PLU-1/JARID1B/KDM5B is required for embryonic survival and contributes to cell proliferation in the mammary gland and in ER+ breast cancer cells

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Abstract. The four members of the JARID1/KDM5 family of proteins, a sub-group of the larger ARID (AT rich DNA binding domain) family, have been shown to demethylate trimethylated lysine 4 on histone 3 (H3K4me3), a chromatin mark associated with actively transcribed genes. In some lower organisms a single homologue of *JARID1* is found, and functions of the four proteins found in mice and humans may be specific or overlapping. To investigate the function of the JARID1B protein we examined the effects of deletion of the gene in mice. Systemic knock out of *Jarid1b* resulted in early embryonic lethality, whereas mice not expressing the related *Jarid1A* gene are viable and fertile. A second mouse strain expressing a *Jarid1b* gene with the *ARID* domain deleted was viable and fertile but displayed a mammary phenotype, where terminal end bud development and side branching was delayed at puberty and in early pregnancy. Since development of terminal end buds are completely dependent on signalling from the estrogen receptor (ERα), we investigated the expression of a target gene (progesterone receptor) in the ΔARID mouse and found levels to be reduced as compared to wild-type. JARID1B is widely expressed in ER+ breast cancers and breast cancer cell lines, and interaction with ERα was demonstrated by co-immunoprecipitations in cells transfected with tagged *ERα* and *JARID1B* genes. Down-regulation of expression of JARID1B using shRNAi in MCF-7 cells resulted in a dramatic decrease in E2 stimulated tumour growth in nude mice. The data demonstrate a specific role for Jarid1B in early embryonic development, in the development and differentiation of the normal mammary gland, and in estrogen induced growth of ER+ breast cancer.

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Introduction

Dynamic changes in histone modifications such as acetylation, phosphorylation and methylation play a significant role in differentiation and development (1-3). In this context the JARID1/KDM5 group of histone demethylases [KDM5:lysine demethylase 5 (4)] have engendered considerable interest as the first identified proteins able, through the jumonji domain, to demethylate tri-methylated lysine 4 on histone 3 (5-9), a mark generally associated with promoters of actively transcribed genes (1). JARID1 homologues are represented by a single gene in lower organisms for example *Lid* in *Drosophila* (10,14) and *rbr-2* in *C. elegans* (6), while 4 proteins are found in mammals. Although some redundancy might be expected, specific function could be generated by differing profiles of expression. SMXC/JARID1C/KDM5C, [a gene on the X chromosome which is not inactivated (16)], is expressed preferentially in neuronal tissue and is associated with neuronal survival and X linked mental retardation (7,13). The RB binding protein RBP2/JARID1A/KDM5A (14), is widely expressed (3,6,8,15), and PLU-1/JARID1B/KDM5B [JARID1B nomenclature used throughout this report], shows a highly restricted expression in normal adult tissues, being largely

confined to the testis and the differentiating mammary gland (16-18). JARID1B is however expressed in breast cancer (16,18) being strongly associated with ER^+ cancers (19), and ectopic expression is also seen in cancers deriving from tissues normally not expressing the protein (20). In melanoma, JARID1B defines a subpopulation of slowly cycling cells necessary for continuous tumour growth (21).

Specificity of function may also depend on sequence differences. Although the four JARID1 proteins show high sequence homology, the conservation is less stringent at the 3'-end that contains the LXCXE RB binding sequence found only in JARID1A (14,16), and two PHD domains. One of these (PHD3) is absent in JARID1C and D and the second (PHD2), present in all four proteins shows sequence differences to JARID1B. PHD domains are involved in interactions with other proteins forming complexes, the composition of which depends on the cell context. Differences in complex formation might therefore be expected among the JARID1 proteins. Studies looking at protein interactions of the JARID1 demethylases have focused on their function in repression of transcription, relating this to the demethylation of H3K4me3 at promoters of down-regulated genes (3,5,22). JARID1B associates with HDACs through the PHD2 and PHD3 domains (17), while JARID1A associates with the Notch-RBP-J repressor complex through the PHD3 but not the PHD2 sequence (23). JARID1A and B have been reported to associate with different components of the repressive polycomb complexes (24,25). Nevertheless, interactions with other transcription factors including myc and steroid receptors can result in activation of expression of target genes (10,26,27).

Differences in tissue tropism are indicated from studies following effects of gene deletion. Thus deletion of *Jarid1a* in the mouse results in defects in development of the hematopoietic system (8), however the homozygous KO is viable and fertile, as opposed to the lid$^{-/-}$ in the fly which is lethal (10). The knock out of the homologue of *JARID1C* in the zebra fish results in defects in neuronal development and dendritic cell morphogenesis is impaired in rat (7). To investigate whether deletion of the *Jarid1b* gene in the mouse would indicate a role for the involvement of JARID1B in mammary gland function, we attempted to develop a Jarid1b$^{-/-}$ mouse. We found that systemic KO of *Jarid1b* results in early embryonic lethality, indicating a crucial role for JARID1B in early embryonic development that cannot be rescued by the other Jarid1 proteins. However, expression of a mutated gene with the *ARID* domain deleted that also causes loss of demethylase activity (5,27), resulted in a mammary gland phenotype, showing a reduction in the number of end buds developing at puberty, and a delay in side branching. This phenotype suggested that an effect on ERα signalling was involved, since an ERα KO mammary gland shows complete inhibition of the development of end buds (28,29). Interaction with ERα is of great of interest, not only for normal mammary gland development but because it could be involved in the proliferation of ER positive cancers, where JARID1B is expressed. JARID1A interacts with ERα (26) and JARID1B with the androgen receptor (27). We therefore analysed interaction of JARID1B with ERα, investigated the effect of JARID1B expression on the growth of an ER^+ breast cancer cell line, and sought to demonstrate a role for JARID1B in ER signalling in the ΔARID mouse.

Materials and methods

Ethics statement. All animal work was under carried out under UK Home Office Project Licence Numbers: PPL 70/5930 and PPL 70/6847, strictly adhering to Home Office guidelines. This study was approved by the King's College London Ethics Review Process Committee and Cancer Research UK Ethics Review Process on 30th October, 2008.

Animal experiments. For staged pregnancies, observation of vaginal plug was designated as day zero. To ensure accuracy of staged pregnancies, embryos were assessed for stage of embryonic development at the point of mammary gland harvest. To study the development of the nulliparous mammary gland the female mice were caged together from birth in order to synchronize the estrous cycle. For the collection of mammary gland tissue or embryos, females were culled by carbon dioxide inhalation.

Mammary gland whole mounts. The right-sided thoracic and inguinal mammary glands (3rd and 4th from the neck respectively) were dissected onto glass slides and fixed flat overnight at room temperature in Carnoy's fixative (75% ethanol, 25% glacial acetic acid). The mammary glands were serially hydrated in decreasing concentrations of ethanol, stained overnight at room temperature in Carmine solution (0.5% carmine dye w/v, 0.2% aluminum potassium sulfate) and then dehydrated in increasing concentrations of ethanol.

Development and screening of transgenic mice
a) Exon 1 KO mouse. A *Jarid1b* targeting vector designed to replace exon 1 was generated by flanking the loxP-neomycin-LoxP expression cassette with genomic sequence amplified from the sv129/ola murine strain. The 5' 2.4 kb homologous region (HR1) and the 3' 5.7 kb homologous region (HR2) comprised of intronic 1 and 2 sequences respectively. After injection of the construct into male C57bl/129/ola ES cells, screening of G418-selected (200 μg/ml) ES clones was performed by Southern hybridization using a PCR generated probe within the 3' homologous region. Two positive clones (1A4, strain 1 and 1A8, strain 2) carrying the integrated targeting vector were injected into either C57BL/6 blastocysts (1A8) or C57BL/6 embryos at the 8 cell stage (1A4) of development. Genotyping on DNA obtained from ear snips or from early embryos was performed by PCR to distinguish the WT allele (TGGATTGTAACTCTGTTCTCCCTAC and TTCTACTAG CAACGGCAACACCTAG) from the allele that had undergone homologous recombination (CATCTGTCAGACCCTTAGTAC GCTA and GCTACCGGTGGATGTGGAATGTGTG) (Fig. 2). For the detection of Jarid1b expression and identification of *Jarid1b*$^{-/-}$ embryos before 7.5 days, individual blastocysts at 3.5 and 4.5 dpc were genotyped by PCR after extraction of DNA with the Red extract-N-Amp tissue kit and amplification with the primers listed above.

b) The Jarid1b ΔARID mouse strain. A *Jarid1b* targeting vector was designed to replace exons 2-4. The targeting vector was generated by flanking the loxP-neomycin-LoxP expression cassette with genomic sequence amplified from the sv129/ola murine strain. The 5' 7.3 kb homologous region (HR1) comprised of intron 2 and 48 bp of exon 2 and the 3' homologous region

(HR2) comprised of 1.2 kb of intron 5 (Fig. 3). After injection of the construct into male 129/ola ES cells, screening of G418-selected (200 μg/ml) ES clones was performed by Southern hybridization using a PCR generated probe (1.2 kb) within the 3' homologous region. A positive clone (2F9) carrying the integrated targeting vector was injected into C57BL/6 blastocyst to generate a single mouse lineage homozygous for the transgene. The C57BL/6/129ola chimeric strain was backcrossed over six generations onto the C57BL/6 genetic background to give the *Jarid1B* ΔARID mouse strain.

Growth of tumours from MCF-7 cell clones in nude mice. Two clones of MCF-7 cells where JARID1B expression was constitutively knocked-down by shRNA (clones 4 and 29), and two lines from MCF-7 cells transfected with empty vector (clone 1 and pSUP mix) were studied (22). Balb/C Nu/Nu mice were implanted with estrogen implants (1.5 mg, 90-day release) 48 h before subcutaneously injecting the cells in matrigel. Appearance and size of tumours were monitored twice weekly. Statistical analysis was carried out using a two-tailed t-test.

End-point PCR. PCR amplification reactions were performed on 2 μl of cDNA using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and primers specific to *Plu-1/Jarid1B* exon 1 (5'-cgctttcatccacaagatcc-3') and exon 5 (5'-cagactg cctctggggaata-3'). PCR products were resolved on a 1.5% agarose gel, excised and purified using Wizard SV gel and PCR clean up systems (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA sequencing reactions were performed using BigDye terminator v3.1 cycle sequencing kit and 3730 DNA analyzer (Applied Biosystems).

Isolation of RNA and real-time PCR. Inguinal mammary glands (4th from the neck) and adult testis were collected, snap-frozen and total RNA isolated using the RNeasy Lipid tissue kit (Qiagen, Crawley, UK). Random hexamers were used to generate cDNA from 1 μg of total RNA using Superscript III transcriptase (Invitrogen, Paisley, UK) according to the manu-facturer's instructions. Relative quantification of target gene levels was achieved using SYBR Green JumpStart ReadyMix (Sigma-Aldrich, Poole, UK) according to the manufacturer's instructions and the Opticom 2 continuous florescence detector. Normalisation was against 18s ribosomal RNA in separate tube reactions. To prevent amplification of genomic DNA PCR primers were designed to span intronic regions. The reactions were visualised by agarose gel electrophoresis to confirm target amplicon size. The $2^{-\Delta\Delta CT}$ method (Applied Biosystems; User Bulletin No. 2) was used to calculate the target gene expression level relative to the mammary gland identified as expressing the highest level of the target gene. Target gene and control gene amplification rates were compar-able. Each group is represented as an average. Statistical analysis was carried out using a two-tail t test. Sense and anti-sense primer sequences are as follows: *Jarid1b*: 5'-CGA TAAACTTCATTTCACCCCG-3' and 5'-ACCCACCTTCTTC TGCGACTAAC-3'; *18s*: 5'-CTTCTTAGAGGGACAAGTGGC GTTCAG-3' and 5'-AACTGATCCTCCAAACCTCTTCTC-3'; *Wnt4*: 5'-GAGTGCCAATACCAGTTCCG-3' and 5'-CCAGCC TCGTTGTTG-3'; *PR*: 5'-CCCACAGGAGTTTGTCAAACT-3' and 5'-TCCGGGATTGGATGAATG-3'; *ERα*: 5'-CTAGCAGA

TAGGGAGCTGGTTCA-3' and 5'-GGAGATTCAAGTCCCC AAAGC-3'.

Cell culture. COS-7 cells were maintained in DMEM supple-mented with 10% fetal calf serum and 0.3 μg/ml glutamine at 37°C in 10% CO_2. MCF-7 cells were maintained in the same medium supplemented with 10 μg/ml insulin. Clones selected by shRNAi or with control vector were grown in 500 μg of G418 (22).

Protein extraction and Western blot analysis. Inguinal mammary glands (4th from the neck) were collected, snap-frozen and nuclear protein extracts isolated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Nuclear protein extract (20 μg) was separated on a 6% polyacrylamide gel, as detailed previously (16).

Antibodies. The JARID1B antibody α-PLU-1C, has been described previously (17,18). Antibodies to Lamin B and HA were purchased from Santa Cruz Biotechnology (Calne, UK) and Covance (Princeton, NJ, USA) respectively. Antibody to the poly-Histidine tag was purchased from Sigma-Aldrich.

Plasmids. pcDNA3.1 Myc-HisA PLU-1/JARID1B was described previously (16). pcDNA3.1 Myc-HisA PLU-1/ JARID1B ΔARID was a kind gift from Dr C.D. Chen (27) and pXJ41neo-HA-ER was a gift from Dr W. Hong (26).

Transient transfections and immunoprecipitations. Cells were cultured to 50-80% confluence in 10-cm plates for preparation of whole cell extracts and transfected using Lipofectamine LTX (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were washed twice with PBS, then lysed for 15 min on ice in 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, plus EDTA-free complete protease inhibitor cocktail (Roche, Lewes, UK). Cells were passed ten times on ice through a fine gauge needle, centrifuged to pellet cell debris and the supernatant pre-cleared with protein A/G agarose beads (Pierce). To 300 μg of supernatant, 30 μl of antibody conjugated agarose was incubated overnight at 4°C.

In situ hybridization. In situ hybridization was carried out as previously described using actin as a positive control to ensure integrity of the blocks (18).

Results

Expression of Jarid1b in the mammary gland. We have previously shown that Jarid1b is expressed in the mammary bud in the mouse embryo and in the pregnant mammary gland in the adult mouse (18). Analysis of levels of expression of Jarid1b mRNA at different stages of differentiation in the mammary gland showed that expression is seen in the virgin gland, but the level is increased at pregnancy, decreased at lactation and re expressed to some degree at involution. Fig. 1A shows the profile of expression in C57BL/6 mice, but a similar pattern of expression is seen in Balb/C mice (30). To look at the expression at the cellular level, we used *in situ* hybridization (16,18) looking at sections of the virgin mammary gland (4 and
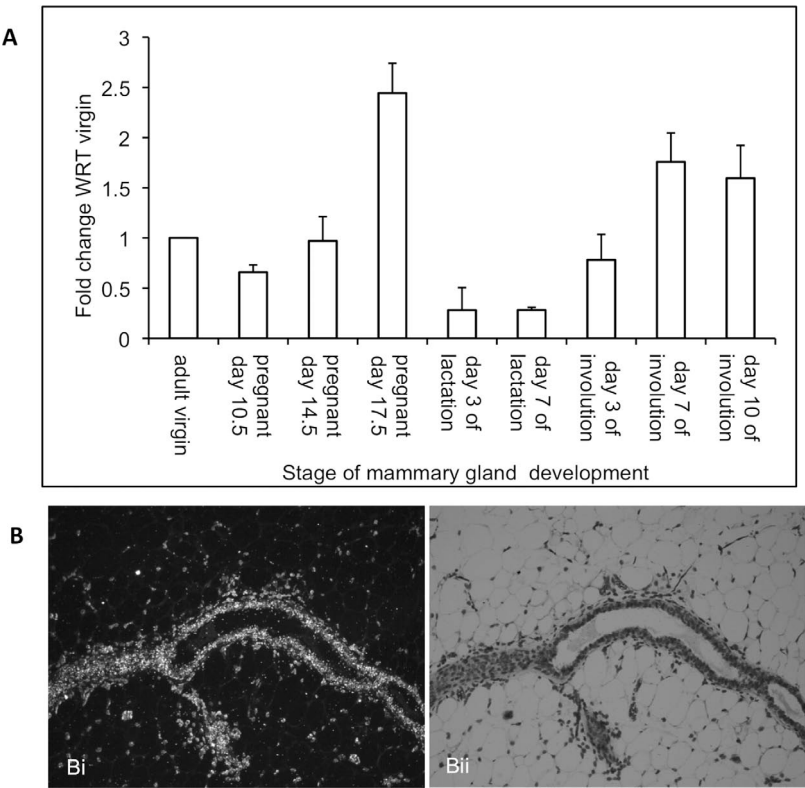
Figure 1. Expression of Jarid1b in the C57BL/6 murine mammary gland. (A) The expression of the Jarid1b transcript was determined by qRT-PCR and the level related to that expressed by the virgin mammary gland. (B) *In situ* hybridization showing that the Jarid1b transcript in mammary epithelial cells. Paired light field H&E staining (Bii) and dark field (Bi) on a 12-week old (adult) C57BL/6 WT nulliparous mammary gland section showing ductal development with side branching and the localisation of the Jarid1b transcript to mammary gland epithelial cells.

Table I. Systemic knock out of *Jarid1b* is an early embryonic lethal.

A, Genotyping of progeny from a heterozygous *Jarid1b/Ex1KO* breeding program

	Number of mice	Wild-type	Heterozygote	Homozygote KO
Strain 1A4 (14 litters)	139	67	72	0
Strain 1A8 (18 litters)	106	57	49	0

B, Genotyping of embryos from a heterozygous *Jarid1b/Ex1KO* breeding program

Strain 1A4	E9.5-10.5	E8.0-8.5	E7.5	Total
Wild-type	4	2	0	6
Heterozygote	4	9	7	20[a]
Homozygote KO	0	0	0	0
Strain 1A8	E9.5-10.5	E8.0-8.5	E7.5	Total
Wild-type	0	2	1	3
Heterozygote	5	5	13	23[a]
Homozygote KO	0	0	0	0

[a]The data indicate that the numbers of WT and heterozygous viable potency do not conform to Mendelian ratios (p-value 1.8×10^{-8}), suggesting that some embryos heterozygous for *Jarid1b* may not survive in late pregnancy.

12 weeks) and in the pregnant gland (pd12 and pd18). The data show that Jarid1b is expressed in the luminal epithelial cells (see Fig. 1B for the 12-day virgin gland), indicating that any effects on regulation of gene expression will be seen in the epithelial compartment, where ERα is also expressed and functional (29).

Lack of expression of Jarid1b leads to embryonic lethality. To study the function of JARID1B *in vivo*, knock out mice were developed, using the strategy illustrated in Fig. 2. Recombination of the injected linearised construct in ES cells resulted in replacement of exon 1 with the neomycin gene. The selected ES cells (Fig. 2B) were injected into C57BL/6 blastocysts or
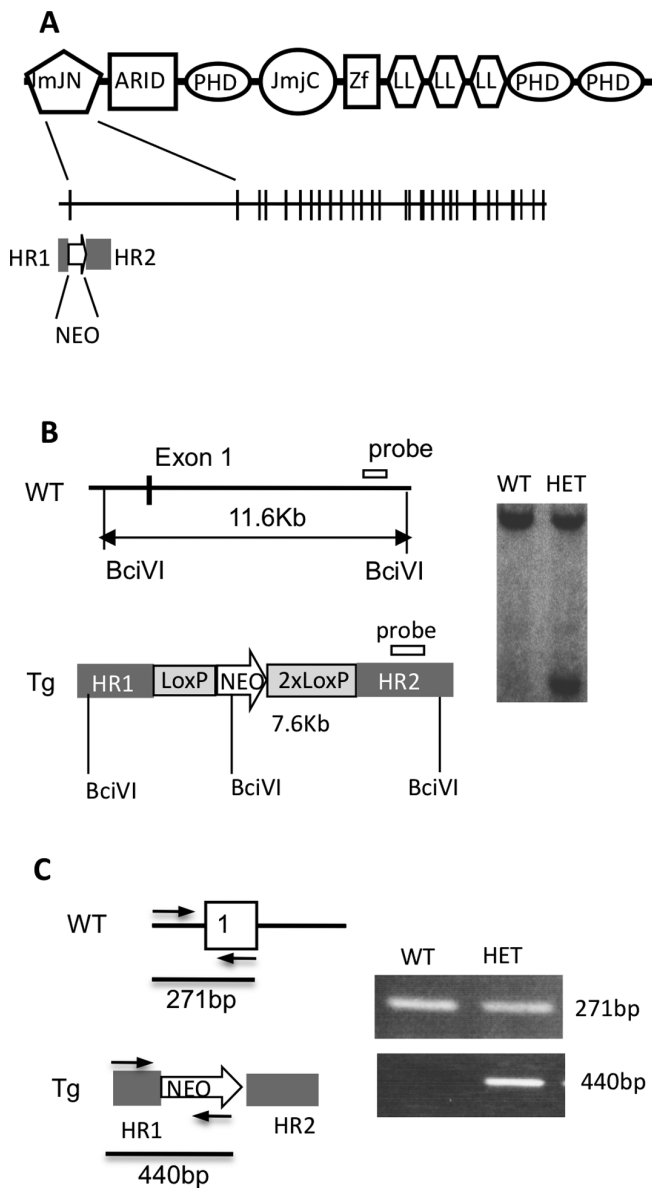
Figure 2. Generation of Jarid1b exon 1 construct and screening strategy used to detect homologous recombination. (A) Schematic representation of Jarid1b depicting protein domains, genomic organisation and the 5'/3' homologous regions (HR1 and HR2). (B) Enlarged schematic depicting the WT and trans-genic allele after homologous recombination and the location of the BciVI restriction sites used to distinguish the WT from the recombined allele. Southern blotting of a BciVI digest of C57bl/sv129 hybrid ES cell genomic DNA showing homologous recombination. (C) Schematic representation showing PCR primer location (arrows) used to screen DNA for homologous recombination (see Materials and methods).

8 cell embryos. Several males carrying the recombined gene were identified, and two strains (1A4 and 1A8) were developed from two of the founders. The recombined and wild-type genes were detected in DNA from ear snips using the PCR assays described in Materials and methods, and Fig. 2C shows the bands detected in WT and heterozygous mice using these assays.

Litters from crosses between heterozygotes were analysed for genotype in both mouse strains and no homozygote offspring were detected in a total of 139 from strain 1A4, and in a total of 106 from strain 1A8 (Table IA). Mice heterozygous for

the defective gene were viable and fertile and showed no abnormalities of internal organs or abnormal behaviour and no obvious difference in mammary phenotype from the WT mice. Embryos from heterozygous crosses were also examined after timed matings, and no homozygous embryos were found for either strain at E7.5 (Table IB). Examination of earlier embryos from crosses between *Jarid1b*^{+/-} heterozygotes showed that *Jarid1b*^{-/-} embryos could be identified up to E4.5, but attempts to develop *Jarid1b*^{-/-} ES cell lines have been unsuccessful. The results indicate that the defect induced by lack of expression of Jarid1b occurs early in embryonic development, before or just after implantation.

Development of the Jarid1b ΔARID mouse. Using a different approach to develop a mouse strain lacking expression of the Jarid1b protein, we replaced exons 2-4 with a floxed neomycin gene, which should have resulted in expression of a truncated mRNA with no translated sequences. Fig. 3 shows the strategy, the construct used, and the Southern blot of a Pst1 digest of the DNA from the injected ES cells showing homologous recombination.

A homozygous mouse strain expressing the modified *Jaridb* gene was developed on the C57BL/6/129 background, with both male and female mice being viable and fertile with pups born according to a Mendelian ratio. However, examination of the mRNA expressed in testis and in the pregnant mammary gland (tissues known to express high levels of Jarid1b), showed that exon 1 was spliced to exon 5, thus removing the neomycin gene. This is illustrated in Fig. 3C for RNA extracted from the mammary gland at day 18 of pregnancy, which shows the reduced size of the PCR product in the transgenic mouse using primers in exons 1 and 5. Exons 2-4 encode 5 amino acids of the JmjN domain and the entire ARID domain. Therefore, in frame splicing of the primary transcript from exons 1-5 results in the truncation of the JmjN domain (deletion of amino acids, Asp69, Trp70, Gln71, Pro72, Pro73) and complete deletion of the ARID domain. This mouse has therefore been termed the Jarid1b ΔARID strain.

Examination of levels of Jarid1b RNA by RTqPCR in the 18.5 day pregnant mammary gland indicated an increase in the level in the Jarid1b ΔARID mouse mammary gland (Fig. 3D), while levels of protein were reduced in comparison with the WT gland (Fig. 3E). The mice were viable and fertile showing no gross abnormalities in size, or differences in the morphology of internal organs and tissues, but the development of the mammary gland appeared to be affected. As this is difficult to quantitate on the mixed background, because of strain differences in profiles of development (31), for detailed examination of the morphology of the developing and differentiating mammary gland the mice were backcrossed on to a C57BL/6 background (6 backcrosses).

The Jarid1b ΔARID mammary gland presents with fewer terminal end buds and a defect in ductal elongation and side branching. To determine whether nulliparous mammary gland development was affected in the Jarid1b ΔARID mouse, thoracic mammary glands at 4 and 12 weeks were whole mounted and stained with carmine (see Materials and methods). Fig. 4A shows that the Jarid1b ΔARID nulliparous gland at 4 weeks of age presents with a less organised ductal system
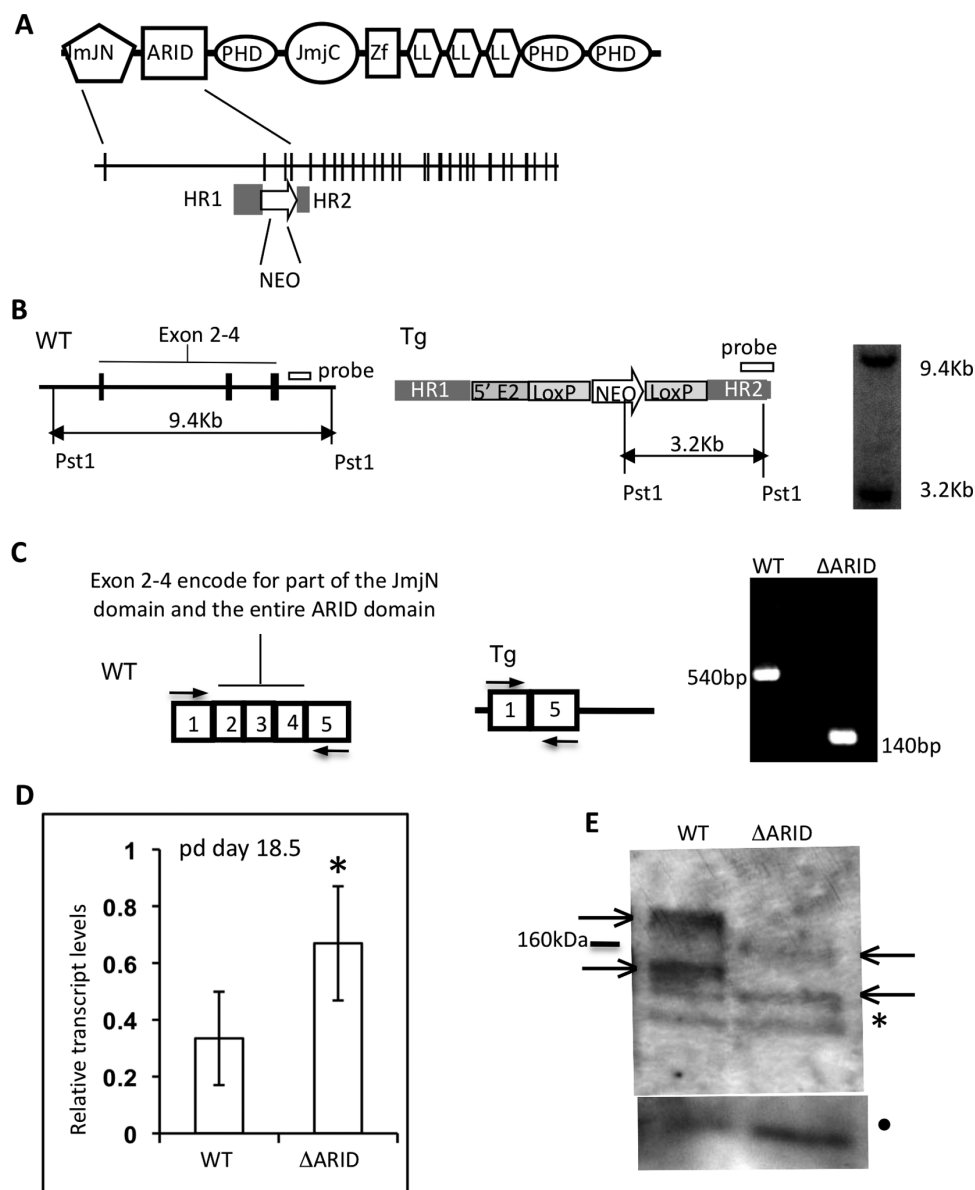
Figure 3. Generation of Jarid1b ΔARID construct and screening strategy used to detect homologous recombination. (A) Schematic representation of *Jarid1b* depicting Jarid1b protein domains, genomic organisation and the 5'/3' homologous regions (HR1 and HR2). (B) Enlarged schematic depicting the WT and transgenic allele after homologous recombination and the location of the Pst1 restriction sites used to distinguish the WT from the recombined allele. Southern blottig shows homologous recombination using sv129 ES cell genomic DNA and PstI digest. (C) Schematic representation of the WT mRNA and after aberrant splicing from exon 1 to 5 removing the neomycin sequences, showing PCR primers (arrows); also RT-PCR using cDNA from the mammary gland at day 18.5 of pregnancy demonstrating exon 1-5 splicing (confirmed by sequencing the PCR product, data not shown). (D) Quantitative RT-PCR using primers downstream of the ARID domain showing an increase in Jarid1b ΔARID transcript compared to WT at day 18.5 of pregnancy. (P<0.02. WT, n=4. ΔARID, n=5). (E) Western blotting of Jarid1b in nuclear extracts from the mammary gland from mice at day 18.5 of pregnancy (arrows), *non-specific signal; ˙Lamin B.

(Fig. 4Aii) than the wild-type gland (Fig. 4Ai), with on average 2.5-fold reduction in TEB number (Fig. 4A top histogram p<0.0005). This effect was also seen in the inguinal gland (data not shown). By adulthood (12 weeks), as compared to the WT gland (Fig. 4Aiii), the mammary gland of the Jarid1b ΔARID adult female (Fig. 4Aiv) had failed to fully ramify the fat pad, presented with fewer ductal bifurcation points (Fig. 4A bottom histogram p<0.005) and showed a distinct lack of secondary side branches trailing the primary ducts (Fig. 4Aiv). By 20 weeks of age the adult nulliparous mammary gland of the Jarid1b ΔARID female had ramified the fat pad but still presented with fewer Y branch points and a lack of ductal side branches (data not shown).

Examination of the morphology of the Jarid1b ΔARID mammary glands during pregnancy showed that a defect in side branching (resulting in the development of fewer alveoli) was still detectable at 12.5 days of pregnancy (compare Jarid1b ΔARID Fig. 4Biii and iv with WT 4Bi and ii). However by day 18.5 of pregnancy, the gross morphology of the Jarid1b mammary glands was comparable to that of the WT glands (compare Jarid1b ΔARID Fig. 4Bvii and and viii with WT 4Bv and vi), indicating recovery of the gland after the initial delay in development. Since the deletion of the ARID domain results in loss of demethylase activity of JARID1B, it is possible that this deficiency is responsible for the ΔARID mammary gland phenotype particularly if the loss is only partial, allowing
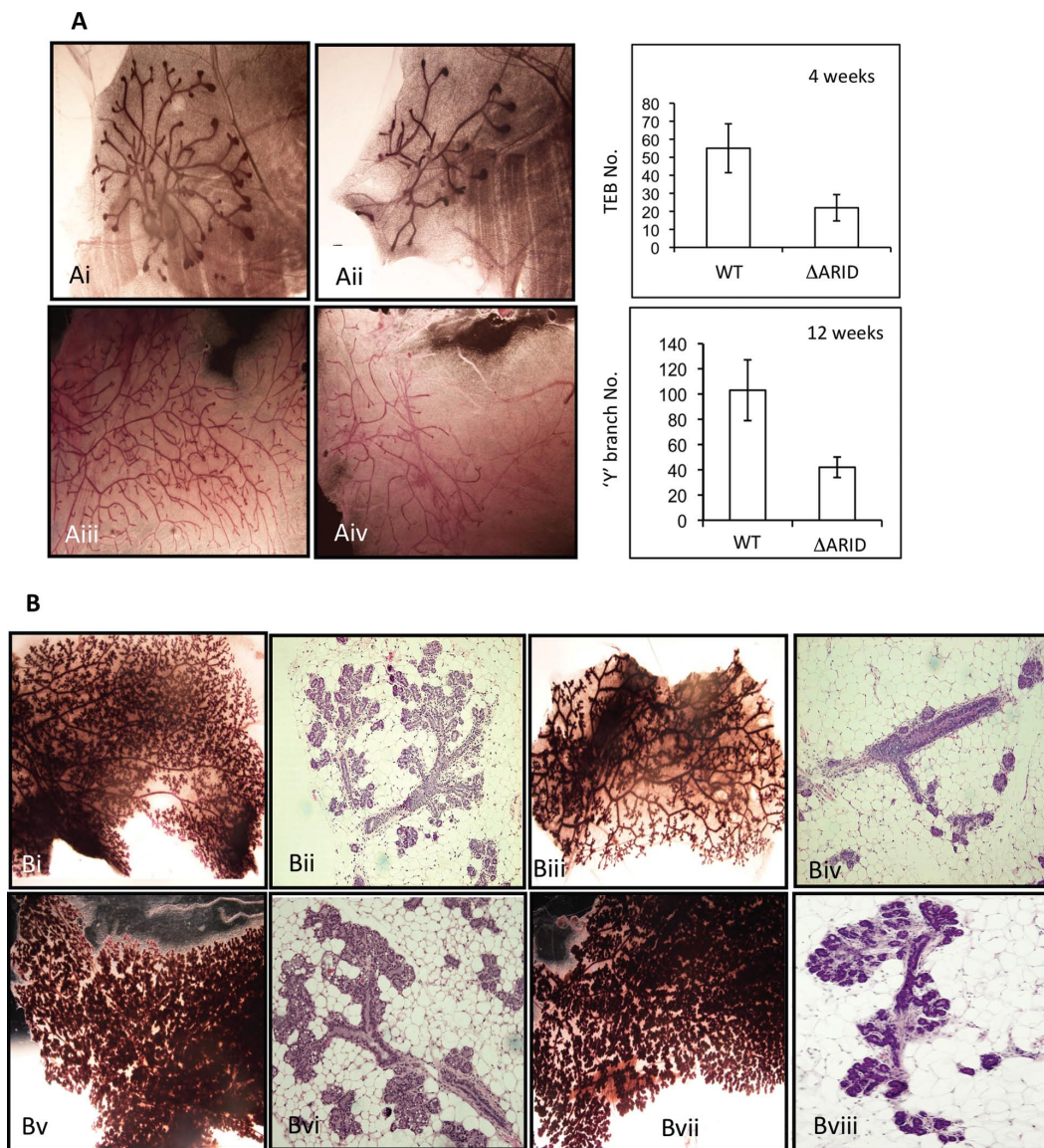
Figure 4. Abnormal mammary gland development in the C57BL/6 Jarid1b ΔARID female mice. (A) Delayed development of the mammary tree in the pubertal gland. Representative carmine staining of thoracic mammary glands of C57BL/6 WT and C57BL/6 Jarid1b ΔARID mice at 4 weeks (Ai and Aii respectively), and 12 weeks of age (Aiii and Aiv respectively). Quantification of the TEB number in the C57BL/6 nulliparous mammary glands at 4 weeks of age, (top histogram n=6 mice per group). Quantification of 'Y' branch points (bifurcation) in the 12-week old adult mammary gland (bottom histogram WT, n=3. ΔARID, n=4). (B) A delay in mammary gland development at mid-pregnancy is over come by late pregnancy. Representative carmine (i, iii, v and vii) and H&E staining (ii, iv, vi and viii) of C57BL/6 WT (i, ii, v and vi) and C57BL/6 Jarid1b ΔARID (iii, iv, vii, viii) thoracic mammary glands at day 12.5 (i-iv) and day 18.5 (v-viii) of pregnancy.
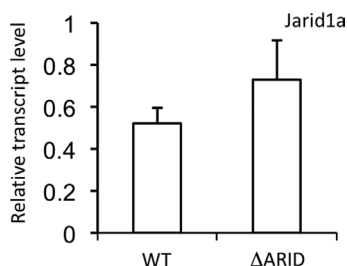

Figure 5. Levels of expression of Jarid1a are not significantly different in the ΔARID mouse. Quantitative RT-PCR showing relative transcript levels (see Materials and methods) of Jarid1a in the mammary gland of C57BL/6 WT (n=4) and Jarid1b ΔARID (n=4) mice at 18.5 days of pregnancy.

for recovery of the gland. The level of Jarid1a expression is not significantly increased (p=0.09) in ΔARID mammary

glands as compared to the WT (Fig. 5), suggesting that compensation for reduced demethylase activity is not occurring.

Nulliparous mammary gland ductal development is crucially dependent upon epithelial ERα signalling: in the ERα knock out mammary gland at 4 weeks of age, terminal end buds (TEB) were absent and no further ductal elongation had occurred by adulthood (28,29). Moreover, the ΔARID mammary phenotype is remarkably similar to that seen in the KO of a co-regulator of the ERα receptor CITEDI (32). A possible explanation for the ΔARID phenotype is that JARID1B interacts with the ERα signalling pathway, and the JARID1B ΔARID protein is less functional in this interaction. We therefore looked at the levels of expression of the ERα itself and of a downstream target, the progesterone receptor (PR), using RT qPCR with RNA from the 12-week nulliparous gland. While the levels of ERα did not appear to be altered in the Jarid1b ΔARID
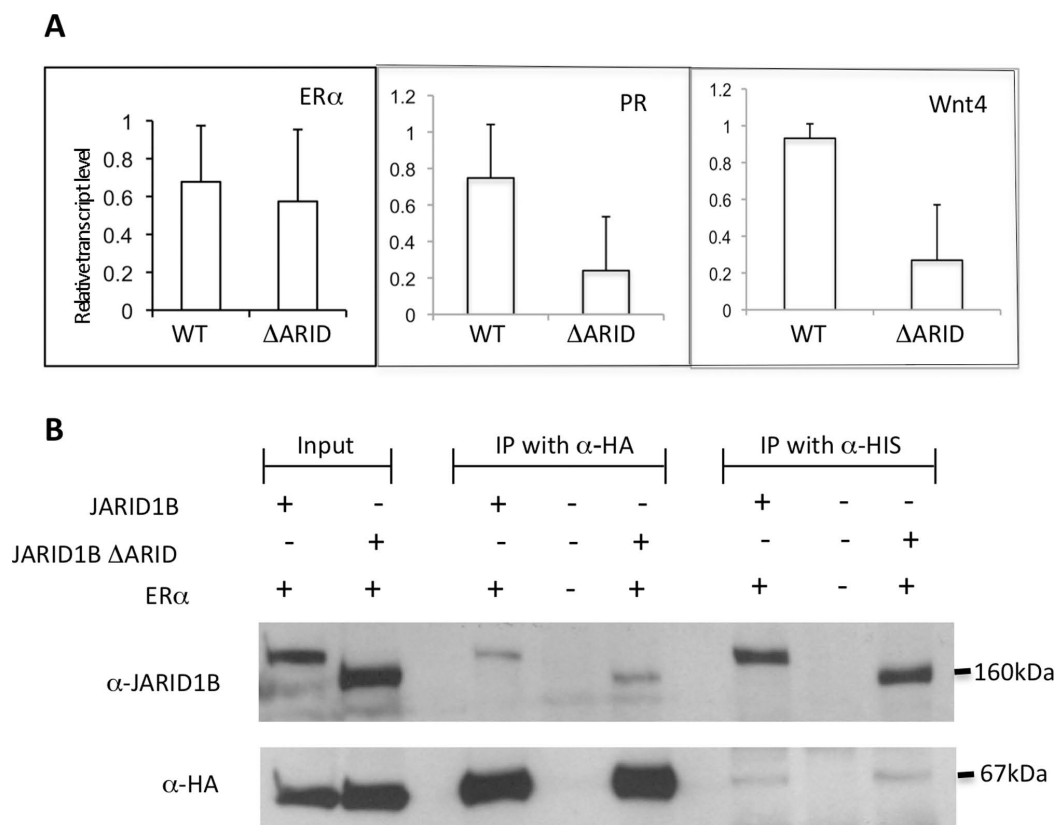
A

B

Figure 6. Jarid1b affects ERα signalling in the mammary gland and interacts with ERα. (A) Quantitative RT-PCR showing relative transcript levels (see Materials and methods) of *ERα, PR*, and *Wnt4* in the nulliparous mammary glands from C57BL/6 WT and Jarid1b ΔARID mice at 12 weeks of age (WT, n=3. ΔARID, n=4). One experiment representative of two is shown. (B) Myc His tagged JARID1B or ΔARID JARID1B and HA tagged ERα, transiently expressed in COS-7 cells were immunoprecipitated with anti-HA or anti-His and subjected to Western blotting using anti-JARID1B antiserum (a-PLU-1C) or anti-HA antibody.

mammary gland, levels of PR mRNA were consistently lower in the Jarid1b ΔARID gland, and almost reached significance (p=0.074), even though a relatively small number of mice were available for analysis. Moreover, the levels of expression of Wnt4 mRNA, a downstream target of PR were significantly (p<0.02) lower in the Jarid1b ΔARID mouse mammary gland (Fig. 6A).

JARID1B interacts with ERα. JARID1A has been shown to interact with ERα (26) and JARID1B with the androgen receptor (27). As shown in Fig. 6B, we now find that JARID1B can interact with ERα as demonstrated by co-precipitation of the two proteins in cells transfected with the tagged genes. Interestingly, interaction with ERα also occurs with the JARID1B ΔARID construct from Xiang *et al* (27). Since the ΔARID JARID1B protein binds to ERα, any impact of deleting the ΔARID domain would have to be on the function of the interacting complex. The reduced level of expression of the defective JARID1B protein (Fig. 3E) is unlikely to be responsible for the effect on mammary gland development, as the glands of the heterozygote mice from the embryonic lethal strain, where reduced levels of JARID1B are expressed, develop normally (data not shown).

Effect of depletion of JARID1B expression on estrogen-dependent growth of MCF-7 cells grown as tumours in nude mice. JARID1B is preferentially expressed in ER+ breast cancers and breast cancer cell lines (16,17,19). To see if JARID1B is involved

in ERα stimulated growth of ER+ cells, we used shRNAi to look at the effect of knocking down expression of wild-type JARID1B on the growth of MCF-7 cells (22) grown as tumours in nude mice. Two MCF-7 lines, where expression of JARID1B was inhibited by shRNA knock-down (clones 4 and 29) and two lines transfected only with empty vector (pSUP mix and clone 1) were used for these experiments.

MCF-7 cells do not form tumours in nude mice without estrogen stimulation, and for analysis of estrogen-dependent growth of the cell lines *in vivo*, estrogen pellets were implanted into the mice 48 h before subcutaneous injection of cells in matrigel. Fig. 7AI shows the growth of shRNA knock-down clone 4 compared to two lines transfected with only the vector (pSUP 1 and pSUP mix) injected at 1x10⁷ cells. Fig. 7AII and AIII representing two different experiments, showing the growth of two RNAi clones and control clone pSUP 1 injected at a cell number of 1x10⁶. Clearly the rate of tumour growth is dramatically faster when JARID1B is expressed with both cell concentrations (Fig. 7AI) shRNAi clone 4 against pSUP 1 and pSUP mix p<0.005 and p<0.02 respectively; Fig. 7AII and AIII, pSUP1 against RNAi clone 4 and clone 29 p<0.005 and p<0.002 respectively).

Discussion

Our studies seeking to understand the function of JARID1B using a mouse model and human cell lines have shown that expression of the protein is required for embryonic survival
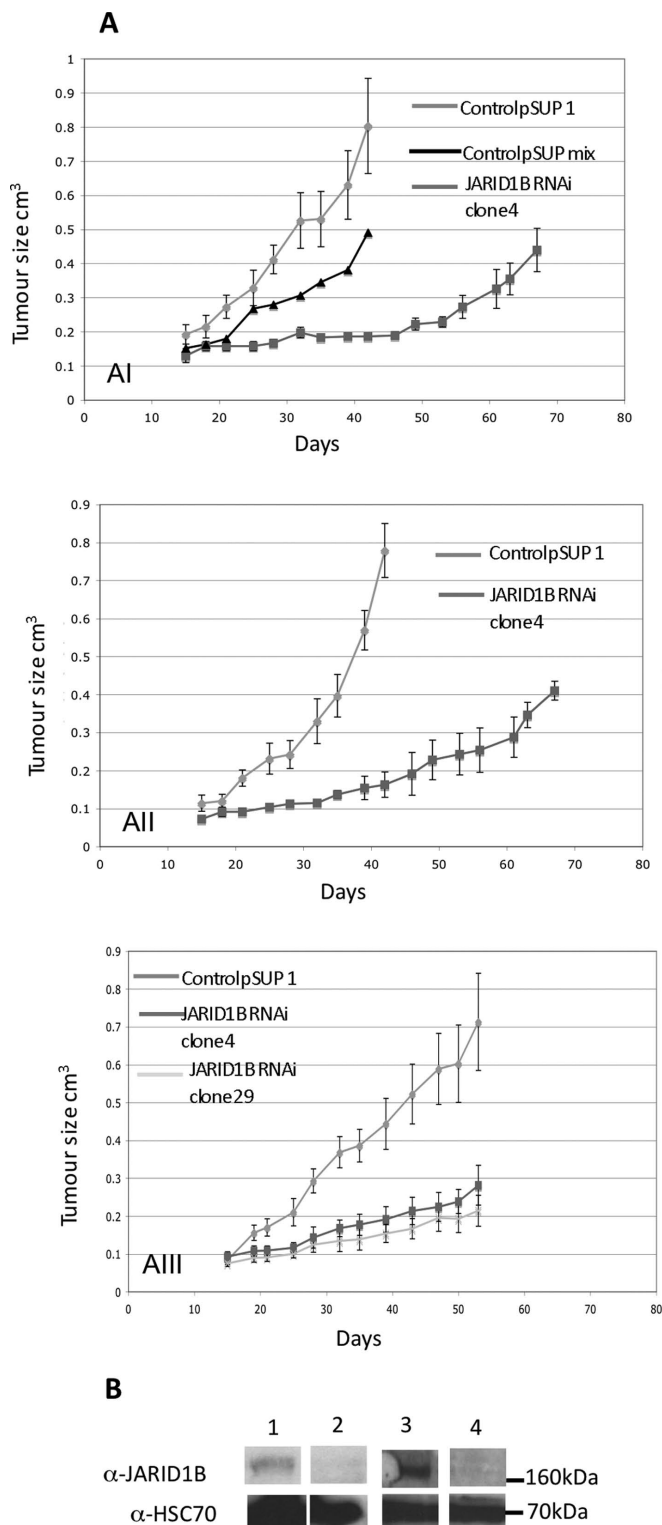
Figure 7. JARID1B enhances E2 dependent tumour growth of MCF-7 cells. (A) MCF-7 cells lines transfected with vector alone [control pSUP1, control pSUP mix) or with JARID1B shRNA (RNAi clone 4, RNAi clone 29 (22)] were subcutaneously injected, in matrigel, into nude mice pre-treated with estrogen pellets. (AI) Indicated cells were inoculated at a cell number of $1x10^7$. (AII and AIII) The results of two independent experiments are shown with mice injected with $1x10^6$ cells. (B) Western blotting with anti-JARID1B (a-PLU-1 C) of: 1, control pSUP mix; 2, JARID1B knock-down clone 4; 3, control pSUP1; 4, JARID1B knock-down clone 29.

and that in the mammary gland, and in breast cancer this nuclear protein can affect cell growth and ERα signalling.

Analysis of the offspring and embryos in the systemic knock out in the mouse showed that no Jarid1b^{-/-} offspring or E7.5 embryos were produced and Jarid1b^{-/-} embryo development was arrested after E4.5, highlighting the requirement of Jarid1b for implantation and/or developmental stages immediately following implantation. The embryonic lethality is in contrast to the result of knocking out the close family member Jarid1a, where mice are viable and fertile but later show defects in the hematopoietic system (8). Clearly Jarid1a or the other two Jarid1 proteins cannot compensate for loss of expression of Jarid1b in embryonic development. Deletion of expression of Jarid1 in lower organisms, where a single gene is operative, can be lethal as with the Drosphila Lid (10), while in C. elegans effects on vulva development are seen (6). Interestingly, deletion of another H3K4 demethylase Lsd1, which can demethylate H3K4me2, also results in embryonic lethality in the mouse. Development was arrested at the same time as seen with the Jarid1b^{-/-} embryos, i.e., before E5.5, and embryos were reabsorbed by 7.5 days (33,34). The data agree with the observation that Jarid1b is strongly expressed in the epiblast of normal E5.5 embryos where uncommitted cells choose between proliferation and differentiation (35). Moreover, the fact that attempts to develop Jarid1b^{-/-}ES cells have been unsuccessful correlates with studies showing that mouse embryo ES cells do not survive upon abolishing Jarid1b expression by shRNA (36).

The viability of the Jarid1b ΔARID mouse strain is somewhat surprising since deletion of the ARID domain is reported to result in loss of demethylase activity (5,27). However, the assessment using immunofluorescence to detect changes in H3K4me3 in cells transfected with the mutant cDNA is not quantitative and a small level of residual activity may be present. If Jarid1b demethylase activity is required for embryonic survival past E4.5, this must be specific to Jarid1b as the other members of the family do not compensate in the systemic KO embryos. This specific requirement is likely to depend on another function-specific to Jarid1b, involving the 3' sequences, where the main differences in sequence with the other JARID1 proteins are seen (16), and on which protein interactions involved in recruitment to specific chromatin domains may depend.

We have previously shown that Jarid1b is expressed in spermatagonia and at specific stages of meiosis (pachytene and diplotene) in the testis (37). However, both male and female Jarid1b ΔARID^{-/-} mice are fertile. Again either the demethylase function of Jarid1b is not required for the development of the male gametes, or a residual activity is present, or activity can be assumed by another H3K4me3 demethylase. Jarid1d (38) is also expressed in the same stages of meiosis as Jarid1b, but not in spermatagonia (39). In C. elegans, the loss of function of the homologue (spr5) of the LSD1 demethylase leads to the development of sterility over many generations (40). We observed no such effect over 10 generations in the ΔARID mouse.

The studies in the Jarid1b ΔARID adult mouse are consistent with the hypothesis that Jarid1B plays a role in the development of the normal mammary gland at a stage where signalling from the ERα receptor is vital for expansion of the mammary tree. The ERα knock out mammary gland shows only a rudimentary ductal system, which does not progress (28) even

when ERα expression is specifically ablated in the mammary epithelial cells (29). The Jarid1b ΔARID mouse shows a delay in the development of the gland at puberty and early pregnancy, and the obvious reduction in end bud development at 4 weeks suggested that signalling from the ERα receptor might involve an interaction with JARID1B that is impaired in the JARID1B ΔARID mammary glands. The reduced levels of the downstream target PR and its downstream target Wnt 4 (41,42) support this idea. It is also interesting to note that a very similar mammary phenotype is seen in a mouse where the expression of the ERα co-regulator CITED1 has been ablated (32). As previously shown for the JARID1A protein (26) we found that an interaction of JARID1b with ERα could be detected by co-precipitation. Interestingly, interaction was also seen with the JARID1B ΔARID construct supporting the idea that deletion of the ARID domain affects the downstream function.

A highly significant association of JARID1B with ER$^+$ cancers is found on analysing microarray data available in the public database (19), and expression of JARID1B is high in ER$^+$ cell lines (16,43). Clearly interaction of JARID1B with ERα could have profound effect on the growth of ER$^+$ cells. That this is indeed the case was demonstrated by finding a pronounced reduction in E2-dependent tumour growth of the ER$^+$ MCF-7 breast cancer cell line when JARID1B expression was silenced by shRNAi.

The function of H3K4 demethylases as activators of transcription is dependent on interactions with specific transcription factors. It has been shown that LSD1, when interacting with AR, can also demethylate H3K9me3, which is normally a repressive mark (44), and this dependence on interacting proteins for substrate specificity could be a general phenomenon. Interaction of the JmjC domain with dmyc in *Drosophila* inactivates the demethylase activity and Lid then becomes involved in activation of myc targeted genes (10). Clearly, the composition of the complexes recruited is crucial to determining the function of the JARID1 proteins and other demethylases (45). JARID1A is a component of the MRG15 complex, when it is involved in activation of transcription, and it is proposed that JARID1A functions to maintain reduced H3K4 methylation ensuring the transcriptional elongation rate (46). Many complexes contain both methylases and demethylases together with components that may be cell specific. JARID1B is found in a complex purified form MCF-7 cells which also contains the H3K9 and H327 methylases G9a and EZH2, and the LSD1 demethylase (47). The formation of a specific complex can also depend on external signals, as with estrogen stimulation of cell growth, making it important to work within the appropriate cell (ER$^+$) and growth environment.

Taken together, our data indicate that JARID1B function is crucial for embryonic development after E4.5, contributes to the differentiation orientated proliferation seen in the normal mammary gland, and to the proliferation of ER$^+$ breast cancer cells. Clearly it will now be important to identify on a global scale, genes which are regulated by ERα and which also require the involvement of JARID1B, and to define complex(es) containing both components. Investigation of the effect of JARID1B expression on the H3K4/H3K9/H327 methylation status of chromatin in different mammary phenotypes, could contribute to the definition of the epigenetic profile of stem and progenitor cells (21). It will also be important to characterise the novel function of JARID1B that is required for embryonic development after E4.5, and for survival in cultured ES cells.

Acknowledgements

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