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related wasp *P. metricus*; this is an early example of the utility of 454 sequencing for transcriptomics (36). Our results demonstrate that it is possible to use species that have had their genomes sequenced as “hubs” to efficiently generate genomic resources for clusters of related species that might each be especially well suited to address particular evolutionary problems. This “hub and spokes” approach should enable genomics to be deployed for a broader range of species than is currently being done, until whole-genome sequencing of eukaryote genomes becomes routine.

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#### Supporting Online Material

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## JMJD6 Is a Histone Arginine Demethylase

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Arginine methylation occurs on a number of proteins involved in a variety of cellular functions. Histone tails are known to be mono- and dimethylated on multiple arginine residues where they influence chromatin remodeling and gene expression. To date, no enzyme has been shown to reverse these regulatory modifications. We demonstrate that the Jumonji domain-containing 6 protein (JMJD6) is a JmjC-containing iron- and 2-oxoglutarate-dependent dioxygenase that demethylates histone H3 at arginine 2 (H3R2) and histone H4 at arginine 3 (H4R3) in both biochemical and cell-based assays. These findings may help explain the many developmental defects observed in the JMJD6<sup>-/-</sup> knockout mice.

Iron- and 2-oxoglutarate-dependent dioxygenases have been shown to oxidize a variety of substrates including metabolites, nucleic acids, and proteins (1). A candidate dioxygenase, JMJD6, shares extensive sequence and predicted structural homology with an asparaginyl hydrox-

ylase (2, 3) as well as the JmjC domains found in several histone lysine demethylases (fig. S1A) (4–8). Given the predicted conservation of structural elements and key residues (9–11), it is likely that JMJD6 retains an analogous catalytic activity. Here we report in vitro and in vivo data that clearly indicate that JMJD6 functions as an arginine demethylase.

To test whether JMJD6 demethylates the N-terminal tails of histone H3 or H4, we incubated bulk histones with JMJD6 in the presence of Fe(II), 2-oxoglutarate, and ascorbate (12). An-

tibodies specific for various methylated sites on histones H3 and H4 were used to assess demethylation. Although no lysine demethylation was observed, a substantial reduction in H3R2me2 and H4R3me2 was observed in the presence of JMJD6 compared with buffer alone (Fig. 1A). These effects were site-specific as no changes in dimethylarginine were seen at positions H3R17 or H3R26. Previously, no enzyme had been shown to reverse regulatory arginine methylation, although deiminases can convert methylarginine to citrulline via demethylimination (13, 14). However, the requisite chemistry is analogous to that demonstrated for demethylation of alkylated nitrogens by other dioxygenases (fig. S1C).

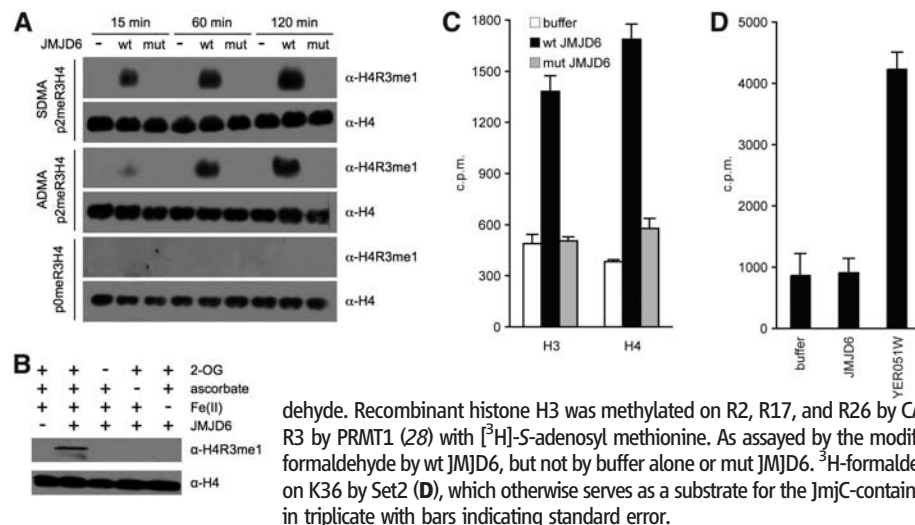
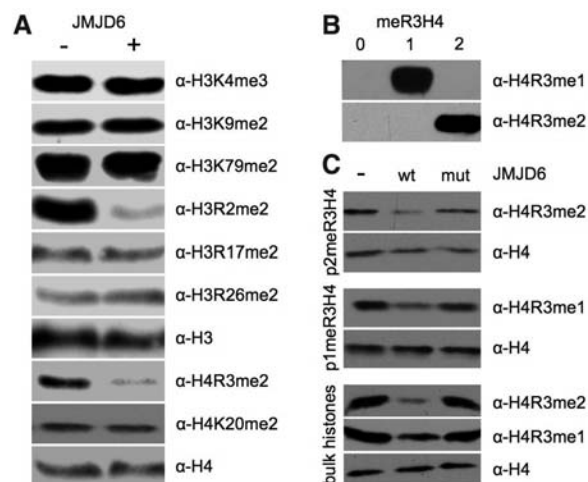
To investigate the preference for the substrate methylation state, we used antibodies specific for either mono- or dimethylated (symmetric) H4R3 (Fig. 1B). The recombinant JMJD6 was able to demethylate H4R3me2 when either heterogeneous bulk histones or synthetic peptides encompassing the N-terminal 30 residues of histone H4 were used as substrates (Fig. 1C). To a lesser extent, JMJD6 could also demethylate H4R3me1-containing substrates (Fig. 1C). Mutation of the residues predicted to mediate Fe(II) binding (mut JMJD6) prevented demethylation (Fig. 1C).

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To confirm that JMJD6 promotes oxidative demethylation of arginine residues, we examined product formation. Appearance of the H3R4me1 product from the dimethylated peptide substrate (p2meR3H4) was observed in the presence of wild-type (wt) recombinant JMJD6, but not the inactive (mut) variant (Fig. 2A). Product formation was dependent on the presence of Fe(II), 2-oxoglutarate, and ascorbate (Fig. 2B), and activity was subject to saturation by substrate (fig. S2C). Generation of a second product, formaldehyde, was measured with recombinant histone H3 and H4 proteins radiolabeled with  $^3\text{H}[\text{CH}_3]$ . Upon demethylation, the released  $^3\text{H}$ -formaldehyde was converted to radiolabeled 3,5-diacetyl-1,4-dihydrolutidine and quantitated. Again, only wt JMJD6 could liberate  $^3\text{H}$ -formaldehyde from histones H3 and H4 methylated on arginine (Fig. 2C), but mut JMJD6 could not liberate  $^3\text{H}$ -formaldehyde from H3 methylated on K36 (Fig. 2D).

**Fig. 1. JMJD6 is a putative Fe(II)- and 2-oxoglutarate-dependent dioxygenase. (A)** JMJD6 demethylates H3R2me2 and H4R3me2. Bulk histones were incubated in the presence (+) or absence (-) of purified recombinant JMJD6. Antibodies (Abcam) specific for the indicated histone methylation sites were used to detect loss of these modifications by Western blot analysis. Recognition of the indicated methylated sites was confirmed with blocking peptides (fig. S2A). Total amounts of histone H3 and H4 are shown as loading controls. Similar results were obtained with antibodies from another manufacturer (fig. S2B). **(B)** The  $\alpha$ -H4R3me1 and  $\alpha$ -H4R3me2 antibodies specifically recognize synthetic peptide substrates, where R3 contains one (1) or two (2) methyl groups, respectively. **(C)** JMJD6 demethylates both mono- and dimethylarginine residues. Bulk histones or histone H4 peptides were synthesized with symmetric dimethylarginine (p2meR3H4) or monomethylarginine (p1meR3H4) and incubated in the absence (-) or presence of wt JMJD6 protein or a catalytically inactive (mut) JMJD6 (H187A; D189A; H273A). Demethylation was assessed by Western blot analysis.

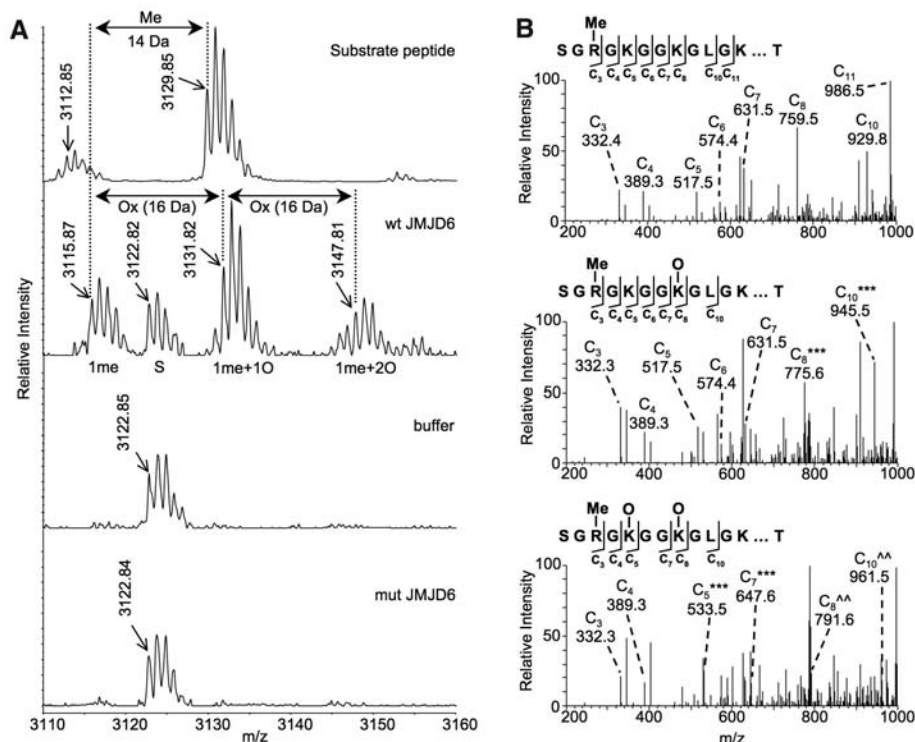


fully demethylated peptide product could not be ascertained under these assay conditions because it was first necessary to enrich the reaction products by immunoprecipitation with the  $\alpha$ -H4R3me1 antibody.

In addition to the expected demethylated (H4R3me1) product, two additional products (+16 and +32 daltons) were observed that likely represent additional oxidation of the monomethylated product. To further validate the demethylation product and identify additional modification sites, we analyzed the electron transfer dissociation (ETD) MS/MS spectra of the products (Fig. 3B, top panel). Although all of the C ions indicated the loss of a methyl group (-14 daltons) upon incubation with JMJD6, the data also revealed that the 3131.82-dalton peak contains a mono-oxidation product of p1meR3H4 with modification preferentially occurring at lysine 8 (Fig. 3B, middle panel). The 3147.81-dalton product was mainly oxidized on lysine 8 and lysine 5 (Fig. 3B; bottom panel). Such oxidation in vitro was also observed with the histone H3 peptide substrate (figs. S3 and S5). Although the physiological significance of the observed lysine oxidation is unknown, it is nevertheless clear that JMJD6 can effect arginine demethylation in vitro.

Unlike previously characterized JmjC-containing lysine demethylases, the JMJD6 catalytic domain is not accompanied by recognizable domains that help target these enzymes to histones (15), perhaps contributing to the low activity of our recombinant protein in vitro. It is possible that JMJD6 is recruited to specific chromatin sites through interactions with other proteins. To determine whether JMJD6 could efficiently promote arginine demethylation in the context of living cells, we transfected V5-tagged expression constructs encoding either wt JMJD6 or an inactive variant that lacks one of the Fe(II) ligands (H187A; mut). Indirect immunofluorescence staining with antibodies recognizing the V5 tag or various histone arginine methylation sites

**Fig. 2. JMJD6 is a Fe(II)- and 2-oxoglutarate-dependent dioxygenase. (A)** Formation of the H4R3me1 product from symmetric (SDMA) or asymmetric (ADMA) dimethylarginine containing p2meR3H4 substrates. Peptide substrates derived from histone H4 containing two or zero methyl groups on R3 were incubated in the absence (-) or presence (+) of wt JMJD6 protein or a catalytically dead (mut) JMJD6 (H187A; D189A; H273A). The  $\alpha$ -H4R3me1 antibody was used to detect formation of this product by Western blot analysis. **(B)** Formation of the demethylated H4R3me1 product from the p2meR3H4 substrate by JMJD6 requires Fe(II), ascorbate, and 2-oxoglutarate (2-OG). **(C)** JMJD6-mediated arginine demethylation generates formaldehyde. Recombinant histone H3 was methylated on R2, R17, and R26 by CARM1 (28), and recombinant histone H4 was methylated on R3 by PRMT1 (28) with  $^3\text{H}$ -S-adenosyl methionine. As assayed by the modified Nash method (29),  $^3\text{H}$ -methyl groups were liberated as formaldehyde by wt JMJD6, but not by buffer alone or mut JMJD6.  $^3\text{H}$ -formaldehyde is not produced by JMJD6 from histone H3 methylated on K36 by Set2 (D), which otherwise serves as a substrate for the JmjC-containing lysine demethylase Yer051w (4). Assays were performed in triplicate with bars indicating standard error.

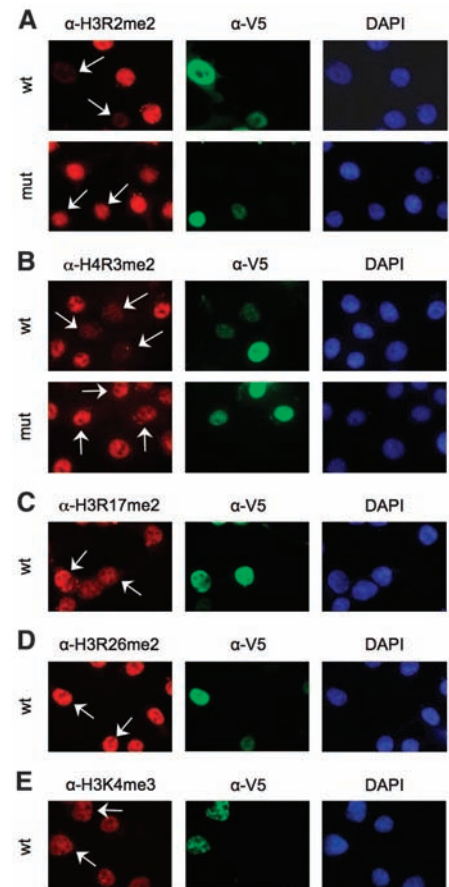


**Fig. 3.** Analysis of demethylation products by mass spectrometry. **(A)** JMJD6 demethylates p2meR3H4 to the monomethylated product (1me). The p2meR3H4 peptide substrate [expected protonated molecular mass ( $M + 1$ ) = 3129.6 daltons] was incubated with buffer alone or JMJD6 (wt or mut). Products were immunoprecipitated with the  $\alpha$ -H4R3me1 antibody before analysis. A p1meR3H4 peptide synthesized with a stable isotope of leucine (containing six  $^{13}\text{C}$  and one  $^{15}\text{N}$  atoms) was added as an internal standard (S) before immunoprecipitation. The peak with  $m/z$  3112.85 in the top panel is a deaminated impurity that was removed during immunoprecipitation. **(B)** ETD fragmentation of the demethylation products (+5 charged ions) indicates that in addition to demethylation, JMJD6 can promote oxidation of nearby lysine residues [1me + 10 or 1me + 20 in (A)]. The relevant ion fragments are labeled and the corresponding peptide positions are illustrated. Fragments containing a single oxidation modification are denoted by “\*\*\*” and fragments containing two oxidation events are denoted by “^^”. Shown are the partial (for full spectra, see fig. S4) MS/MS spectrum of the monomethyl product (top panel), the monomethyl product oxidized on K8 (middle panel), and the monomethyl product with oxidation of both K5 and K8 (bottom panel).

revealed that cells overexpressing wt JMJD6, but not mut JMJD6, displayed a substantial reduction in global H3R2me2 (Fig. 4A) and H4R3me2 content (Fig. 4B), in line with the loss of staining observed in the presence of blocking peptides (figs. S6A and S6B). Consistent with the site selectivity observed with recombinant JMJD6 in vitro (Fig. 1A), JMJD6 overexpression had almost no effect on the global levels of H3R17me2 (Fig. 4C), H3R26me2 (Fig. 4D), or H3K4me3 (Fig. 4E). Collectively, these in vitro and cellular data both indicate that JMJD6 is a histone arginine demethylase.

JMJD6 was previously identified as the phosphatidylserine receptor responsible for recognizing apoptotic cells (16–20). Subsequent studies have challenged these conclusions (21–23), failing to confirm a role for JMJD6 in apoptotic cell clearance or phagocytosis (23, 24). Instead, the findings presented here support a role for JMJD6 in the nucleus as a histone arginine demethylase. In conjunction with other modifications found on

histone tails, methylarginine contributes to the histone code that mediates chromatin remodeling and gene expression (25). In addition, methylarginine residues have been found on a large number of nonhistone proteins (26), though it remains to be seen whether any are substrates for JMJD6. Data are emerging that arginine methylation plays an important role in cellular differentiation and proliferation during development (25). Knockdown or knockouts of JMJD6 in model organisms were accompanied by numerous developmental defects during embryogenesis (18–20, 23) that may result from inappropriate methylation of histones and other proteins. Furthermore, reports that the Hypoxia-Inducible Factor (HIF) hydroxylases serve as cellular sensors by virtue of their substrate and cofactor requirements (27) raises the possibility that numerous cellular processes mediated by arginine or lysine demethylation could be directly regulated in response to dynamic changes in a cell’s metabolic, environmental, or developmental status.



**Fig. 4.** JMJD6 promotes histone arginine demethylation in cultured HeLa cells. Transient overexpression of V5-tagged wild-type (wt), but not an inactive variant (mut) of JMJD6, reduces global amounts of H3R2me2 (A) and H4R3me2 (B), but not H3R17me2 (C), H3R26me2 (D), or H3K4me3 (E). DAPI (4',6-diamidino-2-phenylindole) staining marks the location of nuclei in the field, and arrows indicate transfected cells.

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# Demethylation of H3K27 Regulates Polycomb Recruitment and H2A Ubiquitination

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Methylation of histone H3 lysine 27 (H3K27) is a posttranslational modification that is highly correlated with genomic silencing. Here we show that human UTX, a member of the Jumonji C family of proteins, is a di- and trimethyl H3K27 demethylase. UTX occupies the promoters of *HOX* gene clusters and regulates their transcriptional output by modulating the recruitment of polycomb repressive complex 1 and the monoubiquitination of histone H2A. Moreover, UTX associates with mixed-lineage leukemia (MLL) 2/3 complexes, and during retinoic acid signaling events, the recruitment of the UTX complex to *HOX* genes results in H3K27 demethylation and a concomitant methylation of H3K4. Our results suggest a concerted mechanism for transcriptional activation in which cycles of H3K4 methylation by MLL2/3 are linked with the demethylation of H3K27 through UTX.

The methylation of lysine residues on histones is often associated with either the activation [methylation of histone H3 lysine 4 (H3K4), H3K36, and H3K79] or repression (methylation of H3K9, H3K27, and H4K20) of transcription (1, 2). Methylation offers an additional level of control by permitting single, double, and triple modification of the same lysine residues (1, 2), resulting in differential regulation of many cellular processes (1–3). The methylation of H3K27 is implicated in X chromosome inactivation, imprinting, stem cell maintenance, circadian rhythms, and cancer (4–6) and is carried out by Enhancer of zeste homolog 2 methyltransferase, a component of the mammalian polycomb repressive complexes (PRCs), including PRC2 (7–9). Tri- and dimethyl H3K27 are enriched on inactive X chromosomes, as well as at promoter regions of inactive genes (2, 10, 11). Here, we describe the characterization of UTX, a Jumonji C (JmjC)-domain-containing protein capable of demethylating tri- and dimethyl H3K27.

We affinity-purified recombinant human UTX using a baculovirus expression system (Fig. 1A and fig. S1, A and B) and assessed its activity in demethylation assays. UTX has a specific demethylation activity toward tri- and dimethyl H3K27 without affecting methylation on H3K4, H3K9, H3K36, H3K79, and H4K20 (Fig. 1B and fig. S1C). Monomethylated H3K27 was not affected to the same extent when we used comparable concentrations of UTX that diminished di- and trimethylated species (Fig. 1B). The demethylation activity of UTX toward di- and trimethyl H3K27 was confirmed by mass spectrometric analysis of methylated H3K27 peptides corresponding to the H3 tail (fig. S2). Point mutations (H1146A and E1148A) in the catalytic JmjC-domain of UTX abrogated enzymatic activity, substantiating its role as an H3K27 demethylase (Fig. 1C and fig. S1B). Ectopic expression of wild-type UTX, but not its catalytic mutant, resulted in a global decrease in di- and trimethyl H3K27 levels (fig. S3A). Although the tetratricopeptide repeats (TPRs) present in UTX were not essential for the demethylation activity of UTX, the enzyme without TPRs displayed reduced activity (fig. S3, B and C). As is characteristic for the JmjC class of enzymes (12–22), demethylation activity by UTX required the addition of Fe(II) and ascorbate (Fig. 1D). However, similar to the case of JARID1d (a trimethyl H3K4 demethylase), we did not find a requirement for

exogenously added  $\alpha$ -ketoglutarate for UTX activity (Fig. 1D).

Recent genome-wide mapping of polycomb target genes revealed that polycomb group proteins and trimethyl H3K27 are enriched in *HOXA-D* loci (23), whose activity is required for embryonic development and when misregulated may lead to carcinogenesis. To assess the demethylation properties of UTX *in vivo*, we analyzed expression levels of *HOXA* and *HOXC* cluster genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR) after the depletion of UTX with small interfering RNAs (siRNAs) (fig. S4, A and B). Immunostaining analysis using UTX antibodies revealed nuclear staining for endogenous UTX (fig. S5, A and B). Quantitative analysis of mRNA levels indicated that a number of genes in both *HOXA* and *HOXC* clusters are repressed after the knockdown of UTX (Fig. 1, E and F). Specifically, *HOXA* 6, 7, and 13 as well as *HOXC* 4, 5, 6, 8, and 12 displayed greater than 50% repression after UTX depletion.

To demonstrate that UTX mediates such transcriptional effects on *HOX* genes directly, we examined UTX occupancy on the promoter as well as the body of the *HOXA13* and *HOXC4* genes with the use of a quantitative chromatin immunoprecipitation (qChIP) assay. We found a nearly 20-fold enrichment of UTX on the *HOXA13* and *HOXC4* promoters, but only two- to five-fold enrichment on the coding region (Fig. 2, A to D). The depletion of UTX (ChIP analysis revealed about a 50% decrease of UTX occupancy at the *HOXA13* and *HOXC4* promoters; Fig. 2, I and J) resulted in increased levels of di- and trimethyl H3K27 at the promoters of *HOXA13* and *HOXC4* but not at the 3' end of these genes, consistent with the role of UTX as a H3K27 demethylase at the promoter of these genes (Fig. 2, E to H). We also examined the recruitment of PRC1 to *HOXA13* and *HOXC4*, which is targeted to methylated H3K27 sites and possess H2A ubiquitin ligase activity (24, 25). The depletion of UTX leads to an increased occupancy of the Ring finger components of the PRC1 complex (Bmi1 and Ring1A proteins) and a concomitant enhancement of monoubiquitinated H2A at *HOXA13* and *HOXC4* (Fig. 2, I and J). In contrast, there was no change in the levels of histone H3 or in other histone modifications examined after UTX knockdown (Fig. 2, I and J). Taken together, these results indicate that the demethylation of

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