H3K27 demethylation by JMJD3 at a poised enhancer of anti-apoptotic gene *BCL2* determines ER α ligand dependency

Amy Svtelis¹, Stéphanie Bianco¹,
Jason Madore², Gabrielle Huppé¹,
Alexei Nordell-Markovits¹, Anne-Marie
Mes-Masson² and Nicolas Gévry^{1,*}

¹Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada and ²Centre de recherche du Centre Hospitalier de l'Université de Montréal (CR/CHUM)/Institut du Cancer de Montréal, Montréal, Quebec, Canada

Chromatin represents a repressive barrier to the process of ligand-dependent transcriptional activity of nuclear receptors. Here, we show that H3K27 methylation imposes ligand-dependent regulation of the oestrogen receptor α (ER α)-dependent apoptotic response via Bcl-2 in breast cancer cells. The activation of *BCL2* transcription is dependent on the simultaneous inactivation of the H3K27 methyltransferase, EZH2, and the demethylation of H3K27 at a poised enhancer by the ER α -dependent recruitment of JMJD3 in hormone-dependent breast cancer cells. We also provide evidence that this pathway is modified in cells resistant to anti-oestrogen (AE), which constitutively express *BCL2*. We show that the lack of H3K27 methylation at *BCL2* regulatory elements due to the inactivation of EZH2 by the HER2 pathway leads to this constitutive activation of *BCL2* in these AE-resistant cells. Our results describe a mechanism in which the epigenetic state of chromatin affects ligand dependency during ER α -regulated gene expression.

The EMBO Journal (2011) 30, 3947–3961. doi:10.1038/emboj.2011.284; Published online 12 August 2011

Subject Categories: chromatin & transcription

Keywords: BCL2; H3K27me3; JMJD3; oestrogen receptors

Introduction

Oestrogens (E2) are physiologically important steroid hormones that influence proliferation, differentiation, and apoptosis in hormone-dependent breast cancer (Welboren *et al*, 2009). The functions of E2 are mainly mediated by oestrogen receptor α (ER α), a ligand-dependent transcription factor that functions in conjunction with coregulator proteins to control gene expression by binding to gene regulatory regions that contain an oestrogen response element (ERE; Fullwood *et al*, 2009; Welboren *et al*, 2009). Despite being an important inducer of cellular proliferation, ER α presence

in breast cancers is often prognostic of an increased survival rate (Badve and Nakshatri, 2009). This is in part due to the availability of anti-oestrogens (AEs), synthetic molecules that compete with E2 for binding to ER α , which have shown to be a successful treatment in ER α -positive breast cancers (Osborne and Schiff, 2011). While AE (for example, tamoxifen (TAM) or fulvestrant/ICI182780 (ICI)) has been beneficial to a large number of patients, resistance to these drugs is a major problem in the treatment of breast cancer (Normanno *et al*, 2005; Osborne and Schiff, 2011). AE-resistant breast cancers show a deregulation in their ER α gene expression program, resulting in E2-independent proliferation (Sommer and Fuqua, 2001; Normanno *et al*, 2005; Badia *et al*, 2007; Naughton *et al*, 2007; Riggins *et al*, 2007). However, the genomic events involved in the development of AE resistance in breast cancer are not clearly defined.

ER α regulates target gene transcription by recruiting co-activator proteins to favour the recruitment of the basal transcriptional machinery (Pearce and Jordan, 2004). More specifically, current models of ER α action suggest that this receptor stimulates transcription of RNA polymerase II (RNAPII) target genes by promoting favourable alterations in promoter structure and the formation of stable transcriptional pre-initiation complexes on hormone-responsive promoters embedded in chromatin (Mellor, 2005). The majority of EREs are present in enhancers that are distal from the transcription start site, and lead to the formation of chromatin loops by associating with proximal promoter regions to enhance gene expression of ER α -regulated genes (Fullwood *et al*, 2009). E2 stimulation in cells can also lead to a non-genomic response, in which ER α target gene expression is modified by actions outside the nucleus implicating the association of ER α with membrane proteins or cytoplasmic signalling proteins, such as AKT (Russell *et al*, 2000; Razandi *et al*, 2002; Ordóñez-Moran and Munoz, 2009). These effects are known to stimulate cell-cycle progression and inhibit apoptosis in MCF7 cells (Arold *et al*, 2001; Castoria *et al*, 2001). Interestingly, recent research alludes to the crossover between the genomic and non-genomic pathways of E2 action in the control of chromatin remodelling (Bredfeldt *et al*, 2010). The dynamic nature of chromatin has been brought to light in the last decade, showing that the once believed static nature of regularly spaced nucleosomes along DNA is actually constantly changing in response to the cellular environment. The modification of histones affects nucleosome structure and determines the state of gene regulatory areas such as promoters and enhancers (Luger, 2003; Kouzarides, 2007; Zhou *et al*, 2010).

In particular, several studies have highlighted the importance of histone methylation on specific lysine residues in maintaining transcriptional programs and determining cell fate and identity (Pasini *et al*, 2004; Benevolenskaya, 2007; Cloos *et al*, 2008). For example, methylation of histone H3

*Corresponding author. Département de Biologie, Faculté des Sciences, Université de Sherbrooke, 2500 boulevard de l'Université, Sherbrooke, Quebec, Canada J1K 2R1. Tel.: +1 819 821 8000/ext. 66013; Fax: +1 819 821 8049; E-mail: nicolas.gevry@usherbrooke.ca

Received: 15 March 2011; accepted: 18 July 2011; published online: 12 August 2011

lysine 27 (H3K27) is an important epigenetic modification found at the promoters of many developmentally important genes and is involved in mammalian X chromosome inactivation, germ line development, cell-cycle regulation, and cancer (Cao and Zhang, 2004; Agger *et al*, 2007; Lan *et al*, 2007). Histone H3 trimethylated lysine 27 (H3K27me3) contributes to the recruitment of the Polycomb repressive complexes (PRC1 and PRC2) on chromatin, which in turn mediate gene silencing (Lund and van Lohuizen, 2004; Simon and Kingston, 2009). In the protein complex PRC2, EZH2 is the mammalian methylase that adds a methyl group to H3K27, which leads to the recruitment of other Polycomb-group proteins and silencing of target genes (Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Muller *et al*, 2002; Cao and Zhang, 2004). However, the demethylases JMJD3 and UTX of the KDM6 family contain a well-conserved JmjC domain in their C-terminal regions which catalyses the transition of H3K27me3 and H3K27me2 to H3K27me1, therefore from a repressive to an active chromatin conformation (Agger *et al*, 2007; De Santa *et al*, 2007; Swigut and Wysocka, 2007; Xiang *et al*, 2007). This histone demethylase activity can antagonize the recruitment of the PRC complex, playing a decisive role between cellular proliferation and differentiation in the establishment of cell fate during development (Agger *et al*, 2007; De Santa *et al*, 2007; Jones, 2007). Genes important in the developmental process are often considered poised, or bivalent, in that both silencing (H3K27me3) and activating (monomethylation and trimethylation of histone H3 lysine 4; H3K4me1/me3) marks are present at promoter and enhancer regions (Azuara *et al*, 2006; Bernstein *et al*, 2006; Mikkelsen *et al*, 2007; Rada-Iglesias *et al*, 2011). The simultaneous presence of H3K4me3 and H3K27me3 is characteristic of poised promoters (Azuara *et al*, 2006; Bernstein *et al*, 2006; Mikkelsen *et al*, 2007), while the combination of H3K4me1 and H3K27me3 marks poised enhancers (Rada-Iglesias *et al*, 2011). Moreover, it has also been suggested that the perturbation of both EZH2 and JMJD3 expression and activity, hence potentially H3K27 methylation, is implicated in carcinogenesis (Seligson *et al*, 2005; Ding and Kleer, 2006; Ding *et al*, 2006; Jones, 2007; Cooper and Foster, 2009). Despite the growing evidence for a prominent role of H3K27 methylation status in transcriptional regulation and cellular function, the understanding of their contribution in establishment of hormone-dependent cancer is still limited.

In this study, we show that the anti-apoptotic ER α target gene, *BCL2*, is differentially regulated in different breast cancer cell types, due to a modification in the chromatin template at a poised enhancer element. In hormone-responsive breast cancer cells (MCF7), JMJD3 and ER α work in concert to remove the repressive H3K27me3 mark from the poised (yet silent) enhancer region to permit appropriate gene activation and anti-apoptotic effects following E2 stimulation. However, in cells that are resistant to AE and over-express HER2, the balance shifts towards a non-genomic constitutive activation of *BCL2* by the prolonged HER2/AKT-mediated inactivation of the methyltransferase EZH2. In this case, the structure of the enhancer region is no longer poised but active due to a lack of H3K27 methylation, and the participation of JMJD3 is no longer required. Therefore, we propose a model in which the modification of the chromatin environment of the *BCL2* enhancer region imposes ligand dependency in hormone-responsive cells, and this epigenetic

mark is deregulated in the acquisition of AE resistance in breast cancer.

Results

Depletion of JMJD3 induces apoptosis in ER α -dependent breast cancer cells

To investigate the role of JMJD3 and its H3K27 demethylase activity in E2-dependent cellular responses, we first proceeded with short hairpin-RNA (shRNA)-mediated knock-down of JMJD3 and analysed cell-cycle distribution using fluorescent activated cell sorting (FACS) in the ER α -positive breast cancer cell line, MCF7. The shJMJD3-1 and shJMJD3-2 constructions efficiently depleted both mRNA and protein levels of JMJD3 as compared to the scrambled shRNA (shCTL) and the non-functional construction shJMJD3-nf controls (Figure 1A and B). Following depletion of JMJD3 expression in MCF7 cells in complete media, we observed by FACS analysis the presence of subG1 fluorescence (21% as compared to 0.4% in the shCTL and 0.6% in shJMJD3-nf cells), characteristic of DNA degradation and indicative of cell death (Supplementary Figure S1). Considering that E2 has been shown to inhibit apoptosis in the mammary gland during menstrual cycle and in breast cancer MCF7 cells (Ferguson and Anderson, 1981; Kyprianou *et al*, 1991; Teixeira *et al*, 1995), we investigated whether the degraded DNA content seen in the FACS analysis was due to apoptosis. As described previously, staining for annexin-V/propidium iodide (PI) followed by FACS analysis showed the inhibitory effect of E2 on this marker of apoptosis in MCF7 cells (~19.8% of apoptotic cells to 1.4% in E2-treated cells; Figure 1C and D). However, cellular depletion of JMJD3 using the shJMJD3-1 and shJMJD3-2 constructs dramatically increased apoptosis in E2-treated cells (33.9 and 24.7%, respectively) compared to the shCTL (1.4%) and shJMJD3-nf (0.9%) (Figure 1C and D). Moreover, similar results were obtained in another ER α -positive breast cancer cell line, T47D (Supplementary Figure S2A), but ER α -negative MDA-MB-231 cells did not undergo apoptosis following JMJD3 depletion (Supplementary Figure S2C), suggesting that the effect of JMJD3 is specific to E2-responsive cells. Taken together, these observations suggest that JMJD3 plays an important role in the control of apoptosis in ER α -dependent breast cancer cells.

JMJD3 controls the apoptotic program through the transcriptional regulation of the anti-apoptotic gene *BCL2*

In order to detect the E2-dependent transcription program associated with the observed apoptotic response, we investigated global gene expression in MCF7 cells depleted or not of JMJD3. Surprisingly, 151 of 274 E2-upregulated genes (55%) are negatively affected by the loss of JMJD3 (Figure 2A; Supplementary Figure S3B). We confirmed this result by quantitative PCR (qPCR) in MCF7 cells, where cellular depletion of JMJD3 was found to significantly reduce the E2-dependent induction of well-known ER α -regulated genes, such as *PR*, *TFF1*, *GREB1*, *IGFBP4*, and *CCND1*, while the *GAPDH* housekeeping gene remains unaffected (Figure 2B). We next classified the mRNA enriched in each subclass on the basis of gene ontology annotation using the PANTHER classification system and searched for a functional enrichment in

Figure 1 JMJD3 depletion causes apoptosis in ER α -positive breast cancer cells. The shRNA construct shJMJD3-1 and shJMJD3-2 efficiently reduce both RNA (**A**) (shown by qPCR) and protein (**B**) (shown by western blotting) levels of JMJD3 in MCF7 cells. ER α and actin have been used to show the specificity of the JMJD3 knockdown and as a loading control. (**C**) Representative apoptosis analysis using annexin-V/PI staining and flow cytometry of MCF7 cells treated with shCTL, shJMJD3-nf, shJMJD3-1, or shJMJD3-2 and with vehicle (–E2) or 17 β -estradiol (+E2) for 24 h. Flow cytometry profile represents annexin-V staining in x axis and PI in y axis. (**D**) The graphical representation annexin-V/PI staining and flow cytometry of MCF7 cells. The data shown are the mean $\pm$ s.e.m. of at least three independent experiments (** $P < 0.01$, t -test).

the apoptosis-related genes (Supplementary Figure S3A). Notably, we observed a small subset of genes implicated in apoptosis (2.6% on the total of genes affected by the depletion of JMJD3). Since JMJD3 mostly plays a positive role in gene activation and its depletion causes apoptosis, we then considered genes implicated in the inhibition of apoptosis (Supplementary Figure S3B). Interestingly, in this subset of

genes, we observed the presence of *BCL2*, one of the most important anti-apoptotic genes regulated by ER α in MCF7 breast cancer cells (Perillo *et al*, 2008). Consistent with the previous observation on the positive role of JMJD3 on the regulation of ER α -regulated genes, specific depletion of JMJD3 by shRNA-mediated knockdown blocked the ER α -dependent activation of *BCL2* expression in MCF7

Figure 2 JMJD3 regulates *BCL2* gene transcription in ER α -positive cells. (A) Venn diagram showing the number of E2 upregulated genes significantly affected by the knockdown of JMJD3 in MCF7 cells. (B) Expression levels of *PR*, *TFF1*, *GREB1*, *IGFBP4*, *CCND1*, and *GAPDH* in MCF7 cells depleted or not of JMJD3, and in absence or presence of E2 for 4 h. mRNA expression levels were determined by qPCR and normalized against expression levels of the 36B4 ribosomal gene. (C) Relative expression of *BCL2* in MCF7 cells treated with vehicle or E2 and depleted or not of JMJD3 using specific shRNA constructs. (D) Immunoblot assay showing the effect of JMJD3 and UTX depletion on BCL2 protein expression in MCF7 cells (GAPDH used as loading control). (E) Immunoblot assay showing the overexpression of the Bcl-2 protein in MCF7 cells as compared with the empty vector (GAPDH used as loading control). (F) The graphical representation annexin-V/PI staining and flow cytometry of MCF7 cells that overexpress Bcl-2 following the depletion of JMJD3. The data shown are the mean $\pm$ s.e.m. of at least three independent experiments (* P < 0.05; ** P < 0.01, *t*-test).

(Figure 2C and D) and T47D cells (Supplementary Figure S2B). This effect on *BCL2* is specific to the ER α response, as a decrease in ER α levels by ICI does not lead to a cumulative effect on *BCL2* expression (Supplementary Figure S4), and no effect was observed on *BCL2* by depletion of JMJD3 in MDA-MB-231 cells (Supplementary Figure S2D). Importantly, this effect was specific to the knockdown of JMJD3, as the shRNA-mediated depletion of UTX, another H3K27me3 demethylase of the same family, did not have the same effect (Supplementary Figure S5). To confirm that the apoptotic program affected by JMJD3 depletion is due to the anti-apoptotic effects of Bcl-2, we overexpressed Bcl-2 in JMJD3-depleted MCF7 cells (Figure 2E). We show by FACS analysis following annexin-V/PI staining that the addition of Bcl-2 renders the cells resistant to apoptosis observed in JMJD3-depleted cells (Figure 2F). Collectively, these results suggest that JMJD3 prevents ER α -positive breast cancer cells from entering apoptosis via its control of E2-dependent *BCL2* gene expression.

JMJD3 is recruited to the ER α binding region at the *BCL2* enhancer region upon E2 signalling

To gain insight into how JMJD3 regulates *BCL2* expression, chromatin immunoprecipitation (ChIP) assays were used to

identify the preferential localization of JMJD3 within or near the *BCL2* promoter region. Based on the report indicating that *BCL2* is regulated by two different promoters, P1 (the principal promoter) and P2 and has two EREs in the enhancer present in the coding region of the gene (Perillo *et al*, 2008; Figure 3A), we surveyed these *BCL2* regulatory regions by ChIP. To determine the transcriptional program of *BCL2* following ER α -dependent activation, ChIP assays were performed at 0 and 30 min after E2 stimulation in MCF7 cells. As expected, ER α was recruited to the ERE sites in the coding region of *BCL2* as well as at the P1 promoter area (Figure 3B). JMJD3 colocalized with ER α at both the active P1 promoter and the ERE enhancer regions (Figure 3C). Moreover, this recruitment of JMJD3 upon E2 treatment was associated with a decrease in H3K27me3 (Figure 3D) in proximity of the ERE enhancer region of *BCL2*. Since H3K27me3 is a transcriptionally repressive chromatin mark that has been associated with poised promoters and enhancers at important developmental genes (Bernstein *et al*, 2006; Jorgensen *et al*, 2006; Sharov and Ko, 2007; Burgold *et al*, 2008), we verified the presence of active transcriptional marks such as RNAPII, H3K4me3, H3K4me1, and histone variant H2A.Z, using histone H3 to control nucleosome density. The results demonstrate that RNAPII (Figure 3E), H3K4me3 (Figure 3F), and H2A.Z

Figure 3 JMJD3 colocalizes with ER α at the *BCL2* promoter. (A) Diagram of the *BCL2* promoter regions (P1 and P2), its regulatory elements (ERE), and amplicons used for qPCR. (B–I) ChIP analysis showing the location of ER α (B), JMJD3 (C), H3K27me3 (D), RNAPII (E), H3K4me3 (F), and H2A.Z (G), H3K3me1 (H) and H3 (I) at the *BCL2* regulatory regions treated with vehicle or E2 for 30 min in MCF7 cells. The data shown are the mean $\pm$ s.e.m. of at least three independent experiments (* P < 0.05; ** P < 0.01, t -test).

(Figure 3G) were present before E2 treatment at the P1 promoter and increased at the ERE after induction. The simultaneous presence of ER α , JMJD3, RNAPII, H3K4me3, and H2A.Z at the P1 promoter and the ERE enhancer region after E2 treatment may be at least partly a consequence of the formation of an active DNA loop, an event that has been previously shown to be an important step in *BCL2* gene activation in response to E2 (Perillo *et al*, 2008). Furthermore, H3K4me1, indicative of a poised enhancer, colocalized with H3K27me3 at the *BCL2* enhancer region (Figure 3H). Another characteristic of enhancers is their relative nucleosomal depletion compared to surrounding regions (He *et al*, 2010). Using formaldehyde-assisted isolation of regulatory elements (FAIRE), we observed that the *BCL2* enhancer region is nucleosome depleted both before and after E2 activation, as well as both before and after H3K27me3 removal by JMJD3

(Supplementary Figure S6). Taken together, these results show that JMJD3 is recruited to the ER α -binding sites in the *BCL2* enhancer region, and that the repressive H3K27me3 mark is actively removed upon E2 treatment.

The presence of ER α and JMJD3 at the *BCL2* enhancer region are interdependent

Many studies have shown that ER α can target the recruitment of histone modifying enzymes such as p400, CBP/p300, Tip60, CARM-1, and RNAPII to gene promoters (Metivier *et al*, 2003; Gevry *et al*, 2009). To establish the order in which ER α and JMJD3 are recruited to the *BCL2* enhancer region, we performed ChIP analyses at 0, 15, 30, and 45 min after E2 stimulation. The results show that ER α , RNAPII, and JMJD3 were concomitantly recruited after 15 min, followed by reduction to pre-treatment levels after 45 min (Figure 4A).

Figure 4 Presence of ER α and JMJD3 at *BCL2* is interdependent. (A) Recruitment kinetics of ER α , RNAPII, JMJD3, H3K27me3, and H3K27me1 after E2 treatment in MCF7 cells. (B) ChIP analysis of ER α , JMJD3, and H3K27me3 recruitment before and after treatment with E2 or tamoxifen (TAM) for 30 min, or ICI 180782 for 24 h followed by E2 for 30 min. (C–F) ChIP assay showing the effect of JMJD3 depletion on the recruitment of JMJD3 (C), H3K27me3 (D), ER α (E), and RNAPII (F). The primers used in these experiments correspond to the ERE (ERE2) of the *BCL2* gene. The data shown are the mean $\pm$ s.e.m. of at least three independent experiments (* P < 0.05; ** P < 0.01, *t*-test). (G) Co-immunoprecipitation of endogenous JMJD3 and ER α . MCF7 cells were treated with E2 for 24 h, and whole cell lysates were immunoprecipitated with antibodies for JMJD3 or ER α . The immunoprecipitated material was then subjected to western blotting analysis with anti-JMJD3 or anti-ER α . (H) Schematic representation of ER α and deletion constructs used for the interactions assays. (I) To analyse the functional domains of ER α that interact with JMJD3, interactions between *in vitro* transcribed and translated JMJD3 with ER α deletion GST constructs were assayed by GST pull-down in absence and presence of ligand.

Furthermore, the recruitment of JMJD3 corresponded to an increase in H3K27me1 after 15 min of induction and a decrease in H3K27me3, suggesting that JMJD3 could be responsible for the transformation of the repressive H3K27me3 mark into transcriptionally permissive H3K27me1 (Figure 4A). Surprisingly, H3K27me1 and H3K27me3 were depleted after 45 min of induction (Figure 4A). Considering that the ERE binding sites are located in exon 2 of the *BCL2* gene (Perillo *et al*, 2008), our observation might be explained by the dynamics of H3K27me1 replacement by an unmodified histone H3 during transcriptional elongation by RNAPII. Because these results did not permit us to clearly determine the active recruitment hierarchy of JMJD3 and ER α to the *BCL2* enhancer region, we examined the recruitment of JMJD3, ER α , and H3K27me3 at the ERE following treatment with the ER antagonists, ICI, or TAM (Figure 4B). Mechanistically, ICI causes rapid degradation of ER α by the ubiquitin/proteasome pathway (McClelland *et al*, 1996; Fan *et al*, 2003). TAM acts by competing with E2 as a ligand for ER α thus inducing a conformational change in the AF-2 domain of ER α , which then blocks its interaction with co-activators such as SRC-1, GRIP1, and CBP/p300 (Shang *et al*, 2000). As expected, TAM treatment induced recruitment of ER α to the enhancer region of *BCL2*, while the pre-incubation of MCF7 cells with ICI following E2 treatment abolished its recruitment. MCF7 cells treated with TAM showed slightly lower levels of JMJD3 recruitment compared to E2 with a comparable decrease in H3K27me3. On the other hand, ICI treatment strongly affects JMJD3 recruitment, accompanied by a subtle increase in H3K27me3 status as compared to uninduced levels. These results suggest that JMJD3 recruitment and H3K27me3 demethylation at the *BCL2* enhancer region are regulated in an ER α -dependent manner. Since H3K27me3 is a repressive mark in transcriptional processes, we investigated the role of JMJD3 and H3K27me3 demethylation in the transcriptional regulation of *BCL2* in response to E2 by ChIP experiments in cells depleted in JMJD3. Using antibodies directed against JMJD3 (as a control), H3K27me3, ER α , and RNAPII, we observed that depletion of JMJD3 specifically abolished the recruitment of JMJD3 and the demethylation of H3K27me3 at the enhancer region upon E2 treatment (Figure 4C and D). Notably, JMJD3 depletion in MCF7 cells reduced the recruitment of both ER α (Figure 4E) and RNAPII (Figure 4F) in presence of E2. In addition, a physical interaction between JMJD3 and ER α was observed by co-immunoprecipitation in MCF7 cells, even before E2 stimulation (Figure 4G). This association occurs through a direct interaction between JMJD3 and the A/B region of AF1 in ER α , as shown by GST pull-down *in vitro* (Figure 4H and I). The fact that ER α interacts with JMJD3 and is able to perform this task independent of the AF2 domain strongly suggests a co-dependency between the two proteins, and that both are simultaneously required for efficient E2-dependent *BCL2* transcriptional activation.

Inactivation of EZH2 methyltransferase activity by non-genomic pathways controls *BCL2* transcription and apoptosis in AE-sensitive and AE-resistant breast cancer cells

The removal of H3K27me3 on chromatin is not entirely dependent on the activity of demethylases, as the phosphorylation of serine 21 of EZH2 (pEZH2) via the AKT pathway

impedes EZH2 binding to histone H3, resulting in a global decrease in H3K27me3 (Cha *et al*, 2005). Of note, the AKT pathway is involved in the non-genomic effects associated with ER α target gene expression (Sun *et al*, 2001; Bredfeldt *et al*, 2010; Castaneda *et al*, 2010). In AE-resistant breast cancer cells, the overexpression of HER2 is sufficient to activate the AKT pathway (Kumar *et al*, 1996; Schiff *et al*, 2004; Jones, 2007). We thus aimed to observe the possibility of a negative relationship between the AKT pathway and H3K27me3 in different types of breast cancer. We performed immunohistochemistry analysis of HER2 (AKT pathway) and H3K27me3 levels on a breast cancer tissue microarray (TMA) composed of surgical breast samples. We scored the intensity of immunostaining for H3K27me3 and HER2 using a scale from 0 to 3, and categorized the samples into two main groups, low (0–1) or high (2–3). Hierarchical clustering using Euclidian distance was performed on the data segregating the tumours into four groups based on these two markers. Heat map visualization reveals the largest cluster to present a high presence of H3K27me3 versus a low presence of HER2 with 67/118 samples, or 56.8% (Figure 5A). A χ^2 test of independence on the expression levels ($\times 2$ (1, $N = 118$) = 7.627, $P = 0.006$) suggests a relationship between H3K27me3 and HER2 levels. Representative immunostaining examples of each group are shown in Figure 5B.

The overexpression of HER2 and the AKT response are important in the development of AE resistance (Campbell *et al*, 2001; Stoica *et al*, 2003). Therefore, we questioned the role of H3K27me3 in AE-resistant cells that overexpress HER2. Since no data on AE resistance were available in the TMA, we used three different MCF7-derived AE-resistant cell lines: MCF7/LCC2 (LCC2), which were developed by the selection of MCF7 cells resistant to TAM (Brunner *et al*, 1993), MCF7/LCC9 (LCC9) cells that are resistant to TAM and ICI (Brunner *et al*, 1997) and MCF7/HER2-18 (HER2), which artificially overexpress HER2 (Benz *et al*, 1992). All of these cell lines show a high level of AKT activation and display an E2-independent phenotype (Wang *et al*, 2006). Interestingly, we observed that JMJD3 knockdown in the AE-resistant cell lines LCC2, LCC9, and HER2 did not affect cell survival, as determined by annexin-V/PI and FACS analysis (Figure 5C). To further investigate the observation of a relationship between H3K27me3 and HER2 levels from the TMA, we used cell lines representative of the high-low and low-high clusters. MCF7 is known to exhibit low levels of HER2 (Wang *et al*, 2006), so these cells may correspond to the high-low cluster from the TMA. The non-genomic E2 response leads to the rapid AKT-mediated pEZH2 in MCF7 cells, which we confirmed by western blot analysis of pEZH2 following E2 treatment of 30 min (Figure 5D). This correlated with a global decrease in H3K27me3 after 16 h treatment with E2 which was inhibited by the pre-treatment of MCF7 with the AKT inhibitor LY294002 (Figure 5D). Longer E2 treatment was necessary to observe a change in H3K27me3 status due to the stability of this chromatin mark (Jones, 2007). We monitored the levels of pEZH2 in LCC2 cells under basal media conditions and found that they were much higher in LCC2 than in MCF7 cells, accompanied by a general decrease in H3K27me3 levels in LCC2 cells (Figure 5D). Therefore, MCF7 cells mimic the high-low cluster from the TMA, while MCF7/LCC2 cells represent the low-high category.

Figure 5 AE-resistant cells have a modified H3K27me3 chromatin signature that alters *BCL2* transcriptional activity. **(A)** Heat map representing the hierarchical cluster analysis performed on the breast cancer TMA, separated into four different clusters based on H3K27me3 and Her2 immunoreactivity scores from 0 to 3: low-low, high-low, low-high, and high-high. Percentages were calculated from the number of patients in a category divided by the total number of patients. Representative immunostaining of each cluster is shown in **(B)**. **(C)** Representative plots of annexin-V/PI staining and flow cytometry analysis of MCF7 cells as compared with AE-resistant cells LCC2, Her18, and LCC9 depleted with shCTL or shJMJD3-1 and treated with vehicle or E2 for 24 h. **(D)** Immunoblot assay showing the levels of phospho-EZH2 (pEZH2) as compared with total EZH2 and H3K27me3 (H3 as a loading control) in MCF7 cells treated with vehicle or E2 for 30 min (pEZH2) or 16 h (H3K27me3) and LCC2 treated with AKT inhibitor LY294002 for 72 h. **(E)** Comparison of *BCL2* mRNA expression levels in MCF7 and LCC2 cells depleted or not of JMJD3, and in absence or presence of E2 for 4 h. The data shown are the mean $\pm$ s.e.m. of three independent experiments (** $P < 0.01$, *t*-test).

In order to further investigate the influence of HER2/AKT and H3K27 methylation status on the development of AE resistance, we employed LY294002 to inhibit EZH2 phosphorylation in LCC2 cells. The levels of pEZH2 greatly decreased to an almost undetectable level in the presence of LY294002 in LCC2 cells, which resulted in a corresponding increase in global H3K27me3 (Figure 5D). We then asked whether JMJD3 depletion has an effect on *BCL2* expression in

AE-resistant LCC2 cells. We found that AE-resistant cells constitutively express high levels of *BCL2* that are not altered by E2 or TAM treatments (Figure 5E; Supplementary Figure S7). Moreover, when JMJD3 was depleted in LCC2, we did not observe downregulation of *BCL2* compared to MCF7 cells (Figure 5E), suggesting that the JMJD3-mediated transcriptional regulation of *BCL2* does not occur in the AE-resistant cell line. Overall, these results suggest the

simultaneous implication of the non-genomic and genomic pathways in the control of H3K27me status and perturbation of one of these pathways may affect *BCL2* expression and the apoptosis response.

Inactivation of the EZH2 methyltransferase activity bypasses the need for JMJD3 in the transcriptional regulation of *BCL2* in breast cancer cells

In light of our observations in the LCC2 cell line, we hypothesized that the H3K27me3 repressive mark is absent at the *BCL2* promoter in these cells, leading to uncontrolled constitutive activation of *BCL2*. In support of this hypothesis, by interfering with the activity of EZH2 by the depletion of its essential partner SUZ12 (Pasini *et al*, 2004), we observed an increase in the basal expression levels of *BCL2* in MCF7 cells (Supplementary Figure S8). Furthermore, a ChIP assay comparing the enhancer region of *BCL2* in MCF7 and LCC2 cells (Figure 6A) showed not only that ER α and RNAPII are present prior to E2 stimulation, but also that the repressive H3K27me3 mark is clearly absent, a result consistent with the lower levels of EZH2 binding to the *BCL2* enhancer observed in our ChIP assays in LCC2 (Figure 6B). These findings indicate that the absence of the repressive H3K27me3 mark renders the presence of JMJD3 obsolete. We then blocked EZH2 phosphorylation with LY294002 treatment and observed by ChIP assays that we were able to restore a repressive state to the *BCL2* enhancer region (Figure 6C) and decrease gene expression (Supplementary Figure S9). When AKT activity was inhibited in absence of E2 stimulation, we observed that the binding of ER α and RNAPII was decreased to basal levels in LCC2 (Figure 6C). More importantly, the level of H3K27me3 increased and the binding of EZH2 was restored (Figure 6C and D).

To confirm the importance of EZH2 phosphorylation status on the Bcl-2 regulated apoptotic program in breast cancer cells and AE resistance, we investigated the effects of EZH2 mutants in MCF7 cells. The mutants were created by converting serine 21 either into alanine to block any potential phosphorylation (EZH2-S21A), or into aspartic acid to imitate the phosphorylated state (EZH2-S21D) (Cha *et al*, 2005). The overexpression of EZH2-S21D in JMJD3-depleted MCF7 cells did not lead to apoptosis, while the overexpression of wild-type EZH2 or EZH2-S21A still showed the typical apoptotic pattern of JMJD3-depleted MCF7 cells (Figure 6E and F). This result is corroborated by a restoration of induced levels of *BCL2* in JMJD3-depleted MCF7 cells that overexpress EZH2-S21D (Figure 6G). So, a decrease in H3K27me3 due to EZH2 phosphorylation is, at least in part, responsible for the increased expression of Bcl-2, resulting in cell survival. To further link these observations to AE resistance, we performed these tests in MCF7 cells in the presence of increasing concentrations of TAM (0, 5, 10, 15, 20, and 25 μ M) for 48 h. Concentration-dependent apoptosis was observed in the presence of TAM (Supplementary Figure S8). Due to the high levels of cell death in increased concentrations FACS analysis following annexin-V/PI staining was measured in the cell lines exposed to 0, 5, and 10 μ M TAM. Of note, the overexpression of EZH2-S21D and Bcl-2 led to a resistance to TAM-induced apoptosis (Figure 6H) and increased cellular proliferation as compared with the control (Supplementary Figure S10A and B). Taken together, these results show that perturbation of H3K27 methylation by the phosphorylation of

EZH2 affects *BCL2* gene expression and the regulation of the apoptotic pathway, thus contributing to the acquisition of AE resistance in breast cancer.

Discussion

In breast cancer, E2 both promotes proliferation of abnormal cells by regulating the expression of cell cycle-related genes, and prevents apoptosis in these cells by increasing the expression of the anti-apoptotic gene *BCL2* (Teixeira *et al*, 1995; Perillo *et al*, 2008). Bcl-2, a key player in the regulation of cell cycle and development of carcinogenesis, is often highly expressed in cancer and is associated with ER α status and more malignant breast cancers (Teixeira *et al*, 1995; Perillo *et al*, 2008). In this study, we show the importance of the modification of an epigenetic mark, H3K27me3, in allowing proper ligand-dependent regulation of *BCL2* in breast cancer cells. In AE-sensitive cells, the JMJD3/ER α -dependent regulation of H3K27 methylation is required for *BCL2* activation. Our results also lead us to propose that AE-resistant cells exhibit a chromatin signature that is permissive for *BCL2* transcription in the absence of stimulation. We show that pEZH2 caused by the overexpression of HER2 in AE-resistant breast cancer cells specifically prevents the methylation of H3K27 surrounding the enhancer region of *BCL2*, allowing the formation of an active chromatin conformation and constitutive activation of *BCL2*. The overall consequence is a bypass of the need for JMJD3 for transcriptional activation. Thus, we show that the establishment of the H3K27me3 mark at the *BCL2* enhancer is required for ligand-dependent regulation of this gene (Figure 7).

First, we observed that during the E2-mediated apoptotic response in MCF7 cells, a physical association between JMJD3 and the AF1 of ER α leads to their co-recruitment to the enhancer of the anti-apoptotic gene *BCL2*, resulting in H3K27me3 demethylation and subsequent gene activation. Therefore, we propose that the repressive chromatin state caused by H3K27 methylation in ER α -dependent cancerous cells is permissive for the regulation of *BCL2* following stimulation by E2 transcription by a mechanism that comprises the interdependent recruitment of ER α and JMJD3. The knockdown of JMJD3 in this context causes apoptosis, since the cells are rendered more sensitive to apoptotic demise in the absence of expression of the anti-apoptotic gene *BCL2*. It is of interest that the enhancer region of *BCL2* in unstimulated MCF7 cells shows a poised enhancer signature (H3K4me1/H3K27me3) similar to what is observed in a subset of key developmental regulatory genes (Rada-Iglesias *et al*, 2011). Studies into the control of poised promoters in developmental genes (H3K4me3/H3K27me3) show that their activation requires the actions of cell signals to recruit transcription factors which can bind to and recruit JMJD3 to chromatin (Bernstein *et al*, 2006; Jorgensen *et al*, 2006; Sharov and Ko, 2007). Our results are in agreement with research on developmentally regulated genes, yet provide an intriguing novel role of the demethylation of H3K27me3 outside this subset of genes. We show here for the first time the involvement of a demethylase, JMJD3, in the resolution of a poised enhancer region by its association with a hormone-dependent transcription factor, ER α , on the chromatin template to regulate a gene that is critical in cell survival. Our finding that H3K27me3 acts as a barrier to the binding of ER α

Figure 6 Inactivation of the EZH2 methyltransferase activity by the HER2/AKT pathway bypasses the need for JMJD3 at the *BCL2* enhancer region in AE-resistant cells. **(A)** ChIP analysis comparing the levels of ER α , JMJD3, RNAPII, and H3K27me3 in MCF7 and LCC2 cells in the absence of E2. The qPCR analysis was performed using primers corresponding to the ERE (ERE2) of the *BCL2* gene. **(B)** ChIP assays showing the levels of EZH2 in MCF7 and LCC2 cells at the ERE binding site in absence of E2. **(C, D)** ChIP experiments showing the effects of AKT inhibitor (LY294002) on the recruitment of ER α , RNAPII, H3K27me3 **(C)**, and EZH2 **(D)** at the ERE2 *BCL2* binding site in LCC2 cells. **(E)** Immunoblot assay showing the overexpression of myc-tagged wild-type EZH2 (EZH2) and mutants EZH2-S21A and EZH2-S21D proteins in MCF7 cells as compared with the empty vector control using the antibody against the Myc tag (GAPDH used as loading control). The graphical representation of annexin-V/PI staining and flow cytometry **(F)** and qPCR analysis of mRNA expression of *BCL2* **(G)** in MCF7 cells that overexpress EZH2 and mutants following the depletion of JMJD3. **(H)** Annexin-V/PI staining and flow cytometry analyses of EZH2 and EZH2 mutant MCF7 cell lines following treatment with 0, 5, and 10 μ M TAM for 48 h. The data shown are the mean $\pm$ s.e.m. of at least three independent experiments (*P < 0.05; **P < 0.01, t-test).

in the ERE enhancer region suggests a two-fold role of JMJD3 in the proper regulation of *BCL2* and apoptosis in E2-dependent breast cancer cells. JMJD3 could act by tightly regulating the accessibility to DNA in important distal control regions, and also by altering chromatin structure to permit communication between distal regulatory elements and the proximal promoter region (Figure 7). Interestingly,

LSD-1, another histone demethylase, has been involved in the activation of the *BCL2* gene by affecting chromatin looping between the enhancer region and the proximal promoter region (Perillo *et al*, 2008). This may suggest an interplay between the demethylation of H3K9me2 and H3K27me2 at the enhancer region, leading to the formation of a chromatin active loop.

Figure 7 Summary model of the role of JMJD3 and H3K27 methylation in the control of *BCL2* gene expression in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. In AE-sensitive cells (MCF7), the JMJD3/ER α -dependent regulation of H3K27 methylation is required for *BCL2* activation. However, AE-resistant cells exhibit a chromatin signature that is permissive for *BCL2* transcription in the absence of stimulation, in part caused by the overexpression of HER2 and subsequent pEZH2, which specifically prevents the methylation of H3K27 surrounding the enhancer region of *BCL2*. These events would allow the formation of an active chromatin conformation and constitutive activation of *BCL2*. The overall consequence is a bypass of the need for JMJD3 for transcriptional activation and resulting ligand-independent activation of *BCL2*.

We also observed that the binding of H2A.Z was pre-bound at *BCL2* and colocalized with ER α and JMJD3 binding following E2 stimulation (Figure 3). It is possible that, in addition to JMJD3, H2A.Z is required for the activation of *BCL2* in response to E2 in breast cancer cells, contributing to the anti-apoptotic characteristic of these cells. It is worth mentioning that H2A.Z binds SUZ12 target genes in mouse embryonic stem cells, and the specific depletion in this context leads to the derepression of PcG targets (Creyghton *et al*, 2008). H2A.Z has been highly associated with gene activity in mammalian cells, so this result was surprising. However, it is possible that H2A.Z colocalization to these areas promotes the bivalency of such genes, poising the promoter for activation. One avenue that remains to be explored is the role of H2A.Z in preventing apoptosis. The depletion of p400, catalytic protein responsible of the H2A.Z incorporation into chromatin, has been shown to lead to apoptosis in several cancer cell lines (Mattera *et al*, 2009, 2010), and we observed an increase in TAM-induced apoptosis in MCF7 cells in the absence of H2A.Z (A Svtelits, unpublished results). The role of H2A.Z in pro- and anti-apoptotic gene regulation would merit some investigation, especially considering the known association of H2A.Z with p53 and ER α , two regulators of apoptotic genes (Gevry *et al*, 2007, 2009).

Our studies also demonstrate that the perturbation of the H3K27 methylation pathway considerably affects the apoptosis program in breast cancer cells. Polycomb-group proteins have been associated with carcinogenesis, namely by the control of senescence via the repression of the *INK4/ARF* locus (Agger *et al*, 2009; Barradas *et al*, 2009). In this case, JMJD3 derepresses this locus, leading to cell-cycle arrest and suggesting a tumour-suppressive role for JMJD3. Conversely, our results suggest that in the context of ER α -dependent breast cancer, JMJD3 promotes cellular growth by promoting the expression of the anti-apoptotic gene *BCL2*. Our results with JMJD3 are supported by the increase in the basal expression levels of *BCL2* shown by depletion of *SUZ12* in MCF7 cells (Supplementary Figure S8). Recently, overexpression of JMJD3 and demethylase activity has been shown to contribute to the pathogenesis of Hodgkin's Lymphoma (Anderton *et al*, 2011). In addition, several studies suggest that EZH2 is a promising prognostic biomarker in

breast cancer (Raaphorst *et al*, 2003; Collett *et al*, 2006; Bachmann *et al*, 2006). Moreover, decreased survival is observed in patients with low H3K27me3 levels (Wei *et al*, 2008). In fact, EZH2 overexpression is related more with the aggressive ER-negative breast cancer subtype and breast cancer invasion (Cao *et al*, 2008; Wei *et al*, 2008; Gonzalez *et al*, 2009). However, we (and others; Bredfeldt *et al*, 2010) have shown that the overexpression of HER2 leads to AKT-mediated phosphorylation of EZH2 in more severe, AE-resistant breast cancer, decreasing global H3K27me3 levels. The levels of pEZH2 and global H3K27me3 in the aggressive breast cancers that exhibit increased EZH2 were not verified in previous studies, so it is possible that EZH2 overexpression in conjunction with HER2 overexpression does not lead to increased H3K27me3. Of note, we also observe a negative relationship in breast biopsy samples between H3K27me3 and HER2, a marker of AE-resistant breast cancer (Figure 5A and B).

These results suggest a context-dependent role for JMJD3 and H3K27me3 in different cell types, and led us to speculate on the role of the HER2/AKT inactivation of EZH2 by phosphorylation in AE-resistant cells. The HER2/AKT-dependent modification of EZH2 decreases H3K27 methylation (Cha *et al*, 2005), which has been strongly implicated in the development of AE resistance in breast cancer cells (Schiff *et al*, 2004; Shou *et al*, 2004). The phosphorylation of EZH2 caused by the overexpression of HER2 in AE-resistant breast cancer cells prevents the methylation of H3K27 surrounding the enhancer region of *BCL2*, allowing the formation of an active chromatin conformation and constitutive activation of *BCL2*. The overall consequence is a bypass of the need for JMJD3 for transcriptional activation (Figure 5E). It is of particular interest that the constitutive expression of the proto-oncogene *BCL2* has been shown to prevent apoptosis after AE treatment in breast cancer cells (Figure 6H). Also, ER α is known to be subject to AKT-mediated phosphorylation, which decreases its transcriptional activity, possibly the result of a negative feedback loop to control E2-dependent responses, which may explain the decrease in binding of ER α genome-wide in the presence of HER2/AKT overexpression (Sun *et al*, 2001; Bhat-Nakshatri *et al*, 2008; Park *et al*, 2008). The action of AKT can increase ER α transcriptional activity in

the absence of E2, suggesting a role in breast cancer development and resistance to therapy (Campbell *et al*, 2001; Pietras and Marquez-Garban, 2007). Although not investigated in our study, the contribution of the post-translational modifications of ER α on *BCL2* regulation in AE-resistant cells is worth considering.

Taken together, our results strongly suggest that the regulation of *BCL2* expression in AE-sensitive breast cancer cells requires the simultaneous and coordinated implication of the genomic and non-genomic pathway in the regulation of *BCL2* by the implication of the effectors of H3K27 methylation, EZH2 and JMJD3. In AE-sensitive cells, upon E2 induction, both pathways converge rapidly to activate the *BCL2* gene transcription by suppressing the methyltransferase activity of EZH2 and by recruiting JMJD3 through ER α to remove the H3K27 mark at the enhancer region of *BCL2*. This regulation is sensitive to AE, as observed by a lack in *BCL2* activation in presence of TAM. However, in the acquisition of AE resistance, *BCL2* becomes constitutively activated due to the overactivation of the HER2/AKT pathway, which results in a global decrease in H3K27me3 that circumvents the requirement of JMJD3 at the *BCL2* enhancer region.

To summarize, we propose that there exists a balance between the genomic and the non-genomic pathways in the epigenetic regulation of transcription of ER α target genes during cellular stimulation with E2, as is the case for *BCL2* in the E2-responsive MCF7 cell line (Figure 7). MCF7 cells remain responsive to external stimuli, such as E2, which results in the rapid regulation of *BCL2* expression. First, the induction of AKT activity by the ER α non-genomic pathway results in the phosphorylation of EZH2 and its subsequent removal from the chromatin template, preventing further H3K27 methylation (Figure 7). Second, the ER α genomic response leads to the concomitant binding of ER α and JMJD3 at the *BCL2* enhancer region and the demethylation of H3K27me3, favouring a transcriptionally active state. The contribution of HER2 remains negligible in E2-responsive cells, seeing as HER2 levels are low and the activation of AKT by the ER α non-genomic response is favoured. However, the disruption of the balance between ER α and HER2 signalling by the overexpression of HER2 often observed in AE-resistant cancer cells results in the superactivation of the end result of the ER α non-genomic pathway (pEZH2 by AKT) in the absence of extracellular stimuli. Thus, the continual phosphorylation of EZH2 leads to a global decrease in H3K27me3, affecting the ER α transcriptional program. The key regulator of *BCL2* expression would seem to be the establishment of the H3K27me3 mark, providing a ligand-dependent response. Our findings show that not only modifications in cellular signalling pathways are required to transform cancerous cells into AE-resistant cells, but that an important key to understand this phenomenon lie in the fundamental epigenetic mechanisms and the signature of epigenetic marks on chromatin. This study may also provide JMJD3 and H3K27me3 as alternatives to current detection methods and chemotherapy treatments in breast cancer.

Materials and methods

Cell culture and shRNA lentiviral transduction

MCF7, MDA-MB-231, 293T, and MCF7/HER2-18 (gift from Mien-Chie Hung, University of Texas, USA) cell lines were

maintained in DMEM (Wisent) containing 10% fetal bovine serum (FBS) and antibiotics. Prior to their use in E2 treatment experiments, MCF7 cells were grown in DMEM without phenol red (Wisent) and supplemented with 5% charcoal-dextran-treated FBS for at least 3 days. The LCC2 and LCC9 cells (gift from Robert Clarke, Georgetown University Medical Center, USA) were cultured in DMEM without phenol red containing 5% charcoal-dextran-treated FBS. All cells were mycoplasma free and were kept in a humidified chamber at 37°C in 5% CO₂. 17 β -estradiol, TAM, and ICI 182780 (Sigma) were used at concentrations of 100 nM, 1 μ M, and 100 nM, respectively, unless otherwise stated. The shRNA sequences designed to inhibit JMJD3 (#1, GAGACCTCGTGTGATTAA; #2, GCGCTCGTGTATGTACATT) were cloned into the pLVTHM backbone (gift from Didier Trono, University of Geneva, Switzerland). The shRNA against UTX was purchased from Open Biosystems in the pLKO.1 vector and shRNA lentiviruses were obtained by cotransfection of pLVTHM or pLKO.1 vectors, pMDVSV-G, and psPAX2 into human 293T cell line. The pcDNA3 constructs overexpressing Bcl-2, EZH2, EZH2-21A, and EZH2-21D were transfected in MCF7 cells as described previously (Gevry *et al*, 2009).

Analysis of apoptosis and proliferation

For subG1 analysis, cells were harvested and fixed in 70% ethanol. Fixed cells were stained with PI (50 μ g/ml) after treatment with RNase (100 μ g/ml). Stained cells were analysed for DNA content by FACS in a FACS Calibur (BD Biosciences). For annexin-V/PI analysis, cells were stained with annexin-V coupled to the fluorophore Cy5 according to the manufacturer's instructions (Abcam) and cells positive for annexin-V detection were measured using the CellQuest software (BD Biosciences). Proliferation assays in MCF7 cells were performed as described by the manufacturer's protocol (CellTiter 96[®] Non-Radioactive Cell Proliferation Assay (MTT), Promega), with a starting quantity of 2000 cells seeded in 96-well plates in complete media supplemented with 10 μ M TAM.

ChIP and FAIRE assays

MCF7 cells were hormone deprived for at least 3 days and then treated with 100 nM of E2 for the indicated time. ChIP assays were performed essentially as described previously (Gevry *et al*, 2007) with a panel of specific polyclonal antibodies generated in-house or from commercial sources, as well as pre-immune, and controls without antibodies. FAIRE experiments were performed as previously described (Eeckhoutte *et al*, 2009). Samples were sonicated to generate DNA fragments of <400 bp. qPCR was performed using a set of primers relevant to the promoter regions of the *BCL2* gene. The primers used in qPCR are listed in Supplementary Table S1.

Co-immunoprecipitation and GST pull-down

MCF7 cells were lysed (50 mM Tris-HCl pH 7.4, 180 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.05% SDS, and protease inhibitors) and 2 mg of lysate was pre-cleared with protein A-Sepharose (COM-PANY) for 60 min at 4°C then incubated with 4 μ g of JMJD3 or ER α antibody overnight at 4°C. Immune complexes were captured using protein A-Sepharose for 60 min at 4°C and washed twice with phosphate-buffered saline. Complexes were released by boiling for 5 min in 2 $\times$ SDS loading buffer and separated on 8% SDS-PAGE gels. Proteins were transferred onto a PVDF membrane and blotted for JMJD3 or ER α (sc-8002, Santa Cruz Biotechnology). GST pull-downs were performed essentially as described in Gevry *et al* (2009).

RNA and microarray analysis

Total RNA was isolated from MCF7 cells grown in E2-free medium using the GenElute mammalian total RNA miniprep kit (Sigma). RNA was reverse transcribed into first-strand cDNA using MMLV reverse transcriptase (Promega). Samples were subjected to qPCR using CFX-96 (BioRad) and qPCR for *36B4* and *GAPDH* mRNA was used as an internal controls. The relative abundance of *BCL2* mRNA (or other test genes) was calculated after normalization using *36B4* mRNA, where relative expression levels were calculated as $2^{-\Delta CT}$ where $\Delta CT = CT_{\text{test gene}} - CT_{36B4}$. Primers used are listed in Supplementary Table S2. Microarray analysis using BeadChip Human Genome (Illumina, HT-12) was performed at the Centre d'innovation at Genome Quebec (McGill University, Montreal, QC, Canada). Data were analysed using the FlexArray 1.5 software and as described previously (Blazejczyk *et al*, 2007). Complete results from these analyses are available in Supplementary dataset 1 and 2.

Analysis of the breast cancer TMA

In all, 141 cases were selected from consented patients who had undergone surgical resection with auxiliary lymph node dissection for breast cancer, at the Centre hospitalier de l'Université de Montréal—Hôtel Dieu (CHUM-HD), between 2003 and 2007. Formalin-fixed, paraffin-embedded archival tissue blocks were obtained from the surgical archives at CHUM-HD. Immunohistochemistry for HER2 and H3K27me3 was performed on representative tumour areas, annotated by a pathologist and two single core TMAs were constructed in parallel. Hierarchical clustering was done using the Cluster 3.0 program, Euclidian distance and complete centroid linkage. The original data set was filtered to eliminate individuals with incomplete staining (16 of 142). Data visualization was done using the Java TreeView program (Saldanha, 2004). To test the distribution of the equivalent levels of expression, data were first transformed into high (2, 3) or low values (0, 1). Where expression values were equivalent (high-high or low-low), we attributed a value of 1 to the sample, 0 otherwise. A χ^2 test of independence with this sample was then run using a random distribution as our hypotheses.

Antibodies

A complete list of antibodies used in the present study in ChIP and western blot analysis are shown in Supplementary Table S2.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

References

- Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* **449**: 731–734
- Agger K, Cloos PA, Rudkjaer L, Williams K, Andersen G, Christensen J, Helin K (2009) The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. *Genes Dev* **23**: 1171–1176
- Anderton JA, Bose S, Vockerodt M, Vrzalikova K, Wei W, Kuo M, Helin K, Christensen J, Rowe M, Murray PG, Woodman CB (2011) The H3K27me3 demethylase, KDM6B, is induced by Epstein-Barr virus and over-expressed in Hodgkin's Lymphoma. *Oncogene* **30**: 2037–2043
- Arold ST, Ulmer TS, Mulhern TD, Werner JM, Ladbury JE, Campbell ID, Noble ME (2001) The role of the Src homology 3-Src homology 2 interface in the regulation of Src kinases. *J Biol Chem* **276**: 17199–17205
- Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merckenschlager M, Fisher AG (2006) Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* **8**: 532–538
- Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, Salvesen HB, Otte AP, Akslen LA (2006) EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *J Clin Oncol* **24**: 268–273
- Badia E, Oliva J, Balaguer P, Cavaillès V (2007) Tamoxifen resistance and epigenetic modifications in breast cancer cell lines. *Curr Med Chem* **14**: 3035–3045
- Badve S, Nakshatri H (2009) Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications. *J Clin Pathol* **62**: 6–12
- Barradas M, Anderton E, Acosta JC, Li S, Banito A, Rodríguez-Niendenfuhr M, Maertens G, Banck M, Zhou MM, Walsh MJ, Peters G, Gil J (2009) Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev* **23**: 1177–1182
- Benevolenskaya EV (2007) Histone H3K4 demethylases are essential in development and differentiation. *Biochem Cell Biol* **85**: 435–443
- Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK (1992) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* **24**: 85–95

Acknowledgements

We thank Alain Lavigne, Viktor Steimle, Bruce Murphy, and Mylène Brunelle for critical comments on the manuscript and Benoît Leblanc for the artwork included in Figure 7. We thank Liette Laflamme and Luc Gaudreau for advice with the immunoprecipitation and GST pull-down assays and providing reagents. We are also grateful to Robert Clark, Mien-Chie Hung, and Didier Trono for providing reagents. We would also like to thank Viktor Steimle for the use of the FACS and important advice concerning the analysis, and Luc Paquette for advice on statistical analysis of the breast TMA. This work was supported with fund from the Canadian Institute of Health Research (CIHR) to NG. NG holds a Chercheur boursier (junior 1) award from the FRSQ. AS was the recipient of a doctoral fellowship from NSERC and Réseau Québécois en reproduction.

Author contributions: AS designed and carried out experiments and analysed data; SB and GH assisted with experiments and data analysis; JM performed the TMA experiments; ANM assisted with bioinformatic analysis; NG supervised the project, designed experiments, and analysed data; AS and NG wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

- Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**: 315–326
- Bhat-Nakshatri P, Wang G, Appaiah H, Luktuke N, Carroll JS, Geistlinger TR, Brown M, Badve S, Liu Y, Nakshatri H (2008) AKT alters genome-wide estrogen receptor alpha binding and impacts estrogen signaling in breast cancer. *Mol Cell Biol* **28**: 7487–7503
- Blazeczyk M, Miron M, Nadon R (2007) FlexArray: a statistical data analysis software for gene expression microarrays <http://genomequebec.mcgill.ca/FlexArray>
- Bredfeldt TG, Greathouse KL, Safe SH, Hung MC, Bedford MT, Walker CL (2010) Xenoestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT. *Mol Endocrinol* **24**: 993–1006
- Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SA, Clarke R (1997) MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 162,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. *Cancer Res* **57**: 3486–3493
- Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME, Clarke R (1993) MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 162780. *Cancer Res* **53**: 3229–3232
- Burgold T, Spreafico F, De Santa F, Totaro MG, Prosperini E, Natoli G, Testa G (2008) The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. *PLoS One* **3**: e3034
- Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H (2001) Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for antiestrogen resistance. *J Biol Chem* **276**: 9817–9824
- Cao Q, Yu J, Dhanasekaran SM, Kim JH, Mani RS, Tomlins SA, Mehra R, Laxman B, Cao X, Yu J, Kleer CG, Varambally S, Chinnaiyan AM (2008) Repression of E-cadherin by the polycomb group protein EZH2 in cancer. *Oncogene* **27**: 7274–7284
- Cao R, Zhang Y (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr Opin Genet Dev* **14**: 155–164
- Castaneda CA, Cortes-Funes H, Gomez HL, Ciruelos EM (2010) The phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer. *Cancer Metastasis Rev* **29**: 751–759

- Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV, Auricchio F (2001) PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J* **20**: 6050–6059
- Cha TL, Zhou BP, Xia W, Wu Y, Yang CC, Chen CT, Ping B, Otte AP, Hung MC (2005) Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. *Science* **310**: 306–310
- Cloos PA, Christensen J, Agger K, Helin K (2008) Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev* **22**: 1115–1140
- Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, Aas T, Otte AP, Akslen LA (2006) Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. *Clin Cancer Res* **12**: 1168–1174
- Cooper CS, Foster CS (2009) Concepts of epigenetics in prostate cancer development. *Br J Cancer* **100**: 240–245
- Creyghton MP, Markoulaki S, Levine SS, Hanna J, Lodato MA, Sha K, Young RA, Jaenisch R, Boyer LA (2008) H2AZ is enriched at polycomb complex target genes in ES cells and is necessary for lineage commitment. *Cell* **135**: 649–661
- Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* **111**: 185–196
- De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G (2007) The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell* **130**: 1083–1094
- Ding L, Erdmann C, Chinnaiyan AM, Merajver SD, Kleer CG (2006) Identification of EZH2 as a molecular marker for a precancerous state in morphologically normal breast tissues. *Cancer Res* **66**: 4095–4099
- Ding L, Kleer CG (2006) Enhancer of Zeste 2 as a marker of preneoplastic progression in the breast. *Cancer Res* **66**: 9352–9355
- Eeckhoutte J, Lupien M, Meyer CA, Verzi MP, Shivdasani RA, Liu XS, Brown M (2009) Cell-type selective chromatin remodeling defines the active subset of FOXA1-bound enhancers. *Genome Res* **19**: 372–380
- Fan M, Bigsby RM, Nephew KP (2003) The NEDD8 pathway is required for proteasome-mediated degradation of human estrogen receptor (ER)- α and essential for the antiproliferative activity of ICI 182,780 in ER α -positive breast cancer cells. *Mol Endocrinol* **17**: 356–365
- Ferguson DJ, Anderson TJ (1981) Morphological evaluation of cell turnover in relation to the menstrual cycle in the 'resting' human breast. *Br J Cancer* **44**: 177–181
- Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed YB, Orlov YL, Velkov S, Ho A, Mei PH, Chew EG, Huang PY, Welboren WJ, Han Y, Ooi HS, Ariyaratne PN, Vega VB, Luo Y, Tan PY, Choy PY et al (2009) An oestrogen-receptor- α -bound human chromatin interactome. *Nature* **462**: 58–64
- Gevry N, Chan HM, Laflamme L, Livingston DM, Gaudreau L (2007) p21 transcription is regulated by differential localization of histone H2A.Z. *Genes Dev* **21**: 1869–1881
- Gevry N, Hardy S, Jacques PE, Laflamme L, Svtelits A, Robert F, Gaudreau L (2009) Histone H2A.Z is essential for estrogen receptor signaling. *Genes Dev* **23**: 1522–1533
- Gonzalez ME, Li X, Toy K, DuPrie M, Ventura AC, Banerjee M, Ljungman M, Merajver SD, Kleer CG (2009) Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1. *Oncogene* **28**: 843–853
- He HH, Meyer CA, Shin H, Bailey ST, Wei G, Wang Q, Zhang Y, Xu K, Ni M, Lupien M, Mieczkowski P, Lieb JD, Zhao K, Brown M, Liu XS (2010) Nucleosome dynamics define transcriptional enhancers. *Nat Genet* **42**: 343–347
- Jones RS (2007) Epigenetics: reversing the 'irreversible'. *Nature* **450**: 357–359
- Jorgensen HF, Giadrossi S, Casanova M, Endoh M, Koseki H, Brockdorff N, Fisher AG (2006) Stem cells primed for action: polycomb repressive complexes restrain the expression of lineage-specific regulators in embryonic stem cells. *Cell Cycle* **5**: 1411–1414
- Kouzarides T (2007) Chromatin modifications and their function. *Cell* **128**: 693–705
- Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB (1996) Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. *Clin Cancer Res* **2**: 1215–1219
- Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* **16**: 2893–2905
- Kyprianou N, English HF, Davidson NE, Isaacs JT (1991) Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res* **51**: 162–166
- Lan F, Bayliss PE, Rinn JL, Whetstone JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaani E, Roberts TM, Chang HY, Shi Y (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. *Nature* **449**: 689–694
- Luger K (2003) Structure and dynamic behavior of nucleosomes. *Curr Opin Genet Dev* **13**: 127–135
- Lund AH, van Lohuizen M (2004) Epigenetics and cancer. *Genes Dev* **18**: 2315–2335
- Mattera L, Courilleau C, Legube G, Ueda T, Fukunaga R, Chevillard-Briet M, Canitrot Y, Escaffit F, Trouche D (2010) The E1A-associated p400 protein modulates cell fate decisions by the regulation of ROS homeostasis. *PLoS Genet* **6**: e1000983
- Mattera L, Escaffit F, Pillaire MJ, Selves J, Tyteca S, Hoffmann JS, Gourraud PA, Chevillard-Briet M, Cazaux C, Trouche D (2009) The p400/Tip60 ratio is critical for colorectal cancer cell proliferation through DNA damage response pathways. *Oncogene* **28**: 1506–1517
- McClelland RA, Manning DL, Gee JM, Anderson E, Clarke R, Howell A, Dowsett M, Robertson JF, Blamey RW, Wakeling AE, Nicholson RI (1996) Effects of short-term antiestrogen treatment of primary breast cancer on estrogen receptor mRNA and protein expression and on estrogen-regulated genes. *Breast Cancer Res Treat* **41**: 31–41
- Mellor J (2005) The dynamics of chromatin remodeling at promoters. *Mol Cell* **19**: 147–157
- Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F (2003) Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115**: 751–763
- Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C et al (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**: 553–560
- Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA (2002) Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell* **111**: 197–208
- Naughton C, MacLeod K, Kuske B, Clarke R, Cameron DA, Langdon SP (2007) Progressive loss of estrogen receptor α cofactor recruitment in endocrine resistance. *Mol Endocrinol* **21**: 2615–2626
- Normanno N, Di Maio M, De Maio E, De Luca A, de Matteis A, Giordano A, Perrone F (2005) Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr Relat Cancer* **12**: 721–747
- Ordóñez-Moran P, Munoz A (2009) Nuclear receptors: genomic and non-genomic effects converge. *Cell Cycle* **8**: 1675–1680
- Osborne CK, Schiff R (2011) Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* **62**: 233–247
- Park S, Song J, Joe CO, Shin I (2008) Akt stabilizes estrogen receptor α with the concomitant reduction in its transcriptional activity. *Cell Signal* **20**: 1368–1374
- Pasini D, Bracken AP, Jensen MR, Lazzarini Denchi E, Helin K (2004) Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *EMBO J* **23**: 4061–4071
- Pearce ST, Jordan VC (2004) The biological role of estrogen receptors α and β in cancer. *Crit Rev Oncol Hematol* **50**: 3–22
- Perillo B, Ombra MN, Berton A, Cuzzo C, Sacchetti S, Sasso A, Chiariotti L, Malorni A, Abbondanza C, Avvedimento EV (2008) DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression. *Science* **319**: 202–206
- Pietras RJ, Marquez-Garban DC (2007) Membrane-associated estrogen receptor signaling pathways in human cancers. *Clin Cancer Res* **13**: 4672–4676
- Raaphorst FM, Meijer CJ, Fieret E, Blokzijl T, Mommers E, Buerger H, Packeisen J, Sewalt RA, Otte AP, van Diest PJ (2003) Poorly

- differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. *Neoplasia* **5**: 481–488
- Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, Wysocka J (2011) A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* **470**: 279–283
- Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER (2002) ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Mol Endocrinol* **16**: 100–115
- Riggins RB, Schrecengost RS, Guerrero MS, Bouton AH (2007) Pathways to tamoxifen resistance. *Cancer Lett* **256**: 1–24
- Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR (2000) Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci USA* **97**: 5930–5935
- Saldanha AJ (2004) Java Treeview—extensible visualization of microarray data. *Bioinformatics* **20**: 3246–3248
- Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK (2004) Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clin Cancer Res* **10**: 331S–336S
- Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK (2005) Global histone modification patterns predict risk of prostate cancer recurrence. *Nature* **435**: 1262–1266
- Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103**: 843–852
- Sharov AA, Ko MS (2007) Human ES cell profiling broadens the reach of bivalent domains. *Cell Stem Cell* **1**: 237–238
- Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* **96**: 926–935
- Simon JA, Kingston RE (2009) Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol* **10**: 697–708
- Sommer S, Fuqua SA (2001) Estrogen receptor and breast cancer. *Semin Cancer Biol* **11**: 339–352
- Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC, Winder AD, Reiter R, Wellstein A, Martin MB, Stoica A (2003) Effect of estradiol on estrogen receptor- α gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. *Oncogene* **22**: 7998–8011
- Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV, Cheng JQ (2001) Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ER α) via interaction between ER α and PI3K. *Cancer Res* **61**: 5985–5991
- Swigut T, Wysocka J (2007) H3K27 demethylases, at long last. *Cell* **131**: 29–32
- Teixeira C, Reed JC, Pratt MA (1995) Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res* **55**: 3902–3907
- Wang LH, Yang XY, Zhang X, An P, Kim HJ, Huang J, Clarke R, Osborne CK, Inman JK, Appella E, Farrar WL (2006) Disruption of estrogen receptor DNA-binding domain and related intramolecular communication restores tamoxifen sensitivity in resistant breast cancer. *Cancer Cell* **10**: 487–499
- Wei Y, Xia W, Zhang Z, Liu J, Wang H, Adsay NV, Albarracin C, Yu D, Abbruzzese JL, Mills GB, Bast Jr RC, Hortobagyi GN, Hung MC (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Mol Carcinog* **47**: 701–706
- Welboren WJ, van Driel MA, Janssen-Megens EM, van Heeringen SJ, Sweep FC, Span PN, Stunnenberg HG (2009) ChIP-Seq of ER α and RNA polymerase II defines genes differentially responding to ligands. *EMBO J* **28**: 1418–1428
- Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD (2007) JMJD3 is a histone H3K27 demethylase. *Cell Res* **17**: 850–857
- Zhou VW, Goren A, Bernstein BE (2010) Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet* **12**: 7–18