Jarid2 Methylation via the PRC2 Complex Regulates H3K27me3 Deposition during Cell Differentiation

Graphical Abstract

1. PRC2 methylates Jarid2
2. Jarid2me3 binds Eed aromatic cage potentially competing with H3K27me3
3. Jarid2me3 promotes allosteric activation of PRC2 enzymatic activity

Highlights
- PRC2 methylates Jarid2 on K116
- Jarid2 methylation promotes PRC2 activity
- H3K27me3 and Jarid2-K116me3 bind to the aromatic cage of Eed
- Jarid2 methylation regulates H3K27me3 deposition during ESC differentiation

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In Brief
Methylation of H3K27 by the Polycomb complex PRC2 is essential for maintaining transcriptional silencing. Sanulli et al. report that Jarid2, a cofactor of this complex is also methylated by PRC2. Reciprocally, methylated Jarid2 regulates PRC2 enzymatic activity creating a regulatory loop.
SUMMARY

Polycomb Group (PcG) proteins maintain transcriptional repression throughout development, mostly by regulating chromatin structure. Polycomb Repressive Complex 2 (PRC2), a component of the Polycomb machinery, is responsible for the methylation of histone H3 lysine 27 (H3K27me2/3). Jarid2 was previously identified as a cofactor of PRC2, regulating PRC2 targeting to chromatin and its enzymatic activity. Deletion of Jarid2 leads to impaired orchestration of gene expression during cell lineage commitment. Here, we reveal an unexpected crosstalk between Jarid2 and PRC2, with Jarid2 being methylated by PRC2. This modification is recognized by the Eed core component of PRC2 and triggers an allosteric activation of PRC2’s enzymatic activity. We show that Jarid2 methylation is important to promote PRC2 activity at a locus devoid of H3K27me3 and for the correct deposition of this mark during cell differentiation. Our results uncover a regulation loop where Jarid2 methylation fine-tunes PRC2 activity depending on the chromatin context.

INTRODUCTION

Appropriate gene expression patterns in distinct cell lineages need to be set during embryogenesis and perpetuated during the lifespan of an organism. Polycomb group (PcG) proteins are known to take part in the maintenance of gene repression mostly through chromatin regulation (Margueron and Reinberg, 2011). Polycomb Repressive Complex 2 (PRC2), a key component of the Polycomb machinery, is composed of four core components: the catalytic subunit Ezh1/2, Suz12, Eed, and RbAp46/48. PRC2 is responsible for the di- and tri-methylation of histone H3 at lysine 27 (H3K27me2/3), a histone mark that correlates with silent or poorly transcribed genomic regions (Simon and Kingston, 2013). In addition to the core components of PRC2, several cofactors were shown to interact with this complex and to modulate both its binding to chromatin and its enzymatic activity (Margueron and Reinberg, 2011).

The molecular mechanisms responsible for PRC2 recruitment to chromatin are still unclear. Two models have been proposed (Kose et al., 2013; Voigt et al., 2013). A first “instructive” model proposes that PRC2 recruitment relies either on transcription factors (TFs) or on long non-coding RNAs (lncRNAs). Several studies support this hypothesis, with the examples of the lncRNAs Xist (Maenner et al., 2010; Zhao et al., 2008), HOTAIR (Rinn et al., 2007), or Kcnq1ot1 (Pandey et al., 2008; Redrup et al., 2009) and of TFs such as YY1 (Palacios et al., 2010; Woo et al., 2010, 2013) or Snail (Herranz et al., 2008). However, the nature and the relevance of the interactions between PRC2 and lncRNAs or TFs are not yet clear (Brockdorff, 2013).

A second “responsive” model relies on the observation that chromatin structure modulates PRC2 recruitment and functions. Several studies have shown that PRC2 activity is regulated by marks already present on chromatin. For example, the H3K4me3 and H3K36me3 marks associated to active transcription are reported to prevent the methylation of H3K27 by PRC2 when present on the same histone tail (Schmitges et al., 2011; Voigt et al., 2012; Yuan et al., 2011). In contrast, PRC2 enzymatic activity is stimulated by H3K27me3 via specific interactions between the methylated lysine 27 and the aromatic cage of Eed (Margueron et al., 2009) and by H2A ubiquitination (H2AUb) through a less defined molecular mechanism (Blackledge et al., 2014; Cooper et al., 2014; Kalb et al., 2014).

Other chromatin features have also been shown to impact PRC2/chromatin interaction such as DNA methylation (Bartke et al., 2010) and nucleosome density (Simon and Kingston, 2013; Yuan et al., 2012). In addition, PRC2 cofactors actively contribute to “sensing” chromatin structure. For instance, the
PCL proteins were recently reported to recognize H3K36me3 (Brien et al., 2012; Cai et al., 2013; Musselman et al., 2012; Qin et al., 2013) and both the cofactors Aebp2 and Jarid2 have putative DNA binding domains (Kim et al., 2004, 2009). Of note, transcription can modulate PRC2 function not only through its impact on chromatin but also through PRC2 interaction with nascent RNA transcripts (Davidovich et al., 2013; Kaneko et al., 2013; Kanhere et al., 2010).

Jarid2, a member of the Jumonji family of proteins (Klose et al., 2006), is a developmental regulator, which is necessary for proper mouse development and embryonic stem cell (ESC) differentiation (Landeira and Fisher, 2011). However, unlike other members of the Jumonji family of proteins, Jarid2 has no histone demethylase activity. Previous studies showed that it interacts with PRC2 complex (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). PRC2 and Jarid2 mostly co-localize at chromatin in ESC (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) and Jarid2 depletion reduces PRC2 enrichment at chromatin, leading to the hypothesis that Jarid2 may act to recruit PRC2 (Pasini et al., 2010). In support of this, we recently demonstrated that Jarid2 has a nucleosome-binding domain that stabilizes PRC2 binding to chromatin (Son et al., 2013), interaction that could be modulated by lncRNAs (Kaneko et al., 2014). Notably, reduced occupancy of PRC2 at chromatin in the absence of Jarid2 does not translate into substantial decrease of H3K27me3 enrichment, suggesting that Jarid2 could constrain PRC2 enzymatic activity. However, several studies have now shown that Jarid2 positively regulates PRC2 activity (Li et al., 2010; Son et al., 2013; Zhang et al., 2011). Overall, although there is a consensus on the importance of Jarid2 as regulator of PRC2, how exactly it modulates PRC2 and H3K27me3 deposition is far from clear.

In this study, we investigate the interplay between Jarid2 and PRC2 and explore how this cofactor regulates H3K27me3 deposition and PRC2 function. We show that Jarid2 is a substrate for PRC2 and we characterize Jarid2 methylation in relation to PRC2 activity by a combination of biochemical, genomic, and in vivo approaches. Our study reveals that Jarid2 methylation participates in a unique regulatory mechanism controlling PRC2 catalytic activity and is required for the proper deposition of H3K27me3 during cell differentiation.

RESULTS

PRC2 Methylates Jarid2 at K116 In Vitro

The function of PRC2 in maintaining gene silencing is thought to mostly rely on its methyltransferase activity toward H3K27. Hence, in vivo mutation of H3K27 to arginine leads to the same phenotypes as deletion of PRC2 components (Pengelly et al., 2013). By performing Lysine Methyl-Transferase (KMT) assays in vitro with PRC2 and Jarid2, we observed that Jarid2 is methylated by PRC2 both when this complex is reconstituted around Ezh2 or Ezh1 (Figure S1A).

We produced different Jarid2 recombinant fragments (Figure 1A) that we used as substrates for in vitro KMT assays and we identified the main methylation site within Jarid2 1–232 N-terminal fragment (Figure 1B). Since the methylation is abrogated in the absence of amino acids (aa) 108 to 119 (Figure 1C) and thanks to mass spectrometry analysis, we identified that K116 is methylated in vitro (Figures 1D and S1B). Under the assay conditions, PRC2 catalyzed the mono- and di-methylation of Jarid2. We confirmed that Jarid2-K116 is the main target of PRC2 by generating a point mutation of K116 to alanine (K116A) in Jarid2 fragments or in the full-length protein (Figure 1E). Of note, we could detect the existence of a second minor site of methylation within the C-terminal of Jarid2, which represents about 20% of Jarid2 methylation signal (Figure 1B, fragment 3, Figure 1E). Since we could not confirm its existence in vivo (data not shown), we focused hereafter exclusively on the role of Jarid2-K116 methylation.

Given the conservation of the Polycomb machinery throughout evolution, we asked to what extent the K116 residue is conserved between different species. Strikingly, all the organisms expressing a homolog of Jarid2 that we analyzed harbored an identical stretch of seven amino acids centered on the methylated lysine (Figure 1F). In addition, PRC2 reconstituted with components from Drosophila was able to methylate a fragment of dJarid2 (formerly dJmJ) containing this conserved aa stretch surrounding the K46 residue (Figures S1C and S1D).

Our results therefore demonstrate that an evolutionary conserved lysine localized within the N terminus of Jarid2 is methylated in vitro by PRC2.

Jarid2 Di- and Tri-Methylation Occurs In Vivo

To test whether Jarid2 is methylated in vivo, we generated antibodies recognizing both Jarid2 di-methylated and tri-methylated at K116 (Jarid2-K116me2 and Jarid2-K116me3). We verified the specificity of these antibodies by dot blot on peptides and by western blot on in vitro methylated recombinant proteins (Figures S2A, S2B, and S2C). Western blot on nuclear extract of ESC line (E14) showed a robust signal, both for the di- and tri-methylated Jarid2-K116 antibodies, which was completely lost in ESC constitutively knocked out (KO) for Eed or conditionally KO for Ezh2 (SET domain deletion) (Figure 2A). We additionally performed competition experiment

Figure 1. PRC2 Methylates Jarid2 at K116 In Vitro

(A) Schematic representation of Jarid2 protein showing the annotated domains and the fragments used in KMT assays. PID, PRC2-interaction domain.

(B) KMT assay performed with PRC2-Ezh2 as enzyme and either Jarid2 fragments described in (A) or octamers (Oct) as substrates.

(C) KMT assay performed with PRC2-Ezh2 as enzyme and Jarid2 fragments as labeled on the top.

(D) ESI mass spectrum showing non-(m/z 572.97), mono-(m/z 577.64), and di-(m/z 582.31) methylation at Lysine 116 in the peptide K116KFAQSQPNSPSTTPVK131 of Jarid2 fragment 1–232.

(E) Top: KMT assay performed with PRC2-Ezh2 as enzyme and Jarid2 full-length (FL) and fragments WT or mutants K116A as substrates. (*) indicates Jarid2 fragments. Bottom: quantification of FL Jarid2 methylation in percentage of Jarid2 WT (mean ± SD, n = 3).

(F) Sequence conservation of the amino acids surrounding the methylated lysine across different species. See also Figure S1.
with Jarid2-K116 me2 and me3 peptides and showed that the signal for Jarid2 tri-methylation was competed out by the Jarid2-K116me3 peptide but not by Jarid2-K116me2 peptide (Figure 2B).

After confirming that Jarid2-K116me2 signal is lost in Eed−/− ESC (Figure 2C), we compared the localization of the total and methylated form of Jarid2 by immunofluorescence (IF). We observed a broad nuclear staining for the total Jarid2, similar to the signal observed for Ezh2 (Figure 2D). In contrast, Jarid2-K116me2 shows a more punctuated signal, which overall does not overlap with DAPI-rich regions.

We have recently reported that Jarid2 is recruited during imprinted X chromosome inactivation in the pre-implantation female embryo and acts as bridge between PRC2 and Xist (da Rocha et al., 2014). We could also observe a robust signal for Jarid2-K116me2 by IF at the blastocyst stage, which was dramatically reduced in blastocysts expected to have undergone maternal loss of Ezh2 (Figure S2D). We then analyzed...
late female blastocyst and observed that Jarid2-K116me2 clearly co-localizes with the characteristic Eed cloud marking the inactive X chromosome (Figure 2E).

Consistent with our in vitro data suggesting that Jarid2 could also be methylated in other species, we could detect methylated dJarid2 by western blot on protein extracts from *Drosophila* S2 cells and embryos (Figure 2F). Moreover, polytene chromosome staining revealed that the methylated dJarid2 associates to chromatin and that it only partially co-localizes with the Polycomb protein Ph, in agreement with a previous report studying dJarid2 (Herz et al., 2012) (Figure S2E).

We conclude that Jarid2 is di- and tri-methylated by PRC2 at K116 in mammalian cells and that this post-translational modification occurs in vivo in particular during early mouse development. Finally, Jarid2 methylation is conserved through evolution as it also occurs in *Drosophila*.

**Jarid2-K116me2 Occupancy Co-Localizes with Total Jarid2 at Chromatin**

The IF experiments prompted us to study in more details the distribution of Jarid2-K116me2 at chromatin. We first analyzed the subcellular distribution of Jarid2-K116me2 by performing cell fractionation experiments followed by western blots. Both total Jarid2 and Jarid2-K116me2 are preferentially enriched in the insoluble chromatin fraction, although they are also detected in the other nuclear fractions (Figure 3A).

We then performed ChIP-sequencing (ChIP-seq) experiments in E14 ESC to compare the localization of Jarid2 and of its methylated form. A similar number of reads were generated for both antibodies with more than 95% of reads mapping to the mouse genome (Figure S3A). As expected, almost all regions significantly enriched for Jarid2-K116me2 were also enriched for total Jarid2 (Figure 3B). Conversely, about 80% of the regions significantly enriched for total Jarid2 were enriched for its methylated form (Figure 3B). Jarid2 and Jarid2-K116me2 display the same narrow distribution around the transcription start sites (TSSs) of PRC2 target genes (Figure 3C), which contrast with the more widespread distribution of H3K27me3. Of note, the enrichments for Jarid2 and Jarid2-K116me2 correlate very well (Pearson correlation, R^2 > 0.98, Figure 3D, left) and the peaks apparently specific either for total Jarid2 or for its methylated form are all characterized by a low number of reads for Jarid2 and H3K27me3 (Figures 3D, S3B, and S3C). Thus, rather than being specific to the methylated or total Jarid2, these specific peaks...
most likely result from the variability in sequencing depth and background estimation between the two samples.

In conclusion, the methylated form of Jarid2 localizes at chromatin of PRC2 target genes in ESC with a distribution that overlaps nearly perfectly with total Jarid2.

Jarid2 Methylation Is Required for Efficient H3K27me3 Deposition

It has been previously shown that artificial tethering of Jarid2 to a reporter gene induces transcriptional repression in a PRC2-dependent manner (Li et al., 2010; Pasini et al., 2010). We similarly engineered cell lines stably integrated with a luciferase reporter gene and expressing, in an inducible manner, Gal4-Jarid2 WT or mutants that cannot be methylated, in which K116 was replaced by an alanine or an arginine (K116A or K116R, Figure 4A).

To avoid potential leakiness of the inducible system or contribution of the endogenous Jarid2, we knocked down both the endogenous and Gal4-fusion Jarid2 proteins by RNA interference. All cell lines present substantially reduced Jarid2 protein levels as shown by western blot (Figure 4B). Upon induction, while the Gal4-Jarid2 WT protein led to transcriptional repression of the luciferase reporter, both the Gal4-Jarid2-K116A and K116R only modestly reduced transcription (Figure 4C). To rule out the possibility that the K116 point mutation interfered with Jarid2 binding to PRC2, we performed co-immunoprecipitation in HEK293 cells overexpressing Flag-Jarid2 WT or K116A, followed by WB with the antibodies indicated on the right.

Monitoring H3K27me3 levels at the reporter transgene by ChIP, we found that the expression of Gal4-Jarid2 WT resulted in an increase of H3K27me3 by over 5-fold, while expression
of Gal4-Jarid2-K116A/R only led to an increase of the mark by about 2-fold (Figures 4E and S4). Importantly, the Gal4 fusion proteins and Ezh2 were similarly recruited to the reporter upon doxycycline treatment (Figure 4E). Thus, mutating K116 of Jarid2 does not affect PRC2 recruitment per se but does affect its ability to promote PRC2 enzymatic activity on H3K27.

We conclude that Jarid2-K116 methylation is dispensable for PRC2 recruitment to chromatin but necessary to efficiently promote PRC2-mediated H3K27me3 deposition.

Jarid2 Methylation Regulates PRC2 Enzymatic Activity Depending on the Chromatin Context

Our previous work showed that the C-terminal domain of Eed forms an aromatic cage that recognizes H3K27me3, leading to allosteric changes that stimulate PRC2 enzymatic activity on H3K27 (Margueron et al., 2009). To determine whether Jarid2 could be involved in a similar mechanism, we monitored PRC2 Histone-Lysine-Methyl Transferase (HKMT) activity in presence of peptides mimicking Jarid2-K116 di- and tri-methylation. We observed that both Jarid2-K116me2 and me3 peptides markedly stimulate PRC2 enzymatic activity on recombinant oligonucleosomes, similarly to the H3K27me3 peptide (Figure 5A). This stimulatory activity was lost with a recombinant PRC2 complex carrying a point mutation (Eed F97A) in Eed’s aromatic cage (Figure 5B), indicating specific interaction between the methylated K116 and the aromatic cage.

To confirm the specificity of Eed-Jarid2-K116me2/3 interaction, we measured the binding affinity of Eed (ΔEed residues 77 to 441, see [Margueron et al., 2009]) for Jarid2-K116me2/3 peptides using isothermal titration calorimetry (ITC). Jarid2-K116me3 peptide exhibited ten times more affinity for ΔEed than H3K27me3 peptide, with a dissociation constant (Kd) of 3 μM (Figures 5C and S5). Jarid2-K116me2 peptide affinity for Eed was lower than Jarid2-K116me3 but still higher than H3K27me3.

To further explore Eed-K116me3 interactions, we solved the structure of ΔEed co-crystallized with Jarid2-K116me3 peptide and compared it with the structure of ΔEed-H3K27me3. Within the two structures, the Eed aromatic cages largely superimpose and the Jarid2-K116me3 and H3K27me3 peptide backbones significantly overlap, despite some differences in the aa sequences (Figure 5D; Table 1). However, Jarid2-K116me3 peptide forms additional contacts with Eed (Figure 5D, red circles). In particular, the large hydrophobic side chain of the phenylalanine at position +1 (F117) lies alongside the stem of the methyl-lysine and could contribute to the stabilization of K116me3 within the hydrophobic pocket. Indeed, a peptide in which the F117 was replaced by an alanine (F117A) did not interact with Eed (Figure 5C) and was unable to stimulate PRC2 enzymatic activity (Figure 5E).

The N-terminal region of Jarid2 comprises distinct domains for both PRC2 and chromatin interactions (Son et al., 2013). To evaluate the role of K116me3 in this context, we generated fragments of Jarid2 covering aa 109–450, K116 being fully methylated, unmodified, or mutated to alanine or arginine. The fully methylated Jarid2 fragment was produced by coupling, through native chemical ligation, a peptide containing aa 109–123 (K116me3) with the aa 124–450 fragment. We then monitored PRC2 enzymatic activity on recombinant or native nucleosomes either alone or in the presence of equimolar amounts of Jarid2 fragments (Figure 5F). As expected, all Jarid2 fragments stimulated PRC2 enzymatic activity. However, the methylated fragment was about 5- to 6-fold more efficient as compared to the K116A or K116R fragments when recombinant chromatin was used as substrate (Figure 5F, black bars). In contrast, this stimulation was lost when native chromatin was used as substrate (Figure 5F, gray bars). This result suggests that histones PTMs present on native chromatin could interfere with the binding of the methylated K116 to Eed’s aromatic cage.

To evaluate a potential competition between H3K27me3 and Jarid2-K116me3, we reconstituted chromatin in presence of increasing ratios of octamers fully methylated on K27 and monitored PRC2 activity in presence of Jarid2 fragments (Figure 5G). As expected, the titration of H3K27me3 stimulates PRC2 enzymatic activity. In presence of the methylated form of Jarid2, the titration of H3K27me3 has no effect on the stimulation of PRC2 mediated by Jarid2. In contrast, in presence of the non-methylated or non-methylatable forms of Jarid2, the titration of H3K27me3 limits Jarid2-mediated stimulatory effect. This result suggests the presence of a competition between the stimulatory activity of H3K27me3 and Jarid2. When methylated, Jarid2 controls PRC2 enzymatic activity even in presence of H3K27me3. In contrast, when Jarid2 is not methylated, H3K27me3 is taking over the control of PRC2 activity. Importantly, this assay addresses only the case of Jarid2 methylation versus trans-nucleosomes H3K27me3-mediated regulation of PRC2 enzymatic activity. The outcome of this competition could be different when additional histones PTMs or nucleosomes asymmetrically methylated on H3K27 are present.

Altogether, these experiments show that Jarid2 methylation is recognized by the aromatic cage of Eed and promotes allostERIC activation of PRC2 enzymatic activity. Yet, this mechanism is modulated by the presence of PTMs on chromatin, most likely owing to the competitive binding to Eed.

Jarid2 Methylation Is Required for PRC2 Function upon Cell Differentiation

The results presented so far demonstrate that Jarid2 methylation is important for PRC2 activity in vitro and when artificially tethered to chromatin in a cellular context. To confirm the biological relevance of this regulation, we evaluated the function of Jarid2 methylation in ESC. We used ESC KO for Jarid2 (Shen et al., 2009) and generated cell lines rescued with either WT or K116A mutant forms of Jarid2. Since Jarid2 is dynamically regulated during cell differentiation, the rescues were performed using bacterial artificial chromosomes (BACs) in order to recapitulate the endogenous expression of the Jarid2 gene. Rescued ESC lines expressed Jarid2 homogeneously, with appropriate nuclear distributions as illustrated by IF (Figure 6A) and at similar levels as shown by western blot (Figure 6B). Moreover, all three lines exhibited similar global levels of H3K27me3 and Oct4 protein (Figure 6B). Interestingly, Jarid2 WT rescued ESC presented slight morphological differences, displaying more typical ESC colonies, when compared to Jarid2 KO or K116A mutant cells that tend to show a more flattened morphology (Figure 6C,
Table 1. Data Collection and Refinement Statistics

| EED-Jarid2 |
|-----------------|
| **Data Collection** |
| Beamline | Diamond Light Source IO2 |
| Wavelength | 0.9795 Å |
| Space group | P212121 |
| Cell dimensions |
| a, b, c | 49.419, 58.144, 127.042 Å |
| α, β, γ | 90.00°, 90.00°, 90.00° |
| Resolution (Å) | 30–20 Å |
| (2.09–2.0 Å) |
| R_{sym} | 0.084 (0.365) |
| I/σ | 16.2 (2.23) |
| Completeness | 97.2% (38.2%) |
| Redundancy | 3.9 (1.9) |
| **Refinement** |
| Resolution (Å) | 30–2.3 Å |
| Number of Reflections | 16,463 |
| \( R_{work} / R_{free} \) | 0.174 / 0.224 |
| Number of atoms |
| Protein | 2,972 |
| Solvent | 175 |
| B-factors |
| Protein | 21.15 Å² |
| Solvent | 24.03 Å² |
| RMS deviations |
| Bond lengths | 0.007 Å |
| Bond Angles | 1.074° |
| Ramachandran plot |
| Favored regions | 95.8% |
| Allowed region | 4.2% |
| Outlier region | 0.0% |

Highest-resolution shell is shown in parentheses.

left). They also formed fewer colonies when plated at low density as compared to the K116A rescued line (Figure 6C, right). Nonetheless, all the three ESC lines showed similar levels of alkaline phosphatase activity, comparable proliferation rates, and no major differences in gene expression (Figure 6D and day 0 in Figure 6F), thus confirming that Jarid2 is not necessary for ESC pluripotency and self-renewal (Landeira et al., 2010; Shen et al., 2009).

Since Jarid2 depletion has been reported to cause pronounced defects during ESC differentiation (Landeira et al., 2010; Li et al., 2010; Shen et al., 2009), we induced differentiation of the three ESC lines into embryoid bodies (EBs). All ESC lines could initiate differentiation as illustrated by the decreased protein levels of Jarid2 and Oct4 at day 5 and 10 of differentiation (Figure 6E). Next, using digital multiplexed gene expression analysis (NanoString nCounter), we measured transcript levels, during the course of differentiation, of a set of markers of ESC pluripotency and differentiation toward the different germ layers. Overall, gene expression follows the same trend in all three cell lines but with marked differences in term of kinetics and/or extent of gene regulation (Figures 6F and S6). For instance, most pluripotency markers were downregulated (e.g., Fbx15, Zfp42, Ki67, Essrb, Prdm14, Tcl1); however, downregulation appears earlier and more pronounced in the KO and Jarid2-K116A lines, the latter displaying an even more marked effect. A noticeable exception is the transcript Stella/dppa3, which is upregulated in the Jarid2-WT line but downregulated in the two other lines upon differentiation.

KO and Jarid2-K116A lines also displayed specific differences. In particular, markers of differentiation toward the mesoderm such as Brachyury and Foxa2 are activated later in the Jarid2 K116A cells. We confirmed this data by RT-qPCR and further observed that Jarid2 KO cells seem to more efficiently activate the expression of genes such as Gata4 or Olig1 (Figure S6). Finally, repeats were also found to be differentially regulated depending on Jarid2 status as illustrated by the LINE 1 elements (Figure 6F, bottom).

Taken together, these results indicate that Jarid2 and its methylation play an important role during the establishment of cellular differentiation programs.

Figure 5. Jarid2 Methylation Regulates PRC2 Enzymatic Activity Depending on Chromatin Environment

(A) HKMT assay performed with PRC2-Ezh2 on recombinant oligonucleosomes in the presence of peptides mimicking either Jarid2-K116me2 and me3 or H3K27me3.

(B) HKMT assay as in (A) performed with PRC2-Ezh2 reconstituted with either Eed WT or F97A.

(C) Table indicating the sequences of the peptides and the relative dissociation constant \( K_d \) (μM) for ΔEed binding measured by ITC.

(D) Ribbons representation of ΔEed-Jarid2-K116me3 and ΔEed-H3K27me3 complexes. Eed is in gray; Jarid2 peptide is in yellow while H3 peptide is in purple, both in stick representation. Eed aromatic amino acids are in stick and cyan color. Top: top views. Bottom: zoom-in of side views.

(E) HKMT assay as in (A) in presence of peptides Jarid2-K116me3 WT or F117A.

(F) Left: representation of the chimeric proteins used in the HKMT assay. The red cross indicates the point mutations introduced in the protein to perform native chemical ligation. Red circle indicates K116me3. Right: quantification of PRC2-HKMT activity in the presence of the three Jarid2 fragments (mean ± SD, n = 3). Native (gray bars) or recombinant (black bars) chromatin were used as substrates.

(G) HKMT assay performed with PRC2-Ezh2 in the absence or presence of Jarid2 WT, Jarid2-K116me3, or Jarid2 K116R. Recombinant chromatin reconstituted in presence of increasing amount of H3K27me3 octamers is used as substrate. See also Figure S6.
on the correlation of all peaks found enriched for H3K27me3 in at least one of the six samples (Figure 7A). ESC and EBs formed two very distinct clusters, thus confirming that all three lines engaged differentiation. H3K27me3 genomic distribution between all three ESC lines was strongly correlated and overall differences did not exceed the variability between duplicates (Table S1). This result is consistent with the lack of major effect on H3K27me3 upon Jarid2 deletion in ESC and could explain some of the discrepancies among the reports describing the consequences of knocking down or out Jarid2 on H3K27me3 enrichment (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). In contrast, we observed noticeable differences regarding the distribution of H3K27me3 in EBs depending on Jarid2 status. Overall, the H3K27me3 profiles diverged more when comparing H3K27me3 ChIP-seq between ES and EB in the lines knockout or mutant for Jarid2 (Figure 7B). Consequently, a substantial number of peaks are specifically gained or lost in the Jarid2 mutant lines as compare to the WT during differentiation (Figure 7C).

Genomic ontology (GO) analysis revealed that the peaks losing H3K27me3 display the canonical features of PRC2 target peaks such as strong enrichment for CpG-islands, 5’ UTRs, coding regions, or promoters (Figures 7D and S7B). In contrast, the H3K27me3 peaks that were gained in the Jarid2 KO and K116A cells are not characterized by such a clear enrichment for any specific category, except for a bias toward CpG-islands in the K116A cells. The distinct features of peaks that are gained and lost during transition from ES to EB in the Jarid2 KO and K116A lines is also highlighted by the analyses of the localization of peaks relative to the TSS (Figure 7D). While the majority of the peaks losing H3K27me3 in the mutant cells resides within 5 kb of a TSS, the peaks gaining H3K27me3 are more broadly distributed all over the genome. Hence, we found gains of H3K27me3 corresponding to a broad enrichment in between two genes as well as more localized peaks in genomic regions without any gene annotation (Figure 7D).

Altogether our ChIP-seq data highlight the crucial role of Jarid2 and its methylation for the proper deposition of H3K27me3 during the process of differentiation. We further show that the aberrant loss of H3K27me3 detected in the mutant cells appears to be compensated by gains in this mark elsewhere in the genome. Whereas the loss of H3K27me3-enriched sites in the Jarid2 mutant cells is likely to have transcriptional consequences considering the proximity of such sites to the TSS, it is much less clear what the impact of the peaks gaining H3K27me3 will have on gene expression considering their apparently more random distribution.

**DISCUSSION**

PRC2, as a key component of the PcG machinery, is essential to ensure cellular memory through the maintenance of transcriptional repression. This implies that its genomic targeting should be finely regulated and cell-type specific. However, the mechanisms regulating its activity remain largely obscure. Here, we report that Jarid2, which is known to directly interact with PRC2 and to co-localize genome-wide with this complex (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Shen et al., 2009), is actually a substrate of PRC2’s enzymatic activity. Furthermore, we have shown that Jarid2 methylation modulates the enzymatic activity of PRC2 creating a unique regulatory loop, which impacts on H3K27me3 localization. Consequently, a point mutant preventing Jarid2 methylation leads to defects in cell differentiation with inappropriate H3K27me3 deposition.

Until recently, it was assumed that the sole substrate of PRC2 was the histone H3K27. A few publications have now proposed that Ezh2 could methylate other non-histone substrates and in particular TFs such as GATA4, STAT3, the androgen receptor, and RORα (He et al., 2012; Kim et al., 2013; Lee et al., 2012; Xu et al., 2012). An important difference between these putative PRC2 substrates and both H3K27 and Jarid2-K116 is the fact that the former are not involved in retro-control loops regulating PRC2 enzymatic activity. Hence, both H3K27me3 and Jarid2-K116me3 are able to bind Eed and promote the allosteric activation of PRC2 enzymatic activity. Such a process is unlikely to occur with other substrates since either the methylated lysine is not preceded by an arginine (Stat3 and Gata4), which we previously showed to be required for PRC2 stimulation (Margueron et al., 2009), or the substrate was only found to be mono-methylated (e.g., RORα).

The discovery that H3K27me3 stimulates PRC2 enzymatic activity and that a single mutation disrupting Eed’s aromatic cage causes a global loss of H3K27me3 in vivo, led to the proposal of a model whereby this positive feedback mechanism might account for the maintenance of this histone mark during cell division (Margueron et al., 2009). In view of this model and of our data, we speculate that Jarid2-methylation could constitute an alternative mechanism to prime PRC2 activity when targeted to chromatin regions devoid of H3K27me3. This would explain why Jarid2 is important while cells engage differentiation but is overall dispensable in undifferentiated ESC, which supposedly

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**Figure 6. Jarid2 Methylation Is Required to Set Up Differentiation Programs in ESC**

A) IF for Jarid2 and Oct4 in Jarid2 KO and Jarid2 WT or K116A rescued ESC.
B) WB with the indicated antibodies on nuclear extracts of the three ESC lines.
C) Left: colony morphology quantification of the indicated ESC. Values represent the percentage of alkaline-phosphatase-positive colonies showing a compact, flat, or spread morphology (mean ± SD, n ≥ 2). Right: number of methylene-blue-stained colonies (mean ± SD, n ≥ 2) detected 8 days after plating 100 cells per well.
D) Top: cell growth curve of the ESC over 4 days (mean ± SD, n = 3). Bottom: representative view of phosphatase alkaline staining.
E) WB probing nuclear extracts during ESC differentiation.
F) RNA transcript quantification through multiplexed-digital hybridization-based analysis (mean, n = 2). Heatmap representing the Log2-transformed median centered values. Top: messenger RNA classified by function. Bottom: transcripts from repeated sequences. Values are normalized around a set of housekeeping genes (ActB, Hprt1, Gapdh, Ppia, RplP0, and Rm2). See also Figure S6.
A

Color Key

8 days of diff (Hanging drops)

Peaks losing H3K27me3

Peaks gaining H3K27me3

B

ES vs EB WT

ES vs EB Jarid2-KO

ES vs EB Jarid2-K116A

D

% of peaks

ES

EB

KO

Armp22

Lgr1

Oct4

C1d

K116A gain

KO gain

K116A loss

KO loss

(legend on next page)
mostly maintain H3K27me3 though the positive feedback loop involving H3K27me3 and Eed.

Our results suggest that, in vitro, the methylation of Jarid2 could control the balance between Jarid2me3- and H3K27me3-mediated regulations of PRC2 enzymatic activity due to its higher affinity for Eed. However, it is very likely that in vivo additional histone PTMs modulate the balance between these regulatory loops. Hence, it will be interesting to determine whether H2Aub could, for instance, favor the binding of Eed to H3K27me3 (Kalb et al., 2014). The fact that Jarid2 is dispensable for H3K27me3 deposition in undifferentiated ESC while present at almost all PRC2 targets could be explained by the redundancy between Jarid2-K116me3 and H3K27me3 regulatory loops in this context.

In contrast, upon cell differentiation-induced redistribution of H3K27me3, Jarid2 and its methylation become critical for the appropriate regulation of PRC2 activity. Hence, we observed that in Jarid2 KO and K116A cells H3K27me3 enrichment is lost or reduced during cell differentiation at a subset of PRC2 targets, leaving those genes prone to transcriptional activation. Surprisingly, the losses seem to be compensated by gains of H3K27me3 at unusual genomic location for this mark (e.g., far away from the TSS). Therefore, even though the global level of this mark is unchanged in the Jarid2 mutant cells, the genomic distribution of H3K27me3 is incorrect. The fact that the gains of H3K27me3 retain some selectivity for CpG islands in the Jarid2-K116A, which express Jarid2 with functional DNA-binding domains, suggests that this aberrant targeting is not random but results from the combination of partly functional Jarid2 and other cofactors that misguide PRC2 to new sites. It remains to be determined whether those new H3K27me3 peaks have transcriptional consequences or are constituted of chromatin domains that are somehow acting as a “sink” for the surplus of PRC2 activity. Intriguingly, a similar enrichment for H3K27me3 at intergenic regions was also reported for ESC grown in 2i media (Marks et al., 2012), it would therefore be interesting to investigate whether Jarid2 function could be altered in this context.

In conclusion, Jarid2 protein contains distinct domains that regulate PRC2 function through different mechanisms. Our study provides insights into the molecular mechanisms by which Jarid2 controls PRC2 function during mammalian cell differentiation. Although we now have a deeper understanding of the various functions of the rather unique N-terminal moiety of Jarid2, the role of the C terminus remains unclear. The presence of several conserved domains, such as the enzymatically inactive demethylase-like domain, suggests that this C-terminal portion must have some yet unknown functions. Our study also reveals that Jarid2 and its methylation appear to have a more critical role in a developmental context when compared to undifferentiated cells. Dissecting how the multiple functions of Jarid2 in targeting and modulating PRC2 activity are orchestrated during development remains an exciting challenge for the future.

### EXPERIMENTAL PROCEDURES

Full details on experimental procedures and the list of the used antibodies are available on the Supplemental Experimental Procedures.

### BAC Mutagenesis and Genomic Integration

K116A mutation was generated in the BAC-Jarid2 RP23-152H18 by homologous recombination and counter selection strategy (Bird et al., 2012). The construct was inserted into the genome of Jarid2 KO ESC through transposon-mediated BAC integration as previously described (Rostovskaya et al., 2012).

### Recombinant Proteins Purification

Recombinant Jarid2 fragments 6×His-tag were produced in bacteria. Recombinant mammals and flies PRC2, Aebp2, Jarid2 WT, and K116A were produced in SF9 insect cells as described in Supplemental Experimental Procedures.

### Native Chemical Ligation

Eed-Jarid2K116me3 Crystal

The ΔEed protein was prepared as previously described (Margueron et al., 2009) and detailed in the Supplemental Experimental Procedures.

### Nanostring Quantification of Dene Expression

Direct digital mRNA analysis of expression was performed using a custom set of oligonucleotides synthesized by NanoString Technologies. Hybridization and analysis were done using the Prep Station and Digital Analyzer purchased from the company.

### ACCESSION NUMBERS

The GEO repository accession number for the all ChIP-seq datasets reported in this paper is GSE55630. The Protein Data Bank accession number for the structural data reported in this paper is 4X3E.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.12.020.

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Molecular Cell
Supplemental Information

Jarid2 Methylation via the PRC2 Complex
Regulates H3K27me3 Deposition
during Cell Differentiation

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1) Supplemental Figures and Legends

A

B

C

D

Supplemental Figures and Legends
**Supplemental Figure 1** (Related to Fig 1)

(A) KMT assay using PRC2-Ezh1 or PRC2-Ezh2 as enzyme and Jarid2 fragment aa 1-232 as substrate. (B) MS/MS spectrum of the peptide precursor ion at m/z 572.97, 577.64 and 582.31 determining unmodified, mono- and di-methylated Lysine 116 in the peptide 116KFAQSQPNPSTTPVK131 of Jarid2 fragment 1-232. (C) Schematic representation of human (top) and *Drosophila* (bottom) Jarid2 proteins. dJarid2 fragments used in (D) are shown. (D) KMT assay using mammalian PRC2 or dPRC2 as enzyme and dJarid2 aa fragments 1-232 or 1-516 as substrates, octamers (Oct) are used as positive control.
Supplemental Figure 2 (Related to Fig 2)

(A and B) Dot blots showing the specificity of the Jarid2-K116me2 (A) and Jarid2-K116me3 (B) antibodies. The amounts of peptides correspond to Jarid2-K116 un- (Un), di- (me2) and tri- (me3) methylated peptides are indicated on top. (C) KMT assay using PRC2 as enzyme and Jarid2 fragment 1-232 WT or K116A mutant as substrates, followed by WB with the indicated antibodies, showing the specificity of the antibodies on recombinant methylated proteins. (D) IF staining of Jarid2-K116me2 in mouse blastocyst expected to be either Ezh2 f/- or Ezh2 -/- (maternal deletion using the Zp3-Cre). Nuclei are stained with DAPI. Embryos were harvested at 16 cell-stage. Ezh2 -/- embryos appeared mildly delayed. (E) Drosophila polytene chromosome staining performed on WT flies with the indicated antibodies.
Supplemental Figure 3 (Related to Fig 3)

(A) Number of sequenced and mapped reads in the Jarid2 and Jarid2-K116me2 ChIP-seq experiments. (B) Snapshots of representative peaks common to total Jarid2 and Jarid2-K116me2 ChIP-Seq or specific for one of the two as indicated on the left. (C) Normalized read
counts for Jarid2 vs H3K27me3 ChIP-seq at total and methylated Jarid2 target peaks. Read counts for H3K27 were analyzed after extending Jarid2 peaks of 0.5kb on each side. Total Jarid2 ChIP-seq described in figure 3. H3K27me3 ChIP-seq (rescued Jarid2 WT cell) described in figure 7.
**Supplemental Figure 4** (Related to Fig 4)
ChIP performed on T-rex 293 Gal4-Jarid2 WT, K116A and K116R cell lines. H3K27me2/3 (7B11G5) antibody was used for ChIP. Y-axis represents percent of input (mean ±SD, n≥2).
Supplemental Figure 5 (Related to Fig 5)
ITC curves for Eed binding to the indicated peptides.
Supplemental Figure 6 (Related to Fig 6)

Messenger RNA (mRNA) levels were quantified by RT-qPCR upon differentiation of ES into EB. Values are normalized to TBP mRNA and plotted as $2^{-\Delta\Delta C_T}$ (mean ±SD, n≥2).
Supplemental Figure 7 (Related to Fig 7)

(A) Number of sequenced and mapped reads in the H3K27me3 ChIP-seq experiments (3 cell lines both undifferentiated “ESC” or differentiated “EB”). (B) Genomic ontology of peaks gaining or losing H3K27me3 during cell differentiation specifically in the Jarid2 KO or Jarid2 K116A cells.
2) Supplemental Table and Legend

|          | ES KO 1  | ES KO 2  | ES WT 1  | ES WT 2  | ES K116A 1 | ES K116A 2 | EB KO 1  | EB KO 2  | EB WT 1  | EB WT 2  | EB K116A 1 | EB K116A 2 |
|----------|----------|----------|----------|----------|------------|------------|----------|----------|----------|----------|------------|------------|
| ES KO 1  | 1        | 0.985    | 0.97     | 0.989    | 0.975      | 0.979      | 0.929    | 0.904    | 0.965    | 0.972    | 0.876      | 0.861      |
| ES KO 2  | 0.985    | 1        | 0.928    | 0.993    | 0.953      | 0.978      | 0.932    | 0.912    | 0.964    | 0.87     | 0.881      | 0.872      |
| ES WT 1  | 0.97     | 0.928    | 1        | 0.953    | 0.969      | 0.948      | 0.887    | 0.862    | 0.942    | 0.95     | 0.85       | 0.833      |
| ES WT 2  | 0.989    | 0.993    | 0.953    | 1        | 0.96       | 0.978      | 0.922    | 0.9      | 0.97     | 0.978    | 0.877      | 0.865      |
| ES K116A 1 | 0.975    | 0.953    | 0.969    | 0.96    | 1          | 0.98       | 0.918    | 0.897    | 0.953    | 0.956    | 0.917      | 0.905      |
| ES K116A 2 | 0.979    | 0.97     | 0.948    | 0.978    | 0.99       | 1          | 0.931    | 0.912    | 0.965    | 0.968    | 0.928      | 0.919      |
| EB KO 1  | 0.929    | 0.932    | 0.887    | 0.922    | 0.918      | 0.931      | 1        | 0.996    | 0.971    | 0.967    | 0.933      | 0.929      |
| EB KO 2  | 0.904    | 0.912    | 0.862    | 0.9      | 0.897      | 0.912      | 0.996    | 1        | 0.957    | 0.953    | 0.925      | 0.927      |
| EB WT 1  | 0.965    | 0.964    | 0.942    | 0.97     | 0.953      | 0.965      | 0.971    | 0.957    | 1        | 0.996    | 0.936      | 0.927      |
| EB WT 2  | 0.972    | 0.97     | 0.95     | 0.978    | 0.956      | 0.968      | 0.967    | 0.953    | 0.996    | 1        | 0.923      | 0.914      |
| EB K116A 1 | 0.876    | 0.881    | 0.85     | 0.877    | 0.917      | 0.928      | 0.933    | 0.925    | 0.936    | 0.923    | 1          | 0.995      |
| EB K116A 2 | 0.861    | 0.872    | 0.833    | 0.865    | 0.905      | 0.919      | 0.929    | 0.927    | 0.927    | 0.914    | 0.995      | 1          |

**Supplementary Table 1** (Related to Fig 7)

Pearson correlation between each ChIP-seq samples. 1 and 2 refer to biological replicates.
3) Supplemental Experimental procedures

Cloning

dJarid2 clone LD17709 was obtained from the Drosophila Genomics Resource Center and the fragments 1-232 and 1-516 were cloned in pet102 for bacterial expression.
hJarid2 fragments were cloned in pET102 vector (Invitrogen) for bacterial expression.

Site directed mutagenesis

Lysine 116 was mutated to Alanine and Arginine following the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Cell lines

T-Rex 293 cells (Invitrogen) were grown according to the manufacturer’s instructions. First, 5XGal4RE-tk-Luc-Neo plasmid was stably integrated into the cells and selected by G418. Subsequently, the selected clone was generated by stable transfection of pCDNA4-T0-Gal4-hJarid2 WT and K116A. Single clones are selected with zeocin (300 µg/ml) and screened for the expression of Gal4-Jarid2 48 hours after doxycycline induction at 1µg/ml (Sigma). Stable clones of 293T cell over-expressing pCMV4-HA-Flag-hJarid2 WT or K116A and resistant to G418 were screened for the expression of the Flag-tagged proteins by WB.

ESC were cultured on gelatin-coated dishes in DMEM media supplemented with 15% FBS, 100mM non essential amino acids, 0.1mM 2-mercaptoethanol, 1mM L-Glutamine (Invitrogen) and 103U/ml leukemia inhibitory factor (LIF) (ESG1107, Millipore).

ESC E14 and Eed-/- were cultured as previously described (Li et al., 2010). ΔEzh2 f/f ROSA Cre-ERT2 ESC were generated in E.H. laboratory. Jarid2-/- ESC were generously provided by S. Orkin.

D. melanogaster S2 cells were cultured in Schneider's Drosophila medium (Invitrogen) supplemented with 10% FBS at 25 °C.

ESC differentiation

EBs are formed by hanging drop method. Cells are diluted at 1000 cells/20µl drop in ESC media in absence of LIF. After 2 days EBs are flushed in low adherence plates.

shRNA

ShRNA were designed against hJarid2 accordingly to
Oligonucleotides were annealed and sub-cloned in the pLKO.1 vector (Addgene). Cells were infected and selected with 2µg/ml puromycin.

**Luciferase Assay**

Luciferase reporter activities were measured in whole cell lysates using the Luciferase Assay System (Promega, #E15020) and Fluostar Optima BMG labtech luminometer. All experiments were done in biological and technical triplicates and normalized for protein concentration (Bradford).

**Antibodies**

WB, ChIP and IF were performed using the following antibodies: polyclonal antibodies specific to Jarid2-K116 me2 and me3 were raised against a synthetic peptide H2N-CRLQAQRK(dimethyl)FAQSQ-CONH2 and CRLQAQRK(trimethyl)FAQSQ-CONH2 representing aa 109-121. The serum was first counter-selected on a column containing the unmodified peptide and subsequently affinity purified on the methylated peptide. Antibodies against Ezh2, Suz12, Jarid2 and H3K27me2/3 were previously described (Margueron et al., 2008); total H3 (39163) and H3K4me3 (39159) were purchased from Active Motif; Gal4 (06-262) for ChIP was purchased from Millipore; Gal4 for WB from Santa Cruz Biotechnologies (sc-510); H3K27me3 (ab6002) and Lamin B1 (ab16048) were purchased from Abcam; Flag M2 was purchased from Sigma (F1804); Oct4 (BD 611203) BD Biosciences. Eed antibody (M26) was kindly provided by A. Otte to E. H. Anti d-Jarid2 antibody was kindly provided by M. Yamaguchi and used 1:1000 for WB and 1:200 for polytene staining. Anti Ph antibody was kindly provided by G. Cavalli.

**Nuclear Extract and Immunoprecipitation**

For nuclear extract preparation cells were incubated with buffer A (10mM Hepes pH 7.9, 2.5mM MgCl2, 0.25M sucrose, 0.1% NP40, 0.5mM DTT, 1mM PSMF) for 10 min on ice, centrifuged at 8000 rpm for 10 min, resuspended in buffer B (25mM Hepes pH 7.9, 1.5mM MgCl2, 700 mM NaCl, 0.5mM DTT, 0.1 mM EDTA, 20% glycerol), sonicated and centrifuged at 14000 rpm 15min.

For immunoprecipitation 1mg of nuclear extract was incubated with 1-3µgr of antibody bound to protein A/G overnight. Then beads were washed three times with BC300 (50mM Tris pH7.9, 300mM KCl, 2mM EDTA, 10% Glycerol, and protease inhibitors), and eluted with
0.2 M glycine pH 2.6. For co-immunoprecipitation, samples were first dialyzed against BC250 (50mM Tris pH 7.9, 250mM KCl, 2mM EDTA, 10% Glycerol, and protease inhibitors), and then immunoprecipitated.

**Protein extract fractionation**

Cells are resuspended in buffer A1 (10mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.34M sucrose, 10% glycerol, 0.1% Triton-X 100, 1mM DTT), spin 5min 2000rpm to obtain the cytoplasm fraction. Nuclei are lysed in buffer B1 (3mM EDTA, 0.2mM EGTA, 1mM DTT) for 30 min in rotation and spin 5min at 2500rpm to obtain the nuclear fraction. Pellets are resuspended in buffer C (10mM Pipes pH 6.8, 1mM CaCl₂, 50mM NaCl) in presence of MNase and incubated at 37°C in shaking for 15 min. DNA digestion is stopped with EGTA 1mM final. Centrifugation 5min at 2500rpm separates chromatin soluble (supernatant) and chromatin insoluble (pellet) fractions. The pellet was solubilized in buffer C complemented with NaCl 1M final concentration and sonicated. All the buffers are supplement with protease inhibitors.

**Dot Blot**

1, 5, 20, 100 ng of peptides were spotted on nitrocellulose membrane and let dry for 30 min. After 20 min blocking in 5% milk T-TBS, primary antibodies are incubated in 1% BSA Tween 0,5%-TBS for 30 min at RT, followed by secondary antibodies for 30 min at RT.

**Cell lines**

First, 5XGal4RE-tk-Luc-Neo plasmid was stably integrated into the T-Rex 293 cells (Invitrogen) and selected by puromycin (1 µg/ml). Subsequently, the selected clone was generated by stable transfection of pCDNA4-T0-Gal4-hJarid2 WT and K116A. Single clones are selected with zeocin (300 µg/ml) and screened for the expression of Gal4-Jarid2 48 hours after doxycycline induction at 1µg/ml (Sigma). Stable clones of 293T cell over-expressing pCMV4-HA-Flag-hJarid2 WT or K116A and resistant to G418 were screened for the expression of the Flag-tagged proteins by WB.

**Recombinant proteins purification**

Recombinant hJarid2 fragments contain a 6XHis-tag and were produced in bacteria using the pET102 system. His-tagged proteins were purified on Ni-NTA beads in His buffer (350mM
NaCl, 0.5% NP40, 15% Glycerol, 10mM HEPES pH 7.6) and protease inhibitors. Elutions were performed in His Buffer plus 200mM Imidazole. Recombinant mammals and flies PRC2, Aebp2, full length Jarid2 WT and K116A were Flag-tagged and all produced in SF9 insect cells after infection with the corresponding baculoviruses as described previously (Li et al., 2010; Margueron et al., 2009; Margueron et al., 2008). Lysates containing Flag-tagged proteins were resuspended in BC300, sonicated and clarified by centrifugation before incubation with Flag-beads (M2-beads) and eluted with Flag peptide.

**Baculoviruses production**

hJarid2 WT and K116A baculoviruses were produced accordingly to Bac-to-Bac Baculovirus Expression Systems (Invitrogen) starting from pFASTbac vectors.

**Native chemical ligation**

Sumo-Jarid2 109-450 N123G/S124C, N123G/S124C/K116A, or 124-450 S124C were cloned in pGEX-5X vectors (GE), expressed in bacteria, digested with sumo-protease and purified on SP-HP column (GE). Jarid2 124-450 S124C was purified by HPLC on a RP-C18 column (22 x 250 mm, Vydac) using the gradient 0 to 55% B in A over 30 min with a flow rate of 15 mL min⁻¹ (A = 0.1 % CF₃COOH in H₂O, B = 0.1 % CF₃COOH in CH₃CN). Synthetic peptides were purchased from CS-Bio.

Jarid2 124-450 S124C (1.35 mg, 37 nmol) and the C-terminal thioester peptide K116me3 (1 equiv.) were dissolved in degassed ligation buffer (200 mM sodium phosphate, 6 M Guanidine, 2 mM EDTA, 50 mM tris(2-carboxyethyl)phosphine, 60 mM 4-mercaptoophenylacetic acid, pH 7) to give a final concentration for each fragment of 0.5 mM. The reaction mixture was heated at 40 °C and monitored by analytical HPLC on a diPhenyl column (4.6 x 150 mm, Vydac) using the gradient 0 to 45 % B in A over 40 min with a flow rate of 1 mL min⁻¹. The reaction was completed in 5 hours and the ligation product Jarid2 K116me3 was purified by HPLC and characterized by MALDI-TOF MS. Lyophilized proteins were resuspended in 7M urea and dialyzed against BC250 containing protease inhibitors.

**KMT assay**

KMT assay were performed as described previously (Margueron et al., 2009). Briefly, the reaction containing 200ng of PRC2-Aebp2, 1 µg of substrates, 4mM DTT was incubated in methylation reaction buffer (50mM Tris-HCl pH 8.5, 2.5mM MgCl₂) in presence of ³H-SAM at 30°C for 15 min or 30 min.
Reactions were stopped by boiling 5 min in SDS Laemmli buffer, run on acrylamide gels and transferred on PVDF membranes. When added to the reaction, peptides are at 10-50μM concentrations. Nucleosomes were generated by salt dialysis. H3K27me3 nucleosomes were generated as described in Voigt et al., 2013.

**Mass spectrometry analysis**

Cold-KMT assay was performed as described above in the presence of PRC2 on the recombinant Jarid2 1-232 fragment with 50μM cold SAM (Sigma). The reaction was stopped by boiling 5 min in SDS Laemmli buffer and run on acrylamide gel. After Coomassie coloration, the band was cut out, washed and proteins were reduced with 10 mM DTT prior to alkylation with 55 μM iodoacetamide. After washing and shrinking of the gel pieces with 100% acetonitrile, in-gel digestion was performed using trypsin overnight in 25 mM ammonium bicarbonate at 30°C. The extracted peptides were analysed by nano-LC-MS/MS using an Ultimate3000 system (Dionex S.A.) coupled to a QSTAR Elite mass spectrometer (Applied Biosystems/MDS SCIEX). Samples are loaded on a C18 precolumn (300 μm inner diameter x 5 mm; C18 PepMap™ guard column, Dionex S. A.) at 20 μl/min in 100% solvent A (2% acetonitrile, 0.1% acid formic). After 3 min of desalting, the precolumn was switched on line with the analytical C18 column (75 μm inner diameter x 50 cm; C18 PepMap™, Dionex S. A.) equilibrated in 95% solvent A. Bound peptides were eluted using a 60 min linear gradient (from 5% to 50% (v/v)) of solvent B (80% acetonitrile, 0.085% formic acid) at a 200 nl/min flow rate. TOF-MS survey scan was acquired for 1 s over a mass range of 400-1200 m/z. An information-dependent acquisition method was used to acquire product ion scans on the three most intense ions per cycle over a mass range of 65–2000 m/z, excluding previously gated ions for 60s.

The resulting spectra were then analyzed using the Mascot™ search engine against an in-house database (Jarid2 fragment 1-232) and the SwissProt Mus. musculus (house mouse) Protein Database (2013 06 03, 16620 sequences). For all experiments, a precursor ion mass tolerance of 0.2 Da was applied allowing up to 3 miss-cleavages. The fragment ion mass tolerance was set to 0.2 Da. Protein N-terminal acetylation, carbamidomethylation of cysteines, oxidation of methionine and methylation, dimethylation and trimethylation of Lysines were set as variable modifications and no fixed modifications were set. All peptide matches were validated in myProMS (Poulet et al., 2007) with the estimated false discovery rate (FDR) less than 1%.
**ΔEed-Jarid2K116me3 crystal**

The ΔEed protein was prepared as previously described (Margueron et al., 2009). For the crystallization trials, protein solutions were prepared as a ΔEed complex solution at 1.5 mg ml\(^{-1}\) with peptide at a sevenfold higher molar ratio. All protein solutions contained TCEP at 15 mM concentration. Crystals were grown at 18°C using the vapour diffusion technique in hanging drops. Drops were prepared by mixing equal volumes of ΔEed protein complex with reservoir solution containing 3.7–3.9 M formate solution. Crystals were transferred into mother liquor with 5–10% glycerol before flash cooling in liquid nitrogen. Diffraction data for the Jarid2-K116me3 and me2 protein complex crystals were collected using an in-house MicroMax 007HF rotating anode coupled to a RaxisIV++ detector at Diamond Light Source on Beamline I02 at a wavelength of 0.9795 Angstrom. Data were integrated using Denzo and scaled with Scalepack. The protein complex crystal structures were solved by molecular replacement using PHENIX and the previously published ΔEed structure as the search model. Standard refinement was carried out with reffmac5 together with manual model building with Coot. Figures were created with Pymol (DeLano Scientific; [http://pymol.sourceforge.net/](http://pymol.sourceforge.net/)).

**Alkaline Phosphatase and Colony formation assay**

Alkaline Phosphatase staining was performed with the Stamgent Alkaline Phosphatase Staining Kit (Milteny Biotec SAS). For colony formation assay, 100 or 500 cells were plated on gelatin-coated 6 well plates and let grown for 7 days, before staining with methylene blue solution (0.2% methylene blue in 70% ethanol) and washes with 70% ethanol.

**Cell growth assay**

50000 cells were plated in 6 well dishes in triplicated and counted every 24 hours over 4 days.

**ESC immunofluorescence**

Cells are grown on coverslips, fixed 4% paraformaldehyde 5 min at RT, permeabilized with 0.5% Triton-X 100 in PBS 5 min RT, blocked with 20% goat serum in PBS and incubated overnight with primary antibody: Oct4 1:500, Jarid2 1:500, Ezh2 1:500, Jarid2-K116me2 1:750. Alexa Fluor Dyes secondary antibodies (Invitrogen) are used 1:500.

**Mouse embryo collection and immunostaining**

All animals used in the studies were handled with care and experiments were done according to the guidelines from French legislation and institutional policies. Preimplantation embryos were obtained from superovulated female mice (4-to-8-weeks-old). They were collected in M2
medium (Sigma) by flushing the uterus at 68h (for 16-cell stage) or 92h (for blastocyst) after hCG (human chorionic gonadotropin) injection. Wild-type embryos were obtained from B6D2F1 intercrosses.

Maternal and paternal Ezh2-deficient embryos were generated from mating between Ezh2^Flox/Flox^ Zp3 cre ^+/−^ females with Ezh2^KO/+^ or ^KO/Flox^ males. Immunofluorescence was carried out as described previously (Torres-Padilla et al. 2006), with some modifications. After removal of the zona pellucida with acid Tyrode’s solution (Sigma), embryos were fixed in 4% paraformaldehyde, 0.2% sucrose, 0.04% Triton-X100 and 0.3% Tween20 in PBS for 15 min at 37°C. After permeabilization with 0.5% Triton-X100 in PBS for 30 minutes at room temperature, embryos were washed in PBStp (0.05% Triton-X100; 1mg/ml polyvinyl pyrrolidone (PVP-Sigma)) then blocked and incubated with anti Jarid2-K116me2 (1/400) and anti Eed (1/100; M26) antibodies in 1% BSA, 0.05% Triton-X100 for ~16h at 4°C. Embryos were washed in PBStp twice and blocked 30 minutes in 1% BSA in PBStp and incubated for 2h with Alexa conjugated anti rabbit or anti mouse antibodies (Invitrogen/Molecular probes) at room temperature. After washing, embryos were mounted in Vectashield (Clinisciences) containing DAPI for visualizing the DNA. Image were acquired on a Zeiss LSM700 inverted confocal microscope with a Plan apo DICII (numerical aperture 1.4) 63x oil objective. Z sections were taken every 1 mm Images were analyzed using ImageJ software.

**Fly Embryo and S2 collection and immunostaining**

*Drosophila melanogaster* flies were raised in standard corn meal yeast extract medium at 25°C. The Oregon-R line was obtained from A. Bardin’s laboratory.

For Western blot experiments, 0-12h old Drosophila embryos grown at 25°C were dechorionated and taken up in ice-cold PBS buffer containing 0.01% Triton. Embryos were homogenized with a homogenizer in 2x SDS-Laemmli buffer. For Western blot experiments on S2 cells, cells were detached from a flask, wash once in PBS, resuspended in lysis buffer (10 mM Tris pH8, 150 mM NaCl, 50 mM KCl, 0.3% Triton, 1mM EDTA, 1mM DTT and added 5x SDS Laemmli buffer.

**Immunostaining of polytene chromosome**

Polytene chromosome-staining procedures were adapted from a previously described protocol (Lavrov et al., 2004). For Jarid2-K116me2/PH double immunostaining, rabbit anti-Jarid-K116me2 and goat anti-PH antibodies were used.

**RT-qPCR**
Total RNA was isolated using the Rneasy Mini Kit (Qiagen). cDNA was synthetized using High Capacity cDNA RT kit (4368814-Applied Biosystems) and quantitative PCR was performed with technical triplicate using SYBR green reagent (Roche) on a ViiA7 equipment (Applied Biosystems). At least three biological independent experiments were performed for each assay and negative controls RT are always included. Primers sequences are provided below.

**ChIP**

ChIPs were performed as described previously (Margueron et al., 2008). Cell confluence and amount of starting material were kept constant by plating defined number of cells the day before cross-linking. Primers sequences for ChIP and RT-qPCR are provided below:

| Name       | Application | Sequence                  | Reference                  |
|------------|-------------|---------------------------|----------------------------|
| Luc_FW     | ChIP        | GTGTTGGGCSSGTATTTAT       |                            |
| Luc_Rv     | ChIP        | TACGGTACCTCTCGGAAATGT     |                            |
| Oct4_FW    | RT-qPCR     | CTCGGGAGGGTCCCAGGACAT     | Shen et al, 2009           |
| Oct4_Rv    | RT-qPCR     | GATGCTGGTCTGGCTGAACACCT   | Shen et al, 2009           |
| Gata4_FW   | RT-qPCR     | CACAGATGAACGGCATCAACC     | Pasini et al, 2010         |
| Gata4_Rv   | RT-qPCR     | CAGCGTGGTGGGTTAGTCTG      | Pasini et al, 2010         |
| Pax3_FW    | RT-qPCR     | TCCCATGGTGGGTCTCTAAG      | Pasini et al, 2010         |
| Pax3_Rv    | RT-qPCR     | CTCCACGTAGGCGTTGTC        | Pasini et al, 2010         |
| Foxa2_FW   | RT-qPCR     | GATGGAAGGGGCAGAAGC       | Pasini et al, 2010         |
| Foxa2_Rv   | RT-qPCR     | GTATGTGTTCATGCCATTCGCC    | Pasini et al, 2010         |
| Nestin_FW  | RT-qPCR     | GCCCTAGTTCAGGCCCGCC       | Pasini et al, 2010         |
| Nestin_Rv  | RT-qPCR     | AGACAGGCCGCGTAGCAGAAG     | Pasini et al, 2010         |
| Olig1_FW   | RT-qPCR     | TGAATTCCACCTGTTAGGCC      | Pasini et al, 2010         |
| Olig1_Rv   | RT-qPCR     | CGATGCTACCGGATACGAGAATA   | Pasini et al, 2010         |

**ChIP-Seq**

ChIP was performed as described above starting from 25 µg of chromatin; magnetic Dynabeads coupled to Protein A were used for the IP (Invitrogen). Sonication was performed to obtain fragment size of 150-300 bp. Libraries were prepared accordingly to manufactures (TruSeq ChIP sample Prep Kit, Illumina). Sequencing was performed on a Illumina Hi-Seq 2500. Single-end 100bp reads were mapped on the Mouse reference genome (mm9) using the Bowtie2 software (Langmead and Salzberg, 2012) allowing one mismatch in the seed (22bp) and reporting one location in case of multiple mapping hits. PCR duplicates were then removed using PicardTools (v1.65, http://picard.sourceforge.net).

*Jarid2 and Jarid2-K116me2 ChIP-seq:*
Peak calling was performed with MACS (v1.4.2, (Zhang et al., 2008)) with default parameters, using the Input sample as control. Significant peaks were identified with a minimum FDR of 0.15%. The HOMER software (v3.17, http://biowhat.ucsd.edu/homer/) was used to analyze peaks results. Overlapping peaks between the two conditions were detected, quantified and annotated. A gene Ontology analysis was performed with genes near to peaks and the read count histograms around TSS (+/- 3kb) were generated. The genome tracks were generated using the HOMER software by a normalizing library size of 40M reads.

\textit{H3K27me3 ChIP-seq:}

Peak calling was performed with MACS2 (v2.0.10, (Zhang et al., 2008)) with a DNA fragment size estimated during library preparation, using the Input sample as control. The broad parameter was used to call for modified-histone enriched regions. Significant peaks were identified with a minimum FDR of 5%. The Bioconductor Diffbind package (v1.6.2, (Ross-Innes et al., 2012)) was used to detect differential binding sites, with a minimum FDR of 5% and a minimum fold change of 1.5. The set of peaks used to compare undifferentiated ES and EB cell lines was defined using the higher quality samples (replicates 2). Peaks called differential between ES and EB in one specific condition were annotated with HOMER (v4.3). Genome ontology analysis was done with all differential peaks. Genome tracks were generated using the HOMER software with a DNA fragment size estimated during library preparation.
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