dKDM2 couples histone H2A ubiquitylation to histone H3 demethylation during Polycomb group silencing

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Transcription regulation involves enzyme-mediated changes in chromatin structure. Here, we describe a novel mode of histone crosstalk during gene silencing, in which histone H2A monoubiquitylation is coupled to the removal of histone H3 Lys 36 dimethylation (H3K36me2). This pathway was uncovered through the identification of dRING-associated factors (dRAF), a novel Polycomb group (PcG) silencing complex harboring the histone H2A ubiquitin ligase dRING, PSC and the F-box protein, and demethylase dKDM2. In vivo, dKDM2 shares many transcriptional targets with Polycomb and counteracts the histone methyltransferases TRX and ASH1. Importantly, cellular depletion and in vitro reconstitution assays revealed that dKDM2 not only mediates H3K36me2 demethylation but is also required for efficient H2A ubiquitylation by dRING/PSC. Thus, dRAF removes an active mark from histone H3 and adds a repressive one to H2A. These findings reveal coordinate *trans*-histone regulation by a PcG complex to mediate gene repression.

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Polycomb group (PcG) and trithorax group (trxG) genes encode for antagonistic transcriptional coregulators that play critical roles in stem cell biology, development, and cancer (Ringrose and Paro 2004; Sparmann and van Lo-huizen 2006; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). They were first identified genetically in the fruit fly *Drosophila* as factors required for maintaining the correct expression of homeotic genes throughout development (Maeda and Karch 2006). However, over the years it has become clear that many PcG and trxG proteins play diverse regulatory roles in gene control. Generally speaking, PcG proteins function as transcriptional repressors whereas trxG proteins act as activators.

PcG silencing involves the activities of at least two major types of complexes, PRC1 and PRC2 (Ringrose and Paro 2004; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). *Drosophila* PRC1 contains four core PcG proteins—Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC), and Sex combs extra (SCE), usually referred to as dRING—which constitute the PC core complex (PCC). Mammalian RING1B and fly dRING are

ubiquitin E3 ligases responsible for histone H2A monoubiquitylation (H2Aub), a histone modification associated with transcriptional silencing (Wang et al. 2004; Weake and Workman 2008). PRC2-class complexes harbor the histone H3 Lys 27 (H3K27) methyltransferase Enhancer of zeste [E(z)], extra sex combs (ESC), and CAF1 p55 (Ringrose and Paro 2004; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). Recently, a related complex has been described containing the PcG protein PC-like (PCL) that appears to be particularly important for H3K27 trimethylation (Nekrasov et al. 2007; Sarma et al. 2008). One important but still poorly understood issue remains the question of how PcG complexes are recruited to silence specific genes. In *Drosophila*, a key tethering factor is the sequence-specific DNA-binding PcG protein PHO that binds PcG response elements (PREs) and can initiate the recruitment of PcG silencers (Brown et al. 1998; Fritsch et al. 1999; Mohd-Sarip et al. 2002, 2005, 2006). PHO has also been purified in complex with the INO80 ATP-dependent chromatin remodeler and, as PHORC, in complex with the PcG protein SFMBT (Klymenko et al. 2006).

Modulation of chromatin structure has emerged as a key molecular mechanism through which PcG and trxG proteins control gene expression. Histones are subjected to a wide range of reversible post-translational modifica-

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tions including acetylation, phosphorylation, methylation, and ubiquitylation (Berger 2007; Bhaumik et al. 2007; Ruthenburg et al. 2007). These histone modifications can promote recruitment of specific regulatory factors and modulate chromatin accessibility. For many histone modifications, the transcriptional output is critically dependent on what specific residues are modified. For example, lysine methylation can either be associated with gene silencing (H3K9, H3K27, and H4K20) or with gene activation (H3K4, H3K36, and H3K79). Complicating matters further, these lysines can be mono-, di-, or trimethylated, each of which may have distinct functional consequences. Whereas initially believed to be a permanent mark, recent research has identified a range of specific histone lysine demethylases (KDMs) (Shi 2007). An important group of KDMs are characterized by the presence of a JmjC demethylase domain. Like the lysine methyltransferases (KMTs), KDMs display exquisite substrate specificity and distinct KDMs target different methylated lysines. Depending on what mark they target, KDMs act as either transcriptional corepressors or coactivators.

Another important post-translational histone modification is monoubiquitylation of histones H2A and H2B (Osley 2006; Weake and Workman 2008). H2B monoubiquitylation (H2Bub) at Lys 120 by the E3-ligase BRE1 is an active mark, linked to transcriptional elongation. In contrast, H2Aub at Lys 119 has been implicated in PcG transcription silencing, human X-chromosome inactivation, and heterochromatin formation. Thus, H2Aub and H2Bub appear to be antagonistic histone modifications. The E3 responsible for the majority of H2A ubiquitylation is mammalian RING1B or fly dRING (Wang et al.

2004). The ubiquitylation activity of RING1B is strongly stimulated by association through RING–RING formation with BMI1, a PSC homolog (Cao et al. 2005; Ben-Saadon et al. 2006; Buchwald et al. 2006). We note that H2Aub is the target of diverse regulatory pathways. For example, during the DNA-damage response, histones H2A and H2AX are ubiquitylated by RNF8 (Mailand et al. 2007). Selective H2Aub deubiquitylation can be mediated by a number of ubiquitin proteases involved in cell cycle control and transcription (Joo et al. 2007; Nakagawa et al. 2008; Weake and Workman 2008).

To gain more insight in the enzymatic network underpinning PcG silencing, we characterized dRING and its associated factors. Although it is a bona-fide PRC1 subunit, we found that a significant fraction of dRING is part of a separate assemblage we named dRAF (dRING-associated factors). We identified dKDM2 as a key dRAF subunit that plays a pivotal role in a novel *trans*-histone pathway involving the removal of an active histone H3 methyl mark and formation of the repressive H2Aub mark during PcG silencing.

Results

Identification of dRAF

In the course of our biochemical characterization of *Drosophila* PcG protein complexes in partially purified embryo nuclear extracts (Fig. 1A), we came to realize that several PRC1 subunits exist outside this complex. Immunoprecipitation using affinity-purified antibodies directed against PC yielded the PRC1 subunits dRING, PH, PSC, and PC itself, as established by SDS–polyacryl-

Figure 1. Identification of dRAF. (A) Outline of the chromatographic scheme used to purify dRAF. (B) dRING, PH, and PSC also exist outside PRC1. Protein A Sepharose beads coated with either control anti-GST antibodies (Mock IP) or affinity-purified antibodies directed against PC (α-PC IP) were used to immunopurify PC and associated factors from partially purified and concentrated embryo nuclear extracts (H0.4). Bound and unbound material (FT α-PC IP) was analyzed by Western immunoblotting using antibodies directed against the indicated proteins. (C) dRING and PSC form part of a complex distinct from PRC1 and devoid of PC and PH. The unbound fraction after 2 subsequent PC immunodepletions (FT α-PC IP) was incubated with protein A Sepharose beads coated with affinity-purified antibodies directed against dRING. Bound (α-dRING IP) and unbound (FT α-dRING IP) material was analyzed followed by Western immunoblotting. (D) Identification of dRAF. H0.4, twice PC-immunodepleted (FT α-PC IP) input of the mock-, or dRING immunopurified (α-dRING IP) fractions were resolved by SDS-PAGE and visualized by silver staining. Proteins present in bands excised from a gel run in parallel were identified by nanoflow LC-MS/MS mass spectrometry. Identified proteins are indicated. Their mascot score and number of unique peptides identified are Mtor: 4741 (Mascot score), 55 (number of unique peptides); PSC: 2226, 31; dKDM2 (dRAF1, CG11033): 1920, 23; Ulp1: 1566, 20; dRAF2 (CG4877): 1312, 17; dRING (SCE): 1160, 12. Asterisks indicate background proteins we routinely observe in our immunopurifications: (*1) Heat shock cognate 4; (*2) γ-tubulin; (*3) RanGap; (*4) β-tubulin; (*5) yolk protein 1 and 2; (*6) RFC.

amide electrophoresis (PAGE) followed by Western immunoblotting (Fig. 1B, lane 3). To our surprise, inspection of the PC-depleted flow-through (FT) revealed the presence of significant amounts of dRING, PH, and PSC (Fig. 1B, lane 4). Thus, these proteins are not solely present as stable PRC1 components. Moira (MOR), a core subunit of the *Drosophila* (P)BAP chromatin remodeling complexes, served as a negative control for the PC-immunopurification. In conclusion, these results indicated that substantial amounts of dRING, PH, and PSC might be present either as free molecules or as components of protein assemblages other than PRC1.

To identify a potentially novel dRING-containing complex, we immunopurified dRING from the PC-depleted extracts using protein A beads coated with affinity-purified α -dRING antibodies. After extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, bound and unbound material were resolved by SDS-PAGE and analyzed by Western blotting (Fig. 1C). PSC copurified with dRING (Fig. 1C, lane 3), although a significant amount remained in the unbound fraction (Fig. 1C, lane 4). In contrast to PSC, PH was not associated with dRING. Thus, it appeared that dRING and PSC are part of a protein complex that is distinct from PRC1 and lacks PC and PH. We visualized the dRAFs by SDS-PAGE followed by silver staining (Fig. 1D).

Mass spectrometric analysis confirmed the presence of dRING and PSC. A number of additional proteins were identified suggesting a number of potential links between PcG silencing and distinct cellular processes. (1) As discussed above, dRING and PSC are classic PcG proteins involved in histone H2A ubiquitylation. (2) Megator (Mtor) is a dynamic subunit of the nuclear pore complex that also exists separately in the nucleoplasm and interacts functionally with the MSL dosage compensation complex and has been implicated in mitotic spindle assembly (Qi et al. 2004; Mendjan et al. 2006). (3) Ulp1 is a SUMO peptidase that, like Mtor, is found associated with the nuclear pore complex (Hay 2007). (4) dRAF1 (CG11033), the fly homolog of KDM2 (Tsukada et al. 2006; Frescas et al. 2007; Shi 2007), is a particularly intriguing protein harboring an F-box, CXXC-type zinc finger, PHD finger, and JmjC demethylase domain. (5) Finally, dRAF2 (CG4877) contains a MYND zinc finger, a protein-protein interaction domain implicated in the recruitment of corepressors. In summary, our analysis suggests a greater complexity among PRC1-related PcG complexes than previously appreciated. Below we will focus on the functional characterization of dRAF.

dRAF and PRC1 are separate PcG complexes

First, we set out to confirm that PRC1 and dRAF are indeed separate complexes. We performed a series of co-immunoprecipitations from crude embryo nuclear extracts using antibodies directed against dRING, PC, PH, and PSC. Western immunoblotting showed that dKDM2 and dRAF2 are stably associated with dRING and PSC in crude embryo nuclear extracts (Fig. 2A). Albeit somewhat less efficiently, Mtor and Ulp1 also clearly inter-

acted with dRAF. In contrast, none of these proteins copurified with the PRC1-specific PC or PH. Immunoprecipitations using antibodies against dRAF2, Mtor, and dKDM2 yielded both dRING and PSC, but not PC or PH (Fig. 2B). To investigate the association of dRING with dKDM2 and other interacting proteins further, we followed them over a series of purification steps (Fig. 2C). The core dRAF subunits dRING, PSC, and dKDM2 coelute with dRAF2, Mtor, and Ulp1 during size-exclusion chromatography, behaving as a large multiprotein assemblage (Fig. 2D). These proteins peaked in fractions corresponding to apparent molecular masses ranging from ~600 to 900 kDa. PRC1 subunits PC and PH also migrate as a large complex, although PC displayed significant trailing. The chromatin remodeler ISWI served as a reference and has a smaller apparent molecular mass. Glycerol gradient sedimentation analysis of a Sephacryl S-300 column peak fraction (#14) revealed clearly overlapping cosedimentation of dRAFs as a large complex (Fig. 2E). Again, not all proteins displayed an identical distribution over the gradient, possibly reflecting complex disassembly or heterogeneity. For example, PC, dRING, and to a lesser extent, PSC showed broad distribution profiles compared with dKDM2, dRAF2, and PH, suggesting dissociation from PRC1 and dRAF.

In conclusion, dRAF and PRC1 form two distinct PcG complexes that share PSC and dRING as common subunits. PC and PH are absent from dRAF and define PRC1, whereas dKDM2 is missing from PRC1 and defines dRAF. We consider dRING/PSC/KDM2 the core of the dRAF complex with potential interactions with other factors, which are not explored further here. The intriguing presence of dKDM2, harboring regulatory motifs such as a JmjC type demethylase domain and an F-box, in a complex with dRING raised a number of interesting possibilities. First, the identification of dRAF as a separate entity sharing subunits with PRC1 suggested that these two complexes might work together during transcriptional silencing. If so, the transcriptional profile of cells lacking dKDM2 and other dRAF or PRC1 subunits would be expected to be similar. Second, dKDM2 would be expected to behave genetically as a transcriptional silencer. Thus, *in vivo* dKDM2 is predicted to cooperate with PC but to antagonize gene activation by particular *trxG* methyltransferases. Third and most interestingly, the association between dKDM2 and dRING suggests that dRAF might couple histone demethylation and histone ubiquitylation. In the sections below, we present evidence supporting these three predictions.

dKDM2 and PRC1 control overlapping transcriptomes

To investigate their relative roles in gene expression control, we treated S2 cells with dsRNA directed against dKDM2, dRING, PSC, PC, and PH. As shown in Figure 3A, this caused a significant reduction in the protein level of the targeted factors, without significantly destabilizing associated subunits. However, PSC is up-regulated following a dRING knockdown, suggesting a po-

Figure 2. dRAF and PRC1 are separate PcG complexes that share dRING and PSC. (A) dRING, PSC, dKDM2, dRAF2, Mtor, and Ulp1 associate in crude *Drosophila* embryo nuclear extracts (NE). Nuclear extract was incubated with Protein A Sepharose beads coated with either control anti-GST antibodies (mock) or affinity-purified α -dRING, α -PC, α -PH, or α -PSC antibodies. After extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting. Lane 1 represents 10% of the input material used in the binding reactions. (B) dRING and PSC, but not PC or PH, coimmunoprecipitate with dRAF2, Mtor, and dKDM2. Coimmunoprecipitations were performed and analyzed as described above. (C) Outline of the chromatographic scheme used to characterize the dRAF complex further. (D) The POROS 20 Heparin 400 mM eluate fractionated by Sephacryl S-300 size-exclusion chromatography. The indicated fractions were combined and resolved by SDS-PAGE, followed by immunoblotting with antibodies directed against dKDM2, PSC, dRING, dRAF2, Mtor, Ulp1, Pc, PH, and ISWI. The core dRAF subunits and interactors peaked in fractions corresponding to apparent molecular masses ranging from ~600 to 900 kDa. The elution of the voided volume (void) and the elution of the markers ferritin (440 kDa) and aldolase (158 kDa) are indicated. (E) The dRAF peak fraction #14 from the Sephacryl S-300 column was centrifuged through a 10%–25% glycerol gradient, and collected fractions were examined for the presence of dRAF or PRC1 subunits by immunoblotting.

tential feedback loop via dRING-mediated PSC polyubiquitylation (Buchwald et al. 2006; our unpublished results). For each subunit we performed three fully independent RNAi-mediated knockdown experiments using distinct cell batches. For comparison, we used mock-treated cells and expression analysis of cells depleted for selective subunits of the BAP, PBAP, and ISWI chromatin remodelers (Moshkin et al. 2007).

Previously, we found that principal component analysis (PCA) is an effective mathematical tool to uncover relationships between the transcriptomes of various regulators (Moshkin et al. 2007). PCA is a linear transformation that finds and projects original variables to the fewest principal components (PCs), accounting for most of the variance in the data set. About 77% of the variance in gene expression profiles obtained after individual depletion of the 12 proteins analyzed here is explained by PC1–3. As illustrated by Figure 3B, PCA revealed that the dKDM2, dRING, PC, PSC, and PH expression profiles clearly cluster together, showing that they are highly correlated. As expected of trxG proteins, BRM and its associated proteins anti-correlated with the PcG proteins. Likewise, the ISWI transcription profile was distinct from the PcG proteins. Venn diagram analysis also revealed that dRAF and PRC1 share a large set of target genes (Fig. 3C). Gene ontology analysis suggested the involvement of PcG silencers in a wide range of cellular processes, supporting the notion they play broad roles in transcription control (Ringrose 2007). In conclusion, epi-

static analysis by expression profiling suggested a functional cooperation between PRC1 and dRAF.

dkdm2 is an enhancer of Pc

We next wished to test directly whether dKDM2, like PC, might be involved in silencing of the homeotic loci in vivo. We obtained three independent mutant fly lines harboring distinct P-element insertions in the *dkdm2* gene, which is located on the third chromosome. *dkdm2*^{KG04325} and *dkdm2*^{DG12810} are homozygous lethal, while *dkdm2*^{EY01336} is homozygous viable hypomorphic. Neither homozygous *dkdm2*^{EY01336} nor heterozygous *dkdm2*^{KG04325} and *dkdm2*^{DG12810} animals displayed obvious homeotic transformations. To test the effects of *dkdm2* mutations on homeotic gene silencing, we crossed each of the three alleles into flies carrying either the *Pc*¹ or *Pc*³ allele. We found that in the trans-heterozygous progeny, the *dkdm2* mutant alleles significantly increased the frequency of a range of homeotic transformations (Fig. 4A,B). These include the appearance of sex combs on the second (L2–L1) and third (L3–L1) pairs of legs, transformation of the fourth abdominal segment into the semblance of the fifth (A4–A5), and wing-to-haltere (W–H) transformations. In all cases, the frequency of transformation is much higher in flies trans-heterozygous for one of the *dkdm2* mutants and either *Pc*¹ or *Pc*³ compared with animals carrying only a *Pc* mutation. These strong genetic interactions were ob-

Figure 3. dKDM2 and PRC1 control overlapping transcriptomes. (A) S2 cells were either mock-treated or incubated with dsRNAs directed against selective dRAF or PRC1 subunits dKDM2, dRING, PSC, PC, and PH. Whole-cell extracts were prepared and analyzed by Western immunoblotting. (B) Representation of 12 expression profiles in a three-dimensional transcriptome space, derived after PCA. RNA was isolated, labeled, and hybridized on Affymetrix *Drosophila* Genome 2 arrays. Expression indexes were calculated using the Robust Multichip Average (RMA) algorithm (Irizarry et al. 2003). The Minimum Covariance Determinant algorithm (Rousseeuw and van Driessen 1999) was used to remove genes that were expressed at very low levels. Next, we applied one-way ANOVA on each probe set to identify genes that changed significantly ($P < 0.05$) upon RNAi treatment. For these ~5500 genes, we determined gene expression profiles by taking the ratios between average gene expression indexes obtained from specific RNAi- and mock-treated cells. Expression profiling of subunits of the trxB BAP and PBAP complexes, BRM, MOR, SNR1, OSA, Polybromo, BAP170, and OSA, as well as ISWI has been

described (Moshkin et al. 2007). The expression profiles are shown as a projection on the first three PCs after varimax rotation. Each transcriptome represent significant gene targets after three independent biological replicate experiments. (C) Venn diagrams depicting the overlap and differences between the transcriptional targets of the dRAF-signature subunit dKDM2; dRING and PSC, shared by dRAF and PRC1; and the PRC1-selective subunits PC and PH. Numbers indicate significant target genes affected by depletion of the indicated factor(s).

served using different independent alleles, making it highly unlikely that they were due to nonspecific influences of genetic background. In summary, genetic interaction studies established that *dkdm2* acts as an enhancer of *Pc*, supporting the notion that dRAF and PRC1 cooperate in vivo.

dkdm2 is a suppressor of histone methyltransferases *trx* and *ash1*

We used a genetic approach to test whether, like the *PcG* silencers, dKDM2 might counteract trxB protein-mediated transcriptional activation. The trxB genes *trx* and *ash1* encode for histone methyltransferases that are associated with gene activation. TRX is a well-established histone H3K4 methylase (Bhaumik et al. 2007). Although there appears to be less consensus on the ASH1 target, a recent study made a compelling argument that ASH1 mediates H3K36me2 (Tanaka et al. 2007). Flies carrying a mutant *trx*¹ or *ash1*¹⁰ allele display transformations of abdominal segment A5 toward the likelihood of A4, recognizable by a loss of pigmentation in A5 (Fig. 4C). Moreover, these animals frequently show a partial haltere-to-wing (H-W) transformation. In our crosses we analyzed the progeny of homozygous *trx*¹ or *ash1*¹⁰ females crossed with either wild-type or *dkdm2* mutant males. Trans-heterozygous animals in which a

dkdm2 allele is combined with either a *trx*¹ or *ash1*¹⁰ mutant allele display a strikingly reduced frequency of homeotic transformations (Fig. 4D). We conclude that *dkdm2* acts both as an enhancer of *Pc* silencing and as a suppressor of *trx*¹ and *ash1*¹⁰ mutants. Because dKDM2 is a putative histone demethylase it is highly likely that its function involves the removal of an activating methyl mark.

dkdm2 is required for H3K36me2 demethylation and H2A ubiquitylation in cells

A prominent feature of dKDM2 is the presence of a JmjC demethylase motif. Yeast has a single KDM2 homolog, Jhd1 (yKDM2), but there are two human homologs: hKDM2A (JHDM1a or FBXL11) and hKDM2B (JHDM1b or FBXL10). Both hKDM2A and yKDM2 have been shown to catalyze H3K36me1/2 demethylation, although removal of the me2 mark appeared more efficient than that of me1 (Tsukada et al. 2006). However, hKDM2B was recently found to be a nucleolar protein that mediates selective demethylation of H3K4me3 (Frescas et al. 2007). To determine the histone target of dKDM2 we used RNAi-mediated depletion of S2 cells. In addition to dKDM2 we also depleted S2 cells for dRING, PSC, PC, and PH (Fig. 3A). Next, we isolated bulk histones from mock- and RNAi-treated cells by acid extrac-

Figure 4. *dkdm2* is an enhancer of *Pc* but a suppressor of *trx* and *ash1*. (A) Representative examples of homeotic transformations that were scored in the transheterozygous progeny of a series of crosses in which each of the *dkdm2*^{KG04325} *dkdm2*^{EY01336} or *dkdm2*^{DG12810} mutant alleles or a wild-type (wt) allele were combined with either *Pc*¹ or *Pc*³ mutations. Sex combs are a row of dark, thick bristles, which normally only occur on the first pair of legs of male flies. In flies with defective *Pc* silencing, sex combs also appear on the second (L2–L1) or third (L3–L1) leg. Transformation of the fourth abdominal into the semblance of the posterior fifth (A4–A5) can be detected by the increased pigmentation of A4. Some flies display a defective wing development indicative of a wing-to-haltere (W–H) transformation. (B) Graphical representation of the frequencies of homeotic transformations in flies heterozygous for *Pc*¹ or *Pc*³ mutations but wild type for *dkdm2* (black bars), or transheterozygous animals carrying *Pc*¹ or *Pc*³ combined with either *dkdm2*^{KG04325} (yellow bars), *dkdm2*^{EY01336} (red bars), or *dkdm2*^{DG12810} (orange bars) mutations. The frequency of homeotic transformations is significantly higher in flies transheterozygous for *dkdm2* and *Pc* mutations compared with flies heterozygous for only *Pc* mutations, as determined by Student's *t*-test ($P < 0.05$). Two exceptions are indicated. (C) Representative examples of homeotic transformations observed in the transheterozygous progeny of homozygous *trx*¹ or *ash1*¹⁰ females crossed with either wild-type or *dkdm2* mutant males. Abbreviations of the homeotic transformations: (H–W) haltere-to-wing; (A5–A4) transformation of the fifth abdominal segment into the semblance of the fourth. (D) Graphical representation of the frequencies of homeotic transformations in flies heterozygous for either *Trx*¹ or *ash1*¹⁰ (black bars) or transheterozygous animals carrying either *trx*¹ or *ash1*¹⁰ in combination with the indicated *dkdm2* mutations. The frequency of homeotic transformations is significantly lower in flies transheterozygous for *dkdm2* and either *trx*¹ or *ash1*¹⁰ compared with *trx*¹ or *ash1*¹⁰ heterozygotes wild type for *DKDM2*, as determined by Student's *t*-test ($P < 0.05$).

tion. The levels of H3K36me1, H3K36me2, H3K36me3, and H3K4me3 were examined by Western immunoblot analysis using modification-specific antibodies (Fig. 5A). dKDM2 depletion caused a strong increase in H3K36me2 levels, whereas H3K36me1, H3K36me3, and H3K4me3 remained unchanged. In contrast, depletion of dRING, PSC, PC, or PH did not affect any of these histone marks. We conclude that dKDM2 selectively demethylates H3K36me2.

The second prominent feature of KDM2 is the presence of an F-box, a protein-protein interaction domain found in a class of proteins involved in ubiquitylation target selection. Previous research established that dRING is a key factor in histone H2A ubiquitylation (Wang et al. 2004). Moreover, it seemed plausible that PSC, like its mammalian homolog BMI1 (Cao et al. 2005; Buchwald et al. 2006), will stimulate the activity of dRING. A confounding factor is, however, that dRING and PSC are subunits of two distinct complexes, dRAF and PRC1. This raises the question of the relative importance of dRAF versus PRC1 for histone H2A ubiquitylation. Related to this question, we were particularly interested in the possibility that dKDM2 might modulate histone H2A ubiquitylation by dRING/PSC. Follow-

ing cellular depletion of dKDM2, we observed a striking loss of histone H2Aub, as revealed by Western immunoblotting (Fig. 5B). H2Aub was detected using antibodies directed against histone H2A, conjugated ubiquitin, or the E6C5 monoclonal antibody that can recognize H2Aub. The effect of dKDM2 depletion on H2Aub is highly specific, as we did not detect any change in the levels of H2Bub, as determined using antibodies directed against H2B or ubiquitin. The anti-ubiquitin antibody can be used to distinguish between H2Bub and H2Aub because the former migrates slightly slower than the latter on 18% SDS-PAGE. Importantly, the reduction in H2Aub levels due to loss of dKDM2 was comparable with that observed in cells lacking the H2A E3 ligase, dRING, or PSC. In contrast to loss of dKDM2, depletion of the PRC1 subunits PC or PH had no effect on H2Aub levels. These findings suggest that, at least in these cells, dRAF is more critical for bulk cellular histone H2A ubiquitylation than PRC1. We conclude that dKDM2 has a dual function in chromatin regulation: First, dKDM2 mediates histone H3K36me2 demethylation, removing an active chromatin mark. Second, dKDM2, together with dRING/PSC, is responsible for the majority of histone H2A ubiquitylation in cells, a repressive chromatin mark.

Figure 5. Depletion of endogenous dKDM2 causes increased histone H3K36me2 and loss of H2Aub. (A) Reduction of dKDM2 levels leads to a selective increase in H3K36me2. S2 cells were either mock-treated or incubated with dsRNAs directed against selective dRAF or PRC1 subunits dKDM2, dRING, PSC, PC, and PH (see Fig. 3A). Histones were purified by acid extraction and resolved by SDS-PAGE followed by Western blotting using the indicated antibodies directed against selective methyl marks. (B) dKDM2, dRING, and PSC are required for histone H2A ubiquitylation but do not affect H2Bub. The ubiquitylation status of histones purified from RNAi-treated S2 cells were analyzed by Western blotting using antibodies directed against either H2A, H2Aub, ubiquitin (ub), or H2B. The nonubiquitylated histones as well as H2Aub and H2Bub are indicated.

dRAF mediates H3K36me2 demethylation

Our depletion studies in cells indicated that dKDM2 is a bifunctional enzyme required for H3K36me2 demethylation as well as H2A ubiquitylation. To obtain direct evidence for this notion and to gain insight into the molecular mechanism of action of dKDM2, we decided to reconstitute both reactions *in vitro*. We used the baculovirus expression system to coexpress Flag-tagged dKDM2 (F-dKDM2), dRING, and PSC in Sf9 cells. Immunopurification on an anti-Flag column followed by peptide elution yielded a dRAF core assemblage comprising F-dKDM2 and apparently stoichiometric amounts of PSC and dRING (Fig. 6A). These results showed that dKDM2, dRING, and PSC form a stable trimeric complex. Likewise, we assembled and purified PCC, composed of F-PH, PC, PSC, and dRING, F-dKDM2 alone, a heterodimeric F-dRING/PSC complex, F-PSC alone, F-dRING alone, and heterodimeric F-PH/PC. As a substrate for our reconstituted reactions, we used purified endogenous oligonucleosomes harboring a representative full range of histone modifications. We first tested the ability of the recombinant dKDM2/dRING/PSC complex to demethylate H3K36me2 *in vitro*. Incubation of oligonucleosomes with dKDM2/dRING/PSC resulted in the efficient removal of H3K36me2, but not of H3K36me1, H3K36me3, or H3K4me3 (Fig. 6B). As expected, PCC did not display any demethylase activity. dKDM2 alone also efficiently demethylate H3K36me2,

establishing that dRING and PSC were dispensable for this enzymatic activity (Fig. 6C). In conclusion, as suggested by our RNAi-mediated depletion experiments, these *in vitro* reconstitution assays establish that dRAF mediates the selective removal of the H3K36me2 mark.

dKDM2 stimulates H2A ubiquitylation by dRING/PSC

We next compared the ability of the dRAF core complex and PCC to ubiquitylate histone H2A. Approximately equimolar amounts of either dKDM2/dRING/PSC or PCC were incubated with nucleosomes, followed by SDS-PAGE and Western blotting using antibodies directed against H2A, ubiquitin, or H2Aub. We only observed efficient ubiquitylation in the presence of dKDM2/dRING/PSC, but not in reactions containing PCC (Fig. 6D). The reaction was specific for histone H2A because there was no detectable ubiquitylation of H2B. We note that the endogenous H2Aub or H2Bub is not detectable with the histone amounts loaded on these gels. In conclusion, the results from our *in vitro* reconstitution dovetail perfectly with the results of dKDM2 depletion, which suggested that dRAF, rather than PRC1, is responsible for bulk H2A ubiquitylation.

To investigate the role of dKDM2 in more detail, we incubated nucleosomes with various combinations of dRAF subunits (Fig. 6E). dRING alone mediates only a low level of H2A ubiquitylation (Fig. 6E, lanes 2–4). In agreement with the observations of Buchwald et al. (2006) for BMI1, we detected low amounts of H2Aub in reactions containing PSC (Fig. 6E, lanes 5–7) and a modestly increased efficiency when both PSC and dRING were present (Fig. 6E, lanes 15–17). In contrast, dKDM2 robustly stimulated H2A ubiquitylation by dRING (Fig. 6E, lanes 18–20) as well as by dRING/PSC (Fig. 6E, lanes 21–23). By itself dKDM2 did not affect H2Aub (Fig. 6E, lanes 8–10) nor stimulated PSC (Fig. 6E, lanes 12–14). Again, the reactions were highly selective, as in the same assay histone H2B was not ubiquitylated (data not shown). The PRC1 selective subunits PC/PH did not stimulate H2A ubiquitylation by dRING/PSC (Fig. 6F, lanes 4–11), whereas dKDM2 strongly activated an amount of dRING/PSC that by itself did not yield detectable H2Aub (Fig. 6F, lanes 12–15).

Because dRAF links H3K36me2 demethylation to H2A ubiquitylation, we wondered whether these distinct biochemical reactions might be interdependent. H3K36me2 demethylation and H2A ubiquitylation can occur concomitantly in the same reaction (Fig. 7A). Because dKDM2 alone suffices to efficiently catalyze H3K36me2 demethylation (Fig. 6C), this reaction clearly does not depend on H2A ubiquitylation. However, it remained possible that H2A ubiquitylation might be dependent on H3K36me2 demethylation. To address this issue, we first purified endogenous mononucleosomes to test whether H2Aub and H3K36me2 could coexist within the same nucleosome. We used antibodies directed against H3K36me2 to immunodeplete the mononucleosome pool for this histone mark (Fig. 7B). All

Figure 6. dKDM2 stimulates selective H2A ubiquitylation by dRING-PSC. (A) We (co)expressed and purified dRAF and PRC1 core subunits as various multiprotein assemblies or by themselves using the baculovirus system. Purified factors and complexes include Flag-tagged dKDM2 (F-dKDM2)/dRING/PSC, PCC comprising F-PH/PSC/PC/dRING, F-dKDM2, F-dRING/PSC, F-PSC, F-dRING, and F-PH/PC. Following extract preparation, immunopurification, and elution under native conditions using Flag-peptides, proteins were resolved by SDS-PAGE and visualized by Coomassie staining. Asterisks indicate nonspecific background proteins. (B) dRAF core complex demethylates H3K36me2. Oligonucleosomes were incubated with either buffer control, dKDM2/dRING/PSC, or PCC. Approximately equimolar amounts (~30 nM) of each protein complex were added, as judged by Coomassie staining (shown in A). Reaction mixtures were resolved by SDS-PAGE followed by Western blotting using antibodies directed against the indicated methyl marks or the core domain of histone H3. The bottom panel shows the core histones present in the reaction visualized by Coomassie staining. (C) dKDM2 alone is sufficient to demethylate H3K36me2. Oligonucleosomes were incubated with either a buffer control or increasing amounts of dKDM2 (~10, 20, 40, or 80 nM). Analysis was as described above. (D) dRAF core complex (dKDM2/dRING/PSC) ubiquitylates histone H2A, but not H2B. Oligonucleosomes or a buffer control were incubated in the presence of approximately equimolar amounts (~30 nM) of either dKDM2/dRING/PSC or PCC. Reaction mixtures were resolved by SDS-PAGE followed by Western blotting using antibodies directed against H2A, H2Aub, ubiquitin (ub), and H2B. Note that the amount of histones loaded did not allow detection of endogenous H2Aub or H2Bub. (E) dKDM2 stimulates histone H2A ubiquitylation by dRING and dRING/PSC. Oligonucleosomes were incubated with increasing amounts of dRING, PSC, or dKDM2 (~20, 40, or 80 nM), dKDM2 was also titrated in the presence of ~40 nM (++) PSC, and dKDM2 and/or PSC were titrated in reactions containing ~20 nM (+) dRING. Analysis was as described above. (F) PC/PH does not stimulate H2A ubiquitylation by dRING/PSC. Oligonucleosomes were incubated in the presence of either 20 (+) or 40 nM (++) dRING/PSC and increasing amounts of PC-PH (~10, 20, 40, or 80 nM). As a control, dKDM2 (~10, 20, 40, or 80 nM) was added to reactions containing 20 nM (+) dRING/PSC. Analysis was as described above.

H3K36me2 was recovered in the immunoprecipitate (Fig. 7B, lane 2) and absent from the unbound FT fraction (Fig. 7B, lane 3). Conversely, only nonubiquitylated H2A was present in H3K36me2-selected nucleosomes (Fig. 7B, lane 2) and all H2Aub was detected in the H3K36me2-depleted fraction (Fig. 7B, lane 3). We conclude that the active H3K36me2 mark and the repressive H2Aub mark do not coexist within a single nucleosome. To test directly whether H2A ubiquitylation was dependent on H3K36me2 demethylation, we compared H3K36me2-containing and H3K36me2-depleted mononucleosomes as substrates in this reaction. As shown in Figure 7C, the presence or absence of H3K36me2 did not influence the efficiency of H2A ubiquitylation by dRING/PSC/dKDM2. As a final test, we mutated two key residues of the dKDM2 JmjC demethylase domain and expressed and purified wild-type and mutant versions of dKDM2 (Fig. 7D). Whereas dKDM2(T241A) and dKDM2(H244A) were unable to demethylate H3K36me2, their ability to ubiquitylate H2A remained

unaffected (Fig. 7E). From these results we conclude that stimulation of H2A ubiquitylation by dKDM2 is independent of demethylation of H3K36me2.

Taken together, these results confirmed that dKDM2 is involved in two completely different biochemical reactions. Firstly, dKDM2 is a histone demethylase that specifically removes the active H3K36me2 mark. Secondly, dKDM2 strongly stimulates histone H2A ubiquitylation by dRING/PSC, thus promoting a mark associated with silent chromatin. Collectively, our knock-down studies in cells and our in vitro reconstitution reactions suggested that dRAF rather than PRC1 is the major H2A ubiquitylating enzyme in cells.

Discussion

In this study, we investigated the molecular mechanisms involved in PcG-mediated gene silencing. The major findings of this work are the following. First, we identified a novel PcG silencing complex we named dRAF,

by dRING/PSC. Oligonucleosomes were incubated with ~20, 40, or 80 nM of dKDM2, dKDM2 (T241A), or dKDM2 (H244A), respectively. Reaction products were resolved by SDS-PAGE followed by Western blotting.

harboring core subunits dKDM2, dRING, and PSC. Whereas dRING and PSC are also part of PRC1, the other two PRC1 core subunits, PC and PH, are absent from dRAF. In addition, we found that significant amounts of PSC and PH are not associated with either PRC1 or dRAF, suggesting they might form part of other assemblages. In short, our work suggests a greater diversity among PcG complexes than previously anticipated. Second, genome-wide expression analysis revealed that dKDM2 and PRC1 share a significant number of target genes. Third, we found that *Pc* and *dkdm2* interact genetically and cooperate in repression of homeotic genes in vivo. Fourth, dKDM2 counteracts homeotic gene activation by the *trxG* histone methyltransferases TRX and ASH1. Fifth, we uncovered a novel *trans*-histone pathway acting during PcG silencing. dKDM2 plays a central role by removal of the active H3K36me2 mark and promoting the establishment of the repressive H2Aub mark by dRING/PSC. Finally, our observation that dKDM2 is required for bulk histone H2A ubiquitylation by dRING/PSC, suggests that dRAF rather than PRC1 is the major histone H2A ubiquitylating complex in cells.

The term *trans*-histone pathway was first coined to describe that H2B ubiquitylation is required for H3K4 and H3K79 methylation, whereas the reverse is not the case (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002;

Figure 7. H2A ubiquitylation and H3K36me2 demethylation can occur concomitantly but are not interdependent. (A) H2A ubiquitylation and H3K36me2 demethylation can occur concomitantly in the same reaction. Oligonucleosomes or a buffer control were incubated in the presence of dRING/PSC (~30 nM), dKDM2 (~30 nM), or with increasing amounts of dKDM2 (~10, 20, 40, or 80 nM) in the presence of ~30 nM dRING/PSC. Reaction products were resolved by SDS-PAGE followed by Western blotting using the indicated antibodies. (B) H3K36me2 and H2Aub are mutually exclusive nucleosomal marks in bulk chromatin. Approximately 2 mg of purified endogenous mononucleosomes were immunopurified using Protein A Sepharose beads coated with antibodies directed against H3K36me2. Input, bound, and unbound FT fractions were collected, resolved by 18% SDS-PAGE, and analyzed by Western blotting using antibodies directed against H3K36me2, H2Aub, and H2A. (C) Stimulation of H2A ubiquitylation by dKDM2 is independent of H3K36me2. Mononucleosomes (*left* panel) or H3K36me2-depleted mononucleosomes (*right* panel) were incubated in the presence of either ~30 nM dRING/PSC alone or together with ~40 or ~80 nM dKDM2 or ~80 nM dKDM2. Analysis was as described above. (D) Wild-type Flag-tagged dKDM2 (F-dKDM2) or mutants F-dKDM2(T241A) and F-dKDM2(H244A) were expressed in Sf9 cells using the baculovirus system. Following extract preparation, immunopurification, and elution under native conditions using Flag-peptides, proteins were resolved by SDS-PAGE and visualized by Coomassie staining. (E) H3K36me2 demethylation defective dKDM2 mutants remain fully able to stimulate H2A ubiquitylation

Sun and Allis 2002; Bhaumik et al. 2007; Larabee et al. 2007; Tanny et al. 2007; Weake and Workman 2008). Recently, it was found that H2Bub determines the binding of Cps35, a key component of the yeast H3K4 methylase COMPASS complex (J.S. Lee et al. 2007), providing insight in the molecular mechanism by which two positive marks are coupled. Here, we described a different type of *trans*-histone regulation where the removal of the active H3K36me2 mark is directly linked to repressive monoubiquitylation of H2A. A recent study strongly argued that ASH1 mediates H3K36me2 (Tanaka et al. 2007). Significantly, our genetic and biochemical analysis revealed an in vivo antagonism between dKDM2 and ASH1. Thus, dKDM2 appears to reverse the enzymatic activity of *trxG* protein ASH1 through H3K36 demethylation, whereas it does not affect H3K4 methylation. The observation that chromatin binding of TRX is ASH1 dependent (Rozovskaia et al. 1999) is likely to be part of the explanation of the strong genetic interaction between *dkdm2* and *trx*. The association of the H3K27me2/3 demethylase UTX with the MLL2/3 H3K4 methylase complexes is an example of coupling removal of a repressive mark to the establishment of an active mark (M.G. Lee et al. 2007).

Our work revealed that the key H2A E3 ubiquitin ligase dRING is part of two distinct complexes, PRC1 and dRAF. A previous study identified the mammalian

BCOR corepressor complex, which harbors RING1, NSPC1, and FBXL10 and other proteins, absent from dRAF (Gearhart et al. 2006). These findings suggest that BCOR and dRAF represent a variety of related but distinct silencing complexes. Reduction of dKDM2 caused a dramatic loss of H2Aub levels, which was comparable with that observed after depletion of dRING or PSC. However, knockdown of PRC1 subunits PC or PH had no effect on H2Aub. These observations suggest that dRAF rather than PRC1 is responsible for the majority of H2A ubiquitylation in cells. This notion was reinforced by in vitro reconstitution experiments, suggesting that dRAF is a more potent H2A ubiquitin ligase than PRC1. An unresolved issue remains the molecular mechanisms that underpin the opposing consequences of either H2A or H2B ubiquitylation. It is intriguing that H2Aub appears to be absent in yeast, present but less prominent than H2Bub in *Drosophila* (see Fig. 5; our unpublished results), and abundant in mammalian cells. An attractive speculation is that H2Aub becomes more important when genome size increases and noncoding regions and transposons need to be silenced.

In summary, we identified the PcG complex dRAF, which employs a novel *trans*-histone pathway to mediate gene silencing. dKDM2 plays a pivotal role by coupling two distinct enzymatic activities, H3K36me2 demethylation and stimulation of H2A ubiquitylation by dRING/PSC. Our results indicate that dRAF is required for the majority of H2Aub in the cell. dKDM2 cooperates with PRC1 but counteracts *trxG* histone methylase ASH1. These findings uncovered a repressive *trans*-histone mechanism operating during PcG gene silencing.

Materials and methods

Plasmids, recombinant proteins, immunological procedures, and antibodies

Details of cloning procedures are available upon request. For baculovirus expression, the coding sequence of full-length dKDM2, dKDM2(T241A), and dKDM2(H244A) were cloned into a modified version of the shuttle vector pVL1392 (Pharmingen) expressing an in-frame N-terminal Flag tag. For antibody production, dRING (full length), PSC (amino acids 1107–1602), dKDM2 (amino acids 1–353), dKDM2 (amino acids 647–923), dRAF2 (amino acids 1–440), Mtor (amino acids 367–716 and 1097–1419), and Ulp1 (amino acids 1–493) were cloned in pGEX-2TKN, a derivative of pGEX-2TK (Pharmacia) and expressed as glutathione S-transferase (GST) fusion proteins. Protein purification, immunization, and affinity purifications were as described (Harlow and Lane 1998; Chalkley and Verrijzer 2004). The following antibodies were used: guinea pig: α -dRing: SN11 and SN12; α -dKDM2: GR368; α -PSC: GR254; α -Ulp1: SNC045; rabbit: α -dKDM2: GR207; α -dRAF2: SN1915; α -Mtor: SN1912; α -PC: SN965; α -PH: SN964 (Mohd-Sarip et al. 2002); α -MOR: SN670 and SN671 (Mohrmann et al. 2004); α -ISWI (Kal et al. 2000); α -PSC monoclonal hybridoma supernatant clone 6E8 (Martin and Adler 1993); H3K36me1 (Abcam Ab9048); H3K36me2 (Upstate Biotechnologies 07-369); H3K36me3 (Abcam Ab9050); H3K4me3 (Abcam Ab8580); H3 (Abcam Ab1791); H2A (Upstate Biotechnologies 07-146); H2Aub (E6C5) (Upstate Biotechnologies 05-678); H2B (Upstate Biotechnologies 07-371);

ubiquitin (Affiniti Research Products Ltd. PW8810). Immunoblotting experiments were performed using standard procedures. Coimmunoprecipitation experiments were performed as described (Mohd-Sarip et al. 2002). All critical immunoprecipitations and Western blotting experiments were repeated with different antisera. *Drosophila* embryo nuclear extract fractionation by $(\text{NH}_4)_2\text{SO}_4$ precipitation, POROS Heparin and Sephacryl S-300 size-exclusion chromatography, and glycerol gradient sedimentation were performed as described (Mohrmann et al. 2004).

Drosophila genetics

Flies were maintained under standard conditions. *dkdm2*^{KG04325}, *dkdm2*^{EY01336}, and *dkdm2*^{DG12810} strains were obtained from the Bloomington *Drosophila* Stock Center (<http://flystocks.bio.indiana.edu>). *Pc*¹, *Pc*³, *trx*¹, *ash1*¹⁰ mutant alleles were provided by F. Karch (Geneva). Information on these stocks can be found at FlyBase (<http://www.flybase.org>). Five females heterozygous for either *Pc*¹ or *Pc*³ mutations were crossed with five wild-type or *dkdm2* heterozygous mutant males. For the crosses involving *trx* or *ash1*, we analyzed the progeny of five homozygous *trx*¹ or *ash1*¹⁰ females crossed with five wild-type or various *dkdm2* heterozygous mutant males. All crosses were performed in parallel at 25°C, and the frequencies of homeotic transformations were counted. Significance analysis was performed by Student's *t*-test for proportions.

RNAi-mediated knockdowns and genome-wide expression analysis

RNAi-mediated depletion and expression profiling and statistical analysis were performed as described (Moshkin et al. 2007). Briefly, dsRNA targeting the various subunits was synthesized using an Ambion Megascript T7 kit according to the manufacturer's protocol. Oligonucleotide sequences used to generate dsRNA will be provided upon request. Cells were incubated in the presence of dsRNA for 4 d. RNA samples from three independent biological replicates were isolated, prepared, and hybridized with Affymetrix microarrays. PCA was performed as described (Moshkin et al. 2007). Venn Diagrams were constructed using *t*-test, assigning 1 for significantly up-regulated genes ($P < 0.05$) and -1 for significantly down-regulated genes ($P < 0.05$). Co-occurrence of up- and down-regulated genes is shown as overlap on the Venn diagrams. All statistical analysis was performed using R and Bioconductor packages. Details will be provided upon request.

In vitro demethylation and ubiquitylation assays

Reconstituted PCC, dKDM2/dRING/PSC, dKDM2, dRING/PSC, PSC, dRING, and PH/PC were expressed using the baculovirus system and purified as described previously (Francis et al. 2001; Mohd-Sarip et al. 2002). Mono- and oligonucleosomes were prepared essentially as described with minor modifications (Bulger and Kadonaga 1994). Chromatin was treated with sarkosyl (0.05% final concentration) for 5 min and immediately loaded onto 5%–30% sucrose gradients. After ultracentrifugation in a SW28 rotor for 16 h at 26,000 rpm at 4°C, 3-mL fractions were collected and those containing either mononucleosomes or oligonucleosomes with a repeat length of 10–15 were used. Histone demethylase assays were carried out as described (Tsukada et al. 2006) using ~200 μ g of oligonucleosomes per 400- μ L reaction, incubated for 1 h at 30°C. Ubiquitylation reactions contained 200 μ g of oligonucleosomes in 2-mL reactions containing 25 mM Hepes-KOH (pH 7.6), 2 mM MgCl_2 , 70 mM

KCl, 0.6 mM DTT, 5 mM ATP, 2 mM NaF, 10 mM Okada acid, 0.484 μ g of ubiquitin activating enzyme E1 (Boston Biochem, catalog no. E-305), 2.58 μ g of ubiquitin-conjugating enzyme UbcH5c (Boston Biochem, catalog no. E2-627), and 6.64 μ g of His₆-ubiquitin (Alexis Corporation, catalog no. BSTU-530-M002) and were incubated for 1 h at 30°C. For coupled ubiquitylation/demethylation reactions, the respective buffers were simply combined 1:1. Reaction mixtures were concentrated by standard TCA precipitation and resolved in SDS-loading buffer. Ten percent of each reaction was resolved by 18% SDS-PAGE followed by Western blotting.

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