-7 cells were treated with 1 nM 17β-estradiol for the indicated time periods, total RNA was prepared and the JARID1A, B and C transcript levels were determined by quantitative PCR. **B.** LNCaP cells were treated with 1 nM R1881 for the indicated time periods, total RNA was prepared and the JARID1A, B, C and D transcript levels were determined by quantitative PCR.

3.2 Identification of siRNA specific for JARID1 family

In order to find out whether the JARID1 demethylases played a role in ERα and/or AR function, two siRNA pairs for each JARID1 form were obtained and validated, using MCF-7 as a breast and LNCaP as a prostate cell model. Cells were treated with each siRNA pair and the respective JARID1 mRNA and protein levels were determined at different time-points after transfection, using quantitative PCR and Western blot analysis. In MCF-7 cells, a robust reduction of expression was observed for JARID1A (to ~ 30 %),

1B (to ~ 10 %) and 1C (to ~ 20 %) from two up to four days post-treatment, when compared to cells treated with lipid only or transfected with unrelated siRNAs. A strong decrease of the JARID1A, 1B and 1C protein levels was seen three and four days after siRNA transfection (Fig. 12A-C). In LNCaP cells, a solid reduction of expression to ~ 20 % was observed for all four JARID1 genes two days after transfection. At the protein level a strong reduction of the JARID1A, 1B, 1C and 1D proteins was seen four days post-transfection (Fig. 13A-D).

The selectivity of each siRNA pair for the respective JARID1 mRNA was tested in MCF-7 and LNCaP cells by measuring the expression levels of the closest JARID1 family member (Fig. 14). In both cell lines no significant reduction in JARID1B expression was seen after JARID1A knock-down and vice-versa. The JARID1C-specific siRNAs did not affect the expression levels of JARID1A in MCF-7 cells or of JARID1D in LNCaP cells. Finally, JARID1D siRNAs showed no effect on JARID1C expression. These data document the high selectivity of the siRNAs that were selected for this study.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120
100
80
60
40
20
0
untr.
lipid
siCo
#1
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siJARID1A
JARID1A
GAPDH
B
Expression [%]
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100
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untr.
lipid
siCo
#1
#2
siJARID1B
JARID1B
GAPDH
C
Expression [%]
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120
100
80
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40
20
0
untr.
lipid
siCo
#1
#2
siJARID1C
JARID1C
GAPDH

Figure 12

Knock-down of JARID1A-C in MCF-7 cells. MCF-7 cells were transfected with two siRNA pairs (#1 and 2#) specific for the different JARID1 forms, as indicated. In the controls, cells were transfected with unrelated siRNAs (siCo), with lipid only or left untreated (untr.). JARID1 mRNA levels were measured by quantitative PCR two days later and protein levels determined by Western blot analysis three days later. GAPDH protein levels were assessed as loading control. **A**. Knock-down of JARID1A. **B.** Knock-down of JARID1B. **C.** Knock-down of JARID1C.

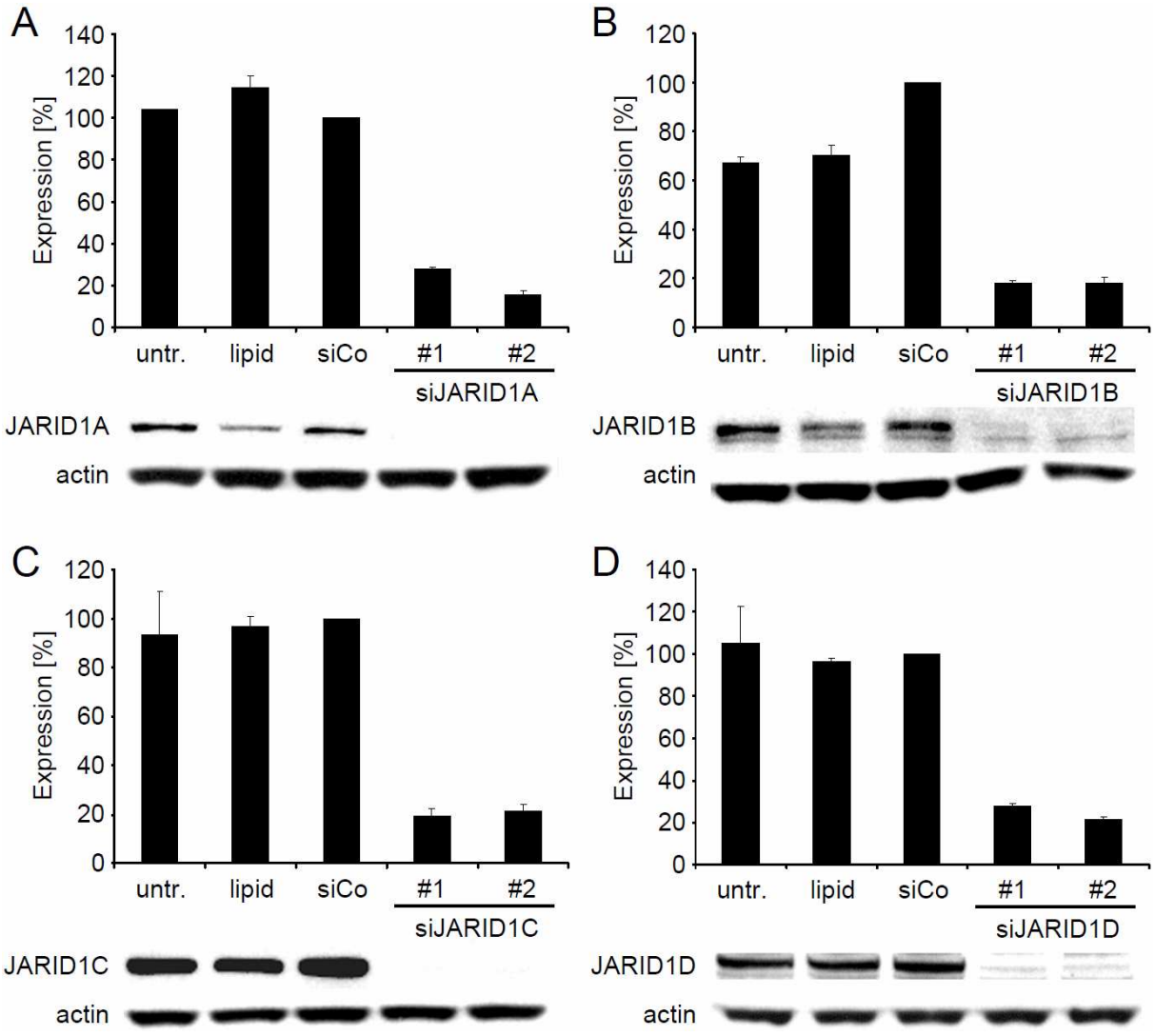


Figure 13

Knock-down of JARID1A-D in LNCaP cells. LNCaP cells were transfected with two siRNA pairs (#1 and 2#) specific for the different JARID1 forms, as indicated. In the controls, cells were transfected with unrelated siRNAs (siCo), with lipid only or left untreated (untr.). JARID1 mRNA levels were measured by quantitative PCR two days later and protein levels determined by Western blot analysis four days later. Actin protein levels were assessed as loading control. **A.** Knock-down of JARID1A. **B.** Knock-down of JARID1B. **C.** Knock-down of JARID1C. **D.** Knock-down of JARID1D.

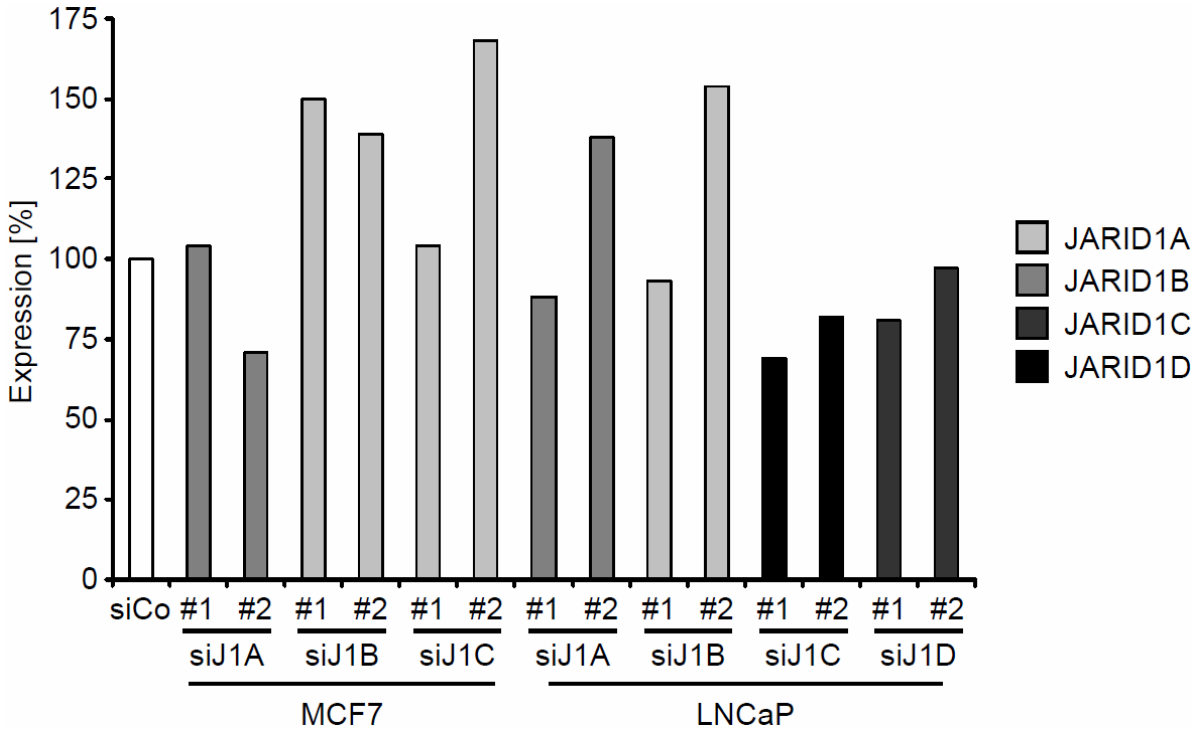


Figure 14

Determination of the selectivity of each siRNA pair in MCF-7 and LNCaP cells. Cells were transfected with two siRNA pairs (#1 and #2) for each JARID1 form, as indicated. In the control, cells were transfected with unrelated siRNAs (siCo). The mRNA levels of the family member with the highest homology were determined by quantitative PCR two days later.

3.3 Role of JARID1 family in ERα function

3.3.1 Effect of JARID1 knock-down on ERα transcriptional activity

After identifying siRNAs specific for each JARID1 form, the effects of JARID1 knock-down on estrogen-regulated genes were examined. In order to characterise and find the optimal conditions for estrogen stimulation, MCF-7 cells were stimulated with 17β-estradiol for different time periods and the expression of four genes known to be estrogen-regulated was measured by quantitative PCR (Fig. 15). The highest increase was found for pS2 expression for which a 30-fold stimulation was observed after 16 hours of estrogen treatment. The expression of another estrogen target gene, the PR gene, was also dramatically increased (25-fold) after estrogen stimulation. The c-myc transcript levels were increased to a lesser extent (5-fold) after 1 hour of estrogen treatment before slowly returning to the basal level within 16 hours. The expression of ERα itself showed a small increase after 1 hour of estrogen treatment, followed by a reduction to basal level after 6 h. Overall the data show the strongest stimulatory effects of estrogen to take place between 16-24 h.

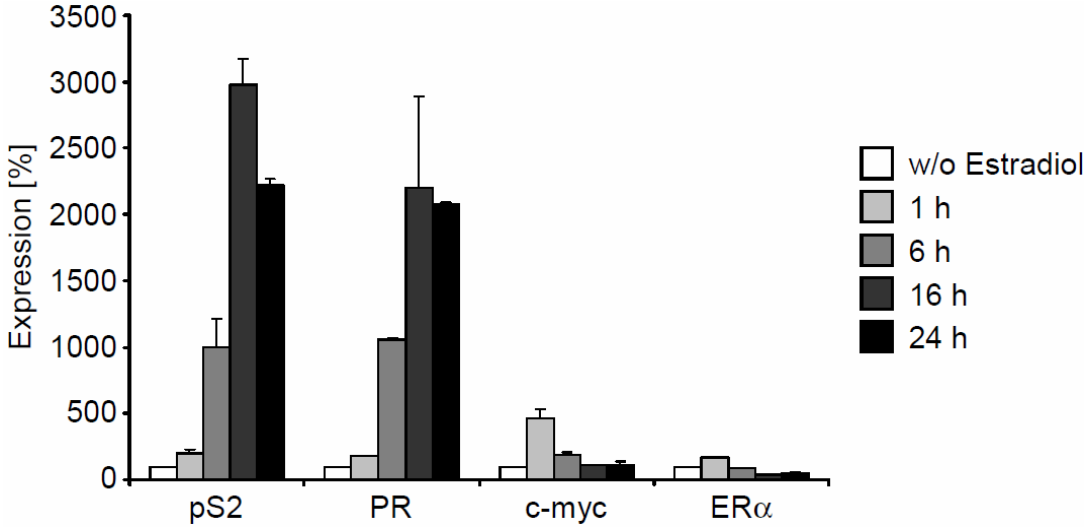
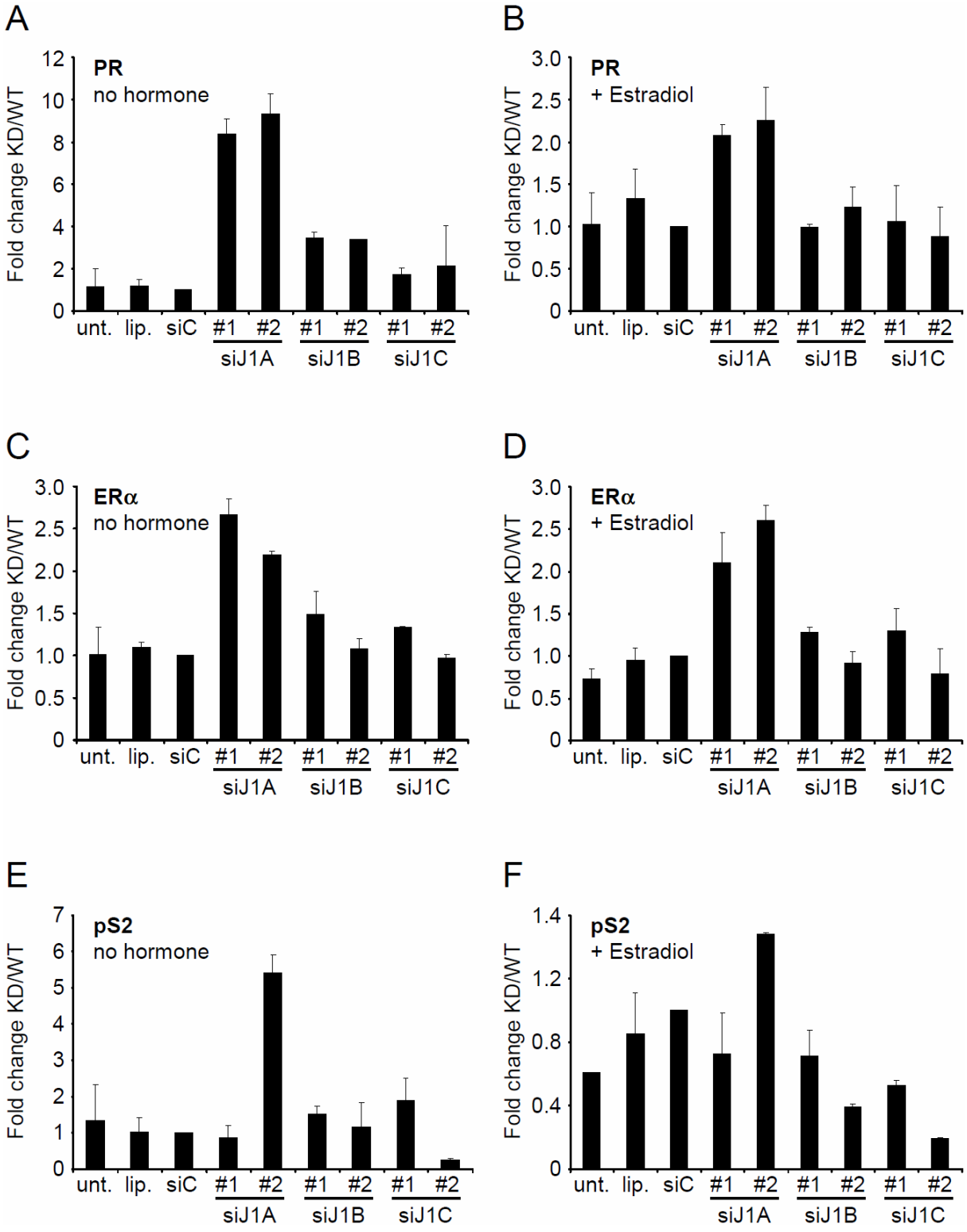


Figure 15

Regulation of estrogen target genes by 17β-estradiol in MCF-7 cells. MCF-7 cells were treated with 1 nM 17β-estradiol for 1, 6, 16 or 24 hours, total RNA was prepared and the pS2, PR, c-myc and ERα transcript levels were determined by quantitative PCR.

After finding the optimal conditions for siRNA treatment and estrogen induction, the effects of JARID1 knock-down on the estrogen regulation of selected genes were investigated. MCF-7 cells were transfected with the siRNAs directed against the different JARID1 forms. The cells were treated or not for 24 hours with estrogen and total RNA was purified four days later. Quantitative PCR showed that knocking-down the expression of JARID1A led up to a 9-fold increase of PR mRNA levels. A much stronger induction was seen in absence than in presence of estradiol (Fig. 16A and B). Reducing the expression of JARID1B or 1C had far less effects on basal PR levels and no effect on estrogen-stimulated PR levels. Concerning ERα, a 2-fold increase was seen after JARID1A depletion, and unlike for PR, a comparable increase was also seen in presence of estradiol (Fig. 16 C and D). For pS2, only one of the JARID1A siRNAs resulted in an induced expression (Fig. 16E and F). No effect of JARID1A knock-down was observed on c-myc expression (Fig. 16G and H). Interestingly, the knock-down of JARID1C decreased the expression of pS2 and c-myc. However, this effect was restricted to siRNA #2. Based on the marked effects of JARID1A knock-down on PR transcript levels, it was decided to concentrate on this form and its role in regulating PR gene expression.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PR
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Fold change KD/WT
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2
4
6
8
10
12
unt. lip. siC #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C
B
PR
+ Estradiol
Fold change KD/WT
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0.5
1.0
1.5
2.0
2.5
3.0
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siJ1A siJ1B siJ1C
C
ERα
no hormone
Fold change KD/WT
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0.5
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1.5
2.0
2.5
3.0
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siJ1A siJ1B siJ1C
D
ERα
+ Estradiol
Fold change KD/WT
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unt. lip. siC #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C
E
pS2
no hormone
Fold change KD/WT
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6
7
unt. lip. siC #1 #2 #1 #2 #1 #2
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F
pS2
+ Estradiol
Fold change KD/WT
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siJ1A siJ1B siJ1C

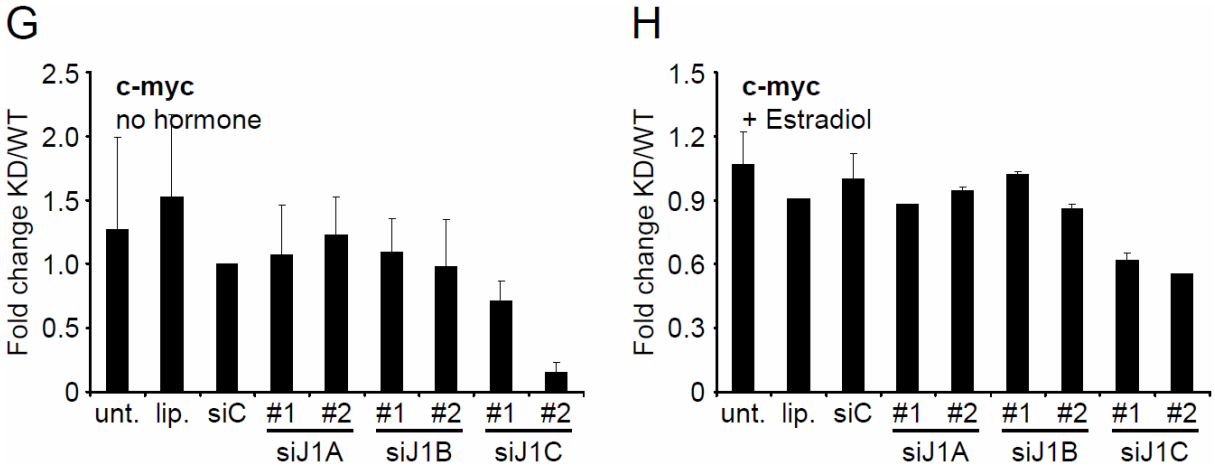


Figure 16

Effects of JARID1 knock-down on estrogen-regulated gene expression. MCF-7 cells were transfected with siRNAs specific for each JARID1 form (siJ1A: JARID1A; siJ1B: JARID1B; siJ1C: JARID1C). In the controls, cells were transfected with unrelated siRNAs (siC), with lipid only (lip.) or left untreated (untr.). PR, ERα, pS2 and c-myc transcript levels were determined by quantitative PCR. MCF-7 cells were treated with vehicle or with 1 nM 17β-estradiol respectively. The fold induction compared to cells treated with unrelated siRNA are given. **A.** PR expression in presence of vehicle. **B.** PR expression after 1 nM 17β-estradiol treatment. **C.** ERα expression in presence of vehicle. **D.** ERα expression after 1 nM 17β-estradiol treatment. **E.** pS2 expression in presence of vehicle. **F.** pS2 expression after 1 nM 17β-estradiol treatment. **G.** c-myc expression in presence of vehicle. **H.** c-myc expression after 1 nM 17β-estradiol treatment.

3.3.2 Effect of JARID1A wild-type and mutant overexpression on PR promoter

In order to determine whether the demethylase activity of JARID1A was necessary for the repressive effect observed on PR expression, a reporter gene assay was performed. A plasmid encoding full-length human JARID1A was generated. Since estrogen treatment causes a selective increase in transcripts derived from promoter B in breast cancer cells (Graham et al. 1995), a reporter plasmid containing the PRB promoter region -711 to +29 placed upstream of a Luc reporter was devised. This region does not contain a canonical ERE but has previously been shown to be strongly induced by estrogen in cell-based transactivation assays (Kastner et al. 1990). As shown in Figure 17A, this observation was confirmed as an approximately 7-fold induction was measured in MCF-7 cells co-transfected with an ERα expression plasmid and treated with 50 nM or higher estrogen concentrations for 24 hours. Then, the effects of wild-type and enzymatically inactive JARID1A forms were compared. For site-directed mutagenesis, an appropriate primer pair was used to replace the histidine codon 483 with an alanine codon in order to abrogate the demethylase activity in JARID1A (Klose et al. 2007). The corresponding expression plasmids were co-transfected with the ERα expression vector and the reporter vector containing the PRB promoter into MCF-7 cells (Fig. 17B). After 5 hours, the cells were treated with different estrogen concentrations for another 24 hours.

Determination of Luc activity revealed that JARID1A wild-type, but not the enzymatically inactive mutant, entirely prevented the estrogen induction of the PRB promoter, even at the highest hormone concentration tested.

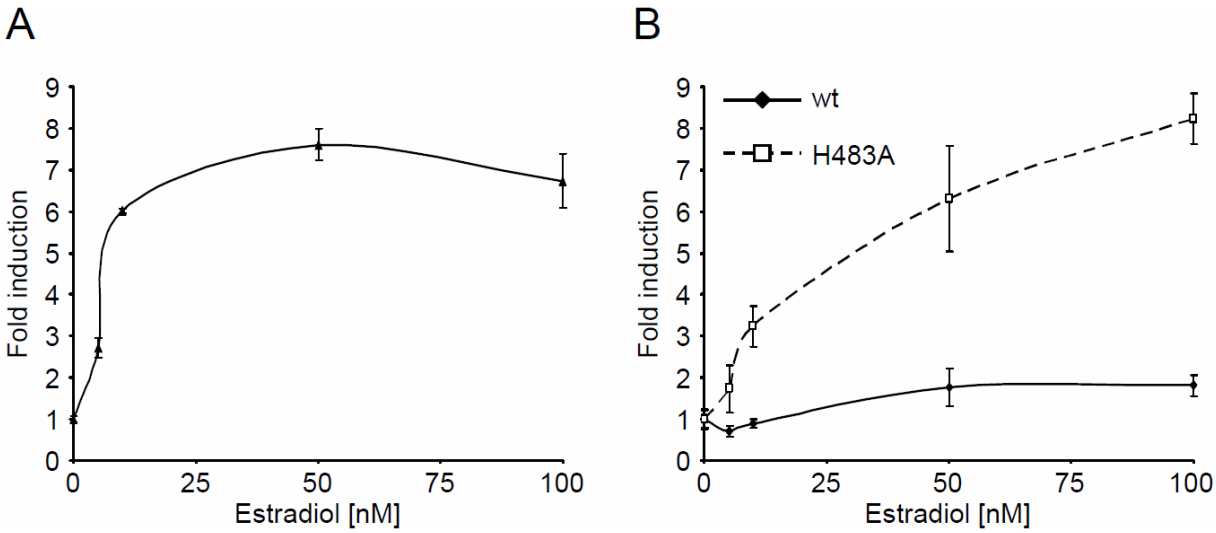


Figure 17

Effects of JARID1A on PR stimulation by estrogen. **A.** MCF-7 cells were transfected with a PRB promoter reporter vector. **B.** MCF-7 cells were co-transfected with a promoter reporter vector and an expression vector for wild-type (wt) or enzymatically inactive (H483A) JARID1A. Stimulation was with 0-100 nM 17β-estradiol and Luc activity was determined 24 h later. The fold inductions compared to cells not treated with estrogen are given.

3.3.3 Interaction of JARID1A and ERα

Several reports show that PR is under estrogen control and that this is mediated by the ERα isoform (Graham et al. 1995; Flototto et al. 2004). As JARID1A may cooperate with ERα to control the levels of PR, it was determined whether an interaction between these two proteins took place in MCF-7 cells. Protein extracts were immunoprecipitated with an ERα antibody and analysed by Western blot using a JARID1A antibody, or conversely immunoprecipitated with a JARID1A antibody and analysed with an ERα antibody. The results showed that a complex formed of JARID1A and ERα can be immunoprecipitated when using an ERα antibody (Fig. 18). This was however not the case in the reverse experiment using a JARID1A antibody. One possible explanation is that the JARID1A antibody used interfered with the JARID1A/ERα interaction.

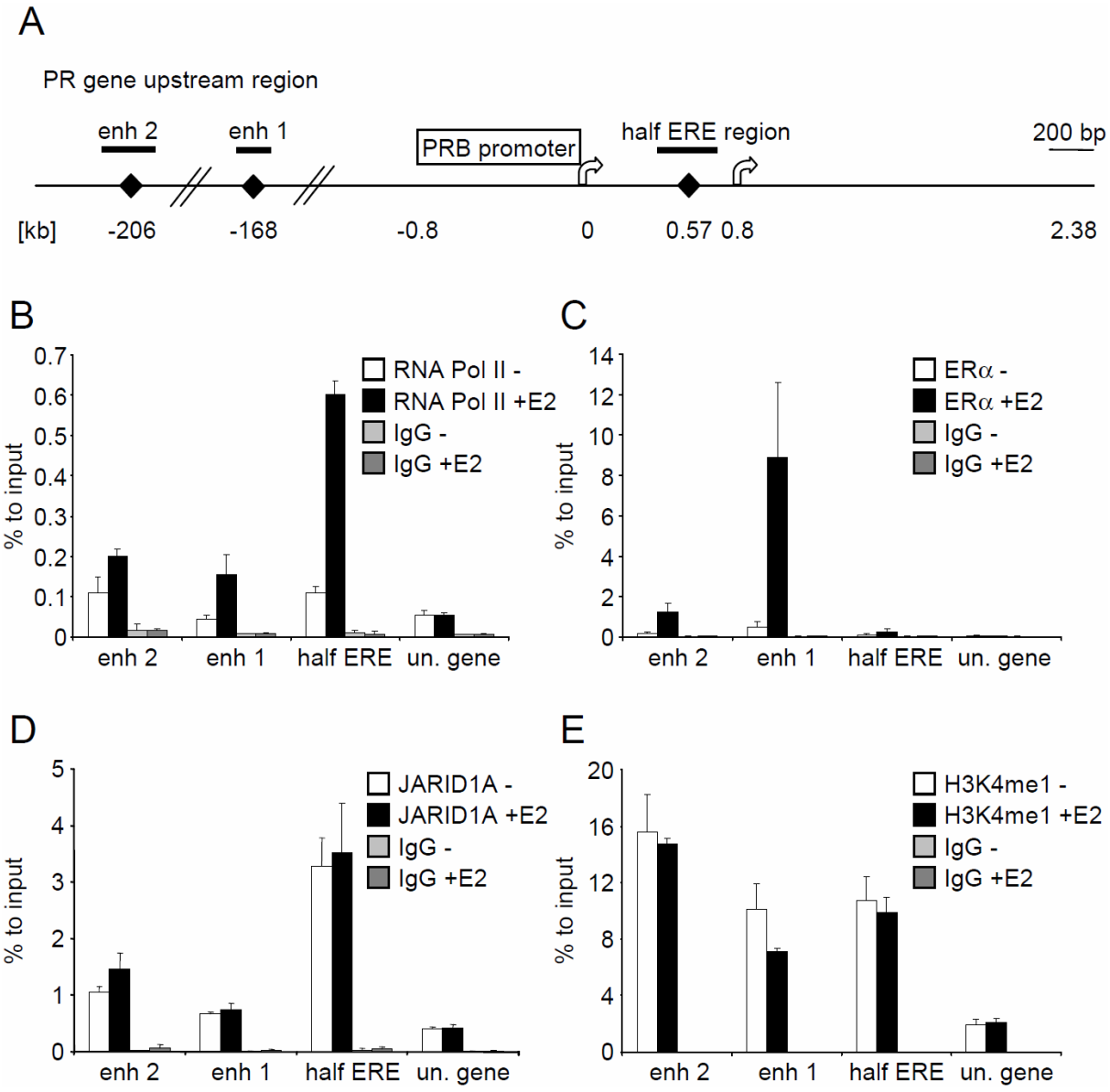
Figure 18

Immunoprecipitation of ERα with JARID1A. Total protein extracts were prepared from MCF-7 cells treated with 1 nM 17β-estradiol and immunoprecipitated with an ERα- or JARID1A-specific antibody. Western blot (WB) analysis of the eluate and supernatant (SN) was performed with the indicated antibodies.

3.3.4 DNA binding of JARID1A and H3K4 methylation to PR regulatory regions

Genome-wide surveys indicate that ER-binding sites are often located far away from the transcriptional start site (tsp) of estrogen-regulated genes. The precise regions involved in the estrogen control of the PR gene have not all been characterised and multiple EREs exist, as shown in several studies. A half-palindromic ERE located 571 bp downstream of the PRB tsp and adjacent to two Sp1-binding sites has been identified (Fig. 19A). Both this ERE half-site and the Sp1 elements are implicated in the estrogen responsiveness of this regulatory region. In addition, two ER-binding sites located 168 kb and 206 kb upstream of the PRB tsp were identified by whole-genome cartography. In order to find out whether JARID1A and the H3K4 methylation status of these different regulatory sequences played a role in estrogen response, ChIP was performed using primers specific for each region. Primers directed against a sequence of an unrelated gene (F-box and leucine-rich repeat protein 11) were used as control. In order to exclude that changes in the H3K4 methylation status were due to different levels of total H3, an antibody directed against H3 was first used for the ChIP experiments. Comparable signals were obtained at all analysed regions. Then, in order to demonstrate that transcription took place, RNA polymerase II (Pol II) occupancy was determined. It was not observed in absence of hormone but increased significantly at the ERE half-site after estrogen treatment (Fig. 19B). Only weak recruitment of Pol II was detected at the enhancer regions after hormone stimulation. Loading of ERα was not observed in the absence of estrogen. Conversely, ERα could be detected at enhancer 1 after hormone treatment (Fig. 19C). Based on the previous results, it could be assumed that JARID1A was located in the PR regulatory regions. Indeed, high JARID1A levels were detected at the ERE half-site region (Fig. 19D). This occupancy of JARID1A was found to be

independent of hormone treatment. Concerning the enhancer regions, JARID1A binding was near control levels in the absence and presence of estrogen at enhancer region 1 and slightly more elevated at enhancer region 2. To analyse the H3K4 methylation status at the PR regulatory regions, antibodies specific for each methylation pattern were used. Mono- and dimethylation were visible at all locations, with no effect of the estrogen treatment (Fig. 19E and F). Interestingly, monomethylation was highest at the enhancer 2 region and dimethylation at the half-ERE region. Trimethylation was limited to the half-ERE region and, in line with its activating function, was significantly increased by estrogen treatment (Fig. 19G).



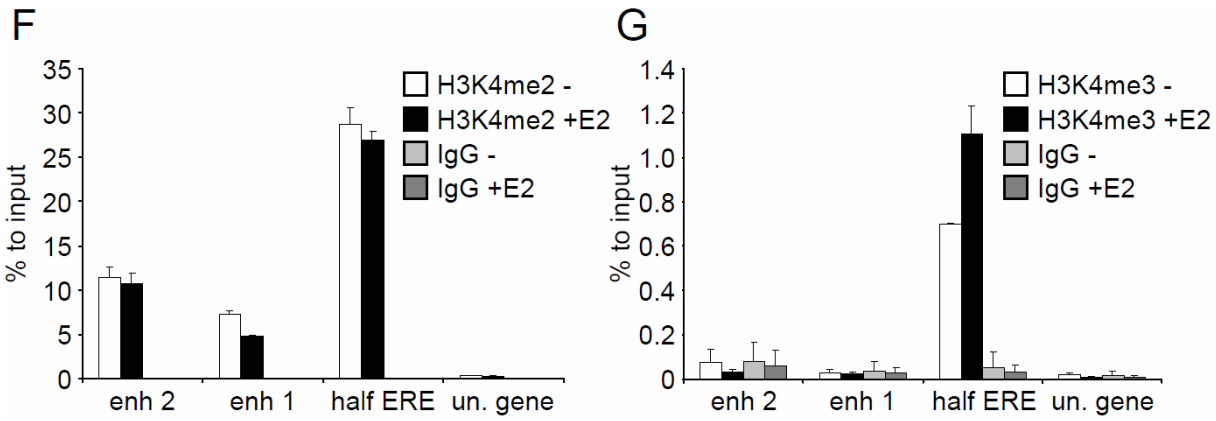
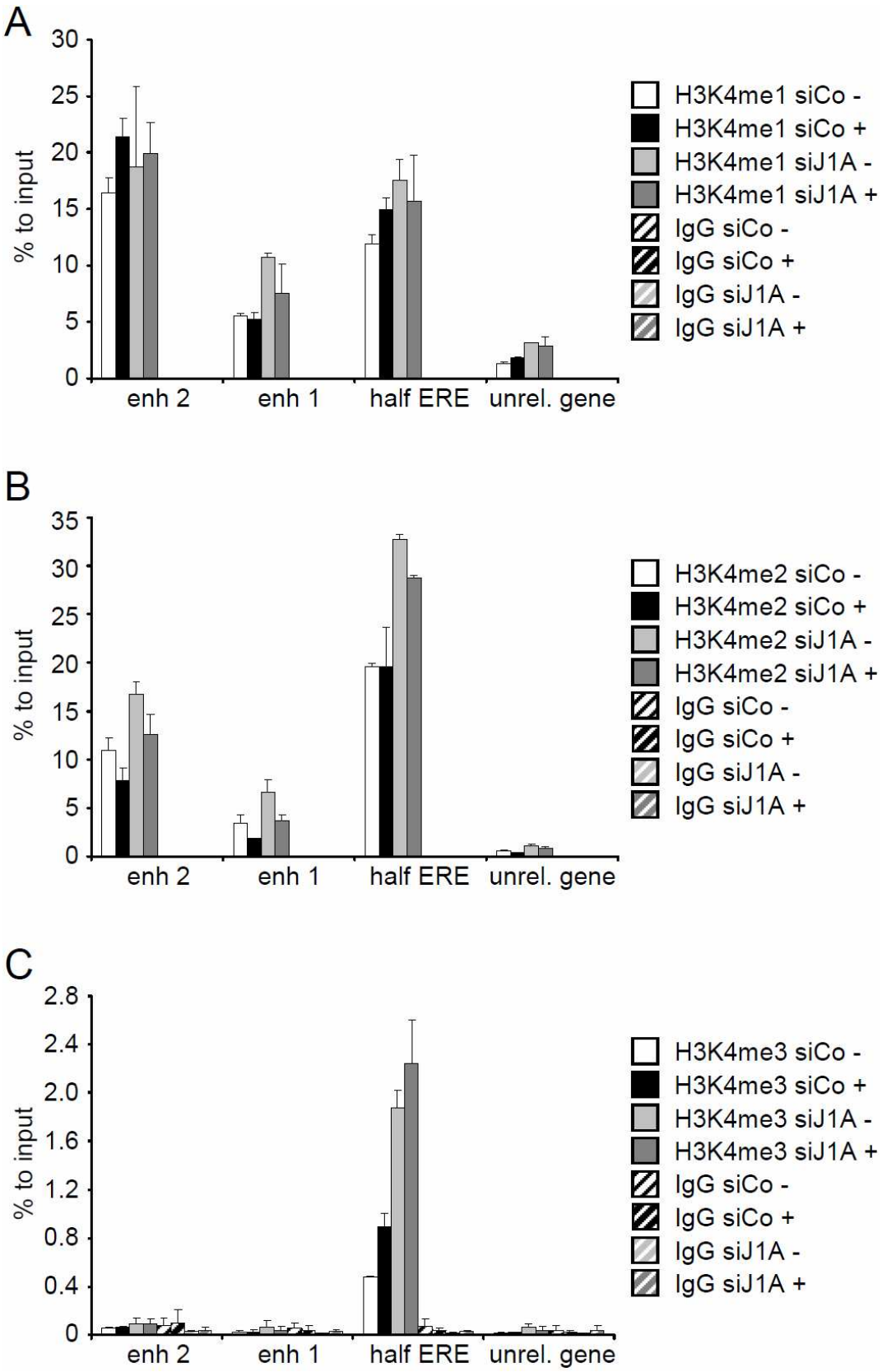


Figure 19

ChIP analysis of PR regulatory regions in MCF-7 cells. **A.** Schematic representation of the PR gene upstream region. The tsp for the PRB and PRA transcripts are indicated with arrows. Distal ER-binding sites and a proximal half-ERE located 571 bp downstream of the PRB tsp are highlighted with black diamonds. The respective DNA regions amplified with the primer pairs used in ChIP are shown above the sites with black bars. The white box indicates the PRB promoter region that was subcloned upstream of the Luc reporter construct. **B.** ChIP assay of Pol II binding. **C.** ChIP assay of ERα binding. **D.** ChIP assay of JARID1A binding. **E.** ChIP assay of H3K4 monomethylation. **F.** ChIP assay of H3K4 dimethylation. **G.** ChIP assay of H3K4 trimethylation. Chromatin isolated from MCF-7 cells treated for 1 h with 1 nM 17β-estradiol (black bars) or untreated (white bars) was immunoprecipitated with the appropriate antibodies and the bound DNA analysed by quantitative PCR amplification. An IgG-specific antibody was used in control experiments (light and dark grey bars).

3.3.5 Accumulation of H3K4 methylation upon JARID1A depletion

The ChIP results showed an enrichment of JARID1A and H3K4 di- and trimethylation at the half-ERE. However, as the modification in H3K4 trimethylation did not parallel a change in JARID1A binding, it was analysed whether a reduction in the cellular JARID1A levels affected H3K4 methylation at the PR gene upstream region. MCF-7 cells were transfected with JARID1A-specific siRNAs and the PR regulatory regions analysed by ChIP, as before. No effect on H3K4 monomethylation was observed (Fig. 20A). Concerning H3K4 dimethylation, a general increase was seen at all analysed regions (Fig. 20B). The most dramatic changes were observed for H3K4 trimethylation at the ERE half-site region. Here, a strong increase was detected following JARID1A knock-down both in the non- and hormone-stimulated cells (Fig. 20C). This finding reflects the colocalisation seen for JARID1A and di- and trimethylation at the ERE half-site, and leads to the conclusion that JARID1A modulates PR expression by keeping di- and especially trimethylation at a minimal level.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15
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enh 1
half ERE
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H3K4me1 siCo +
H3K4me1 siJ1A -
H3K4me1 siJ1A +
IgG siCo -
IgG siCo +
IgG siJ1A -
IgG siJ1A +
B
% to input
35
30
25
20
15
10
5
0
enh 2
enh 1
half ERE
unrel. gene
H3K4me2 siCo -
H3K4me2 siCo +
H3K4me2 siJ1A -
H3K4me2 siJ1A +
IgG siCo -
IgG siCo +
IgG siJ1A -
IgG siJ1A +
C
% to input
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2.4
2.0
1.6
1.2
0.8
0.4
0
enh 2
enh 1
half ERE
unrel. gene
H3K4me3 siCo -
H3K4me3 siCo +
H3K4me3 siJ1A -
H3K4me3 siJ1A +
IgG siCo -
IgG siCo +
IgG siJ1A -
IgG siJ1A +

Figure 20

Influence of JARID1A levels on H3K4 methylation of the PR regulatory regions in MCF-7 cells. JARID1A-specific siRNAs (mixed pair #1 and #2) were used for expression knock-down (light and dark grey bars). An unrelated siRNA pair was used in the control experiments (white and black bars). Chromatin isolated from MCF-7 cells treated with 1 nM 17β-estradiol (black and dark grey bars) or untreated (white and light grey bars) was immunoprecipitated with antibodies specific for H3K4 methylation and the bound DNA analysed by quantitative PCR amplification. An IgG-specific antibody was used as negative control (striped bars). **A.** ChIP assay of H3K4 monomethylation. **B.** ChIP assay of H3K4 dimethylation. **C.** ChIP assay of H3K4 trimethylation.

3.3.6 H3K4 methylation of PR regulatory regions in MDA-MB-231 cells

To further characterise the importance of H3K4 methylation in the control of PR expression and the role of JARID1A in this process, the PR regulatory regions in the ERα-negative cell line MDA-MB-231 were examined. This cell line represents a suitable system for the approach, since in addition to the ERα gene, the PR gene is silenced (Ferguson et al. 1998). First, JARID1A protein levels in MDA-MB-231 and MCF-7 cells were determined by Western blot analyses, and comparable signals were found (Fig. 21A). Next, it was intended to find out whether the local H3K4 methylation patterns in the PR gene regulatory region differed between these two cell lines. ChIP analysis indicated H3 levels to be comparable at all sites examined. No significant Pol II occupancy was detected at any analysed site, in line with the absence of transcriptional activity (Fig. 21B). H3K4 mono- and dimethylation were seen at the half-ERE region (Fig. 21C and D) but no trimethylation was detected at any region (Fig. 21E). In order to find out whether JARID1A was responsible for the lack of histone trimethylation in the PR regulatory regions and therefore possibly involved in the silencing of the PR gene in MDA-MB-231 cells, occupancy of JARID1A was determined. ChIP results showed no occupancy of JARID1A at the PR regulatory regions in MDA-MB-231 cells (Fig. 21F). These data further underline the importance of H3K4 methylation in controlling PR gene expression and strongly suggest that the presence of ERα is mandatory for JARID1A to control PR gene expression.

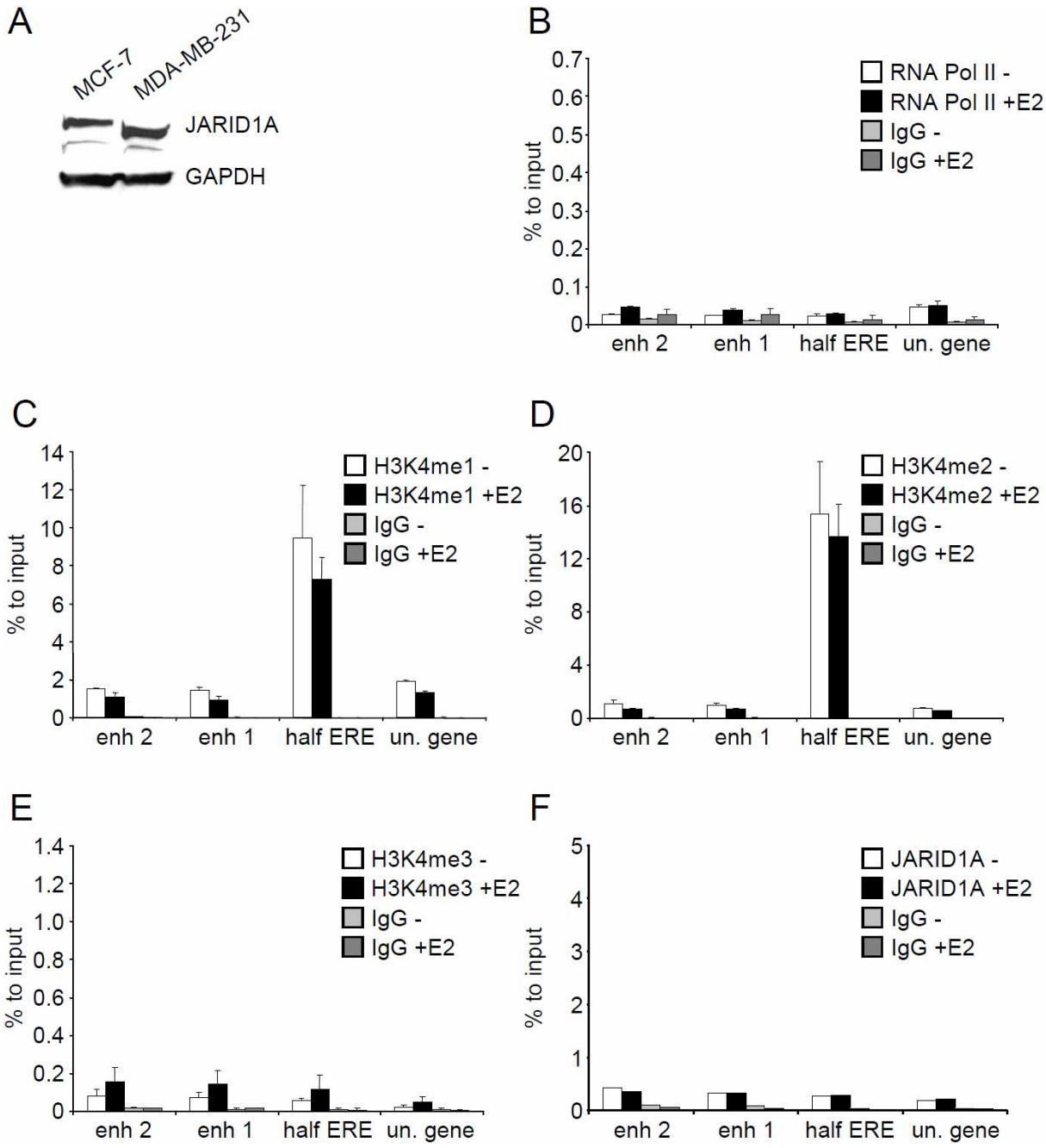


Figure 21

ChIP analysis of PR regulatory regions in MDA-MB-231 cells. **A.** JARID1A protein levels were determined by Western blot analysis in MDA-MB-231 cells, in comparison to MCF-7 cells. Protein extracts were prepared from cells grown in presence of 17β-estradiol and separated by gel electrophoresis. GAPDH protein levels were measured as loading control. **B.** ChIP assay of Pol II binding. **C.** ChIP assay of H3K4 monomethylation. **D.** ChIP assay of H3K4 dimethylation. **E.** ChIP assay of H3K4 trimethylation. **F.** ChIP assay of JARID1A. Chromatin isolated from MDA-MB-231 cells treated with 1 nM 17β-estradiol (black bars) or untreated (white bars) was immunoprecipitated with the appropriate antibodies and the bound DNA analysed by quantitative PCR amplification. An IgG-specific antibody was used in control experiments (light and dark grey bars).

3.3.7 Effect of SMYD3 overexpression on PR gene expression

SMYD3 is one of several H3K4 methylases that may counteract the effects of JARID1 demethylases. Interestingly, it is upregulated in breast cancer and involved in the proliferation of breast cancer cell lines (Hamamoto et al. 2004; Hamamoto et al. 2006). In order to find out whether the overexpression of this enzyme had effects comparable to the knock-down of JARID1A, three expression vectors coding for SMYD3, either the wild-type or two HMTase-inactive mutants (SMYD3-ΔEEL and SMYD3-ΔNHSC), were generated. SMYD3 contains a SET domain which mediates lysine-directed histone methylation. The mutants generated contained a deletion of two different conserved motifs of the SET domain known to be necessary for HMTase activity (Hamamoto et al. 2004). MCF-7 cells were transfected with these plasmids and treated or not with estrogen. Total RNA and protein were extracted two days later.

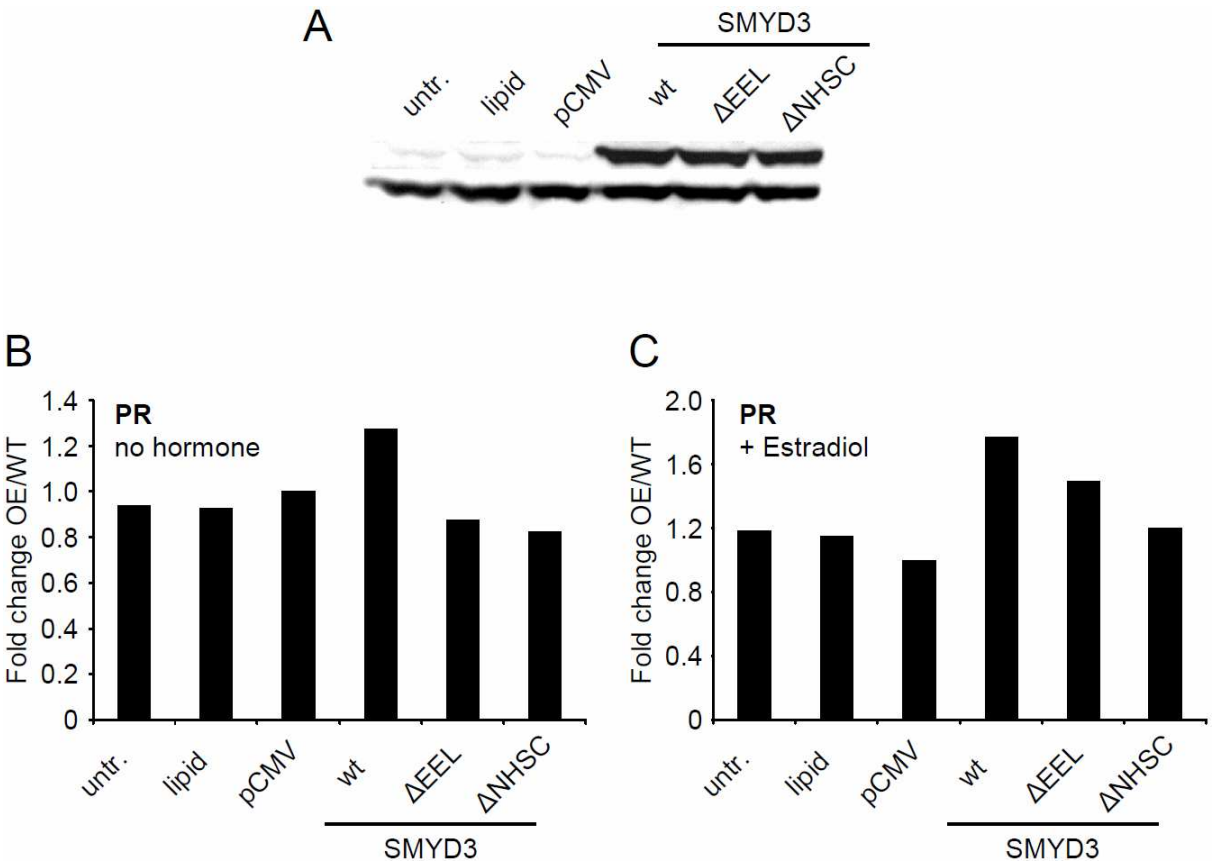


Figure 22

Effects of SMYD3 overexpression on PR expression. **A.** SMYD3 wild-type (wt) or one of the two mutants ΔEEL and ΔNHSC were overexpressed in MCF-7 cells. The extent of overexpression (OE) at day two post-transfection was measured by Western blot analysis. **B./C.** The mRNA level of PR was determined by quantitative PCR at day 2 after plasmid transfection in the absence (B) or presence (C) of estradiol. In the controls, cells were transfected with lipid only, empty vector (pCMV) or left untreated (untr.). The fold inductions compared to cells treated with empty vector are given.

Western blot analysis showed similar overexpression of wild-type and mutants SMYD3 in tranfected cells, when compared to controls treated with lipid only or transfected with empty plasmid (Fig. 22A). Quantitative PCR measurement showed that transfection of SMYD3 wild-type slightly stimulated (1.4-1.8 fold) PR expression, when compared to cells treated with mutant- or empty plasmids (Fig. 22B-C). This was seen both in absence and in presence of estradiol, and is in line with a role of H3K4 methylation in controlling PR expression.

3.4 Role of JARID1 family in AR expression and function

3.4.1 Effect of JARID1 knock-down on AR transcriptional activity

In this part of the work it was examined whether JARID1 family members played a role in regulating AR function. In order to test this, the effects of JARID1 knock-down on the expression of four androgen-regulated genes were examined in the prostate cancer cell line LNCaP. First, the androgen induction of these target genes was measured at five different time-points using quantitative PCR measurement in order to find the optimal time of R1881 treatment (Fig. 23).

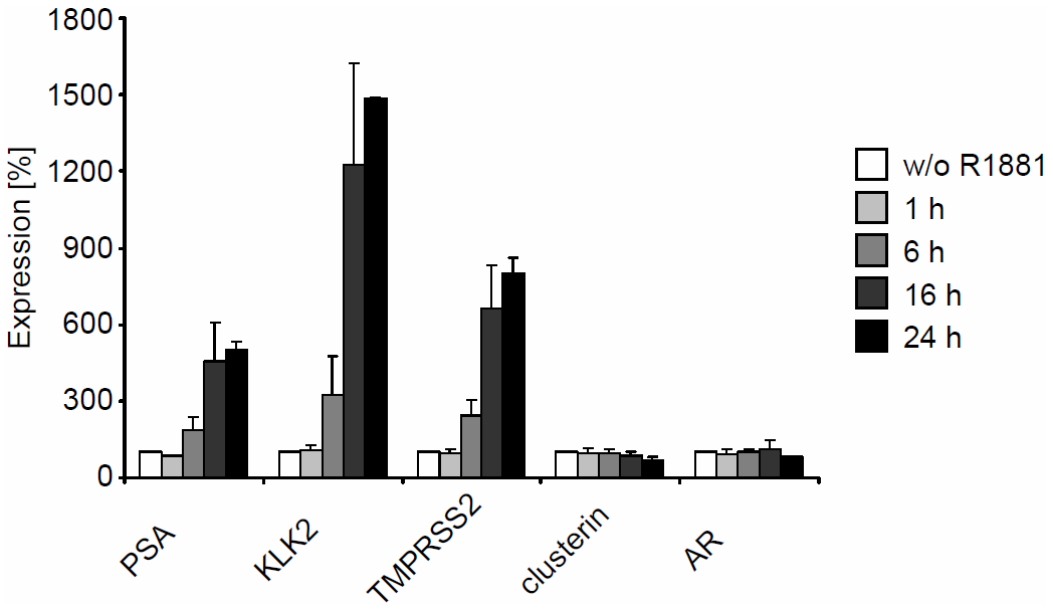
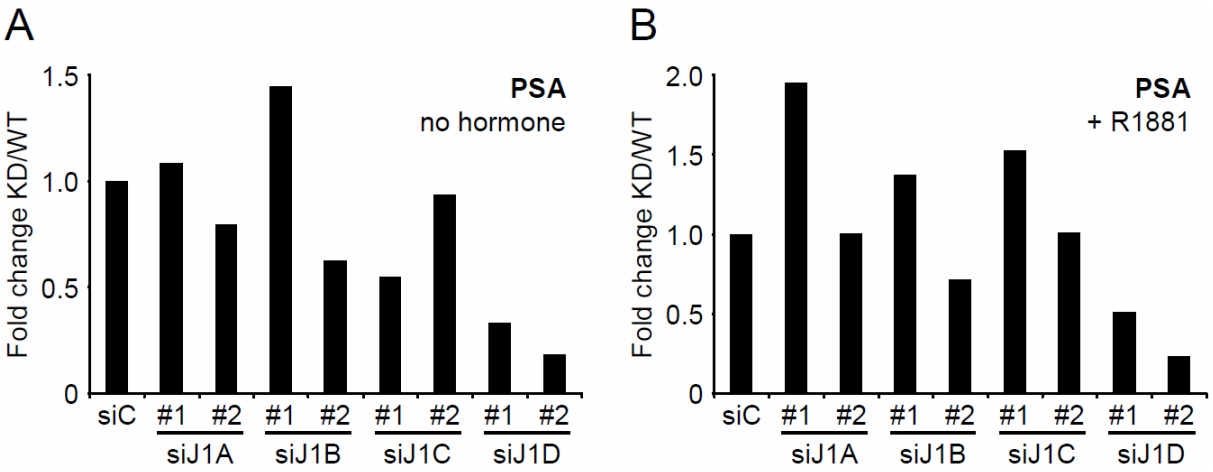


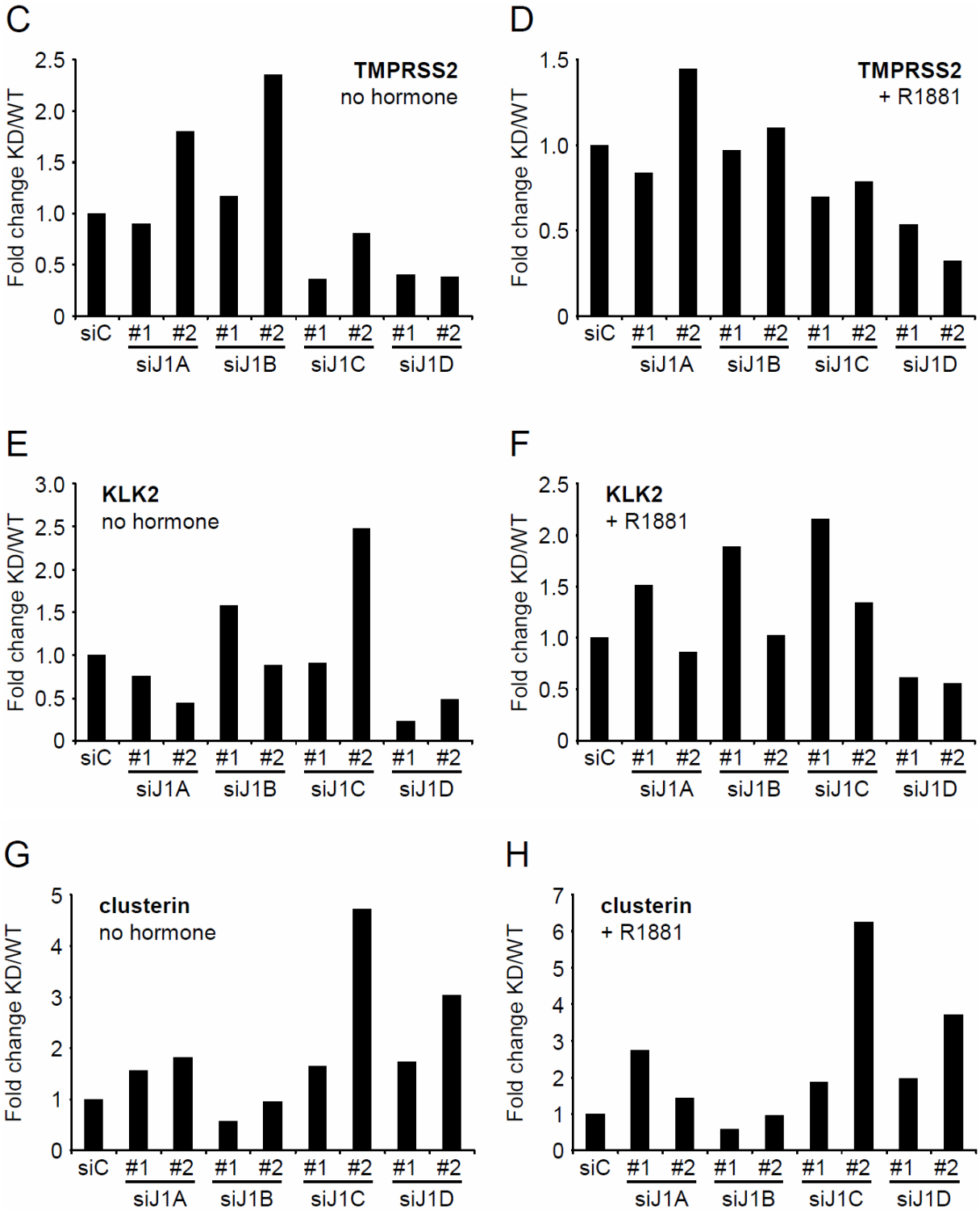
Figure 23

Regulation of androgen target genes by R1881 in LNCaP cells. LNCaP cells were treated with 1 nM R1881 for 0, 1, 6, 16 and 24 hours, total RNA was prepared and the PSA, KLK2, TMPRSS2, clusterin and AR transcript levels were determined by quantitative PCR.

A high increase of KLK2 expression with a 15-fold-maximum after 24 hours of R1881 stimulation was observed. PSA and TMPRSS2 expression also showed a significant increase peaking at 5-fold and 8-fold at the 24 h time-point. Conversely, expression of the androgen-repressed gene clusterin was reduced to about 40 % after 24 hours of androgen treatment. No significant regulation by androgen was observed for AR expression. The optimal androgen treatment time was thereby defined as being 24 h.

In order to find out whether androgen-dependent gene regulation was influenced by any of the JARID1 family members, LNCaP cells were transfected with the previously identified siRNAs directed against each JARID1 form (Fig. 13). The cells were treated or not with R1881 and total RNA was purified five days later. Quantitative PCR showed the most interesting effects for JARID1D. Knocking-down JARID1D led to a strong decrease of all analysed androgen-stimulated genes PSA (~ 70 %), TMPRSS2 (~ 60 %) and KLK2 (~ 50 %) (Fig. 24A-F). These effects were seen in the absence and in the presence of R1881. Conversely, clusterin showed a 2 to 4-fold increased mRNA level after JARID1D knock-down (Fig. 24G and H). In contrast, the reduction of JARID1A, 1B and 1C did not give clear-cut results for PSA, KLK2, TMPRSS2 or clusterin expression. This might be due to the difficulties to transfect LNCaP cells or to off-target side-effects (Scacheri et al. 2004). In order to further substantiate the specificity of the effects observed after JARID1D knock-down, the expression levels of the p21 gene which is not regulated by androgen was measured. JARID1D knock-down had no effect on p21 expression (Fig. 24I and J).




C
TMPRSS2
no hormone
Fold change KD/WT
2.5
2.0
1.5
1.0
0.5
0
siC
#1 #2 #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C siJ1D
D
TMPRSS2
+ R1881
Fold change KD/WT
1.5
1.0
0.5
0
siC
#1 #2 #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C siJ1D
E
KLK2
no hormone
Fold change KD/WT
3.0
2.5
2.0
1.5
1.0
0.5
0
siC
#1 #2 #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C siJ1D
F
KLK2
+ R1881
Fold change KD/WT
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0.5
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siC
#1 #2 #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C siJ1D
G
clusterin
no hormone
Fold change KD/WT
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siC
#1 #2 #1 #2 #1 #2 #1 #2
siJ1A siJ1B siJ1C siJ1D
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clusterin
+ R1881
Fold change KD/WT
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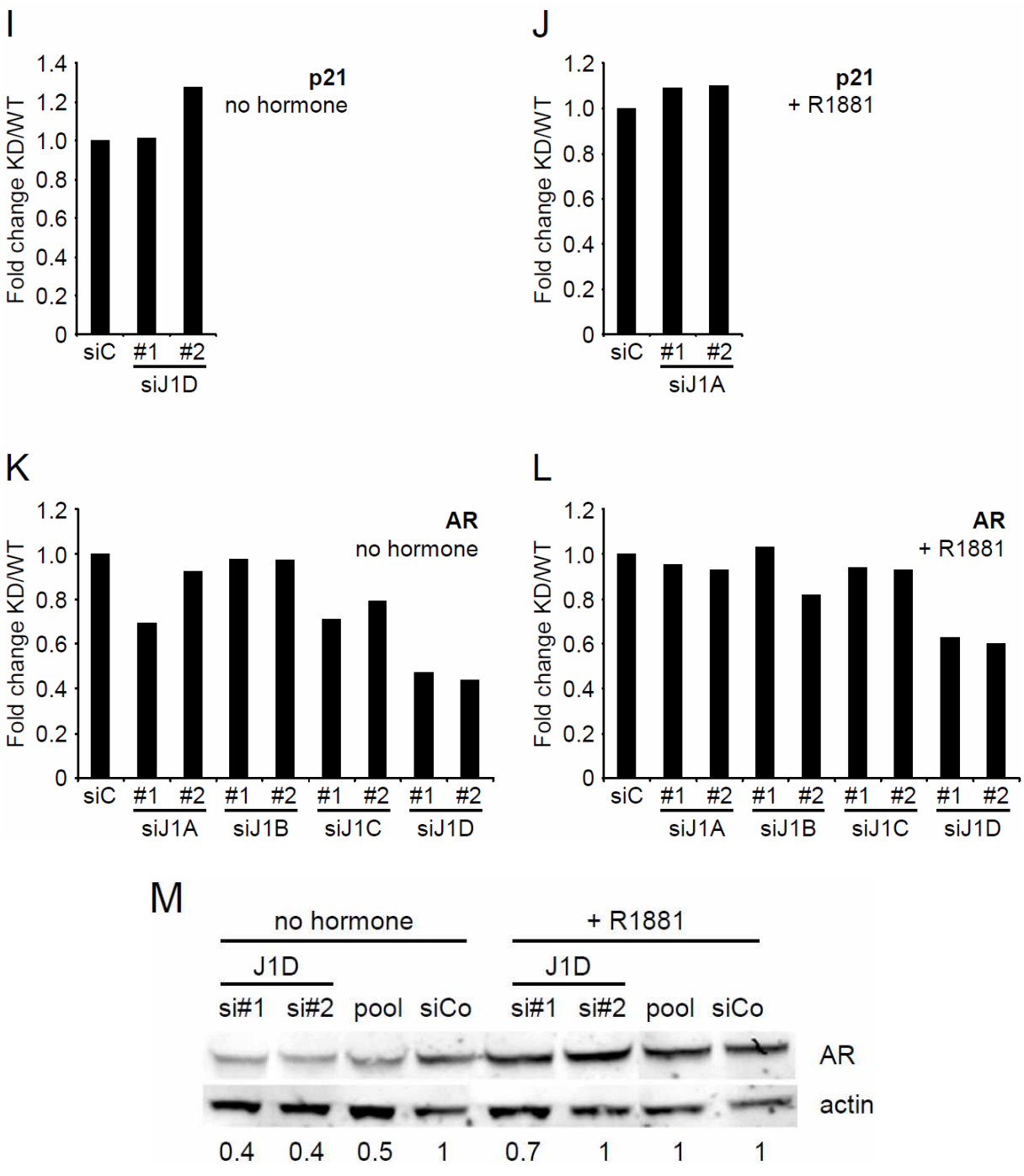


Figure 24

Effects of JARID1 knock-down on androgen-regulated gene expression. LNCaP cells were transfected with siRNAs specific for each JARID1 form (siJ1A: JARID1A; siJ1B: JARID1B; siJ1C: JARID1C; siJ1D: JARID1D). The control cells were transfected with unrelated siRNAs (siC). PSA, TMPRSS2, KLK2, clusterin, AR and p21 transcript levels were determined by quantitative PCR. In the case of AR, the protein levels were determined as well. LNCaP cells were treated with vehicle or with 1nM R1881 respectively. The fold induction compared to cells treated with unreleated siRNA are given. **A.** PSA expression in the presence of vehicle. **B.** PSA expression after 1 nM R1881 treatment. **C.** TMPRSS2 expression in the presence of vehicle. **D.** TMPRSS2 expression after 1 nM R1881 treatment. **E.** KLK2 expression in the presence of vehicle. **F.** KLK2 expression after 1 nM R1881 treatment. **G.** clusterin expression in the presence of vehicle. **H.** clusterin expression after 1 nM R1881 treatment. **I.** p21 expression in the presence of vehicle. **J.** p21 expression after 1 nM R1881 treatment. **K.** AR expression in the presence of vehicle. **L.** AR expression after 1 nM R1881 treatment. **M.** AR protein level in the presence of vehicle and after 1 nM R1881 treatment. The numbers underneath each bar indicate the fold change in intensity in comparison to control.

Interestingly, JARID1D depletion also led to a decrease of AR expression itself (Fig. 24K and L). A slightly stronger suppression was seen in absence than in presence of androgen. Conversely, reducing the expression of JARID1A, B or C had nearly no effects on basal AR levels and no effect on androgen-stimulated AR levels. This observation was confirmed at the protein level (Fig. 24M). A 50 % reduction of AR protein was seen five days post-transfection using siRNA pairs #1, #2 or both together in the absence of hormone, when compared to cells treated with unrelated siRNAs. No change in the actin level was observed. No such effect was seen in presence of R1881, probably due to the stabilising effect of this ligand.

Taken together these results show that JARID1D modulates the expression of several androgen-regulated genes as well as the basal expression of AR.

3.4.2 Interaction of JARID1D and AR

The effects of JARID1D knock-down on transcription of AR target genes suggest that JARID1D may directly cooperate with AR to control the expression levels of androgen target genes. To determine whether an interaction between these two proteins took place protein extracts prepared from LNCaP cells were immunoprecipitated with an AR antibody, or conversely with a JARID1D antibody. The eluates were analysed by Western blot using either antibody. The results showed that a complex formed of JARID1D and AR was precipitated when using an AR antibody (Fig. 25). However, no interaction could be demonstrated in the reverse experiment using a JARID1D antibody. One possible explanation is that the JARID1D antibody used interfered with the JARID1D/AR interaction.

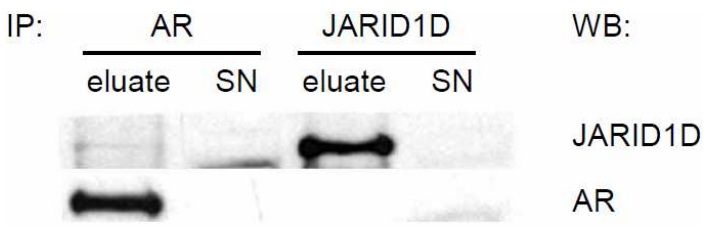
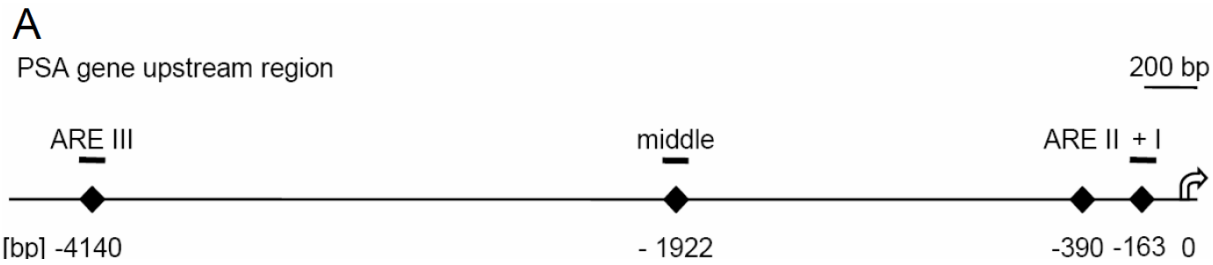


Figure 25

Immunoprecipitation of AR with JARID1D. Total protein extracts were prepared from LNCaP cells treated with R1881 and immunoprecipitated with an AR- or JARID1D-specific antibody. Western blot (WB) analysis of the eluate and supernatant (SN) was performed with the indicated antibodies.

3.4.3 DNA binding of JARID1D and H3K4 trimethylation status at the PSA regulatory regions

The PSA gene is one of the best-characterised androgen-responsive genes in the prostate gland. Three AREs have been identified: ARE I and ARE II are located in the promoter region, whereas ARE III is in the enhancer region located ~ 4 kb upstream of the PSA transcriptional start site (Cleutjens et al. 1997) (Fig. 26A). Little is however known about the role of histone modifications in the control of this gene. In order to find out whether JARID1D binding took place at these elements and if this correlated with H3K4 methylation levels and AR binding, ChIP assays were performed using primers specific for the ARE I promoter and the ARE III enhancer region. Control primers directed against a non-ARE-region located between these two elements and called PSA middle were additionally used (Fig. 26A). First, the occupancy of AR after treatment with R1881 was determined. A specific antibody against AR was used to immunoprecipitate AR-bound genomic DNA fragments. AR binding was detected at the enhancer region already in the absence of hormone (Fig. 26B) and much increased in the presence of hormone. Much weaker but still significant AR binding was also detected at the ARE I in absence and more so in presence of R1881. No signal was obtained when using primers directed against the PSA middle region. Then the binding of JARID1D was examined. The highest enrichment was seen in the ARE III enhancer region at levels that were comparable in the absence and the presence of R1881. JARID1D binding was also detectable at the promoter, regardless of the androgen status (Fig. 26C). Again, no signal was detected in the PSA middle region. Finally the H3K4me3 levels were determined. They were find enriched mostly at the ARE I region whereas at the enhancer they were much lower, comparable to the control middle region (Fig. 26D). These results show that JARID1D is located in the regulatory region of the PSA gene and suggest a direct influence of JARID1D in the repression of androgen-regulated gene expression.



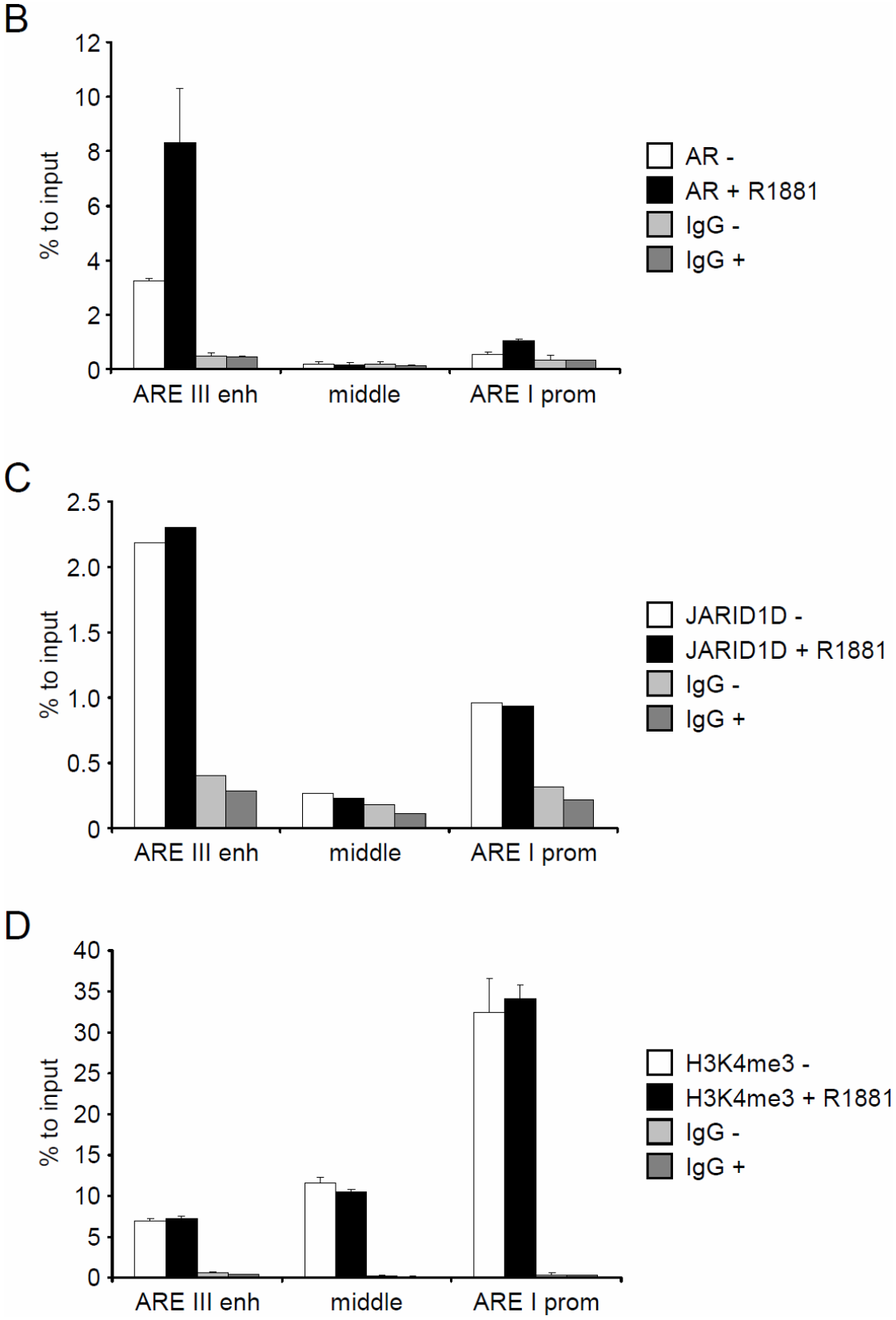

B
% to input
12
10
8
6
4
2
0
ARE III enh
middle
ARE I prom
AR -
AR + R1881
IgG -
IgG +
C
% to input
2.5
2.0
1.5
1.0
0.5
0
ARE III enh
middle
ARE I prom
JARID1D -
JARID1D + R1881
IgG -
IgG +
D
% to input
40
35
30
25
20
15
10
5
0
ARE III enh
middle
ARE I prom
H3K4me3 -
H3K4me3 + R1881
IgG -
IgG +

Figure 26

ChIP analysis of PSA regulatory regions in LNCaP cells. **A.** Schematic representation of the PSA gene upstream region. The tsp for the PSA transcript is indicated with an arrow. The distal enhancer (ARE III), the control PSA middle fragment (middle) and the proximal promoter AREs (ARE II + I) are highlighted with black diamonds. The respective DNA regions amplified with the primer pairs used in ChIP are shown above the sites with black bars. **B.** ChIP assay of AR binding. **C.** ChIP assay of JARID1D binding. **D.** ChIP assay of H3K4 trimethylation. Chromatin isolated from LNCaP cells treated for 1 h with 1 nM R1881 (black bars) or untreated (white bars) was immunoprecipitated with the appropriate antibodies and the bound DNA analysed by quantitative PCR amplification. An IgG-specific antibody was used in control experiments (light and dark grey bars).

4 DISCUSSION

4.1 Role of JARID1A in ERα function

4.1.1 JARID1A is an ERα cofactor regulating PR expression

Little is known about posttranslational modifications associated with ERα-mediated transcription. In this work the role of the H3K4 demethylating enzymes of the JARID1 family in modulating ERα function was studied. Using the breast cancer cell line MCF-7 as model, it was found that JARID1A regulated expression of the PR gene and fine-tuned its control by ERα. Reducing JARID1A expression by specific siRNAs dramatically enhanced PR expression at the basal and less so at the estrogen-stimulated levels. Conversely, overexpressing the JARID1A wild-type enzyme, but not its inactive form, suppressed the activity of the PRB promoter in transient transfection experiments. This and the interaction of JARID1A with ERα prompted an analysis of the potential binding of JARID1A to the PR regulatory regions and of the role of H3K4 methylation in regulating PR expression. Using chromatin immunoprecipitation, binding of JARID1A close to the transcriptional start point of the PRB promoter was evidenced. In addition, an increase in H3K4 trimethylation was observed in the same region, following hormone stimulation. Despite the fact that JARID1A levels were not affected by estrogen treatment, this demethylase is likely to be involved in controlling the H3K4 methylation status of the PR gene, as shown by knocking-down JARID1A and performing ChIP experiments in MCF-7 cells, which led to an increase of di- and especially of trimethylation.

Among estrogen-regulated genes some such as *PGR* and *pS2/TFF1* are linked to cellular differentiation whereas others such as *c-myc*, *cathepsin D (CTSD)* and *cyclin D1 (CCND1)* are implicated in cellular proliferation (Dalvai and Bystricky). Since JARID1A is important for cellular differentiation, its role in ERα-mediated gene control may be restricted to genes involved in this process. Results obtained in this study showed an important role of JARID1A in mediating ERα transcriptional activity for the regulation of PR gene expression. Additional, preliminary knock-down experiments showed an upregulation of pS2 after JARID1A knock-down, indicating a possible involvement of JARID1A also in the transcriptional control of this gene. These results need however to be further substantiated.

JARID1B is overexpressed in all kinds of breast cancers, primary and metastatic, and also in derived cell lines (Lu et al. 1999; Barrett et al. 2002). No direct link between JARID1B levels and ERα-mediated transcription was however observed in the present

work. Yamane and colleagues showed that JARID1B promotes tumour progression by repressing tumour suppressor genes such as BRCA1 (Yamane et al. 2007), which is not regulated by estrogen (present study). Taken together these observations imply that the involvement of JARID1B in breast cancer does not directly result from a regulation of the expression of estrogen target genes.

4.1.2 H3K4 methylation modulates PR expression

The role of the H3K4 methylation status in controlling gene transcription is documented by several studies (Santos-Rosa et al. 2002; Bernstein et al. 2005; Heintzman et al. 2009), but how far nuclear receptors use this histone mark for the regulation of downstream target genes has not been extensively analysed. By ChIP experiments, the H3K4 methylation pattern of three PR regulatory regions, two distal enhancers and one in the promoter region, was analysed.

Recent genome-wide studies reported the occurrence of H3K4 monomethylation at enhancers, of dimethylation preferentially at promoters and of trimethylation exclusively at promoters of active genes (Heintzman et al. 2009; Hublitz et al. 2009). Unlike at promoters, histone modifications at enhancers are highly cell-type specific (Heintzman et al. 2009). The mechanisms responsible for the establishment of the differential histone marks are unknown. Recently, it was shown that H3K4 methylation at enhancers is recognised by the Forkhead factor (FoxA1) via its winged-helix DNA binding domains that mimic histone linker proteins, thus facilitating the recruitment of the steroid receptors ER and AR (Carroll et al. 2005; Wang et al. 2009). FoxA1 binding is essential for ER-chromatin interactions and subsequent stimulation of estrogen target genes. H3K4 dimethylation is found only in euchromatic regions but at both inactive and active euchromatic genes, whereas H3K4 trimethylation is present solely at active genes (Santos-Rosa et al. 2002). The data obtained here showed an overall H3K4 methylation pattern at the PR gene that fits well with these studies: monomethylation accumulated at the enhancers, dimethylation was mainly seen at the promoter and less so at the enhancers, and trimethylation exclusively at the promoter. In addition, an increase in H3K4 trimethylation at the proximal region was associated with hormone stimulation. This change in H3K4 trimethylation corroborates previous findings showing the presence of this mark in the 5’ region of genes to be essential for gene activity (Sims et al. 2007; Heintzman et al. 2009).

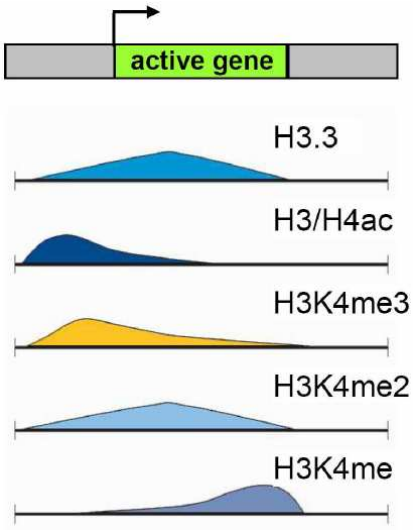


Figure 27

H3K4 methylation and acetylation signature at gene structures (modified from abcam).

The role of H3K4 methylation in regulating PR gene expression was further characterised by ChIP experiments addressing the PR regulatory region in MDA-MB-231 cells, which do not express PR or ERα. Interestingly, the group of estrogen-regulated genes involved in differentiation (*PGR* and *pS2*) are silenced in MDA-MB-231 cells, whereas the group of genes implicated in cellular proliferation (*c-myc*, *CTSD, CCND1*) are constitutively active (Dalvai and Bystricky). Re-expression of *ESR1* (Estrogen receptor α gene) is not sufficient to restore hormonal control (Giamarchi et al. 1999; Fleury et al. 2008), indicating the involvement of epigenetic mechanisms in controlling PR expression. Indeed, previous data generated in MDA-MB-231 cells showed that DNA methylation of the PR promoter at a specific CpG island led to repression and that a reactivation of PR and ER can be achieved by the DNMT inhibitor 5-aza-2'-deoxycytidine (Ferguson et al. 1998). However, when blocking the reactivated ER using a combination of 5-aza-2'-deoxycytidine and antiestrogen, the unmethylated PR gene remains in the repressed state, showing that the removal of DNA methylation alone is not sufficient for PR gene expression in MDA-MB-231 cells. It was further shown that the interaction of the coactivator SRC-1A which contains a histone acetyltransferase activity and the ER is required for PR reactivation (Ferguson et al. 1998). These findings underline the importance of epigenetic mechanisms in controlling PR gene expression.

The results obtained here revealed that Pol II did not bind to the regulatory regions examined, in line with the fact that PR was not expressed in these cells. No H3K4 trimethylation was detected and di- and monomethylation marks were limited to the

proximal region. These data strongly suggest that absence of H3K4 trimethylation is an important factor preventing PR expression in MDA-MB-231 cells and indicate a possible role of JARID1A in the silencing process in this cell line. Since no occupancy of JARID1A in the PR regulatory region could be detected, the role of JARID1A may be that of an early regulator responsible for the active establishment of the silenced status. For the later maintenance of this status, DNA methylation and the absence of ER might be sufficient. Future experiments will show whether for the regulation of PR expression a cross-talk exists between H3K4 methylation and DNA methylation or histone acetylation, as recently reported for other genes (Okitsu and Hsieh 2007; Hung et al. 2009; Otani et al. 2009).

4.1.3 Distal binding sites are involved in the transcriptional control of PR gene expression by ERα and JARID1A

During the last years, ChIP-chip analyses performed to identify the binding sites for AR (Bolton et al. 2007; Massie et al. 2007; Wang et al. 2007), ER (Carroll et al. 2005; Carroll et al. 2006; Kininis et al. 2007) and GR (So et al. 2007) have revealed three main aspects: (1) the presence of binding sites far away from proximal upstream regulatory regions, (2) the significant divergence of receptor-binding sites from the canonical HRE sequence and (3) the presence of composite receptor-binding sites for other transcription factors, including Sp1, AP-1, ETS proteins, Foxo1 (forkhead), GATA-2 and HNF-4. The results achieved in this work confirm the existence of ERα binding sites containing no canonical ERE and located far away from the transcriptional start point. Three PR regulatory regions possibly involved in the control by estrogen, namely enhancer 1 (168 kb), enhancer 2 (206 kb) and the half-ERE region located 571 bp downstream of the PRB tsp were analysed by ChIP experiments. The changes observed in the PR locus upon estrogen stimulation were an increase in Pol II binding to the half-ERE region and of ERα binding to enhancer 1. Interestingly, the enhancer 1 region amplified in the ChIP experiments is directly adjacent to a motif recently found to represent a *bona fide* ERE (Boney-Montoya et al.). Concerning JARID1A, a hormone-independent binding to the half-ERE was evidenced.

JARID1A and ERα formed a complex in MCF-7 cells but their binding to the three regulatory regions examined did not follow the same pattern. A possible explanation is that in the absence of estrogen, JARID1A is needed to shut down PR gene expression and that following hormone treatment, long-range interactions between distantly spaced cis-acting elements take place, thus allowing cross-talk between ERα and JARID1A. Indeed numerous long-range chromatin interactions have been detected for ERα (Pan et

al. 2008; Fullwood et al. 2009), including interactions with the promoter-bound transcription factor Sp1 (Parisi et al. 2009). Finally, it is possible that additional PR regulatory regions not examined in our ChIP experiments undergo changes in their H3K4 methylation levels as a response to estrogen treatment. Indeed a few novel response elements bound by ERα have recently been described in the PR gene proximal and distal regions (Boney-Montoya et al.).

4.1.4 Model of cofactor dynamics at the PR promoter locus

In 2000, Brown and colleagues reported a cyclic model of ER transcription complex assembly in breast cancer cells, in which first the liganded ER binds to the regulatory region, followed by recruitment of histone acetylases and RNA Pol II (Shang et al. 2000). Recent work indicates that transcriptional regulation by estrogen is much more complicated as it involves long-range chromatin interactions that are mediated by multiple EREs (Pan et al. 2008; Fullwood et al. 2009). The work presented here expands this model by the finding of the important role of H3K4 methylation in estrogen regulation of PR expression and the identification of the histone demethylase JARID1A as the cofactor responsible for the modutation of this regulatory feature.

SMYD3 is one of several H3K4 methylases that may counteract the effects of JARID1A. Interestingly, it is upregulated in breast cancer and involved in the proliferation of breast cancer cell lines (Hamamoto et al. 2004; Hamamoto et al. 2006). A recent study additionally reveals that it directly interacts with ERα and stimulates its activity in presence of estrogen (Kim et al. 2009). Examination of different estrogen-regulated genes such as pS2, CTSD and GREB1 indicates that this is linked to elevated H3K4 di- and trimethylation at ERE-containing regulatory regions (Kim et al. 2009). Here a HMTase-dependent role of SMYD3 also in the estrogen-mediated regulation of the PR gene was indicated by overexpression experiments. The respective levels of SMYD3 and JARID1A as well as their interaction with liganded ERα may govern the local H3K4 methylation levels at estrogen-regulated genes, and thereby their expression levels.

A possible model for the regulation of the PR gene promoter is that in the absence of hormone JARID1A represses PR gene expression by keeping H3K4 di- and especially trimethylation marks at a minimal basal level via its demethylase activity. After estrogen stimulation, the liganded-ERα moves into the nucleus and binds as a dimer to primarily distal binding sites. The bound receptor then interacts with cofactors including JARID1A close to the transcriptional start site, thereby forming a long-range chromatin looping structure and a platform that leads to recruitment of RNA Pol II, and ultimately to transcriptional activation. As reported for the pRB-JARID1A interaction, binding of ERα to

JARID1A may result in the prevention of the repressive role of JARID1A and thereby promote transcription. Indeed, it was found that long-range interactions occur only in the presence of estrogen, suggesting that ERα is required and may act as anchor point (Pan et al. 2008).

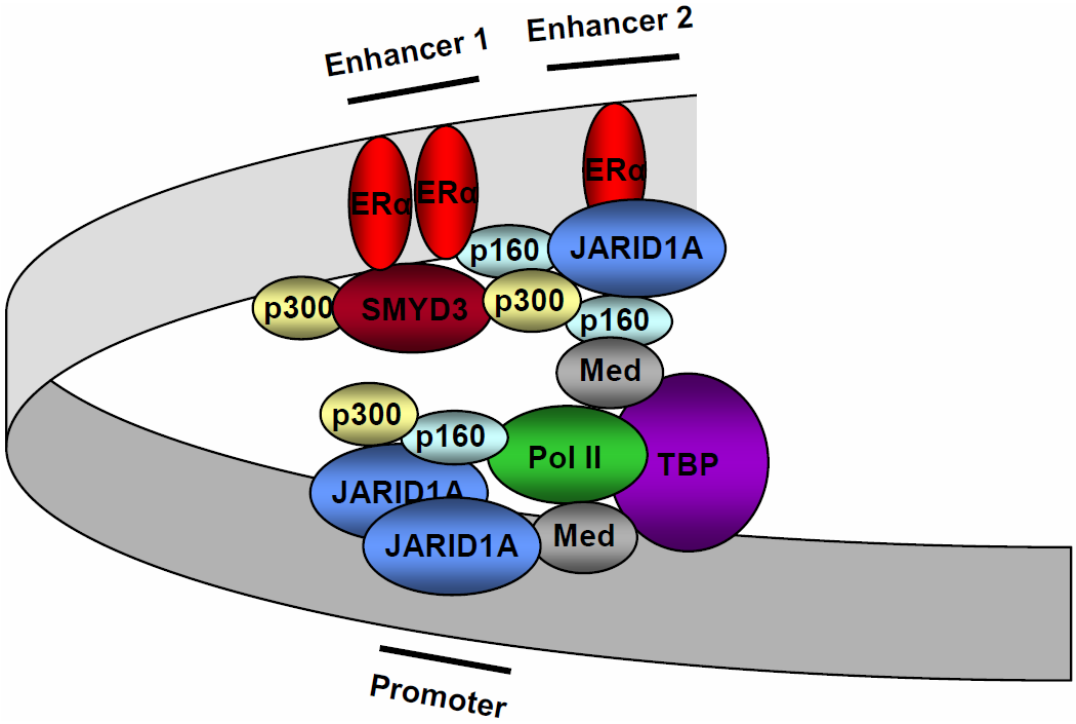


Figure 28

Schematic model of ER coactivator complex assembly on PR regulatory regions. After estrogen stimulation, ER coactivator complex is predominantly recruited to the PR enhancer, which communicates with the PR promoter by long-range chromatin looping structure.

This model is supported by the MDA-MB-231 ChIP results. No occupancy of JARID1A in the PR regulatory region was observed in this ERα-negative cell line, suggesting that its role in PR gene modulation depends on the presence of the ERα.

Métivier and colleagues reported transcriptional activation to be a cyclical process that requires both activating and repressive epigenetic processes (Metivier et al. 2003). Using the pS2 promoter in MCF-7 cells as model, they describe “waves” of transcription which allow the cell to continuously adapt the transcription rate of a gene in response to differing situations through modulating the duration of activation. Each transcriptionally productive cycle needs to be followed by a reinitiation cycle, where complete remodification of nucleosomal structure occurs prior to the initiation of subsequent cycles. JARID1A depletion resulted, also in the presence of hormone, to a doubling of

PR gene expression. It can be assumed that a fraction of JARID1A that is not bound to ERα still limited activation. Another possibility is that in addition to its repressive function in the absence of hormone, JARID1A is needed as part of “quick” silencing in this tightly controlled process.
A dysbalance of this complex system may lead to aberrant control of estrogen target genes and thereby play a role in breast cancer progression.

4.2 Role of JARID1D in AR function

4.2.1 JARID1D is an AR regulator

Little is known about the regulation of the AR gene, which is located on the X-chromosome. The few reports published show that it is controlled by a single promoter lacking typical TATA or CAAAT box motifs, but containing cis-acting sequences including binding sites for Sp1 (Faber et al. 1993; Chen et al. 1997) which contribute to AR promoter activity. The AR mRNA levels are known to be regulated by androgens (autoregulation) – however, no functional AREs have been identified in the AR gene promoter or its 5’-flanking region (Blok et al. 1992; Takane and McPhaul 1996). The reported effects of androgens on AR mRNA levels are diverse and seem to be cell- and tissue-specific. A 2-fold down-regulation of AR mRNA has been reported in LNCaP and the breast cancer cell line T47D following androgen treatment (Trapman et al. 1990; Krongrad et al. 1991; Wolf et al. 1993). In contrast, Takeda and colleagues described that AR mRNA levels were reduced in rat or mouse prostate ventral lobe epithelial cells after castration, and that the levels got restored by androgen treatment (Takeda et al. 1991). An up-regulation of AR mRNA by androgens was also reported for other cell lines, such as human hepatocellular carcinoma and osteoblastic cell lines (Yu et al. 1995; Wiren et al. 1997). At the protein level the AR is stabilised by its ligand (Zhou et al. 1995). In this work, no significant regulation by androgen was observed for AR expression in LNCaP cells. Nevertheless, JARID1D was found to be an activator of AR gene expression. Knock-down experiments of JARID1D led to a significant decrease of AR mRNA and protein levels. These findings fit into what is already known on JARID1D function. The JARID1D gene is located on the Y-chromosome and thus thought to have a function related to male reproduction. Indeed, the involvement of JARID1D in chromatin condensation during spermatogenesis by formation of a complex with the DNA repair related factor MSH5 has been evidenced (Akimoto et al. 2008). This function is different from the transcriptional role described for JARID1D (Lee et al. 2007) and is possibly modulated by associated proteins found in the complex. Likewise, the function

of JARID1D in the control of AR expression might necessitate interaction with another set of proteins. Further studies are now needed to address the mechanism of activation and to clarify whether the demethylase activity is needed.

4.2.2 Role of JARID1D in modulating the transcriptional control of androgen-regulated genes

The complex mechanisms responsible for the modulation of chromatin structure and the recruitment and activation of RNA polymerase II involve many different coregulators, each of which appears to play a specific role in transcriptional activation. Many cofactors of the androgen receptor exhibit enzymatic activity. Recent examples include the H3K9 histone methylase G9a and the H3K9 histone demethylase JHDM2A which interact with the AR and modulate its activating function (Lee et al. 2006; Yamane et al. 2006). However little is known about the role of H3K4 methylation in AR transcriptional gene regulation.

Using the prostate cancer cell line LNCaP as model, it was found in this work that JARID1D regulated the control of several androgen-regulated genes. Deletion of JARID1D by using specific siRNAs resulted in a significant hormone-independent decrease of PSA, KLK2 and TMPRSS2 gene expression, which were previously shown to be highly androgen-regulated. Additionally it was shown that JARID1D forms a complex with the AR in the presence of hormone. In ChIP experiments colocalisation of JARID1D with the AR at the distal enhancer region of PSA was determined.

Little is known about the role of JARID1D in transcriptional regulation. Lee and colleagues showed that JARID1D is a histone demethylase with activity for trimethylated H3K4 (Lee et al. 2007). Additionally, they showed that the polycomb-like protein Ring6a/MBLR binds to JARID1D thereby facilitating its demethylase activity. The complex of both represses the expression of *Engrailed 2.* As this report shows a repressive function for JARID1D and in view of numerous data showing that di- and trimethylation of H3K4 is generally low in inactive genes but elevated in active genes (Santos-Rosa et al. 2002), it was surprising to find an activating effect of JARID1D in correlation with AR function. However, there are other examples of enzymes that affect histone methylation and usually act as transcriptional repressor, while having a transcription-promoting activity in the context of AR signalling. Interestingly this activity was reported to be independent from their enzymatic activity. One example is G9a which acts as a corepressor through methylation of H3K9, which is known to be generally high in inactive genes (Litt et al. 2001; Noma et al. 2001). Concerning AR-mediated transcriptional control, G9a however acts as a coactivator. This function does not require

the enzymatic activity of G9a but the interaction with another histone modifier, namely the arginine-specific protein methyltransferase CARM1. It was found that G9a methyltransferase activity is inhibited by histone modifications associated with transcriptional activation and assumed that this allows G9a to operate as a coactivator (Lee et al. 2006). Another example is the histone demethylase LSD1 which usually represses gene expression by removing mono- and dimethylation marks of H3K4. When associated to the AR, LSD1 is an activator which demethylates the repressive histone marks mono- and dimethyl H3K9 (Metzger et al. 2005). Here also, the activity as a gene silencer or activator seems to depend on the specific interacting partners.

To get a detailed knowledge about the processes occurring at the regulatory regions of the AR target genes after androgen stimulation, ChIP experiments were performed using the well-characterised androgen-regulated gene PSA as model. Two AREs in the PSA gene, one in the distal enhancer and the other in the proximal promoter region, were analysed with regard to AR recruitment, JARID1D binding and H3K4 trimethylation pattern. Associated with the androgen stimulation, the occupancy of AR increased at the PSA enhancer and at a lower level also at the PSA promoter. JARID1D colocalised with the AR in all regions, with the highest accumulation at the PSA enhancer. As already reported for G9a, binding of JARID1D occurred in a hormone-independent manner. This finding and the IP result which indicates an interaction between JARID1D and AR, support a function of JARID1D as an important AR cofactor. A high level of H3K4 methylation at the PSA promoter region was measured but no increase was associated with androgen stimulation. Low levels of H3K4 trimethylation correlated with the presence of JARID1D, in line with a demethylating activity of JARID1D. In order to test this hypothesis, JARID1D wild-type and an inactive mutant were overexpressed in LNCaP cells. This did however not significantly affect the expression of AR target genes, possibly due to the relatively high expression of endogenous JARID1D in these cells. Further experiments are therefore needed to address the requirement of enzymatic activity in the newly identified AR coactivator function of JARID1D and also to find interaction partners possibly involved in the control of this activity.

4.3 Expression of JARID1s is differentially controlled by steroid hormones

4.3.1 JARID1A and JARID1B are regulated by steroid hormones

The expression of several steroid receptor cofactors is under control of steroid hormones (Urbanucci et al. 2008). For instance, the histone demethylases JMJD1A and 2C were

identified as AR cofactors and found to get slightly induced by androgen treatment (Urbanucci et al. 2008). Here, in addition to the identification of JARID1A as a corepressor of the ERα, it was found that JARID1A gene expression was down-regulated after estrogen treatment. This repression of JARID1A expression by estradiol may balance the repressive function of JARID1A at the regulatory regions of estrogen-regulated genes and represent a mechanism for fine-tuning of JARID1A activity.

Another JARID1 family member, JARID1B, was found to get suppressed after estrogen and after androgen treatment. Results obtained in MCF-7 and LNCaP cells show a time-dependent repression pattern. Interestingly, roles for JARID1B and for AR in breast cancer have been reported. In breast cancer, JARID1B promotes progression by repression of tumour suppressor genes such as BRCA1 and in prostate cancer it regulates AR transcriptional activity (Xiang et al. 2007; Yamane et al. 2007). Other genes which play a role in ERα as well as in AR signalling pathways have also been shown to be regulated by both estrogen and androgen. For instance the cell cycle regulator cyclin D1 is induced by both hormones (Gruvberger et al. 2001; Recchia et al. 2009). Expression of the immunophilin FKBP5, which is part of steroid receptor complexes in the cytoplasm, is regulated by several steroid hormones. A strong stimulation by glucocorticoids, progestins and androgens has been reported (Hubler and Scammell 2004). These findings indicate that autoregulation through feedback loops as well as cross-talk between the different steroid receptor pathways are common features of steroid receptor signalling which also affect cofactors such as JARID1 family members.

4.4 Outlook

The fundamental role of histone-modifying enzymes in the process of transcriptional control has become an exciting and promising research field during the last years. More and more proteins have been identified as enzymes able to modify histones. The finding that steroid hormone receptors use histone modifications such as H3K4 methylation for the regulation of gene expression and that proteins responsible for addition or removal of these histone marks act as coregulators of steroid receptors raises the possibility that histone methylases/demethylases are essential factors for the modulation of steroid receptor signalling. Some of them may represent new suitable targets for the treatment of hormone-dependent cancers and steroid receptor-related pathologies. Future work will have to characterise the mechanisms underlying coregulator functions in more detail. Also, selective inhibitors will need to be identified in order to have specific tools at hands. As the specific blocking of steroid receptors represents the standard anti-hormonal treatment of breast and prostate cancer and is associated with few side-effects, targeting

essential cofactors of these receptors that are involved in chromatin modulation may represent an interesting new strategy with great potential for synergy and also for cancer stages that have become refractory.

5 SUMMARY

Steroid receptor activity necessitates the interaction with various coactivators or corepressors. Among these cofactors, enzymes have been identified that locally modify histone tails, mainly histone 3 (H3) and histone 4 (H4). The H3K4 methylation mark is often found in the regulatory regions of transcriptionally active genes. However, how far steroid receptors use this histone mark for the regulation of downstream target genes has not been extensively analysed. The objective of this work was to study the possible role of the JARID1 family members, a subgroup of the JmjC family known to have a H3K4me2/3 demethylase activity, in ERα and AR function.

Using MCF-7 breast cancer cells as model, it was found that JARID1A was involved in the fine-tuning of PR gene expression. Reduction of JARID1A led to enhanced PR expression, both at the basal and estrogen-stimulated levels. Conversely, overexpression of JARID1A wild-type, but not of the enzymatically inactive form, suppressed PR promoter activity. Chromatin immunoprecipitation (ChIP) experiments showed JARID1A to bind in a ligand-independent manner to a region located downstream of the PRB transcription start point and containing an estrogen response element (ERE) half-site. Estrogen treatment led to increased RNA polymerase II binding to this region and to increased ERα binding to the PR enhancer region 1 located more upstream. In addition, an increase in H3K4 trimethylation was detected at the ERE half-site region. Reduction of JARID1A expression led to increased H3K4 di- and especially trimethylation in this region. Analysis of MDA-MB-231 cells which do not express the PR indicated that H3K4 trimethylation did not take place in the regulatory regions examined.

In the second part of this work, JARID1D was found to modulate the expression of several androgen-regulated genes. Expression knock-down in the prostate cancer cell line LNCaP resulted in a ligand-independent decrease of PSA, KLK2 and TMPRSS2 gene expression. AR levels were also repressed, both at the mRNA and protein levels. JARID1D formed a complex with the AR in the presence of hormone. ChIP assays showed that both proteins colocalised at the distal enhancer region of PSA.

Altogether this work shows H3K4 tri- and dimethylation to play an important role in mediating ERα function, and JARID1A to be the histone-demethylating enzyme responsible for the removal of this mark. In addition, JARID1D was identified as a regulator of AR expression and function. The results of this work underscore the importance of epigenetic modifications in steroid receptor function and provide new insights in JARID1 family function.

5.1 Zusammenfassung

In ihrer Funktion als Transkriptionsfaktoren benötigen Steroidrezeptoren die Wechselwirkung mit unterschiedlichen Koaktivatoren und Korepressoren. Unter diesen Kofaktoren wurden Enzyme identifiziert, die die Enden der Histone, hauptsächlich Histon 3 (H3) und Histon 4 (H4), modifizieren. Methylierung am Lysin 4 des Histon 3 wird häufig bei aktiven Genen gefunden. Es ist jedoch nicht klar, inwieweit Steroidrezeptoren diese Modifikation für die Regulation von Zielgenen verwenden. Daraus resultierend beschäftigt sich die vorliegende Arbeit mit der Untersuchung einer möglichen Rolle der JARID1 Familienmitglieder, einer Untergruppe der JmjC Familie mit Demethylase-Aktivität für das Histon 3 am Lysin 4 (H3K4), im Estrogen- (ER) und Androgenrezeptor (AR) Signalweg.

Im ersten Teil der Arbeit wurde unter Verwendung der Brustkrebszelllinie MCF7 als Modell herausgefunden, dass JARID1A an der Regulation der Genexpression des Progesteronrezeptors (PR) beteiligt ist. Die Reduktion von JARID1A führte sowohl auf basalem als auch auf Estrogen-stimuliertem Level zu einer erhöhten PR Expression. Umgekehrt wurde die PR-Promotoraktivität durch Überexpression des JARID1A Wildtyps, nicht aber der enzymatisch inaktiven Form, unterdrückt. Chromatin-Immunoprezipitationsexperimente (ChIP) zeigten, dass JARID1A in einer liganden-unabhängigen Weise an eine Region bindet, die dem transkriptionellen Startpunkt von PRB nachgelagert ist und ein halbes estrogen response element (ERE) enthält. Estrogen-Behandlung führte zu einer erhöhten Bindung der RNA Polymerase II an diese Region und zu einer erhöhten ERα-Bindung an die vorgelagerte PR enhancer Region 1. Zusätzlich wurde ein Anstieg der H3K4 Trimethylierung an dem halben ERE detektiert. Die Reduktion der JARID1A Expression führte zu einer erhöhten H3K4 Di- und vor allem Trimethylierung in dieser Region. Untersuchungen in MDA-MB-231 Zellen, die den PR nicht exprimieren, zeigten keine Trimethylierung in den untersuchten Regulationsregionen.

Im zweiten Teil der Arbeit wurde herausgefunden, dass JARID1D die Expression von mehreren Androgen-regulierten Genen moduliert. Eine Verringerung der JARID1D Expression in der Prostatakrebszelllinie LNCaP führte zu einer liganden-unabhängigen Unterdrückung verschiedener AR-Zielgene. Sowohl auf mRNA- als auch auf Proteinebene konnte zusätzlich eine Verringerung in den AR Levels nachgewiesen werden. JARID1D bildete in Anwesenheit des Hormons einen Komplex mit dem AR. ChIP Experimente zeigten, dass beide Proteine an der distalen enhancer Region des PSA Gens kolokalisieren.

Insgesamt konnte mit der vorliegenden Arbeit gezeigt werden, dass H3K4 Tri- und Dimethylierung eine wichtige Rolle bei der Vermittlung der ERα Funktion spielen. Insbesondere wurde dabei JARID1A als das Histon-demethylierende Enzym charakterisiert, das für das Entfernen dieser Markierungen verantwortlich ist. Zusätzlich konnte JARID1D als Regulator der AR Expression und Funktion identifiziert werden. Die Ergebnisse dieser Doktorarbeit unterstreichen daher die Bedeutung von Histonmodifikationen in der Funktion von Steroidrezeptoren und liefern in diesem Zusammenhang neue Erkenntnisse zu der JARID-Proteinfamilie.

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7 APPENDIX

7.1 Abbreviations

Chemicals/Materials

cFBS	charcoal-stripped FBS
DNase	Deoxyribonuclease
E2	17β-estradiol
ECL	Enhanced Chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FBS	fetal bovine serum
HA	hemagglutinin
HRP	Horseradish peroxidase
LDS	lithium dodecyl sulfate
LNCaP	Lymph Node Carcinoma of the Prostate
luc	luciferase
MCF	Michigan Cancer Foundation
MOPS	morpholinopropane sulfonic acid
M-Per	Mammalian Protein Extraction Reagent
PBS	phosphate buffered saline
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PVDF	polyvinylidene fluoride
R1881	methyltrienolone
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA
Taq	*Thermophilus aquaticus*
TE	Tris-EDTA
TOPO	topoisomerase
Triton X-100	4-(1,1,3,3-Tetramethylbutyl) cyclohexyl-polyethylene glycol

Terms

AIS	androgen insensitivity syndrome
al.	alii
AR	androgen receptor
ARE	androgen response element
ARID	AT-rich interactive domain
ATP	adenosine triphosphate
BRCA	breast cancer
CARM	coactivator-associated arginine methyltransferase
cDNA	complementary DNA
CHIP	chromatin immunoprecipitation
DBD	DNA-binding domain
DNA	deoxyribonucleic acid
DOT	disruptor of telomeric silencing
ER	estrogen receptor
ERE	estrogen response element
EZH	enhancer of zeste homologue
Fig.	Figure
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLP	G9a-related protein
H	histone
HDAC	histone deacetylase
HDM	histone demethylase
HMT	histone methyltransferase
HRE	hormone response element
hsp	heat-shock protein
hu	human
Ig	Immunoglobulin
IP	immunoprecipitation
JARID	jumonji AT-rich interactive domain
JHDM	JmjC-domain-containing histone demethylase
Jmj	jumonji
JMJD	jumonji-domain-containing protein
K	lysine
KDM	lysine demethylase

KLK	Kallikrein
LBD	ligand-binding domain
LSD	lysine-specific demethylase
me	methyl
minP	minimal promoter
mRNA	messenger RNA
myc	v-myc myelocytomatosis viral oncogene homolog
NLS	nuclear localisation signal
NR	nuclear receptor
NTD	N-terminal domain
PADI	petidylarginine deiminase
PAGE	polyacrylamide gel electrophoresis
PcG	polycomb group
PCR	polymerase chain reaction
pH	pondus Hydrogenii
PHD	plant homeo domain
Pol	polymerase
PR	progesterone receptor
pRB	retinoblastoma binding protein
PRC	polycomb receptor complex
PRMT	protein arginine methyltrasferase
qPCR	quantitative PCR
RBP	retinoblastoma binding protein
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-PCR
SET	Su(var)3-9, Enhancer of Zeste, Trithorax
siRNA	small interfering ribonucleic acid
SRC	steroid receptor coactivator family
SR	steroid receptor
SMYD	SET and MYND domain-containing protein
TMPRSS	Transmebrane Protease, Serine
tsp	transcriptional start site
WB	western blot
wt	wild type
XLMR	X-linked mental retardation

Units

bp	base pairs
℃	degree Celsius
g	gram
h	hours
k	kilo
kb	kilobases
l	liter
µ	micro
M	molar
min	minutes
ml	milliliter
mM	millimolar
nM	nanomolar
rpm	rounds per minute
RT	room temperature
s	seconds
V	Volt

7.2 Publications

Publications in journals

Stratmann A. and Haendler B.. The histone demethylase JARID1A regulates progesterone receptor expression. *In review*.

Poster presentations

Stratmann A., Faus H., Haendler B.: Histone-modifying enzymes and androgen receptor function. *Androgens 2008*. Rotterdam, Netherlands, October 2008.

Stratmann A. and Haendler B.: Histone demethylation and steroid receptor function. *Gordon Research Conference Chromatin Structure & Function*. Il Ciocco Lucca (Barga), Italy, May 2008.

Stratmann A.: Androgen receptor and histone methylation. *Spetses Summer School on Nuclear Receptor Signalling: From Molecular Mechanisms to Integrative Physiology*. Spetses, Greece, August 2007.

7.3 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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7.5 Declaration – Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig durchgeführt und verfasst habe. Dabei wurden keine anderen, als die angegebenen Quellen und Hilfsmittel verwendet.

Hereby I declare that all of the following material was written by myself and is properly referenced.

Antje Stratmann

Berlin, Mai 2010