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Histone H2A monoubiquitination promotes histone H3 methylation in Polycomb repression

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A key step in gene repression by Polycomb is trimethylation of histone H3 K27 by PCR2 to form H3K27me3. H3K27me3 provides a binding surface for PRC1. We show that monoubiquitination of histone H2A by PRC1-type complexes to form H2Aub creates a binding site for Jarid2–Aebp2–containing PRC2 and promotes H3K27 trimethylation on H2Aub nucleosomes. Jarid2, Aebp2 and H2Aub thus constitute components of a positive feedback loop establishing H3K27me3 chromatin domains.

Nucleosomes constitute the building blocks of eukaryotic chromosomes. They consist of a core of histone proteins around which DNA is wrapped in two helical turns. The post-translational modification of histones is a key step for the regulation of diverse processes that occur on nucleosomal DNA. Specific histone modifications often decorate arrays of nucleosomes that comprise many kilobases of DNA, but how such extended stretches of chromatin become modified is not well understood. A paradigm for a long-range chromatin-modification mechanism is transcriptional repression by Polycomb protein complexes[1,2]. The Polycomb system generates two distinct histone modifications: methylation of K27 in histone H3 and monoubiquitination of K119 in histone H2A in vertebrates and of the corresponding K118 in *Drosophila* H2A. Polycomb repressive complex 2 (PRC2) catalyzes mono-, di- and trimethylation at H3 K27 (refs. 1,2). At inactive Polycomb-target genes, H3 K27 trimethyl marks typically decorate nucleosomes across the entire upstream, promoter and coding region (http://www.modencode.org/) and are essential for repression of these genes[3]. The H3K27me3 modification is recognized by Polycomb, a subunit of the canonical Polycomb repressive complex 1 (PRC1), and is thought to promote PRC1 interaction with chromatin across the entire length of repressed genes.

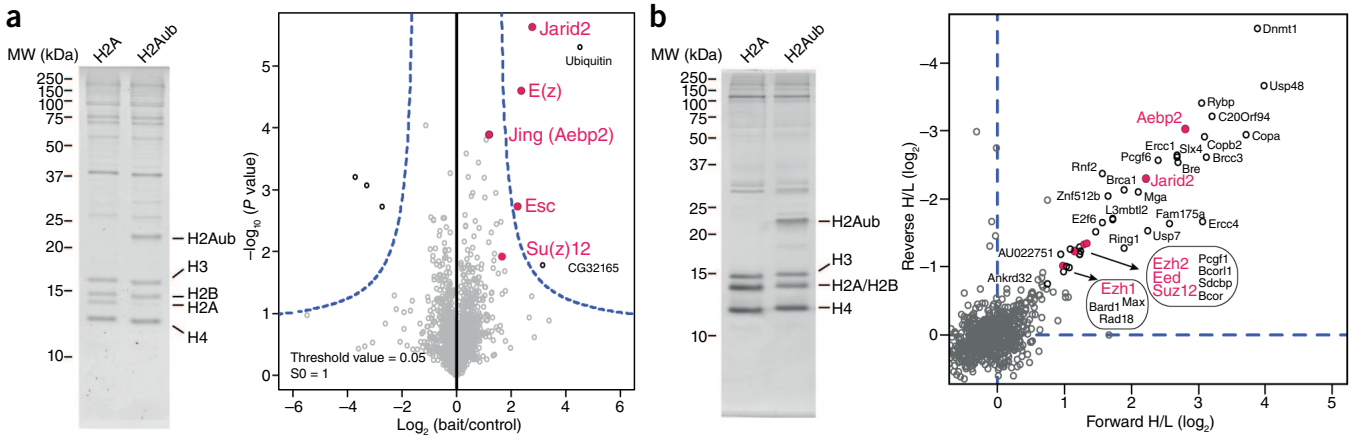

Figure 1 H2Aub nucleosomes bind Jarid2–Aebp2–PRC2. (**a**) Jarid2–Aebp2–PRC2 interaction with H2Aub in *Drosophila*. Left, SDS-PAGE and Coomassie staining of *Drosophila* embryo nuclear extracts, affinity purified with unmodified (left lane) or H2Aub (right lane) nucleosomes. **Supplementary Figure 3** shows all lanes of the triplicate pulldown reactions. MW, molecular weight. Right, H2Aub interactors identified by MS with permutation-based false discovery rate–corrected, two-tailed *t* test (threshold (dashed blue line), $P = 0.05$; S0 = 1; $n = 3$ independent pulldown experiments). *x* axis, $\log_2$-transformed ratio of proteins in the H2Aub pulldown over the H2A pulldown; *y* axis, $-\log_{10}$-transformed *P* values. The label-free quantification intensity of proteins in the H2Aub pulldown over the H2A control is plotted against the $-\log_{10}$ (*P* value) of the *t* test in a volcano plot. The blue line is the permutation-based false-discovery-rate threshold, which indicates Esc, E(z) and Jarid2 as significant H2Aub interactors. (**b**) Jarid2–Aebp2–PRC2 interaction with H2Aub in mammalian cells. Left, as in **a**, but with proteins from mouse embryonic stem cell nuclear extracts that were stable isotope–labeled by amino acids in cell culture (SILAC)[16]. H2A/H2B indicates H2A and H2B. **Supplementary Figure 3** shows all lanes of the SILAC pulldown. Right, proteins identified by MS in the H2A and H2Aub pulldowns, plotted by their $\log_2$-transformed normalized SILAC heavy/light (H/L) ratios in the forward (*x* axis) and reverse (*y* axis) experiment (Online Methods). SILAC data, which additionally identify PRC1.1 and PRC1.6 subunits and Dnmt1 as specific H2Aub interactors, are in **Supplementary Table 1**.

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PRC1 has been proposed to repress transcription through chromatin compaction and also through its ubiquitin-ligase activity for H2A monoubiquitination[1,2]. To gain insight into the function of H2Aub, we set out to identify interactors of this modification.

We reconstituted arrays of four nucleosomes (referred to as oligonucleosomes) with recombinant *Drosophila* or *Xenopus* histones and monoubiquitinated H2A in these templates, using appropriate recombinant enzymes (**Supplementary Fig. 1**). We then used *Drosophila* monoubiquitinated H2A K118 (H2AK118ub) oligonucleosomes and the corresponding unmodified oligonucleosome control template for affinity purification of H2AK118ub-binding proteins from *Drosophila* embryo nuclear extracts (**Fig. 1a**). In parallel, we used *Xenopus* monoubiquitinated H2A K119 (H2AK119ub) and unmodified control oligonucleosomes to identify vertebrate H2AK119ub interactors in nuclear extracts from mouse embryonic stem cells (**Fig. 1b**). In both experiments, quantitative MS analyses identified PRC2 subunits as being among the most highly enriched H2Aub interactors (**Fig. 1a**,**b**). Jarid2 and Aebp2 were the PRC2 subunits showing highest enrichment in both cases (**Fig. 1a**,**b**).

The identification of PRC2 as an H2Aub interactor in both flies and vertebrates prompted us to analyze PRC2 histone methyltransferase (HMTase) activity on H2Aub nucleosomes. We reconstituted recombinant human PRC2 containing EED, EZH2, SUZ12 and RBBP4 (here referred to as PRC2) and assemblies of the same complex that in addition contained AEBP2 (AEBP2–PRC2), JARID2 (JARID2–PRC2) or both JARID2 and AEBP2 (JARID2–AEBP2–PRC2) (**Supplementary Fig. 2a**). For substrates, we used *Xenopus* mononucleosomes that were either unmodified or monoubiquitinated at H2A K119 (**Supplementary Fig. 2b**), and in all cases we used western blot analyses with antibodies against either monomethylated H3 K27 (H3K27me1) or H3K27me3 to monitor PRC2 activity. We first performed a time-course experiment to compare the activity of PRC2 and JARID2–AEBP2–PRC2 on H2A and H2Aub nucleosomes. We found that, consistently with earlier reports[4,5], the catalytic activity of PRC2 alone is largely unchanged on H2Aub nucleosome templates (**Fig. 2a**, lanes 1–8). As expected[6–9], inclusion of JARID2 and AEBP2 in PRC2 resulted in stronger activity for H3 K27 methylation on unmodified nucleosome templates (**Fig. 2a**, comparison of lanes 9–12 and 1–4; refs. 6–9). However, we observed a much stronger increase in H3K27me3 formation when we used JARID2–AEBP2–PRC2 for HMTase reactions on H2Aub nucleosomes (**Fig. 2a**, comparison of lanes 13–16 and 9–12). We estimate that JARID2–AEBP2–PRC2 trimethylates H3K27 in H2Aub nucleosomes with an efficiency 25-fold higher than that of PRC2 (**Fig. 2a**, comparison of lanes 4 and 16; **Supplementary Fig. 2c**,**d**). To assess the contributions of JARID2 and AEBP2 to this stimulation of HMTase activity, we next compared the catalytic activity of all four forms of PRC2 on H2A and H2Aub nucleosome substrates (**Fig. 2b**). JARID2–PRC2 showed higher H3K27 methyltransferase activity than did PRC2 on unmodified nucleosomes, as previously reported[8,9], but this was not further increased on H2Aub nucleosomes (**Fig. 2b**, comparison of lanes 5–8 and 21–24). In contrast, AEBP2–PRC2 methylated H3K27 in H2Aub nucleosomes with considerably higher efficiency than in unmodified nucleosomes (**Fig. 2b**, comparison of lanes 9–12 and 25–28). This suggests that AEBP2 is critical for the specific activation of PRC2 by H2Aub, whereas JARID2 has a more general function in boosting PRC2 HMTase activity, independently of the H2A modification state.

The work reported here uncovers that Jarid2–Aebp2–containing PRC2 binds to H2Aub nucleosomes and demonstrates that H3K27 trimethylation by this complex is strongly enhanced on H2Aub nucleosomes. This establishes H2Aub, Aebp2 and Jarid2 as components of a positive feedback loop in which H2Aub promotes PRC2 binding and H3K27 trimethylation, and H3K27me3 in turn promotes binding of the canonical PRC1 via the chromodomain of Polycomb (**Fig. 2c**). It is currently not clear whether canonical PRC1 indeed has E3 ligase activity for H2A monoubiquitination or whether this modification is generated only by forms of PRC1 lacking Polycomb (examples

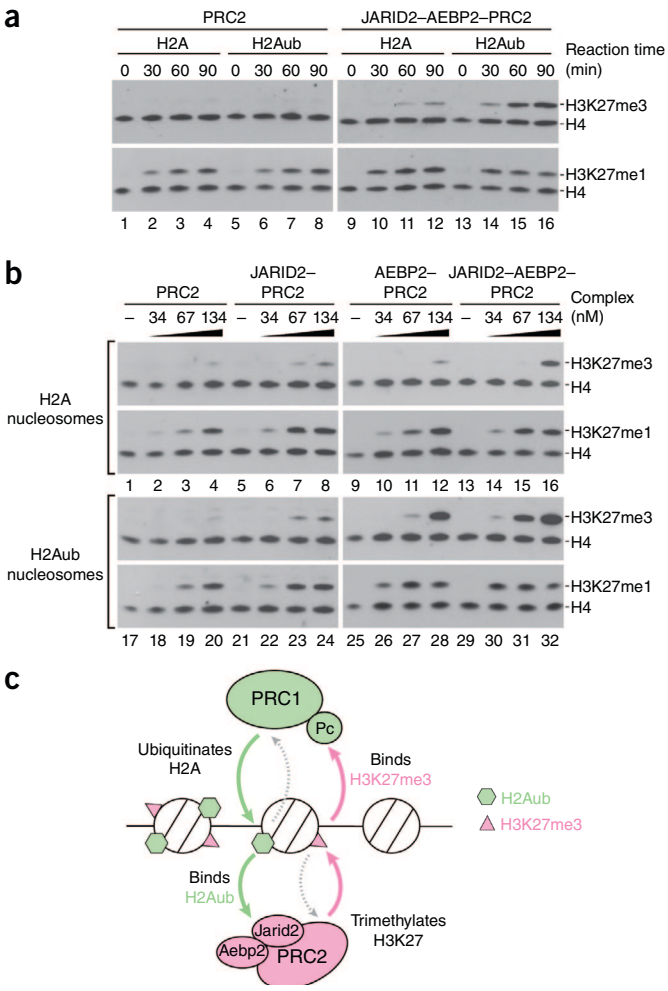

Figure 2 Aebp2 and Jarid2 stimulate H3K27 methylation by PRC2 on H2Aub nucleosomes. (**a**) Western blot analysis of H3K27me1 and H3K27me3 formation in HMTase reactions with PRC2 (134 nM, left) or JARID2–AEBP2–PRC2 (134 nM, right) on unmodified or H2Aub mononucleosomes (460 nM) after the indicated reaction times. Histone H4 signal served as a loading control. Left, blots showing comparable PRC2 activity on both H2A and H2Aub substrates. Right blots showing higher H3K27me3-formation activity by JARID2–AEBP2–PRC2 on H2Aub substrates (lanes 13–16) than on unmodified nucleosomes (lanes 9–12). (**b**) Western blot analysis, as in **a**, showing that only the AEBP2–PRC2 and JARID2–AEBP2–PRC2 complexes exhibit higher activity on H2Aub nucleosomes. Samples are HMTase reactions with increasing amounts of the indicated PRC2 complexes on unmodified (top) or H2Aub (bottom) mononucleosomes (460 nM); reaction time was 90 min in all cases. Results on oligonucleosome substrates are shown in **Supplementary Figure 2c**,**d**, and original blots are shown in **Supplementary Figure 4**. (**c**) Positive-feedback-loop model for generating Polycomb-repressed chromatin. Monoubiquitination of H2A by PRC1-type complexes promotes Jarid2–Aebp2–PRC2 binding and H3K27 trimethylation; H3K27me3 in turn promotes binding of canonical PRC1 via its Polycomb (Pc) subunit (solid green and magenta arrows). PRC1.1 and PRC1.6 are additional H2Aub interactors, and PRC2 binds to and is stimulated by H3K27me3 (refs. 1,2), thus suggesting additional positive feedback loops for H2Aub and H3K27me3 formation (dashed arrows).

in refs. 10,11). Intriguingly, in embryonic stem cells, we also identified the PRC1-type complexes PRC1.1 and PRC1.6 (refs. 12,13) as H2Aub interactors (**Fig. 1b**), results suggesting an additional feedback loop for H2A ubiquitination in vertebrates. The positive feedback loop for H3K27me3 formation by H2Aub uncovered here provides a rationale for how extended domains of Polycomb-repressed chromatin could be generated in both *Drosophila* and vertebrates (**Fig. 2c**). Our findings could explain why H3K27me3 levels at Polycomb-target genes are reduced in mouse embryonic stem cells in which H2AK119ub has been depleted[14]. However, we previously found that bulk H3K27me3 levels were undiminished in late-stage *Drosophila* larvae in which bulk H2Aub levels had been depleted[15], thus suggesting that maintenance of H3K27me3-containing chromatin domains does not strictly depend on H2Aub. The H2Aub-mediated feedback loop may thus primarily be required for the initial formation of H3K27me3 chromatin domains when Polycomb repression is first established during the early stages of embryogenesis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.K. generated the unmodified and H2Aub nucleosome substrates, prepared *Drosophila* nuclear extracts and performed the pulldown experiments and the HMTase assays. H.I.B. and P.W.T.C.J. performed the MS analyses. S.L. prepared PRC2 and AEBP2-containing PRC2, and R.K. prepared JARID2. J.M., M.V. and C.W.M. supervised the project. J.M. and R.K. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reconstitution of recombinant mono- and oligonucleosomes. Nucleosomes with recombinant *Drosophila* or *Xenopus* histones were reconstituted as described[17]. Mononucleosomes were assembled on a 5′-biotinylated 288-bp DNA fragment containing the 601 sequence in the 3′ portion. Oligonucleosomes were assembled on a 5′-biotinylated 854-bp DNA fragment containing an array of four 200-bp repeats containing the 601 sequence. Completeness of reconstitution was monitored by analysis on native agarose gels.

***In vitro* ubiquitination of recombinant nucleosomes.** Recombinant human UBCH5C and $Ring1b_{1–159}$–$Bmi1_{1–109}$ complex were expressed and purified as described[18]. The quality and purity of all proteins was checked by analysis on polyacrylamide gels (**Supplementary Fig. 1**), and determination of total mass was by MS. In the case of the Ring1b–Bmi1 complex, we found that after proteolytic removal of the GST tag and size-exclusion chromatography, the Ring1b protein contained a C-terminal truncation of 29 amino acids, such that the purified dimer comprised $Ring1b_{1–130}$–$Bmi1_{1–109}$. Human UBE1 and ubiquitin were purchased from Boston Biochem.

For *in vitro* ubiquitination of H2A, assembled mono- or oligonucleosomes (400–800 nM) were incubated with UBE1 (21 nM), UBCH5C (150 nM), $Ring1b_{1–130}$–$Bmi1_{1–109}$ complex (86 nM) and ubiquitin (3 μM) in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 μM $ZnCl_2$, 0.5 mM DTT and 2 mM ATP for 90 min at 30 °C. Reactions were terminated by addition of an equal volume of ubiquitination buffer containing 500 mM NaCl. Completion of H2A monoubiquitination was monitored by separation of reaction products on polyacrylamide gels and Coomassie staining or western blot analysis with antibodies against unmodified H2A (Millipore, 07-146; 1:2,000). For pulldown and histone methyltransferase assays, unmodified and H2Aub nucleosome templates were then coupled to streptavidin-coupled Dynabeads (Dynabeads M-280 Streptavidin, Invitrogen), and reaction components were removed by multiple washes with ubiquitination buffer (500 mM NaCl) before equilibration of the bead-bound templates in binding buffer (i.e., for pulldown) or histone methyltransferase reaction buffer.

Preparation of nuclear extracts. Nuclear extracts from wild-type *Drosophila* embryos (10–14 h old) were prepared as described[19]. Nuclear extracts from SILAC-labeled mouse embryonic stem cells (mESCs) were generated as described[20].

Pulldown with H2Aub nucleosomes. Ubiquitination reactions were optimized to obtain oligonucleosome templates in which more than 90% of H2A was monoubiquitinated. Oligonucleosomes immobilized on Dynabeads were washed with binding buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40 substitute, 1 mM DTT and protease inhibitors). For both unmodified and H2Aub nucleosome templates, bead suspensions were distributed into two equal aliquots to obtain two technical replicates for pulldown with SILAC-labeled nuclear extracts from mouse embryonic stem cells. For pulldown with label-free nuclear extracts from *Drosophila* embryos, unmodified and H2Aub nucleosome templates were each distributed into three equal aliquots to obtain three technical replicates for pulldown reactions. To inhibit ubiquitin protease activity present in nuclear extracts, the extracts were pretreated with 5 μM VME-ubiquitin (Boston Biochem) for 10 min on ice before addition to binding reactions. Individual binding reactions were performed in 500-μl volumes containing 30 μg bead-bound oligonucleosomes and 500 μg nuclear extract, with incubation for 4 h on a rotating wheel at 4 °C. Beads were then washed five times with binding buffer, resuspended in LDS gel-loading buffer and analyzed by MS (described below). After binding, integrity of H2Aub and other histones and sample quality were checked by analysis of an aliquot of the binding reaction on a 16% Tris-glycine polyacrylamide gel (Coomassie staining and western blot analysis with an antibody against unmodified H2A (described above).

Mass spectrometry and data analysis. In-gel digestion, peptide desalting, purification, LC-MS/MS and data analyses were performed essentially as described[21,22]. For the SILAC pulldowns, immobilized H2A nucleosomes were incubated with light nuclear extract, and the immobilized H2AUb nucleosomes were incubated with heavy nuclear extract. After incubation and washes, beads from both pulldowns were combined, and bound proteins were subjected to in-gel digestion and LC-MS/MS analysis (forward experiment). A second set of pulldowns were performed, in which the light extract was incubated with the H2AUb nucleosomes, and the heavy extract was incubated with H2A nucleosomes (reverse experiment). Data were visualized by plotting the $\log_2$-transformed ratios of proteins in the forward experiment against their ratio in the reverse experiment. Box-plot statistics, using an in house–generated R script (available upon request), was used to determine statistically significant H2AK119Ub interactors. For the label-free pulldowns performed with *Drosophila* nuclear extracts, the $\log_2$-transformed LFQ intensities of triplicate H2A and triplicate H2AUb pulldowns were calculated, and a permutation-based false discovery rate–corrected *t* test was performed to determine statistically significant H2AK119Ub interactors. Results were visualized in a volcano plot.

Production of recombinant AEBP2–PRC2 and JARID2. Expression and purification of PRC2 comprising EZH2, SUZ12, EED and RBBP4 and containing or lacking AEBP2 was performed with previously reported procedures[23] with the following minor modifications. Sf21 insect cells were grown in suspension culture in Sf-900 III serum-free medium (Life Technologies) and infected at a density of 0.6×10^6 mL^{-1} with viruses corresponding to each PRC2 subunit. After lysis, the sample was subjected to a Ni-affinity purification step. Prior to gel filtration, the sample was loaded onto a HiTrap 5-mL Heparin HP column (GE Healthcare) and eluted with a NaCl gradient of 0.15–2 M.

The cDNA of human JARID2 was cloned into pFastBac-1 as an N-terminal StrepII fusion protein. StrepII-JARID2 was expressed with the Hi5 expression system (Life Technologies). Insect cells were lysed in Buffer A (10 mM MOPS, pH 7.6, 500 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.05 mM EGTA, 5% glycerol, 0.3% NP-40 substitute, 4 mM mercaptoethanol, and protease and phosphatase inhibitors) and then subjected to Strep-Tactin affinity purification (Strep-Tactin Superflow, IBA). After several washes, the bound protein was released in buffer B (10 mM MOPS, pH 7.6, 350 mM NaCl, 5% glycerol, 0.1% NP-40 substitute, 4 mM mercaptoethanol and protease inhibitors) supplemented with 5 mM desthiobiotin (Sigma).

Histone methyltransferase assays. HMTase assays on mononucleosomes or oligonucleosomes were performed with the concentrations of PRC2, JARID2 and nucleosomes indicated in **Figure 2** and **Supplementary Figure 2**, in a reaction buffer containing 10 mM HEPES, pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA, pH 8.0, 2 mM ATP, 0.5 mM DTT, 5% glycerol and 80 μM *S*-adenosylmethionine in a total volume of 20 μl or 60 μl. JARID2–PRC2 and JARID2–AEBP2–PRC2 complexes were assembled by mixture of purified PRC2 or AEBP2–PRC2 complexes with equimolar amounts of JARID2 in reaction buffer, incubated for 5 min on ice and then used for HMTase reactions. Reactions were incubated for the indicated time at 25 °C and stopped by addition of 6.6 or 20 μl 4× LDS gel-loading buffer. Proteins were separated on 4–12% or 12% Bis-Tris MES polyacrylamide gels, transferred to nitrocellulose membrane and probed with antibodies against H3K27me3 (Millipore, 07-449) or H3K27me1 (Millipore, 07-448) at a dilution of 1:3,000. Antibody against H4 (Abcam, ab10156) was added at a dilution of 1:200,000. After incubation with HRP-linked anti-rabbit secondary antibodies (GE Healthcare, NA934; 1:5,000), signals were detected after standard ECL reaction (GE Healthcare) and exposure to film. In addition, detection by the ImageQuant LAS4000 imaging system was used for quantification; signal intensities were analyzed with ImageJ. Original images of gels and blots used in this study can be found in **Supplementary Figures 3–5**.

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