JMJD6 Regulates ERα Methylation on Arginine

Coralie Poulard1,2,3,4,5, Juliette Rambaud1,2,3,4,5, Nader Hussein6, Laura Corbo1,2,3,4,5, Muriel Le Romancer1,2,3,4,5*

1 Université de Lyon, F-69000 Lyon, France, 2 Université Lyon 1, F-69000 Lyon, France, 3 Inserm U1052, Centre de Recherche en Cancérologie de Lyon, F-69000 Lyon, France, 4 CNRS UMR5286, Centre de Recherche en Cancérologie de Lyon, F-69000 Lyon, France, 5 Equipe Labellisée « La Ligue », 6 Lebanese University, Faculty of Sciences, Doctoral School of Sciences and Technology, PRASE, Hadath, Lebanon

Abstract

ERα functions are tightly controlled by numerous post-translational modifications including arginine methylation, which is required to mediate the extranuclear functions of the receptor. We report that upon oestrogenic stimulation, JMJD6, the only arginine demethylase described so far, interacts with and regulates methylated ERα (metERα) function. Moreover, by combining the silencing of JMJD6 with demethylation assays, we show that metERα is a new substrate for JMJD6. We propose that the demethylase activity of JMJD6 is a decisive regulator of the rapid physiological responses to oestrogen.

Citation: Poulard C, Rambaud J, Hussein N, Corbo L, Le Romancer M (2014) JMJD6 Regulates ERα Methylation on Arginine. PLoS ONE 9(2): e87982. doi:10.1371/journal.pone.0087982

Editor: Jean-Marc Vanacker, Institut de Génomique Fonctionnelle de Lyon, France

Received September 17, 2013; Accepted January 2, 2014; Published February 3, 2014

Copyright: © 2014 Poulard et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding for this study was provided by: Fondation Arc Cancer, Ligue Nationale Contre le Cancer, Fondation de France, and Ministère de la Recherche. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: muriel.leromancer@lyon.unicancer.fr

Introduction

Oestrogen (17β-oestradiol, E2) a member of the steroid hormone family, plays a crucial role in many physiological processes and in disease, namely in breast cancer. The biological actions of oestrogens are mediated through ERα and ERβ, which function in the nucleus as ligand-dependent transcription factors promoting gene transcription and the stimulation of cell growth in various tissues, including breast epithelial cells [1,2].

In addition to these well-documented effects, oestrogens also activate multiple signal transduction cascades outside of the nucleus via nongenomic signalling. This nongenomic pathway involves growth factor-dependent kinases and adaptor proteins leading to downstream activation of signalling molecules, such as MAPK and Akt [3–6]. Cellular responses to oestrogens are highly controlled and require the regulation of ERα function through numerous post-translational modifications that regulate both genomic and nongenomic pathways [For a review, [7]]. Most nongenomic effects of oestrogen are mediated through the recruitment of the tyrosine kinase Src and PI3K [3,4]. Our team has contributed to the understanding of this pathway by demonstrating that arginine methylation of the receptor is prerequisite to oestrogen-induced formation of the ERα/Src/PI3K complex which activates Akt [8]. Recently, we also showed that this pathway is activated in aggressive breast tumours and could constitute a new potential target for therapy [9].

Our finding of ERα methylation being a dynamic process strongly suggests the involvement of an enzyme that reverses this methylation. We therefore investigated if the only arginine demethylase identified so far, JMJD6, plays a role in this process. The enzymatic activity of this protein was described by Buick’s team on histones displaying asymmetrical as well as symmetrical demethylation on arginine residues [10]. Indeed, three types of arginine methylation exist in mammalian cells: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). PRMT1 is the major Type 1 arginine methyltransferase that adds the ADMA mark and PRMT5 is the principal Type 2 enzyme which catalyzes SDMA [11].

Recent publications have shown that JMJD6 also possesses lysyl hydroxylase activity [12–14].

In this paper, we demonstrate that JMJD6 demethylates the ERα methylated on R260, thereby regulating oestrogen nongenomic signalling. Moreover, global approaches suggest that JMJD6 regulates other arginine methylated-proteins and further studies should be done to validate this point, based on the data obtained by mass spectrometry.

Results and Discussion

JMJD6 Specifically Interacts with the Methylated Form of ERα

To investigate a functional link between JMJD6 and ERα, we firstly analysed whether the two proteins could interact. GST pull-down assays indicated that JMJD6 directly associates with ERα (Figure 1A) specifically within the hinge domain (Figure S1 A and B). To assess if JMJD6 interacts in vitro with metERα, we performed competition experiments adding the peptide containing R260 methylated or not within the reaction, however, it does not modify the interaction (Figure S2). Next, we wished to validate the binding between endogenous JMJD6 and ERα. Because ERα is rapidly methylated after E2 treatment, we performed reciprocal immunoprecipitation on MCF-7 cells treated with E2 for the indicated times (Figure 1B and Figure S3). Oestrogen treatment induced a rapid and transient interaction between ERα and JMJD6. Interestingly, the kinetics of interaction were reminiscent of those previously described for ERα methylation on R260 [8]. However, while the ERα methylation may vary between five and
fifteen minutes after estrogen treatment along the experiments, the kinetics of interaction of ERα with JMJ6 were concomitant with the kinetic of ERα methylation, suggesting that JMJ6 could specifically interact with methylated ERα (metERα). Several additional findings supported this hypothesis: i) siRNA-mediated reduction of the arginine methyltransferase PRMT1 responsible for ERα methylation, impaired both ERα methylation and the ERα/JMJ6 interaction (Figure 1C); ii) depletion of metERα by...
Figure 2. JMJD6 interacts with ERα within the complex ERα/Src/PI3K. (A) ERα, Src and PI3K coimmunoprecipitate with JMJD6. Lysates of MCF-7 cells, control cells (shCtrl), and JMJD6 knockdown cells (shJMJD6) were immunoprecipitated with JMJD6 antibody. The immunoprecipitates were then blotted with antibodies against PI3K (p85), Src, ERα and JMJD6. The amount of ERα, Src, p85 (PI3K) and GAPDH in the different samples was determined by western blot. (B) Detection of endogenous interaction between JMJD6 and ERα by PLA. MCF-7 cells were incubated with E2 10^{-7} M for 5 min. After fixation, in situ PLA for JMJD6/ERα was performed using antibodies raised against JMJD6 and ERα (panels a,b). The detected dimers are represented by red dots. The nuclei were counterstained with DAPI (blue) (Obj: X63). The lower panel shows
immunoprecipitation using an antibody specifically recognizing the ER\(\alpha\) methylated on R260, also disrupted the ER\(\alpha\)/JMJD6 interaction (Figure 1D); and finally, iii) analysis in other breast cancer cell lines showed that the interaction of ER\(\alpha\) with JMJD6 was restricted to cells expressing metER\(\alpha\), reinforcing the idea that JMJD6 interacts specifically with metER\(\alpha\) (Figure S4).

Altogether these data clearly establish that contrarily to in vitro results showing the interaction between JMJD6 and ER\(\alpha\) was estrogen independent, in cell lines JMJD6 interacts specifically with metER\(\alpha\) upon E\(_2\) stimulation.

**JMJD6 Interacts with metER\(\alpha\) within the Complex metER\(\alpha\)/Src/PI3K**

The methylation of ER\(\alpha\) is a critical upstream signal, required to mediate the interaction of the receptor with Src and the p85 subunit of PI3K, which propagates the signal to downstream transduction cascades responsible for orchestrating cell proliferation and survival. Disappearance of the methylated ER\(\alpha\) occurs concomitantly with the dissociation of the complex, leading to the extinction of downstream kinase activation [8].

In view of these earlier findings, we wondered whether JMJD6 might participate towards regulating the E\(_2\) nongenomic signaling. To test this hypothesis, we performed co-immunoprecipitation experiments on E\(_2\)-treated cells. We found that JMJD6 co-precipitated ER\(\alpha\) with Src and PI3K. In addition, these interactions were clearly reduced in cells in which JMJD6 had been invalidated (shJMJD6) (Figure 2A). Interestingly, the kinetics of interactions were concomitant with the kinetics of ER\(\alpha\) methylation suggesting that the JMJD6/metER\(\alpha\) interaction occurs when the receptor is in complex with Src and PI3K (Figure 2A). We then examined in which compartment this interaction occurred by Proximity Ligation Assay (PLA). Figure 2B shows that JMJD6 interacted with ER\(\alpha\) (panel a) in the cytoplasm of MCF-7 cells, as indicated by the presence of red dots. The signals strongly decreased in MCF-7 cells in which expression of JMJD6 was downregulated (Figure 2B, panel b). The interactions were quantified by counting the number of dots per cell (Figure 2B, lower panel). Moreover, knockdown of PRMT1 strongly decreased JMJD6/ER\(\alpha\) interaction analyzed by PLA (Figure 2C), supporting that JMJD6 specifically interacts with ER\(\alpha\) which is localized in the cytoplasm of the cells [8]. The recruitment of JMJD6 seemed to be mainly mediated by ER\(\alpha\) since JMJD6 could only weakly interact or not at all with Src or p85 of PI3K (Figure S5A and B). However, we cannot exclude that other cofactors are important to recruit JMJD6 within the complex.

To assess whether JMJD6 affects the methylation of ER\(\alpha\) on R260, we used JMJD6-knocked down cells (shJMJD6). After E\(_2\) treatment, ER\(\alpha\) methylation was strongly increased in shJMJD6 cells compared with in control cells (Figure 2D). However, ER\(\alpha\) methylation still decreased after 30 min of E\(_2\) treatment probably due to the residual presence of JMJD6. Of note, this phenomenon occurred concomitantly with an increase in metER\(\alpha\)/Src/PI3K complex (Figure 2E).

Taken together, these data strongly suggest that JMJD6, by regulating the methylation of ER\(\alpha\), could play a role in the oestrogen rapid signalling pathway.

**JMJD6 Preferentially Demethylates Asymmetric Dimethylation on Arginine Residues**

In light of our finding that JMJD6 binds to metER\(\alpha\) and regulates its methylation, we wondered whether JMJD6 could demethylate ER\(\alpha\) in vitro. We performed a demethylation assay using recombinant JMJD6 on ER\(\alpha\) methylated in vitro by PRMT1 as already described [8]. Using a specific antibody directed against ER\(\alpha\) asymmetrically methylated on R260 (Figure 3A left upper panel), we found that JMJD6 reduced the methylation of ER\(\alpha\) R260 (Figure 3A, right panel). We obtained the same demethylation results when we used [\(^3\)H]. AdoMet for the methylation assay and the methylation was visualized by autoradiography excluding possible artefactual antibody detection (Figure 3B).

However, as described by Bruick, its enzymatic activity remained faint, probably due to the need of a coactivator to potentiate its activity, as has been described for PRMT5 [15,16] and for the lysine demethylase LSD1 [17]. In the same type of assay, JMJD6 was not able to demethylate the peptide of ER\(\alpha\) containing monomethylated K302 (K302me1) [18] (Figure 3A, lower panel), thus highlighting the specificity of the catalytic activity of the recombinant protein.

Considering that histone H4 at arginine 3 (H4R3) is a substrate of JMJD6 [10] and that JMJD6 is able to demethylate ER\(\alpha\) in vitro as shown above, we tested whether JMJD6 can remove dimethylation from histone H4 methylated in vitro by recombinant PRMT1. As shown in Figure 3C, JMJD6 was indeed able to decrease H4R3 ADMA in vitro. These results support the role of JMJD6 in the demethylation of the ADMA R3 residue on histone H4.

After validation of H4R3me2a antibody (Figure S6), concordant results were obtained in our in vivo test in which, strikingly, knockdown of JMJD6 significantly increased H4R3me2a, detected by a specific antibody, when compared with the control cells (Figure, 3D, compare lane 2 and 3 to lane 1), while the histone H4 level remained unchanged. Again, immunofluorescence analysis showed an enhanced staining of H4R3me2a in the nuclei of knocked down cells compared to control (Figure 3E).

To provide further evidence for the demethylating function of JMJD6, we used two complementary approaches. Firstly, we analysed the global ADMA of total proteins in MCF-7 cells depleted for JMJD6. Cell lysates of cells invalidated for JMJD6 with three different shRNAs were immunoblotted with an antibody recognizing specifically ADMA (meRG). As shown in Figure 3F, knockdown of JMJD6 had a strong effect on the amount of dimethylated proteins (compare lanes 2–4 to lane 1). We confirmed these results with another antibody recognizing ADMA (ASYM24). However, the same cell extracts analyzed with an antibody directed against SDMA did not show any difference in the amount of methylated proteins, suggesting that JMJD6 may have more affinity for target asymmetric dimethylation (Figure 3E).
Figure 3. JMJD6 demethylates asymmetric dimethylation on arginine residues. (A) GST-ERα fragment (251–305) was methylated by PRMT1 in the presence or in absence of cold methyl donor, SAM. Then, a demethylation assay was performed adding GST (−) or GST-JMJD6 (+). Western blot was performed with the anti-ERαR260me2 antibody and an anti-ERα antibody recognizing the hinge domain (upper panel). Under the same conditions, demethylation was assessed on an ERα peptide methylated on K302 and recognized by the antibody anti- ERα K302me1. The lower panel shows the relative quantification of ERαR260me2/ERα and of ERαK302me1/ERα. (B) Hinge ERα was incubated with GST-PRMT1 in the presence of [methyl-3H] SAM. A demethylation assay was then performed in the presence of GST-JMJD6 or GST. Reaction products were analysed by SDS-PAGE followed by fluorography. The coomassie staining of hinge ERα is also shown. The lower panel shows the relative quantification of metERα/ERα. (C) The experiment of demethylation was performed for H4 methylation as in B (D) Acid extracted-histones from MCF-7 cells, control cells (shCtrl) and JMJD6 Regulates ERα Methylation
JMJ6 knockdown cells (shJMJ6A and B) were analysed by western blot with an antibody recognizing specifically the asymmetric methylation of arginine 3 of Histone H4 anti-H4R3me2a. Control western blotting was performed with anti-histone H4. The lower panel shows the relative quantification of H4R3me2a/H4. E) H4R3 dimethylation was also analysed by immunofluorescence in the same cells as described above (panel a,b). DAPI staining is shown in panels c and d. (F) Lysates of control and shJMJ6 cells were analysed by western blotting with antibodies recognizing asymmetric dimethylation (MeRG and ASYM24) and symmetric dimethylation (SYM10). Controls were performed with anti PRMT1, JMJ6 and GAPDH antibodies.

doi:10.1371/journal.pone.0087982.g003

Of note, the overexpression of JMJ6 reduced global asymmetrical dimethylation assessed with ASYM24 antibody (Figure S7).

Next, we identified putative JMJ6 partners by immunoprecipitation of MCF-7 transfected with JMJ6, coupled to mass spectrometry analysis. In agreement with data in the literature [14,19,20] most of the identified proteins were RNA binding proteins implicated in RNA processing. Interestingly, 23/25 proteins identified have been shown to be methylated on arginine residues (Table 1). Of interest, one of the identified putative partners of JMJ6 was the arginine methyltransferase CARM1, adding further evidence for its role in ADMA regulation. This interaction has been validated by immunoprecipitation (Figure S8).

Altogether, these data support the notion that JMJ6 has a dual enzymatic role, as arginine demethylase and hydroxylase, and participate in various regulation pathways. Significantly, our results have shown that JMJ6 may function as an arginine demethylase for histone and, for the first time, for a non-histone target. Specifically JMJ6 negatively regulates ERα nongenomic signalling. After E2 treatment the methylation of ERα at R260 is crucial to propagate the signal to downstream transduction cascades. We therefore propose that the demethylase activity of JMJ6 is a decisive regulator of the rapid physiological responses to oestrogen, inducing the extinction of downstream kinases activation. Knowing that JMJ6 has been demonstrated to be a factor of poor prognosis in breast cancer [21], we could hypothesize that its demethylase activity could be involved in this effect maybe in part by regulating metERα/Src/PI3K/Akt signalling.

Materials and Methods

Plasmids and GST-fusion Proteins

GST-parallel-JMJD6 was a gift from Bruck [10]. GST-PRMT1, GST-ER176-302, GST-ER297-595, GST-ER176-251, GST-ER251-305 and GST-ER251-305 were produced as described [22].

Table 1. Potential interacting proteins of JMJ6 identified by affinity purification and mass spectrometry.

| Protein Name | Accession No | Process | DMA | MMA |
|--------------|--------------|---------|-----|-----|
| hnRNP U-like protein 1 | Q9BU2 | mRNA processing | DMA, MMA [24] |
| hnRNP K | P61978 | mRNA processing | DMA, MMA [24,25] |
| hnRNP L | P14866 | mRNA processing | MMA [26] |
| hnRNP Q | O60506 | mRNA processing | DMA, MMA [26] |
| U1 snRNP 70 | P08621 | mRNA processing | MMA [26] |
| RNA helicase (DDX3) | O00571 | mRNA processing | DMA, MMA [26] |
| RNA helicase (DDX5) | P17844 | mRNA processing | DMA, MMA [24] |
| RNA helicase (DDX17) | Q92841 | mRNA processing | MMA [26] |
| Paraspeckle protein 2 (PSP2) | Q96PK6 | mRNA processing | MMA [26] |
| PTB-associated-splicing factor (SFPQ) | P23246 | mRNA processing | DMA, MMA [24,27] |
| Non-POU domain-containing octamer-binding protein | Q15233 | mRNA processing | MMA [26] |
| RNA-binding protein (FUS) | P35637 | mRNA processing | DMA, MMA [24,27] |
| RNA-binding protein (EWS) | Q01844 | mRNA processing | DMA, MMA [28,29] |
| Splicing factor HCC1 | Q14498 | mRNA processing | DMA, MMA [24] |
| Poly(U)-binding-splicing factor (PUF60) | Q9UHX1 | mRNA processing | |
| Paraspeckle component 1 (PSPC1) | Q8WXF1 | transcription | DMA, MMA [24] |
| TATA-BP-associated factor 2N TAF15 | Q92804 | transcription | DMA, MMA [24,27] |
| Arg-methyltransferase (CARM1) | Q86X55 | transcription | DMA, MMA [30] |
| Poly(A)-binding protein 1 (PABP1) | P11940 | translation | DMA, MMA [24] |
| Poly(A)-binding protein 4 (PABP4) | Q13310 | Translation | DMA, MMA [27] |
| Ras GTPase-activating BP1 | Q13283 | translation | DMA, MMA [24] |
| SMARCD2 | Q92925 | Chromatin structure | DMA [26] |
| nucleolin | P19338 | mRNA processing/nucleolus | DMA [31] |
| Protein transport protein Sec23B | Q15437 | Endosome trafficking | |
| TFG | Q92734 | unknown | DMA, MMA [24] |

MMA: monomethylarginine. DMA: dimethylarginine. The bold characters highlight arginine methylated proteins.

*indicate proteins already identified as JMJ6 putative partners [14].

doi:10.1371/journal.pone.0087982.t001
Cell Culture and Transfections

MCF-7, ZR75-1 and Cama-1 mammary cells were maintained at 37°C in the appropriate medium supplemented with 10% foetal calf serum 1% non-essential amino acids. Prior to experiments, cells were grown for 48 hr in phenol red-free medium and 10% charcoal-treated serum (Biowest). MCF-7, ZR75-1 and Cama-1 cells come from ATCC. MCF-7 and ZR75-1 have been certified by CelluloNet, Lyon, France.

The siRNA sequences targeting PRMT1 correspond to the coding regions 650–668 and have already been described [8]. 50 nM of PRMT1 specific siRNAs or the scrambled siRNA (Eurogentec) were transfected into MCF-7 cells (1×10^6) using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s guidelines. 72 hr after transfection, cells were lysed in RIPA buffer containing 50 mM Tris HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.25% deoxycholate.

Antibodies

See Table 2.

JMJD6 Stable Knockdown Using Lentiviral Short Hairpin RNA

Three pre-made lentiviral short hairpin RNA (shRNA) constructs targeting human JMJD6 and one negative control construct created in the same vector backbone (pLKO.1-Puro) were purchased from SIGMA. Puromycin selection (1μg/mL) was started 48 h after lentiviral infection.

GST-pull-down Assay

Psg5-ERα and pCDNA3-JMJD6 plasmid were transcribed and translated in vitro using T7-coupled reticulocyte lysate (Promega), in the presence of [35S]methionine. Labelled proteins were incubated with 10 μg of purified recombinant GST- fusion proteins in 200 μl of binding buffer (Tris 20 mM pH 7.4, NaCl 0.1 M, EDTA 1 mM, glycerol 10%, Igepal 0.25%) with 1 mM DTT and 1% milk for 2 h at room temperature. In competition experiments, peptide containing R260 of 1μM of ERα methylated or not was added to the reactions [8]. Beads were washed three times in binding buffer, and then bound proteins were resolved on SDS-polyacrylamide gel electrophoresis (PAGE), and visualized by autoradiography.

Affinity Purification and Mass Spectrometry

MCF-7 cells were transiently transfected with V5-tagged JMJD6. Cell extracts were purified with agarose beads coupled to V5 antibody and loaded on SDS-PAGE. After elution, proteins were digested by trypsin and peptides were analysed by LC/MS/MS at IBCP, Lyon.

Immunoprecipitation and Western Blotting

To study the effect of oestrogen, the cells were treated for different times with E2 (Sigma). After treatment, cells were lysed in RIPA buffer. Protein extracts were incubated with primary antibodies overnight at 4°C with shaking. Protein A-agarose or protein L-agarose beads were added and incubated 1 h at 4°C. The immunoprecipitates were separated on SDS-PAGE. The proteins were visualized by an enhanced chemiluminescence kit (Roche Molecular Biochemicals) following the manufacturer’s instructions.

For histone extraction, cells were lysed in a solution containing 3% of SDS and 10% of β-mercaptoethanol for 2 min at 95°C, then cooled in ice. After addition of DNAase, the samples were loaded on SDS-PAGE.

Immunoblot images were digitized and quantified using the Image J Software.

Methylation and Demethylation Assays

The in vitro methylation protocol has been described previously [8]. The demethylation assay was performed as described for methylated histones [10]. We also used GST-hinge-ERα or histone H4 methylated by PRMT1 and purified on microspin columns to remove S adenosylmethionine (SAM) or a peptide corresponding to amino acids 297–308 non methylated or monomethylated on K302 [18]. Some experiments were per-

| Table 2. List of antibodies and their application. |
|-----------------------------------------------|
| **Antibodies**         | **Species** | **Company**     | **Dilution** | **WB** | **Dilution** | **IF/PLA** |
|------------------------|------------|----------------|--------------|--------|--------------|------------|
| GAPDH (H86504M)        | Mouse      | BD International | 1/25000      |        |              |            |
| ER (04-820)            | Rabbit     | Millipore      | 1/1000       |        |              |            |
| ER (sc-8005)           | Mouse      | Santa Cruz     | 1/1000       |        |              |            |
| ER (sc-544)            | Rabbit     | Santa Cruz     | 1/1000       |        |              |            |
| JMJD6 (ab10526)        | Rabbit     | ab10526 Abcam  | 1/1000       | 1/400  |              |            |
| JMJD6 (sc-28348)       | Mouse      | Santa Cruz     | 1/1000       |        |              |            |
| PRMT1, (24333)         | Rabbit     | Millipore      | 1/4000       |        |              |            |
| H4R3me2as (j39705)     | Rabbit     | Active motif    | 1/1000       | 1/200  |              |            |
| melFGR (j#000002)      | Rabbit     | CH2 Biosystems | 1/1000       |        |              |            |
| Asym24 (07-414)        | Rabbit     | Millipore      | 1/4000       |        |              |            |
| Sym10 (07-412)         | Rabbit     | Millipore      | 1/1000       |        |              |            |
| ERαR260me2 or metERα   | Mouse      | Home made      | 1/1000       |        |              |            |
| PI3K p85 (06-195)      | Rabbit     | Millipore      | 1/9000       |        |              |            |
| ERαK302me1             | Rabbit     | Gift from P. Vertino | 1/5000 |        |              |            |
| Src (sc-8056)          | Mouse      | Santa Cruz     | 1/1000       | 1/150  |              |            |
| Src (2109)             | Rabbit     | Cell Signaling Technology | 1/1000 |        |              |            |

doi:10.1371/journal.pone.0087982.t002
formed with [\(^3\)H]SAM and others with cold SAM. Then, GST-JMJD6 or GST was incubated at 37°C for 1 h and the reaction products were assayed by western blot analysis or by autoradiography.

**Proximity Ligation Assay**

This technology developed by Olink Bioscience (Sweden) allows the visualisation of protein/protein interactions *in vitro* and was first published in 2006 [23]. The experiments were performed following the manufacturer’s instructions and have been previously described [9].

**Image Acquisition and Analysis**

The hybridized fluorescent slides were viewed under a Leica DM6000B microscope. Images were acquired under identical conditions at objective X63. For each sample, at least one hundred cells were counted. Analyses and quantifications of these samples were performed using Image J software.

**Supporting Information**

**Figure S1** Identification of domains of ER\(\alpha\) interacting with JMJD6. A) The organization of ER\(\alpha\) protein showing the functional domains ER\(\alpha\) displays conserved functional domains. A/B including AF-1 (Activation Function 1), C containing the DBD (DNA binding domain), D called Hinge domain including nuclear localization signals, E containing the LBD (Ligand binding domain) and AF-2 (Activation Function 2) and F allowing agonist/ antagonist regulation. B) Radioactive JMJD6 (*) was incubated with GST and with the different domains of ER\(\alpha\) coupled with GST, and the bound proteins were visualized by autoradiography. The lower panel shows the coomassie staining of the gel. (DOC)

**Figure S2** Competition experiment of metER\(\alpha\) peptide with ER\(\alpha\)/GST-JMJD6 interaction. Radioactive ER\(\alpha\) (*) was incubated with GST or GST-JMJD6 in the presence or in the absence of the peptide containing metER\(\alpha\) [already described in (8)] and the bound proteins were visualized by autoradiography. The lower panel shows the coomassie staining of the gel. * indicates the different GST proteins. (DOC)

**Figure S3** JMJD6/ER\(\alpha\) interaction in MCF-7 cells. Immunoprecipitation was performed from E\(_2\)-treated MCF-7 cell extracts with anti-ER\(\alpha\) antibody and revealed with anti-ER\(\alpha\) and anti-JMJD6 antibodies. (DOC)

**Figure S4** JMJD6/ER\(\alpha\) interaction in human breast cancer cells. ZR75-1 (A), and Cama-1 (B) cells were analyzed for ER\(\alpha\) methylation and JMJD6/ER\(\alpha\) interaction. Immunoprecipitation of JMJD6 from extracts of estrogen-deprived cells (t = 0) stimulated with 10\(^{-8}\) M E\(_2\) for the indicated times was performed followed by western blotting with antibody against ER\(\alpha\) and JMJD6. On the same extract metER\(\alpha\) was analyzed by immunoprecipitation with the anti metER\(\alpha\) revealed with an anti-ER\(\alpha\). PRMT1 expression was also analyzed by western blotting. (DOC)

**Figure S5** JMJD6/Src and JMJD6/PI3K interaction in vitro. A) GST pull down assay of *in vitro* translated \(^35\)S-labeled Src or p85 (PI3K) (*) was incubated with GST and GST-JMJD6 and the bound proteins were visualized by autoradiography. Luciferase was used as a negative control. B) The same experiments were performed in the presence or in absence of *in vitro* translated cold ER\(\alpha\) to investigate if ER\(\alpha\) could be the bridge mediating the interactions. The lower panel shows the coomassie staining of the gel. * indicates the different GST proteins. (DOC)

**Figure S6** Validation of anti-H4R3me2a specificity. Extracts from MCF-7 cells transfected with scrambled siRNA or siRNA targeting PRMT1 were assessed by western blotting for Histone H4 methylation using the anti-H4R3me2a. Controls were performed using anti-histone H4 and anti-PRMT1 antibodies. (DOC)

**Figure S7** Role of JMJD6 on global arginine methylation. MCF-7 cells were transfected with pcDNA3 empty vector or pCDNA3-JMJD6-V5. Cell extracts were analyzed by western blotting with an antibody recognizing asymmetric dimethylation (ASYM24). Controls were performed with anti-JMJD6, PRMT1 and GAPDH antibodies. A shorter exposition of the gel is shown in the right-hand panel (*). The lower panel shows quantification of protein methylation in cells transfected with JMJD6 versus mock. (DOC)

**Figure S8** Interaction between JMJD6 and CARM1. MCF-7 cells were transfected with pcDNA3 empty vector or pCDNA3-JMJD6-V5. Cell extracts were immunoprecipitated with V5 antibody and revealed for the presence of JMJD6 and CARM1 with the corresponding antibodies. (DOC)

**Acknowledgments**

We thank R. Bruck for providing JMJD6-expressing plasmids. We also thank P. Bertino for the ER\(\alpha\) peptides and anti-metER\(\alpha\) on lystate. Thanks are also due to Cécile Languilaire and Farida Nasri for their technical assistance.

**Author Contributions**

Conceived and designed the experiments: MLR LC NH. Performed the experiments: CP JR. Analyzed the data: CP MLR LC. Wrote the paper: MLR LC.

**References**

1. Deroo BJ, Korach KS (2006) Estrogen receptors and human disease. J Clin Invest 116: 561–570. doi:10.1172/JCI27987.
2. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, et al (2001) Mechanisms of estrogen action. Physiol Rev 81: 1535–1565.
3. Cabodi S, Moro L, Baj G, Smeriglio M, Di Stefano P, et al (2004) p130Cas interacts with estrogen receptor alpha and modulates non-genomic estrogen signaling in breast cancer cells. J Cell Sci 117: 1605–1611.
4. Cabodi S, Moro L, Baj G, Smeriglio M, Di Stefano P, et al (2004) PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. EMBO J 20: 6050–6059.
5. Hammes SR, Levin ER (2007) Extracellular steroid receptors: nature and actions. Endocr Rev 28: 726–741.
6. Song RX, Zhang Z, Santen RJ (2005) Estrogen rapid action via protein complex formation involving ERalpha and Src. Trends Endocrinol Metab 16: 347–353. S1043-7260(05)00184-0 [pii]; doi:10.1016/j.tem.2005.06.010.
7. Le Romancer M, Pouillard C, Cohen P, Senis Y, Renouf JM, et al (2011) Cracking the estrogen receptor’s posttranslational code in breast tumors. Endocr Rev 32: 597–622. er.2010-0016 [pii]; doi:10.1210/er.2010-0016.
8. Le Romancer M, Treilleux I, Lecomte N, Robin-Lepinasse Y, Senis S, et al (2008) Regulation of estrogen rapid signaling through arginine methylation by PRMT1. Mol Cell 31: 212–221.
9. Pouillard C, Treilleux I, Lavergne E, Bouchekia-Bouzaghou K, Godlard-Leon S, et al (2012) Activation of rapid oestrogen signalling in aggressive human breast cancers. EMBO Mol Med, doi:10.1002/emmm.201201613.
10. Chang R, Chen Y, Zhao Y, Bruick RK (2007) JMJD6 is a histone arginine demethylase. Science 318: 444–447.
11. Bedford MT, Clarke SG (2009) Protein arginine methylation in mammals: who, what, and why. Mol Cell 33: 1–13. S1097-2765(08)00856-5 [pii]; doi:10.1016/j.molcel.2008.12.013.
12. Mantri M, Krojer T, Ragg EA, Webby CJ, Butler DS, et al (2010) Crystal structure of the 2-oxoglutarate- and Fe(II)-dependent histyl hydroxylase JMJD6. J Mol Biol 401: 211–222.
13. Unoki M, Masuda A, Dobmae N, Ariga K, Yoshimatsu M, et al (2013) Lysyl 5-hydroxylation, a novel histone modification, by Jumonji domain containing 6 (JMJD6). J Biol Chem 288: 6053–6062. M112.433284 [pii]; doi:10.1074/jbc.M112.433284.
14. Webby CJ, Wolf A, Gromak N, Dreger M, Kramer H, et al (2009) Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. Science 325: 90–93.
15. Friensen WJ, Panthikun S, Wyre A, Manoret S, Pesiridis GS, et al (2001) The methylosome, a 20S complex containing [BPI] and pICln, produces dimethylarginine-modified Sm proteins. Mol Cell Biol 21: 8289–8300. doi:10.1128/MCB.21.24.8289-8300.2001.
16. Ho MC, Wilczek C, Bonanno JB, Xing L, Seznec J, et al (2013) Structure of the arginine methyltransferase PRMT5-MEP50 reveals a mechanism for substrate specificity. PLoS One 8: e57008. doi:10.1371/journal.pone.0057008; PONE-D-12-34203 [pii].
17. Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437: 432–435. nature04021 [pii]; doi:10.1038/nature04021.
18. Subramanian K, Jia D, Kapoor-Vazirani P, Powell DR, et al (2008) Regulation of estrogen receptor alpha by the SET7 lysine methyltransferase. Mol Cell 30: 336–347. S1097-2765(08)00292-8 [pii]; doi:10.1016/j.molcel.2008.03.022.
19. Boeckel JN, Guarani V, Koyanagi M, Roexe T, Lengeling A, et al (2011) Jumonji domain-containing protein 6 (Jmj6) is required for angiogenic sprouting and regulates splicing of VEGF-receptor 1. Proc Natl Acad Sci U S A 108: 3276–3281. 1008098108 [pii]; doi:10.1073/pnas.1008098108.
20. Hong X, Zang J, White J, Wang C, Pan CH, et al (2010) Interaction of JMJD6 with single-stranded RNA. Proc Natl Acad Sci U S A 107: 14568–14572. 1008032107 [pii]; doi:10.1073/pnas.1008032107.
21. Lee YF, Miller LD, Chan XB, Black MA, Pang B, et al (2012) JMJD6 is a driver of cellular proliferation and motility and a marker of poor prognosis in breast cancer. Breast Cancer Res doi:10.1186/bcr3200.
22. Robin-Lespinasse Y, Sentis S, Kolytcheff C, Rostan MC, Corbo L, et al (2007) bCAF1, a new regulator of PRMT1-dependent arginine methylation. J Cell Sci 120: 638–647. jcs.03357 [pii]; doi:10.1242/jcs.03357.
23. Soderberg O, Gullberg M, Jarvis M, Riddlerstrale K, Leachowski KJ, et al (2008) Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 5: 995–1000.
24. Uhlmann T, Gregehegan VL, Thomas B, Rindova G, Trudgian DC, et al (2012) A method for large-scale identification of protein arginine methylation. Mol Cell Proteomics 11: 1489–1499. M112.020743 [pii]; doi:10.1074/mcp.M112.020743.
25. Kofel K, Bling C, Bellmann-Sickert K, Neundorf I, Beck-Sickinger AG, et al (2009) Type I Arginine Methyltransferases PRMT1 and PRMT3 Act Distributively. J Biol Chem 284: 8274–8282. M109547200 [pii]; doi:10.1074/jbc.M109547200.
26. Hornbeck PV, Kornhauser JM, Tkachev S, Zhang R, Skrzypek E, et al (2012) PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res 40: D261–D270. gkr1122 [pii]; doi:10.1093/nar/gkr1122.
27. Ong SE, Mittler G, Mann M (2004) Identifying and quantifying in vivo methylation sites by heavy methyl SILAC. Nat Methods 1: 119–126. nmeth715 [pii]; doi:10.1038/nmeth715.
28. Belyanskaya LL, Gehrig PM, Gehring H (2001) Exposure on cell surface and extensive arginine methylation of ewing sarcoma (EWS) protein. J Biol Chem 276: 18681–18687. doi:10.1074/jbc.M011446200;M011446200 [pii].
29. Pahlich S, Bschir K, Chiavi C, Belyanskaya L, Gehring H (2005) Different methylation characteristics of protein arginine methyltransferase 1 and 3 toward the Ewing Sarcoma protein and a peptide. Proteins 61: 164–175. doi:10.1002/prot.20579.
30. Jung SY, Li Y, Wang Y, Chen Y, Zhao Y, et al (2008) Complications in the assignment of 14 and 28 Da mass shift detected by mass spectrometry as in vivo methylation from endogenous proteins. Anal Chem 80: 1721–1729. doi:10.1021/ac7021025.
31. Lapreye B, Alamri F, Ghaffari SH, Rao SV, Dumbar TS, et al (1986) Protein and cDNA sequence of a glycine-rich, dimethylarginine-containing region located near the carboxyl-terminal end of nucleolin (62 kDa and 100 kDa). J Biol Chem 261: 9167–9173.