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RBP2 Belongs to a Family of Demethylases, Specific for Tri- and Dimethylated Lysine 4 on Histone 3

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SUMMARY

Methylation of histones has been regarded as a stable modification defining the epigenetic program of the cell, which regulates chromatin structure and transcription. However, the recent discovery of histone demethylases has challenged the stable nature of histone methylation. Here we demonstrate that the JARID1 proteins RBP2, PLU1, and SMCX are histone demethylases specific for di- and trimethylated histone 3 lysine 4 (H3K4). Consistent with a role for the JARID1 *Drosophila* homolog Lid in regulating expression of homeotic genes during development, we show that RBP2 is displaced from *Hox* genes during embryonic stem (ES) cell differentiation correlating with an increase of their H3K4me3 levels and expression. Furthermore, we show that mutation or RNAi depletion of the *C. elegans* JARID1 homolog *rbr-2* leads to increased levels of H3K4me3 during larval development and defects in vulva formation. Taken together, these results suggest that H3K4me3/me2 demethylation regulated by the JARID1 family plays an important role during development.

INTRODUCTION

A strict regulation of gene-expression patterns is essential for cellular identity and differentiation during development of eukaryotic organisms. Eukaryotic chromatin is marked with a vast variety of posttranslational histone modifications. These modifications have been proposed to represent a combinatorial "histone code" specifying the function of the affected genomic regions in terms of chromosome segregation, DNA repair, and transcriptional activity (Strahl and Allis, 2000; Turner, 2000). Thus, some of these modifications appear to play important roles in dividing the genome into transcriptionally active and inactive areas.

As an example of this, tri- and dimethylation of histone H3 at lysine 4 (H3K4me3/me2) is restricted to euchromatin and is generally associated with active transcription (Sims and Reinberg, 2006). The H3K4 tri- and dimethylation marks may facilitate transcription by the recruitment of nucleosome remodeling complexes and histone-modifying enzymes or by preventing transcriptional repressors from binding to chromatin. In agreement with this, factors involved in transcriptional activation have been shown to bind to the H3K4me3 and H3K4me2 marks (Pray-Grant et al., 2005; Sims et al., 2005; Wysocka et al., 2005, 2006). H3K4 methylation appears to occur after the assembly of the preinitiation complex, suggesting that the methylation of H3K4 is not sufficient to initiate transcription but rather that it is involved in other processes like transcriptional elongation, mRNA processing, or maintenance of gene expression (Pavri et al., 2006).

In *Drosophila,* methylation of H3K4 is catalyzed by the two Trithorax group (TrxG) proteins, Ash1 and Trithorax, that fulfill key developmental functions and are vital for the establishment and maintenance of lineage-specific gene-expression patterns through mitotic cell division (Ringrose and Paro, 2004). The Polycomb group (PcG) proteins function antagonistically to the TrxG proteins and catalyze the methylation of the repressive H3K27 mark. The TrxG and PcG proteins maintain the transcriptionally active and repressive states of the *Hox* genes, respectively; consistent with a crucial role of these proteins and the histone marks in maintaining the correct expression pattern of the *Hox* genes, mutations of these lead to abnormal development (Ringrose and Paro, 2004).

The mammalian Trithorax homolog, MLL, also regulates *Hox* expression, and it catalyzes the trimethylation of H3K4 (Milne et al., 2002; Nakamura et al., 2002). Moreover, like *Drosophila* Trithorax, MLL is required for normal development (Yu et al., 1995). In addition to MLL,

mammals have at least three other SET-domain-containing proteins, SET1, SET7, and SMYD3, that all catalyze the methylation of H3K4 (Martin and Zhang, 2005).

Genome-wide studies of the H3K4 methylation status in mammalian cells support a key role for the H3K4me3/me2 marks in *Hox* gene regulation, cellular differentiation, and development (Bernstein et al., 2005; Margueron et al., 2005). Accordingly, H3K4 methylation is modulated in a lineage-specific manner on a variety of genes during embryonic stem (ES) cell differentiation (Bernstein et al., 2006). Moreover, in contrast to the vast majority of genes that display a discrete H3K4 methylation pattern, with methylations occurring in the proximity of promoters of transcriptionally active genes and in stretches of less than 2kb, the *Hox* gene cluster displays large cell-type specific H3K4me3/me2 regions overlapping several *Hox* genes (Bernstein et al., 2005, 2006; Guenther et al., 2005). These areas are believed to constitute transcriptional accessible chromatin regions set up during embryonic development to preserve *Hox* expression (Chambeyron and Bickmore, 2004).

In contrast to other histone modifications such as acetylation and phosphorylation, methylation, and in particular trimethylation, has until recently been regarded as irreversible, because of the high thermodynamic stability of the N-CH_3 bond. The identification of the amine oxidase LSD1 as an H3K4me2/me1 specific demethylase changed this dogma (Shi et al., 2004). However, LSD1 is unable to demethylate trimethylated H3K4, most likely due to the absence of a protonated nitrogen required for oxidation. Recently, it was hypothesized that the proteins harboring a Jumonji C (JmjC) domain might possess histone lysine demethylase activity (Trewick et al., 2005). The proposed reaction mechanism theoretically permits the demethylation of not only tri-, di-, and monomethylated lysine residues but also of arginine residues and nucleotides. The ability of the Jumonji proteins to demethylate trimethylated variants of histone lysines was verified experimentally by the identification of the JMJD2 protein family as histone demethylases with specificity for tri- and dimethylated H3K9 and H3K36 (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006b; Whetstine et al., 2006). JmjC-domain-containing histone demethylases catalyze demethylation of methylated histone lysines through an oxidative mechanism requiring iron and α-ketoglutarate (α-KG) as cofactors and most likely occurs through direct hydroxylation of the affected methyl group (Cloos et al., 2006; Tsukada et al., 2006; Whetstine et al., 2006). The catalytical core of these enzymes, conferring the proteins with iron and α-KG binding ability and demethylation activity, is located within the conserved JmjC domain.

Although several biological processes, including stages involved in X-chromosome inactivation (Heard et al., 2004) and circadian transcription of specific genes (Ripperger and Schibler, 2006), are characterized by a fast turnover of the H3K4me3 mark, no enzymes have so far been reported that can demethylate H3K4me3. In this report we demonstrate that the JmjC-containing JARID1 protein family including the RB binding protein 2 (RBP2, JARID1A), PLU1 (JARID1B), and SMCX (JARID1C) is a family of histone demethylases with specificity for tri- and dimethylated H3K4. Moreover, chromatin immunoprecipitation (ChIP) studies suggest that RBP2 mediates transcriptional repression of *Hox* genes through the modulation of H3K4me3. In addition, we provide compelling evidence indicating that the JARID1 family plays an important role in cellular differentiation and development.

RESULTS

Ectopic Expression of the JARID1 Family Members Reduces Tri-, Di-, and Monomethylated H3K4 In Vivo

In an attempt to identify additional demethylases amongst the Jumonji protein family, we undertook a candidate approach. We generated a phylogenetic tree of the Jumonji proteins based on an alignment of their respective JmjC domains (Figure 1A). This analysis revealed that the proteins segregate into distinct clusters. So far, three families of JmjC histone demethylases have been described (reviewed in Klose et al., 2006a): the FBXL10/11 family, the JMJD1 family, and the JMJD2 family. Interestingly, all submembers within each family share the same enzymatic specificity for methylated histone lysine residues. Based on this, we speculated that members of the other families would exhibit novel but similar enzymatic specificities.

Several structural features suggest that the four members of the JARID1 family (JARID1A/RBP2, JARID1B/PLU1, JARID1C/SMCX, and JARID1D/SMCY) are potential histone demethylases. First, the domains present in the JARID1 family include a JmjC, JmjN, Arid/Bright, and C5HC2 zinc finger domain in addition to two or three plant homeodomaine (PHD) domains that all are hallmarks of chromatin modifiers (Figure 1B). Secondly, these enzymes feature the canonical HX(E/D)X_nH motif, the so-called "facial triad," which is predicted to be involved in iron binding, is conserved in almost all JmjC proteins, and has been shown to be required for their activity (Klose et al., 2006a) (Figure 1C; for simplicity only the JMJD2 and the JARID1 families are shown).

To examine the catalytic activity of the JARID1 family, we ectopically expressed HA-tagged versions of RBP2, PLU1, and SMCX proteins in U2OS cells and analyzed histone methylation by immunofluorescence. As shown in Figure 2, overexpression of RBP2 and PLU1 led to a strong decrease in tri-, di- and monomethylated H3K4 (H3K4me3/me2/me1). Similarly, ectopic expression of SMCX resulted in a significant decrease in H3K4me3 staining (Figure 2C); however, in this experiment, the decrease in H3K4me2/me1 levels were lower than that observed for RBP2 and PLU1. We did not detect any significant decrease in H3K9me3 levels or other marks tested (Figure 2A and data not shown). To test for the specificity of the reduction in H3K4me3 levels, we generated an RBP2 mutant (H483G/E485Q), in which two of the three amino acids believed to be involved in Fe(II) binding were mutated. Indeed, when this mutant was ectopically

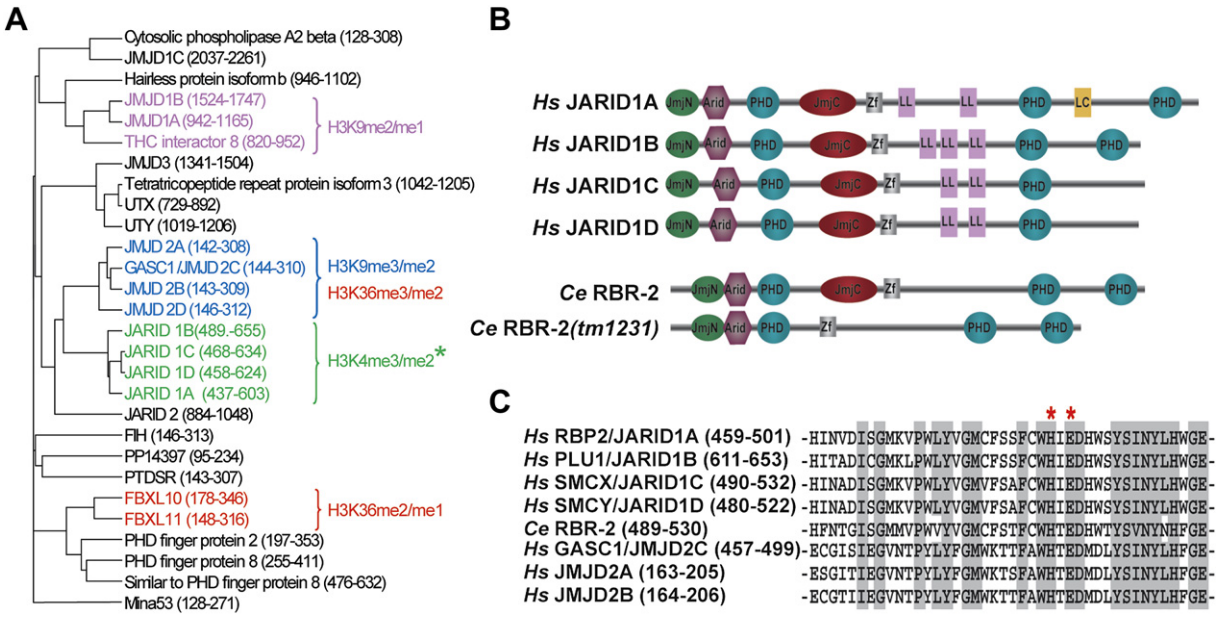


Figure 1. Domain Structure of the JARID1 Family Members

(A) Phylogenetic analysis of the human JmjC family. Alignment of the JmjC domains and construction of the phylogenetic tree were conducted using the neighboring-joining method (Saitou and Nei, 1987). The reported specificities of the different JmjC subfamilies are indicated. The green asterisk indicates the specificity reported in this study.

(B) Schematic representation of the human JARID1 family members, JARID1A, JARID1B, JARID1C, and JARID1D. Shown are ARID, AT-rich interactive domain; JmjN, jumonji N domain; JmjC, jumonji C domain; PHD, plant homeotic domain; Zf, C5HC2 zinc finger domain; LL, LXXLL; and Ce RBR-2, the only JARID1 family member in *C. elegans* and the putative structure of a *C. elegans* mutant, *tm1231*, lacking the JmjC domain used in this study.

(C) Sequence alignment of the JmjC domains from the JARID1 and the JMJD2 families. The highly conserved histidine (H) and glutamic acid (E) residues involved in iron binding and required for the catalytic activity of the JmjC enzymes, are indicated by red asterisks.

expressed in U2OS cells, no reduction in H3K4 methylation levels was observed (Figure 2A), demonstrating that the demethylating activity of RBP2 is dependent on a catalytically active JmjC domain.

We confirmed the global reduction of H3K4 methylation by immunoblotting of cell lysates prepared from transfected cells. To do this, we transfected 293 human embryonic kidney cells with expression vectors for RBP2, RBP2(H483G/E485Q), PLU1, and SMCX. Western blots were probed with antibodies against a panel of different histone lysine marks. As shown in Figure 2D, ectopic expression of RBP2 reduced global levels of H3K4 tri-, di-, and monomethylation. Similar results were obtained for PLU1 and SMCX (Figure S1). In contrast, the catalytically inactive RBP2 mutant increased methylation of H3K4. While a reduction of H3K36 methylation was observed, other tested epigenetic marks were not decreased by RBP2 overexpression. Although the decrease in H3K4 methylation may be an indirect consequence of JARID1 overexpression, these data, taken together with the structural features of the JARID1 proteins, suggest that they are histone demethylases with specificity for methylated H3K4.

RBP2 and SMCX Demethylate H3K4me3/me2 In Vitro

To determine the intrinsic enzymatic specificity of the RBP2-catalyzed demethylation reaction, we expressed human recombinant His-tagged RBP2 from baculovirus-infected insect cells and purified the recombinant protein by a combination of affinity and size-exclusion chromatography (Figures 3A and 3B). The incubation of histones with increasing amounts of highly purified RBP2 led to a progressive reduction in H3K4me3/me2 marks (Figure 3C). In contrast, we did not detect any activity of the recombinant RBP2 protein toward monomethylated H3K4. This is in contrast to the observation that overexpression of RBP2 results in a significant reduction of H3K4me1 in vivo, indicating that the latter is an indirect consequence of overexpression.

To gain further insight into the specificity of RBP2, synthetic H3K4me3/me2/me1 histone peptides were incubated alone or in the presence of purified RBP2 and analyzed by mass spectrometry. In agreement with the specificity determined for histones, we observed a shift in the mass of the peptides incubated with recombinant RBP2 corresponding to H3K4me3/me2 demethylation in vitro (Figure 3D). Thus, approximately 50% of H3K4me3 peptide was converted to H3K4me2 and 10% further to H3K4me1 after incubation with recombinant human RBP2 using an enzyme-to-substrate ratio of 1:20. Ten percent of H3K4me2 was converted to H3K4me1 under the same conditions, indicating H3K4me2 is also a substrate for RBP2 but that the trimethylated H3K4 peptide is

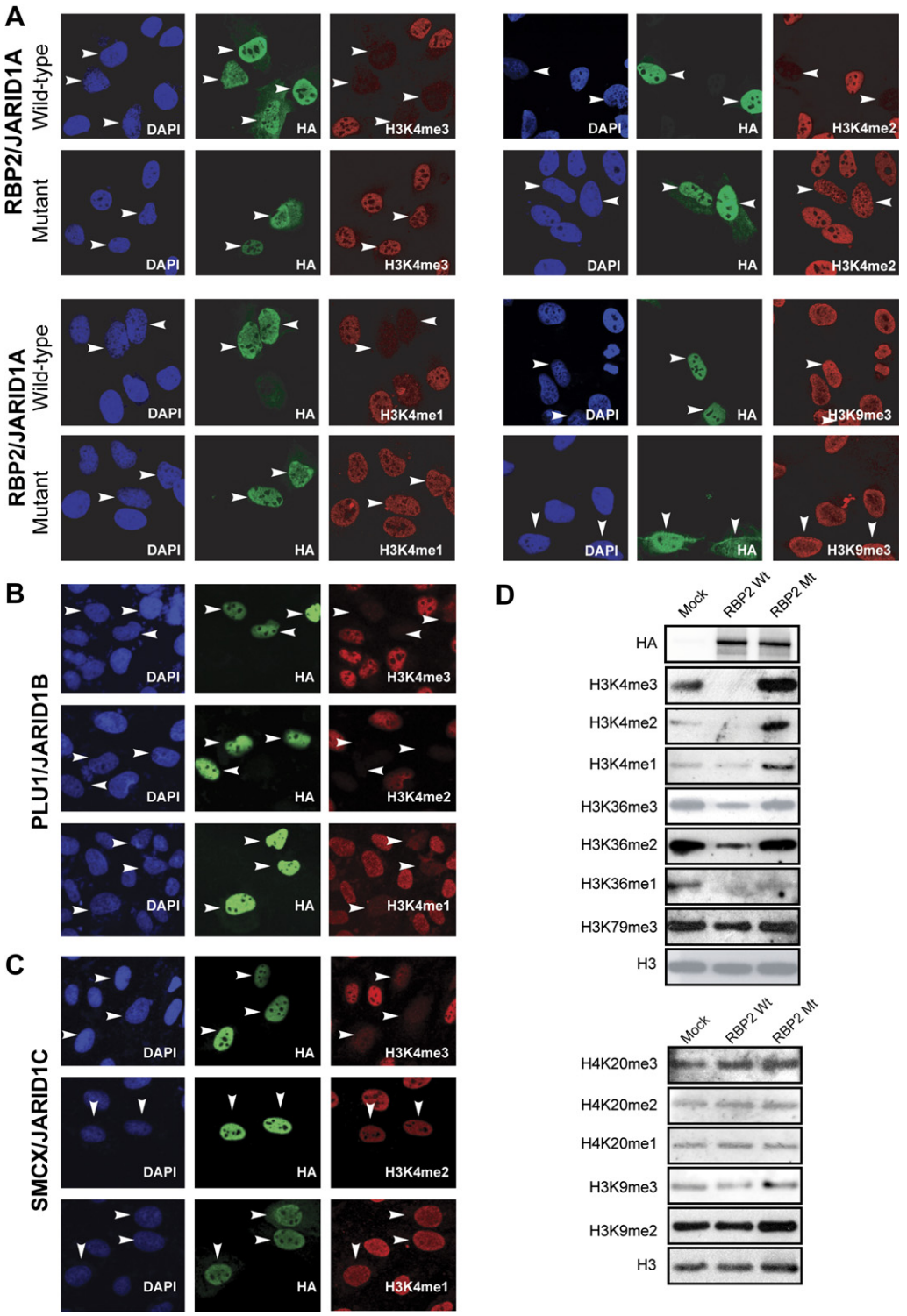


Figure 2. Members of the JARID1 Family Demethylate H3K4me3/me2/me1 In Vivo

(A) U2OS cells were transfected with HA-tagged RBP2 or RBP2 mutant. The transfected cells were fixed, costained for the indicated histone modifications and for the expression of the proteins (anti-HA), and analyzed by confocal microscopy. White arrows indicate cells expressing the tested protein. The cells were counterstained with DAPI to visualize cell nuclei.

(B) U2OS cells transfected with HA-tagged PLU1 were processed as described in (A).

(C) U2OS cells transfected with HA-tagged SMCX were processed as described in (A).

a better substrate for RBP2 in vitro. No unmethylated peptide was detected in any of the reactions, suggesting that monomethylated H3K4 is not a substrate for RBP2.

The specificity of RBP2 appeared to be identical for nucleosomes incubated with increasing amounts of RBP2 (Figure 4A). To further examine the enzymatic specificity of RBP2, histones were incubated with saturating amounts of RBP2 for the H3K4me3/me2 mark and tested for demethylating activity for the lysine-methylated forms of H3K9, H3K27, H3K36, and H4K20me3 (Figure 4B). No significant demethylation was detected for any of these marks. Taken together, these results show that RBP2 is a histone demethylase, which specifically catalyzes the demethylation of H3K4me3 and H3K4me2.

To test if other members of the JARID1 family also demethylate tri- and dimethylated H3K4 in vitro, we affinity-purified full-length recombinant SMCX/JARID1C from insect cells (Figure S2). Purified SMCX was incubated with histones, and, as shown in Figure 4C, SMCX catalyzed the demethylation of tri- and dimethylated H3K4 in vitro but not of H3K36me3/me2 and H3K9me3/me2. These results demonstrate that SMCX, as RBP2, is a demethylase that specifically catalyzes the demethylation of H3K4me3/me2 and suggest that the members of the JARID1 family all are demethylases having this specificity.

RBP2 Target Genes and Their H3K4me3 Status

The *Drosophila* RBP2 homolog *Lid* has been classified as a Trithorax-like protein. *Lid* is required for the correct regulation of homeotic genes during development, and homozygous *Lid* mutations results in larval lethality (Gildea et al., 2000). The function of Trithorax proteins is conserved during evolution, and we therefore speculated whether mammalian RBP2 could have a role in regulating *Hox* gene expression during development and differentiation. To investigate this, we stimulated mouse ES cells to differentiate using all trans retinoic acid (ATRA). As shown in Figure 5A, the expression of the ES cell markers *Oct4* and *Nanog* is rapidly repressed in response to ATRA treatment. Moreover, *Hoxa1*, *Hoxa5*, and *Hoxa7*, but not *HoxA11*, are rapidly activated upon differentiation (Figure 5B). To understand if RBP2 could be involved in the regulation of the *Hox* genes, we tested if RBP2 could bind to their promoters by ChIP analysis (Figure 5C). As a positive control for the ChIP experiments we used *Brd8*, which is a known target for RBP2 in human cells (Benevolenskaya et al., 2005). We found that RBP2 was present on the *Hoxa1*, *Hoxa5*, *Hoxa7*, and *Hoxa11* promoters in undifferentiated ES cells and that the binding to the *Hoxa1*, *Hoxa5*, and *Hoxa7* promoters is lost upon ES cell differentiation, correlating with their transcriptional activation. Consistent with the lack of *Hoxa11* activation during differentiation, RBP2 binding to the *Hoxa11* promoter was not altered. Moreover, in agreement with RBP2 being an H3K4me3 demethylase, loss of RBP2 binding to the *Hoxa1*, *Hoxa5*, and *Hoxa7* promoters correlates with increased H3K4me3 levels (Figure 5D). Further characterization of the RBP2 interaction with *Hoxa5* confirmed this result and showed that both H3K4me2/me3 levels increase upon loss of RBP2 binding, while the nucleosomal density of the promoter decreased in differentiating ES cells (Figure S3). Consistent with the lack of expression and maintenance of RBP2 binding during ES cell differentiation, the H3K4me3 levels of the *Hoxa11* promoter were not affected (Figure 5D). Taken together, these results suggest that RBP2 participates in the transcriptional regulation of *Hox* expression during differentiation.

The RBP2 *C. elegans* Homolog RBR-2 Regulates Global Levels of H3K4 Trimethylation and Vulval Development

Human *RBP2* is evolutionarily conserved, with homologs in mouse (*Jarid1a*), *Drosophila* (*Lid*), *C. elegans* (*rbr-2*) and *S. pombe* (*Lid2*). To examine the biological role of JARID1-type demethylases and their global impact on H3K4 trimethylation in vivo, we turned to the nematode *C. elegans*, which only encodes one single JARID1-type gene, *rbr-2* (ZK593.4). The predicted RBR-2 protein displays significant homology and shares the domain structure with human RBP2 and PLU1 (Figure 1B). To determine whether RBR-2 also catalyzes H3K4me3/me2 demethylation, we purified full-length recombinant RBR-2 from insect cells (Figure 6A) and tested its activity by incubation with bulk histones and the relevant cofactors. As shown in Figure 6B recombinant His-RBR-2 specifically demethylates H3K4me3/me2 in vitro.

Of special interest, a *C. elegans* mutant strain *rbr-2*(*tm1231*) has been isolated carrying an in-frame deletion of 648 bp in the *rbr-2* gene. The mutant transcript can give rise to a protein of 1261 amino acids lacking the entire JmjC domain (Figure 1B) and should therefore be devoid of demethylase activity. To investigate the putative effect of the *rbr-2* mutation on the global H3K4 methylation status, we analyzed the status of the H3K4me3 mark in wild-type (N2) animals and in the *rbr-2*(*tm1231*) mutant in the four larval developmental stages L1-4 and in adult animals by western blotting. This analysis revealed that the global H3K4me3 levels were significantly increased at all developmental stages in the *rbr-2*(*tm1231*) mutant when compared to N2 (Figure 6C). Moreover, when RBR-2 was inhibited by RNA interference, the H3K4me3 levels were also increased, albeit to a lesser extent than for the *rbr-2*(*tm1231*) mutant (Figures 6D and 6E), further supporting the notion that RBR-2 is functioning as a demethylase in vivo. Interestingly, the *rbr-2*(*tm1231*) mutant exhibited a complex phenotype displaying a highly penetrant vulval phenotype with 80% of the animals either having

(D) Ectopic expression of RBP2 and RBP2 mutant in 293 cells. Protein lysates from 293 cells transfected with empty vector (mock), HA-tagged wild-type RBP2 (RBP2 WT), or mutant RBP2 (RBP2 Mt) analyzed by immunoblotting. The blots were probed with the indicated antibodies.

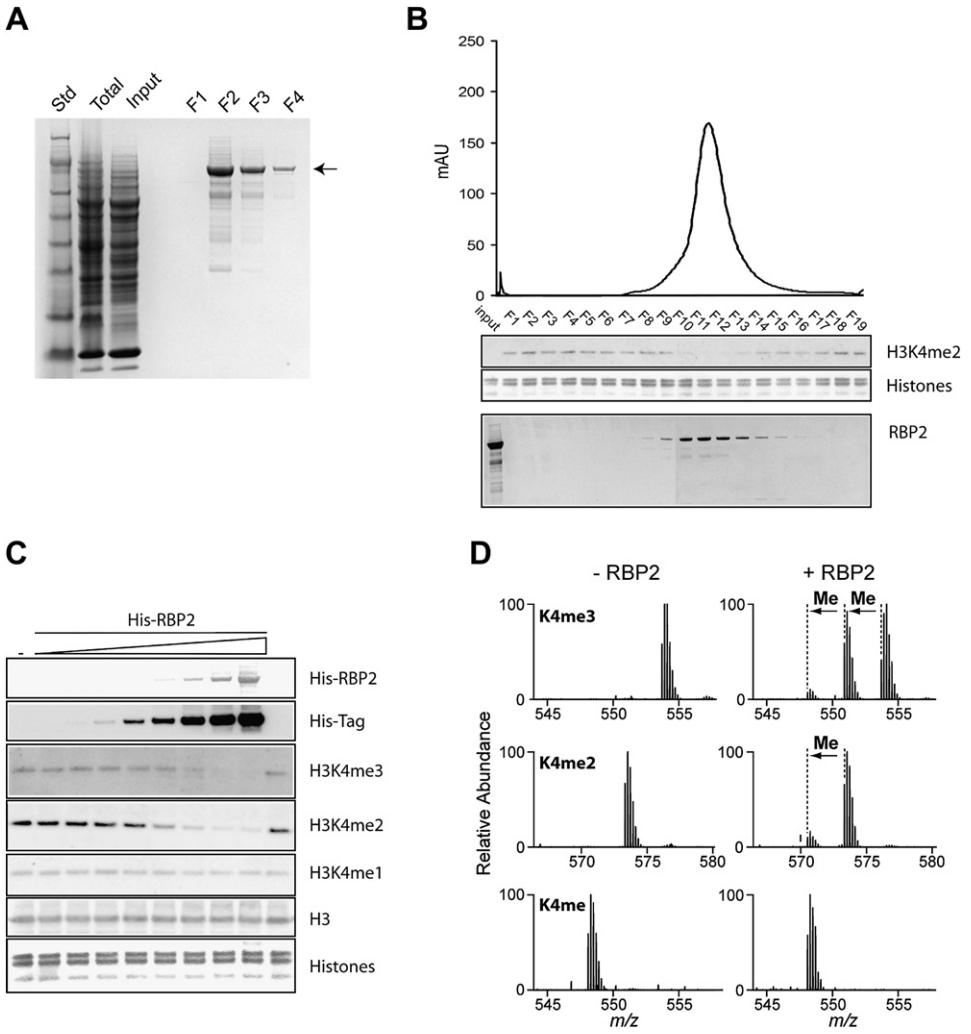


Figure 3. RBP2 Demethylates H3K4me3/me2 In Vitro

(A) SDS-PAGE analysis of purified His-tagged recombinant RBP2 expressed in insect cells. Shown are Std, molecular weight standard, total, total lysate; cleared input, input; F1–F4, fractions 1–4 eluted from a cobalt-affinity column. Samples were subjected to SDS-PAGE and stained using Coomassie blue. The arrow indicates the position of recombinant RBP2 (90 kDa).

(B) Size-exclusion chromatography of recombinant RBP2. Fraction F2 from cobalt-affinity chromatography was further purified by size-exclusion chromatography to obtain highly pure recombinant RBP2. Fractions from size-exclusion chromatography of recombinant RBP2 were incubated with histones for 30 min at 37°C, and demethylation activity was assayed by immunoblotting using an antibody specific for H3K4me2. A Ponceau stain of the input histones analyzed by immunoblotting is shown below the immunoblot. The lower panel of the chromatogram shows SDS-PAGE analysis stained by Coomassie blue of the RBP2 eluted from the size-exclusion column.

(C) Demethylation assay of histones incubated with 0.03, 0.06. 0.125, 0.25, 0.5, 1, 2, and 4 μg of recombinant RBP2; top and bottom panels show Ponceau stain of the RBP2 and histones present in the assays, respectively. The middle panels show the reactions probed with the indicated antibodies and assayed by immunoblotting.

(D) H3K4me3/me2/me1 peptides (2.5 μg) were incubated with or without recombinant RBP2 (4 μg) and analyzed by mass spectrometry. The masses of tri-, di-, and monomethylated H3K4 peptides were 2765.3, 2864, and 2737.2 Da, respectively. A shift in mass equivalent to one methyl group is indicated as "Me."

undeveloped vulvas or being multivulva (12.5%, n > 500) (Figure 6F). In contrast, although *rbr-2*(*RNAi*) treatment resulted in approximately 90% knockdown of *rbr-2* transcripts in the F1 generation (Figure 6D) and apparently increased global levels of H3K4me3, less than 2% of treated animals displayed a multivulva phenotype (data not shown), suggesting insufficient knockdown of *rbr-2*. Nevertheless, the appearance of a low-penetrant vulva phenotype in *rbr-2* RNAi-treated animals and the phenotype of the *rbr-2*(*tm1231*) mutant indicates a role of *rbr-2* in vulva development.

A group of genes collectively termed synthetic multivulva (*SynMuv*) genes act redundantly to negatively control cell-fate specification of vulva-precursor cells

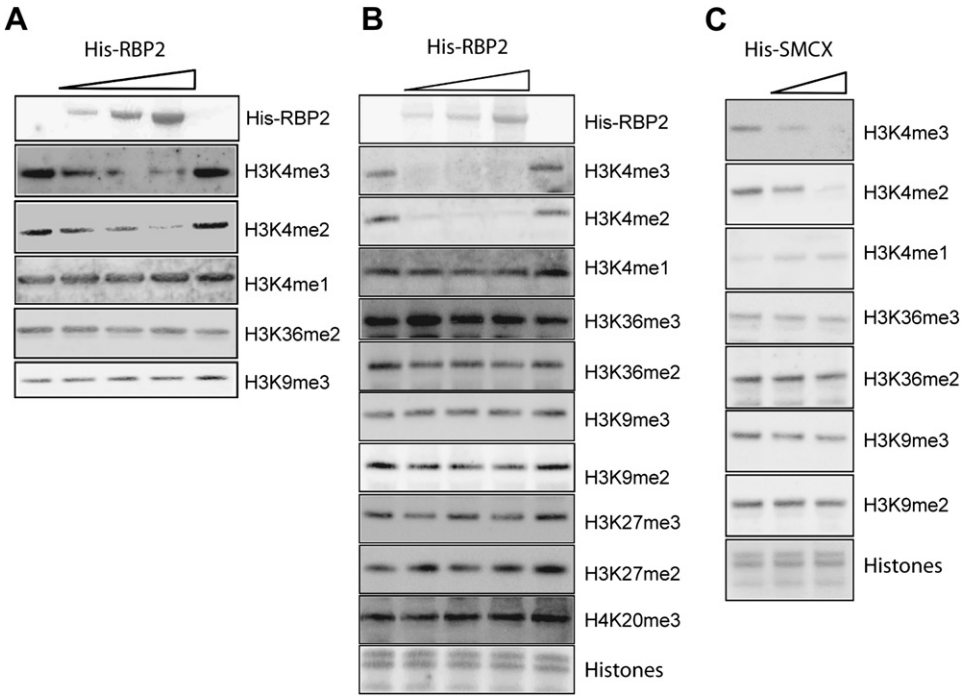


Figure 4. RBP2 Histone Demethylation Is Specific for H3K4me3 and H3K4me2

(A) Demethylation assay of nucleosomes isolated from HeLa cells incubated with 2, 4, and 8 μg of recombinant His-RBP2; top panel shows Ponceau stain of the His-RBP2. The lower panels show the reactions probed with the indicated antibodies and assayed by immunoblotting.

(B) Demethylation assay of histones incubated with 2, 4, and 8 μg of His-RBP2. Top and bottom panels show Ponceau stain of the His-RBP2 and histones present in the assays, respectively. The middle panels show the reactions probed with the indicated antibodies and assayed by immunoblotting.

(C) Demethylation assay for histones incubated with 2 and 4 μg of recombinant His-SMCX. The reaction mixture was separated by SDS-PAGE and assayed for demethylation of the histones with the indicated antibodies by immunoblotting.

(VPCs). Importantly, a large fraction of *SynMuv* genes encodes transcription factors including those homologous to the mammalian pRB/E2F protein complex (Fay and Han, 2000). Given that RBP2 can bind to pRB, we speculated that RBR-2 might be involved in transcriptional repression of genes acting in vulva development. Therefore we tested the effect of *rbr-2* knockdown in different classes of *SynMuv*. We used *lin-15*(*n767*) and *lin-38*(*n751*) as representatives of class A mutations and *lin-35*(*n745*), *lin-15*(*n744*), and *efl-1*(*se1*) as representatives of class B mutations. We also performed *rbr-2*(*RNAi*) in the *lin-15*(*n765ts*) genetic background that carries mutations in both class A and class B genes, but showing the synthetic multivulva phenotype only at the restrictive temperature (above 20°C) (Clark et al., 1994). Strikingly, *rbr-2*(*RNAi*) interference of *lin-15*(*n765ts*) animals gave rise to a multivulva phenotype (40%, n ≥ 800 in three independent experiments) at the permissive temperature, suggesting that *rbr-2* negatively regulates vulva induction (Figures 6G and 6H). Consistently, the multivulva phenotype was also observed in the double-mutant *lin-15*(*n765ts*);*rbr-2*(*tm1231*) at permissive temperature (Figure 6H). In contrast, *rbr-2*(*RNAi*) did not exert apparent effects on vulva phenotypes in class A or class B genetic backgrounds, and *rbr-2* therefore does not function as a class A or class B *SynMuv* gene (data not shown). In the *lin15*(*n765ts*) genetic background, *rbr-2*(*RNAi*) also caused an increase in H3K4me3 levels (Figure 6I).

The requirement for a sensitized genetic background for the efficient induction of a multivulva phenotype is most probably reflected by insufficient knockdown of *rbr-2*. However, the difference in the severity of phenotypes observed between *rbr-2*(*tm1231*) mutant animals and *rbr-2*(*RNAi*) might be suggestive of a dominant-negative function of the RBR-2 mutant protein. Taken together, the results support the importance of H3K4 demethylation by RBR-2 in the regulation of specific genes during development and differentiation.

DISCUSSION

Using a combination of in vivo and in vitro assays, we have demonstrated that the JARID1 proteins, RBP2, PLU1, and SMCX, are histone demethylases specific for tri- and dimethylated H3K4. The enzymatic activity of the JARID1 family and the fact that RBP2 binding to target genes correlates with decreased levels of associated H3K4me3 and mRNA levels is consistent with the fact that the

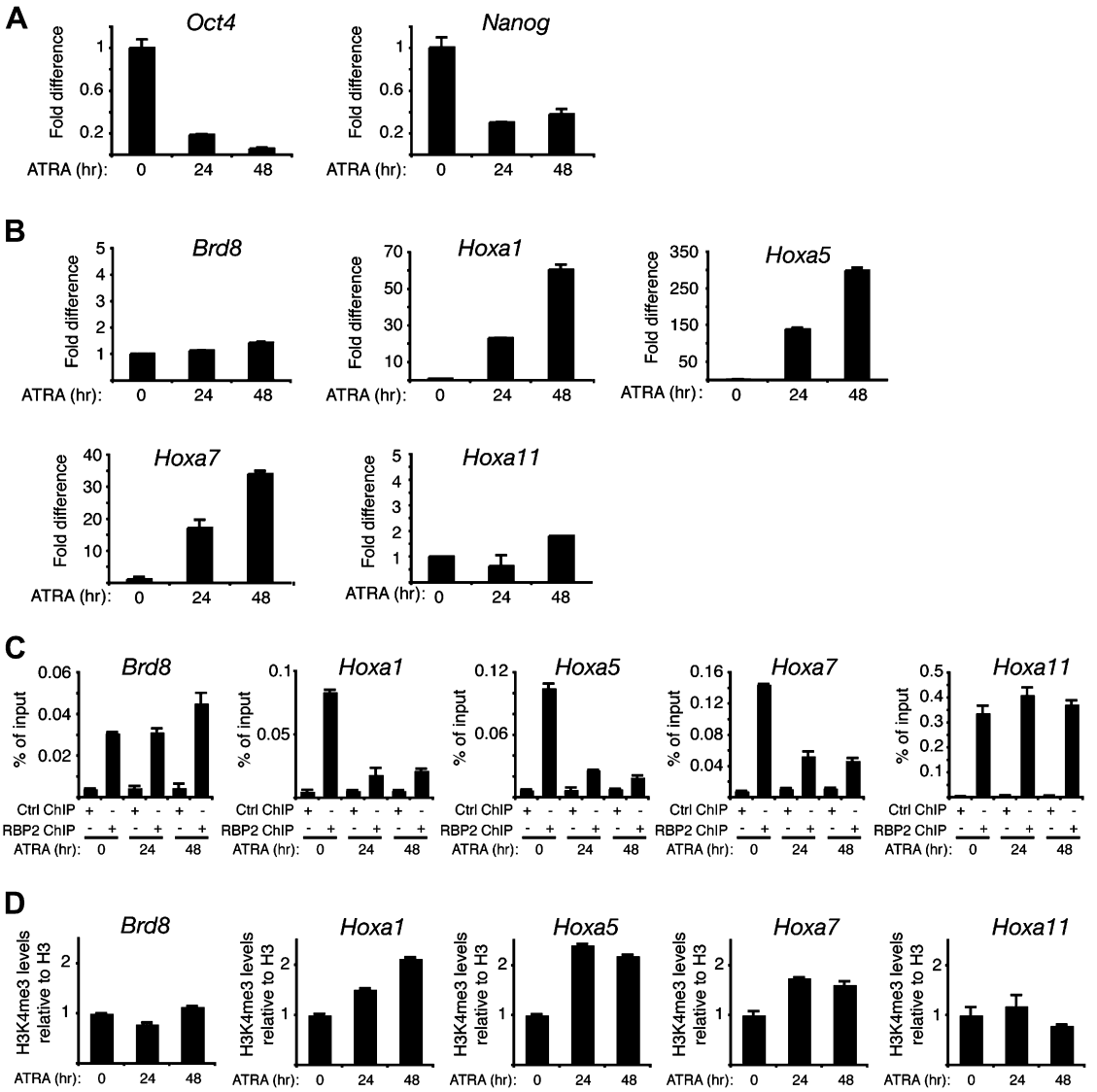


Figure 5. *Hox* Genes Are Targets of RBP2

(A) Expression levels of the ES cells specific markers *Oct4* and *Nanog* in growing or differentiating ES cells treated with ATRA (1 μM) for the indicated times.

(B) Expression levels of *Brd8, Hoxa1, Hoxa5, Hoxa7*, and *Hoxa11* during the differentiation of the ES cells presented in (A).

(C) ChIP analysis using RBP2-specific antibodies and an antibody to HA as a negative control. The same samples as used in (A) and (B) were used for these experiments.

(D) ChIP analysis using H3K4me3-specific antibodies and an antibody to HA as a negative control. The results are presented as "% bound" and normalized to the levels of histone H3 bound. The same samples as used in the other experiments presented in this figure were used for these experiments. The error bars represent the standard deviation of the mean.

JARID1 proteins primarily function as transcriptional repressors. Moreover, our results on the only JARID1 homolog in *C. elegans*, RBR-2, show that the protein is required for the regulation of H3K4 trimethylation levels during larval development and for vulva formation. This suggests an essential role for the JARID1 family as chromatin modifiers regulating H3K4me3 levels in eukaryotic development and differentiation.

The Specificity of the JARID1 Family in H3K4me3/me2 Demethylation

LSD1, a member of the amine oxidase family, catalyzes the demethylation of mono- and dimethyl H3K4 but lacks the ability to demethylate trimethylated substrates (Shi et al., 2004). In contrast, the JmjC family of proteins has the capacity of demethylating tri-, di-, and monomethylated substrates using an oxidative hydroxylation reaction

mechanism (Cloos et al., 2006; Klose et al., 2006b; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Previously, three families of JmjC demethylases have been identified: (1) the FBXL10/FBXL10 family having specificity for H3K36me2/me1, (2) the JMJD1 family including JMJD1A, JMJD1B, and TRIP8 with specificity to H3K9me2/me1, and (3) the JMJD2 family, which shows dual-substrate specificity demethylating both H3K9me3/me2 and H3K36me3/me2 (Cloos et al., 2006; Klose et al., 2006b; Whetstine et al., 2006). In this report, we demonstrate that RBP2 and SMCX catalyze the reversal of H3K4me3/me2 with high specificity in vitro. Furthermore, the members of the JARID1 family show similar specificity for H3K4 demethylation in vivo. We also observed that ectopic expression of the JARID1 family could reduce H3K36 methylation. Since H3K36 methylation is linked to active transcription, the reduction of methylation could be a consequence of inhibition of transcription caused by H3K4 demethylation. In agreement with this, overexpression of RBP2 in cell lines having an integrated luciferase reporter caused a general reduction of transcription (data not shown).

Apparently, the demethylation activity for the JARID1 family overlaps with the specificity observed for LSD1 and may predict redundant or cooperative functions of LSD1 and the JARID1 proteins. Interestingly, the facts that SMCX mutations are found in patients with mental retardation (Jensen et al., 2005) and that the Co-REST repressor complex, which contains LSD1, plays a role in neurogenesis (Shi et al., 2005) may suggest that the two demethylases cooperate as part of the same complex (Co-REST).

The JARID1 Protein Family

The best-described members of the JARID proteins are RBP2 and PLU1. PLU1 expression is progressively decreased in advanced and metastatic melanomas. Likewise melanoma cell lines in general exhibit low expression levels of RBP2 suggesting a possible role in tumorigenesis (Roesch et al., 2005, 2006; Vogt et al., 1999). To date no cellular function has been ascribed for SMCX and SMCY; however, studies have shown that mutations in SMCX are frequently found in patients with X-linked mental retardation, suggesting a role for these proteins in development (Jensen et al., 2005).

RBP2 was originally identified by virtue of its ability to bind the retinoblastoma protein (pRB) (Defeo-Jones et al., 1991; Fattaey et al., 1993). RBP2 has been suggested to function as a transcriptional repressor and to play a role in differentiation. Consistent with this, interference of *RBP2* expression using siRNA results in the transcriptional upregulation of two homeotic genes *BRD2* and *BRD8* in U937 cells (Benevolenskaya et al., 2005). However, in the presence of pRB, RBP2 appears to function as a coactivator of transcription, probably by either modulating or dissociating RBP2 from the target-gene promoter (Benevolenskaya et al., 2005; Chan and Hong, 2001; Mao et al., 1997). Our demonstration that RBP2 is an H3K4me3 demethylase taken together with the fact that it is dissociated from genes activated during differentiation strongly suggests that RBP2 is a transcriptional repressor. This suggestion is further supported by the observation that another JARID1 family member, PLU1, works as transcriptional repressor when fused to the DNA binding domain of GAL4 (Tan et al., 2003).

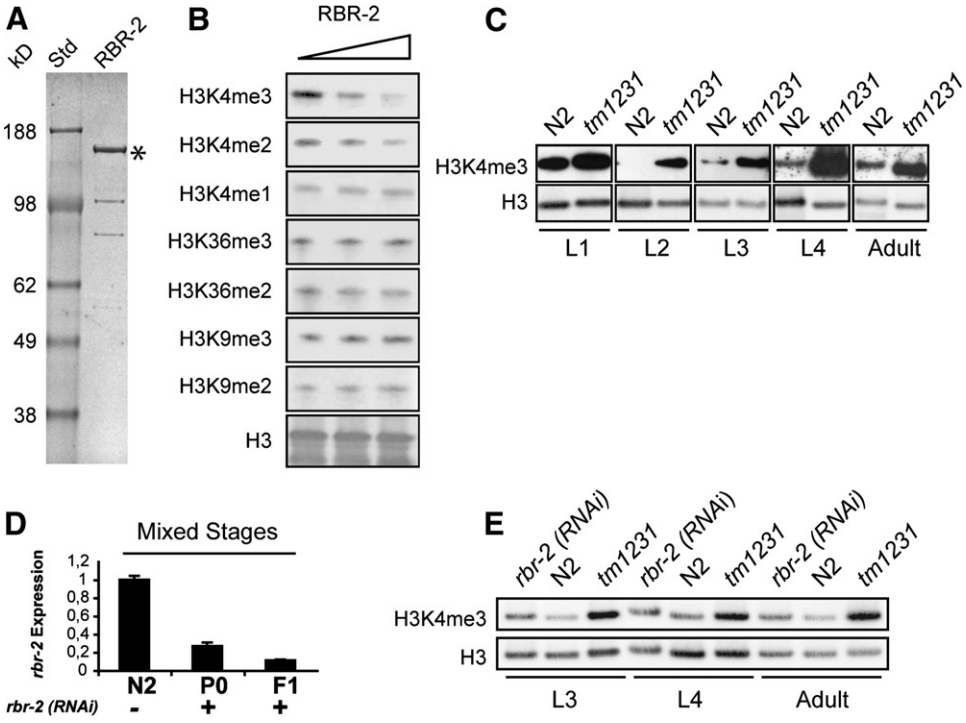
The Function of JARID1 Family Homologs in *S. pombe*, *D. melanogaster*, and *C. elegans*

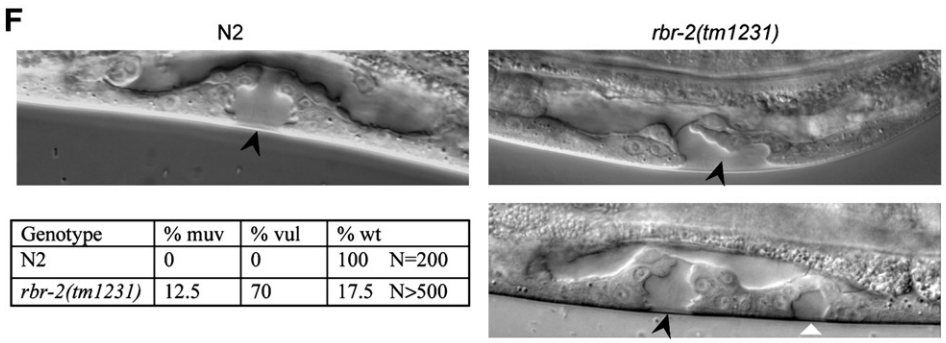
Interestingly, the *S. pombe* RBP2 homolog, Lid2, copurifies with Ash2 and Sdc1 the yeast orthologs of human ASH2L and DPY30 (Roguev et al., 2003). Ash2 and Sdc1 are found in the yeast COMPASS H3K4 methyltransferase complex, thus providing the possibility that histone methyltransferases and demethylases with specificity for the same histone mark have common complex partners (Steward et al., 2006). *Drosophila lid* was initially identified in a genetic screen for identification of new members of the trithorax group. Interestingly, the screen was performed in a genetic background having a Trithorax gene mutation in *Ash1* (Gildea et al., 2000), a histone methyltransferase with specificity for H3K4. Of further interest, Ash1 has been shown to be necessary for *Hox* gene expression (Klymenko and Muller, 2004). Taken together with the fact that Trithorax is required for the maintenance of *Hox* expression, these data suggest a role for RBP2 in the regulation of *Hox* genes. In agreement with this, we found that RBP2 is specifically associated with several genes of the *Hoxa* cluster in murine ES cells.

In this study, we took advantage of the fact that *C. elegans* only encodes a single member of the JARID1 family. Interestingly, our results also point toward an important role for the *C. elegans* RBP2 homolog, *rbr-2*, in development and differentiation. The demonstration that deletion of the JmjC domain of RBR-2 or RNAi-mediated knockdown of *rbr-2* results in a significant increase of H3K4me3 levels strongly suggests that RBR-2 is an important regulator of H3K4 trimethylation in vivo. Moreover, the fact that the functional inactivation of *rbr-2* results in specific defects in vulva development suggests that the regulation of H3K4me3 levels is essential for normal differentiation. The stronger vulva phenotype observed in the *rbr-2*(*tm1231*) mutant may suggest that the residual protein levels in the RNAi-treated worms are sufficient for some stages of vulva development. In support of this suggestion, the increase in H3K4me3 levels in the RNAi-treated worms was not as pronounced as in the mutant animal.

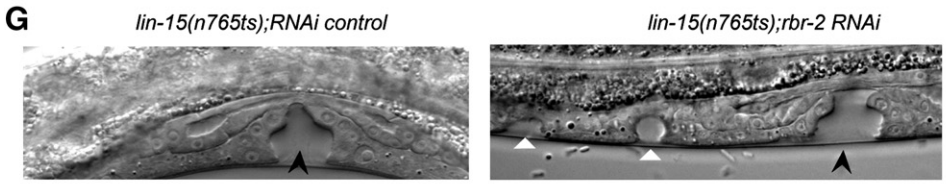
H3K4me3 Turnover in Mammalian Organisms

Modulation of H3K4 methylation has also been observed in other biological systems. One example is the circadian variation of the transcription of the albumin D-element binding protein gene (*Dbp*) in the mouse liver (Ripperger and Schibler, 2006). Here, during transcriptional repressive phases, H3K4 trimethylation at the *Dbp* gene is markedly reduced in the matter of hours. X-chromosome





Genotype	% muv	% vul	% wt
N2	0	0	100 N=200
rbr-2(tm1231)	12.5	70	17.5 N>500



H

Genotype	%muv at 15°C	
N2	0	N=200
rbr-2(tm1231)	12.5	N>500
lin-15(n765ts)	4	N=154
lin-15(n765ts) RNAi control	2	N>500
lin-15(n765ts); rbr-2(RNAi)	40	N>800
lin-15(n765ts); rbr-2(tm1231)	32	N=200

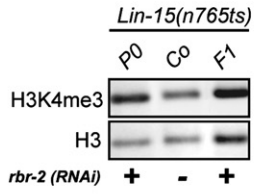


Figure 6. Mutation or Knockdown of the *RBP2* Homolog *rbr-2* in C. *elegans* Increases Global Levels of H3K4 Trimethylation and Results in Aberrant Vulval Development

(A) His-tagged recombinant RBR-2 purified from insect cells was separated by SDS-PAGE and stained by Coomassie blue. An asterisk indicates RBR-2. A molecular weight standard (Std) was included.

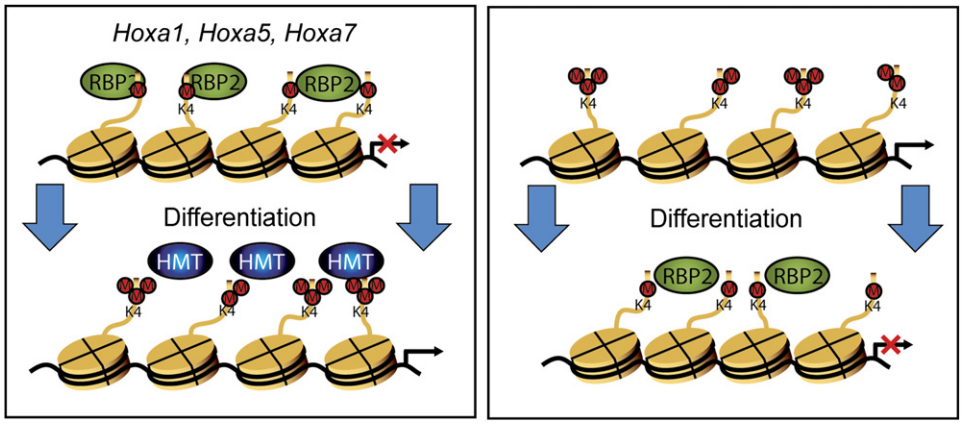


Figure 7. Putative Model for RBP2 Modulation of Chromatin and Transcription in Cellular Differentiation

inactivation (XCI) represents another example of a biological process featuring a rapid loss of H3K4 trimethylation. XCI occurs in the early embryonic development of female mammals, where epigenetic silencing of one X chromosome takes place to attain dosage parity between XX females and XY males. One of the earliest and most characteristic epigenetic features of XIC is loss of tri- and dimethylation of H3K4, concomitant with transcriptional silencing of the affected X-linked genes (Heard et al., 2004). The mechanism causing the loss of the activating H3K4me3 mark is presently unknown but has been speculated to involve demethylating enzymes (Heard et al., 2004).

In mice, only a very limited number of genes have been reported to evade XIC (http://www.informatics.jax.org/). Strikingly, two of the seven genes known to escape silencing are JmjC-domain proteins: the ubiquitously transcribed tetratricopeptide repeat gene on X chromosome (*Utx*) and *Smcx* (Agulnik et al., 1994; Greenfield et al., 1998). The human orthologs of these two genes likewise evade epigenetic silencing, and given the putative demethylating activity of these proteins it is tempting to speculate on their involvement in establishing or maintaining XIC, perhaps through removal of the activating H3K4me3/me2 marks.

Model for the Role of RBP2 in Transcriptional Regulation

In Figure 7 we propose a model for how RBP2 could be involved in regulating the transcription of genes during cellular differentiation. We suggest that RBP2 is implicated in this modulation in two ways, either (1) by being released from genes that have been kept silenced, such as the *Hox* genes, which require activation during differentiation, or (2) by being recruited to genes that require silencing after cellular differentiation. Such genes may also include silent developmental genes carrying a bivalent H3K4me3/H3K27me3 epigenetic signature and that are "poised for transcription" (Bernstein et al., 2006). In this way, RBP2 may contribute to transcriptional silencing by keeping the levels of H3K4me3 low. Thus, RBP2 could constitute one of several mechanisms by which chromatin-modifying enzymes, including histone acetylase/deacetylase complexes and the Polycomb group proteins, orchestrate the fine-tuned epigenetic regulation of genes during cellular differentiation.

An open question is how RBP2 and other JARID1 proteins are recruited to certain chromatin areas. For PLU1 two interacting proteins, brain factor 1 (BF1) and paired box 9 (PAX9), both of which are developmental transcription factors, have been identified (Tan et al., 2003). BF1

(B) Demethylation assay of histones incubated with purified recombinant RBR-2. Reactions were probed with the indicated antibodies and assayed by immunoblotting.
(C) Levels of H3K4me3 in *C.elegans* larval stages and adults. Synchronized WT and mutant animals were collected and processed for immunoblotting at different stages of development using an antibody specific for H3K4me3. H3 staining was used to quantify the loading.
(D) Expression levels of *rbr-2* mRNA, quantified by real-time RT-PCR in WT animals (N2) fed with bacteria carrying an empty vector (–) or a portion of *rbr-2* gene (+). Both P0 and F1 were tested for the efficiency of RNA interference. The error bars represent the standard deviation of the mean.
(E) Levels of H3K4me3 in *C. elegans* late larval stages (L3 and L4) and adults. Synchronized WT (N2), *rbr-2*(*tm1231*) mutants, and N2 interfered for *rbr-2*(*RNAi*) were collected and processed as described in (C).
(F) DIC microscopic images of developing vulva in WT (N2) (top left image) and *rbr-2*(*tm1231*) mutant (right images). Black arrowheads indicate the vulval invagination (at the "Christmas tree" (L4) stage) and white arrowheads indicate extrainvagination. In *rbr-2* mutants both defective vulva opening and pseudovulval invagination are observed. A table providing an overview of phenotypes is given.
(G) *rbr-2*(*RNAi*) in *lin-15*(*n765ts*) induces multivulva phenotype at permissive temperature (15°C). *lin-15*(*n765ts*) interfered with control bacteria (control RNA) (left) and *rbr-2*(*RNAi*) (right). Black arrowheads indicate the vulval invagination and white arrowheads indicate extrainvaginations.
(H) Table providing percentage of animals with a multivulva phenotype in the different genotypes.
(I) H3K4me3 levels in *lin-15(n765ts)* L3 larvae fed at 15°C with bacteria expressing *rbr-2*(*RNAi*) (+) or control (–) and assessed by immunoblotting. Both P0 and F1 were analyzed. H3 staining was used as loading control.

and PAX proteins interact with members of the Groucho corepressor family, suggesting that PLU1 has a role in Groucho-mediated transcriptional repression (Tan et al., 2003). Thus, most likely, JARID1 proteins take part of specific chromatin-remodeling complexes homing to certain genomic areas. Alternatively, JARID1 proteins may bind directly to chromatin; in this context, it is pertinent to note that all JARID1 proteins contain an ARID/BRIGHT domain, which can bind to DNA. In addition, the JARID proteins feature PHD and C5HC2 zinc fingers, which may mediate interactions with specific histone marks. Further studies are required to unravel the link between JARID1 proteins and transcriptional regulation of developmental genes. It would also be of great interest to study the role of these proteins in stem cell differentiation, where epigenetic changes are important players.

EXPERIMENTAL PROCEDURES

Cloning Procedures

The open-reading frame (ORF) of human RBP2 and a RBP2 deletion mutant (nucleotides 150–2543) was generated by PCR amplification using pcDNA3-HA-RBP2 (a generous gift from W.G Kaelin) as a template. SMCX was PCR amplified from a human fetal brain cDNA library (Invitrogen). The amplified fragments were cloned into the pCR8/GW gateway entry vector Invitrogen (Carlsbad, CA), and the DNA sequences were verified by sequencing. To generate expression vectors, the appropriate entry clones were transferred into gateway-compatible pCMV-HA, pBabepuro, and Baculovirus virus transfer vector, pACHlt-A. A CMV-driven expression vector encoding Myc-tagged PLU1 was a generous gift from J. Taylor-Papadimitriou.

Recombinant RBP2, SMCX, and RBR-2

Recombinant baculoviruses were generated by cotransfection of baculovirus transfer vector containing either a deletion mutant encoding N-terminally hexahistidine-tagged human RBP2 (amino acids 1–797) or full-length SMCX or RBR-2 and Bsu36I linearized Bakpak6 baculovirus DNA· Histidine-tagged proteins were expressed in *Trichoplusia ni*, High Five, and purified by Cobalt-Sepharose. Selected fractions were further purified by size-exclusion chromatography as described previously (Cloos et al., 2006).

Generation of Antibodies to RBP2

Polyclonal antibodies were generated by immunizing rabbits with affinity-purified RBP2 (amino acids 1–797). The antibodies were affinity purified using an affigel-resin (Biorad) coupled with RBP2. Antibody specificity was confirmed by immunoblotting and immunoprecipitation (data not shown).

Demethylation Assay

Bulk histones, synthetic histone peptides, or nucleosomes were incubated with purified His-RBP2, His-SMCX, or His-RBR-2 for 30 min at 37°C in demethylation buffer (50 mM Hepes-KOH, pH 7.7, 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-ketoglutarate, 40 μM $FeSO_4$, 2 mM ascorbic acid). $MgCl_2$ was omitted in the nucleosome assays). Reaction mixtures were analyzed by either western blotting using specific antibodies or mass spectrometry. Antibodies used in the study were as follows: anti-H3K9me3 (Upstate 07-523), anti-H3K9me2 (Upstate 07-212), anti-H3K27me3 (Upstate 07-449), anti-H3K27me2 (Abcam 24684), anti-H4K20me3 (Upstate 07-463), anti-H3K4me3, (Abcam ab8580), anti-H3K4me2 (Upstate 07-030), anti-H3K4me1 (Upstate 07-436), anti-H3K36me3 (Abcam ab9050), anti-H3K36me2 (Upstate 07-369), anti-H3 (Abcam Ab1791-100), anti-His (Upstate 05-531), and anti-HA (CRP Inc. AFC-101P).

Mass Spectrometry Analysis

Four micrograms of recombinant RBP2, was incubated with 2.5 μg of either H3K4me3, H3K4me2, or H3K4me1 peptide (Upstate 12-564, 12-460, and 12-563) in demethylation buffer in a final volume of 90 μl for 30 min at 37°C. Reaction mixtures were brought to 4 M Urea, left for 30 min at room temperature, diluted ten-fold using 0.1% trifluoroacetic acetic (TFA), and desalted on a C8-StageTip (Cloos et al., 2006). Peptides were eluted in 10 μl (60% methanol, 0.1% TFA) and analyzed by nanoelectrospray on a LTQ-Orbitrap mass spectrometer (ThermoElectron, Bremen, Germany).

Expression Analysis and ChIP Assays

Expression analysis and ChIP assays were performed and analyzed as previously described (Bracken et al., 2003). In addition to the generated RBP2 antibody, rabbit anti-HA (Santa Cruz), rabbit anti-H3K4me3 (Abcam), rabbit anti-H3K4me2 (Upstate), and rabbit anti-H3 (Abcam) were used for ChIP.

C. elegans Methods

Maintenance, culturing, and genetic manipulations of *C. elegans* strains were carried out according to standard procedures (Brenner, 1974) and conducted at 15°C.

Supplemental Data

Supplemental Data include three figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/128/6/1063/DC1/.

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