A Jumonji (Jarid2) Protein Complex Represses cyclin D1 Expression by Methylation of Histone H3-K9*

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Covalent modifications of histone tails have critical roles in regulating gene expression. Previously, we identified the jumonji (jmj, Jarid2) gene, the jmjC domain, and a Jmj family. Recently, many Jmj family proteins have been shown to be histone demethylases, and jmjC is the catalytic domain. However, Jmj does not have histone demethylase activity because the jmjC domain lacks conserved residues for binding to cofactors. Independently of these studies, we previously showed that Jmj binds to the cyclin D1 promoter and represses the transcription of cyclin D1. Here, we show the mechanisms by which Jmj represses the transcription of cyclin D1. We found that a protein complex of Jmj had histone methyltransferase activity toward histone H3 lysine 9 (H3-K9). We also found that Jmj bound to the H3-K9 methyltransferases G9a and GLP. Expression of Jmj recruited G9a and GLP to the cyclin D1 promoter and increased H3-K9 methylation. Inactivation of both G9a and GLP, but not of only G9a, inhibited the methylation of H3-K9 in the cyclin D1 promoter and repression of cyclin D1 expression by Jmj. These results suggest that Jmj methylates H3-K9 and represses cyclin D1 expression through G9a and GLP, and that Jmj family proteins can regulate gene expression by not only histone demethylation but also other histone modification.

EXPERIMENTAL PROCEDURES

Plasmids and Transient Transfection—The following expression vectors were used: mammalian expression vectors for only FLAG epitope (pFLAG), FLAG-Jmj (pFLAG-Jmj), myc-G9a, FLAG-GLP (16), FLAG-SETDB1 (17) (a kind gift from Dr. Nakao), myc-SET7, myc-Suv39h1 (kind gifts from Dr. Urano), and bacterial expression vectors for glutathione S-transferase (GST)3-histone H3 N-tail mutants (18). In addition, cDNA for only FLAG epitope or FLAG-Jmj was inserted into pCAG-IRE-Puro vector (a kind gift from Dr. Niwa), and

VoC

3 The abbreviations used are: GST, glutathione S-transferase; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ChIP, chromatin immunoprecipitation assay; CBB, Coomassie Brilliant Blue; HMT, histone methyltransferase.

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designated to be pFLAG-IP or pFLAG-Jmj-IP, respectively. These vectors were used for selection of transfectants. Transfection into 293T and NIH3T3 was performed using the TransFast (Promega) and FuGENE HD (Roche Applied Science), respectively. The procedure was performed according to the manufacturer’s instructions.

**Antibodies**—We used antibodies against FLAG (M2, Sigma), T7 (Novagen), Myc (9E10, Santa Cruz Biotechnology) epitopes, G9a (16), GLP (C-7-5, MBL), Jmj (NB100–2214, Novus), SETDB1 (ab12317, abcam), histone H3 (ab1791, abcam), histone H3-K9me1–3 (me1, 07–450; me2, 07–441; me3, 07–442; Upstate), mouse IgG (Sigma), and guinea pig IgG (Santa Cruz Biotechnology) for immunoprecipitation, Western blotting, immunostaining, and ChIP assay.

**Histone Methyltransferase (HMT) Assay**—The 293T cells were transfected with pFLAG or pFLAG-jmj vectors. Cell extracts and immunoprecipitated complex with FLAG were prepared using the same method described under “Protein-Protein Interaction.” The immunoprecipitated complex was washed and incubated at 30 °C for 60 min in a HMT reaction buffer (50 mM Tris-HCl pH 8.5, 20 mM KCl, 10 mM MgCl2, 10 mM 2-ME, 250 mM sucrose) containing 250 nCi of S-adenosyl-[methyl-3H]-methionine and 5–10 μg of histone substrate. The substrate was core histones (Roche Applied Science) or GST-histone H3-tail fusion proteins (18). The reactions were terminated by the addition of 2 × SDS sample loading buffer and boiling for 5 min. The reaction products were separated by 15% SDS-PAGE and visualized by CBB staining. The gels were incubated in Enlight solution (MoBiTec), dried, and then the methylated products were detected by autoradiography.

**Protein-Protein Interaction**—NIH3T3 cells were transfected with various expression vectors. Cell extracts were prepared with Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40) containing Complete protease inhibitor mixture (Roche Applied Science) from NIH3T3 transfectants or mouse embryos at embryonic day 14.5. The extracts were incubated with 5–10 μg of anti-FLAG, anti-G9a, or anti-GLP antibodies, and immunoprecipitated with protein G-Sepharose (GE Healthcare). Immunoprecipitates were analyzed by Western blot analysis.

**ChIP Assay**—The assays were performed using a ChIP Assay kit (Upstate) according to the manufacturer’s instructions and as previously described (14). DNA was analyzed by PCR using specific primers to mouse cyclin D1 promoter, P1 (-33): 5’-CCACTTGGAGAGGCAAGC-3’ and 5’-AATAAATTCCTGTAGTCCGTTG-3’, P2 (-591): 5’-CCACGGGAGAGATGATG-3’ and 5’-AGCCTCTGTCTTCTCCT-3’. The reactions were incubated with 0.1% Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40) containing Complete protease inhibitor mixture (Roche Applied Science) from NIH3T3 transfectants or mouse embryos at embryonic day 14.5. The extracts were incubated with 5–10 μg of anti-FLAG, anti-G9a, or anti-GLP antibodies, and immunoprecipitated with protein G-Sepharose (GE Healthcare). Immunoprecipitates were analyzed by Western blot analysis.

**Histology and Immunostaining**—Embryos were dissected in Dulbecco’s phosphate-buffered saline (PBS) and then fixed overnight in 4% paraformaldehyde in PBS for immunostaining, or for 30 min to 2 h in 0.2% glutaraldehyde-1% formaldehyde-0.02% Nonidet P-40 in PBS for X-gal staining. Embryos were sectioned transversely. X-gal staining was performed as described previously (19). For immunostaining of G9a and GLP, the same antibodies as for the ChIP assay were used. The immunohistochemistry procedure was described in a previous study (20).

**Statistical Analysis**—The experimental data for multiple comparison tests were analyzed using Fisher’s least significant difference (LSD) test after obtaining a significant difference with one-way analysis of variance (ANOVA). Values of p < 0.05 were considered statistically significant.

**RESULTS**

The Jmj Protein Complex Has Histone Methyltransferase Activity toward Histone H3-K9—Previously we showed that a protein complex including Jmj protein binds to the cyclin D1 promoter and represses the transcriptional activity. The repression is required for repression of cell proliferation of cardiac myocytes during development (14). However, the molecular

**Knockdown by siRNA**—NIH 3T3 cells were used for the knockdown experiments and transfected using Lipofectamine RNAiMAX according to the manufacturer’s instructions. pFLAG-IP or pFLAG-jmj-IP was transfected 24 h after siRNA transfection. The transfectants for expression vectors were selected in the presence of 1–2 μg/ml puromycin (Sigma) and used for the ChIP assay and RT-PCR analysis. Three duplex small interfering RNAs (siRNAs) for mouse G9a and GLP were tested (G9a and GLP Stealth RNAi 3 Select, Invitrogen) and two oligonucleotides showed strong effects on knockdown (G9a, MSS201293 and MSS201295; GLP, MSS234056, MSS234057). Stealth RNAi negative control duplexes (Invitrogen) were also used.

**Real-time PCR**—Total RNA was isolated using RNeasy (Qiagen). First strand cDNA synthesis was performed with Superscript II and oligo (dT)12–18 primer per the manufacturer’s instructions (Invitrogen). The cDNA, LightCycler 480 SYBR Green I Master (Roche Applied Science) and ABI 7300 (Applied Biosystems Japan) were used. Real-time PCR was carried out with the following primers, GAPDH: 5’-GGTGTAGGAGCCCAACGGGTTCAT-C-3’ and 5’-GCCAGTGACTCTCCGTTTGCGC-3’, cyclin D1: 5’-CTGGCCATGTAACATCTGGA-3’ and 5’-ATCCGGCTCTGGCATTTG-3’, G9a: 5’-CTATGC- GCCCGTTACTATGGC-3’ and 5’-GCTTCCAGGGATGCTCCTCGCAATGC-3’, GLP: 5’-CTATGCTTGGGAGAGAGGAC-3’ and 5’-TTCGCACTGGTTGTATGTA-3’. The expected amplifications without other products including primer dimers were checked by melt curve analysis and gel electrophoresis. For determination of the relative amount of products, standard curve or comparative CT methods were used as per the instructions of Applied Biosystems. To standardize the amount of sample cDNA, GAPDH was used as the endogenous control.

**Histone DEP Interaction**—The experimental data for multiple comparison tests were analyzed using Fisher’s least significant difference (LSD) test after obtaining a significant difference with one-way analysis of variance (ANOVA). Values of p < 0.05 were considered statistically significant.
mechanisms of transcriptional repression by Jmj remain unknown. To investigate the mechanisms, we examined the possibility that Jmj is involved in modification of the histone. A Jmj protein complex was collected from cells transfected with a FLAG-Jmj expression vector, and we examined the activities of the complex with respect to histone modification in vitro. Deacetylation and methylation of several histone residues are well known as histone modifications involved in transcriptional repression (for review, see Ref. 1). We could not detect any apparent histone deacetylation activity (data not shown). On the other hand, histone methyltransferase assay showed that the Jmj complex had histone methyltransferase activity toward bulk histone H3 (Fig. 1A).

Next, we examined the residues of histone H3 targeted by the Jmj complex. For this purpose, we used a normal H3 polypeptide (WT, residues 1–57) and several mutant polypeptides, in which K9/K27 (N4), K4/K27 (N9), K4/K9 (N27), or K4/K9/K27 (NT) were substituted for arginine, as substrates (Fig. 1B). Signals were detected strongly in WT and N9 and very weakly in N27, but were not detected in N4 and NT (Fig. 1C). No signals in NT showed that the Jmj complex did not transfer methyl groups to K36. These results indicated that the Jmj complex has histone methyltransferase activity to H3-K9 in vitro.

We next investigated histone methyltransferase activity of the Jmj complex to H3-K9 in vivo. cyclin D1 is a direct target of Jmj (14). Probably because Jmj represses cell proliferation (14, 21), there are no appropriate cell lines which express Jmj strongly. It is virtually impossible to analyze the hearts of jmj mutant embryos by ChIP assay, because the mutant dies around embryonic day 11.5 (E11.5) and the heart before E11.5 is very small. Therefore, we examined by gain of function but not loss of function.

We examined the effects of expression of Jmj on histone methylation of cyclin D1 promoter using ChIP assay (Fig. 2). Four regions in the promoter (P1, around –33; P2, around –591; P3, around –982; P4, around –2707) were analyzed. We could not detect any methylation states (mono-, di-, and tri-methylation) of H3-K9 in the four regions when control vector (only FLAG was expressed) was transfected (Fig. 2, lane FLAG). On the other hand, mono- or di-methylation (me1 or me2, respectively) of H3-K9 was observed in three regions (P2–P4) by Jmj expression (Fig. 2, lane FLAG-Jmj). Tri-methylation (me3) was also detected in the three regions, but the signals were weaker. Very weak signals for H3-K9me1–3 were also observed in P1. These data in vitro and in vivo showed that the Jmj complex has histone methyltransferase activity toward H3-K9.

Jmj binds to the H3-K9 Histone Methyltransferases G9a and GLP and Recruits Them to the cyclin D1 Promoter—H3-K9 histone methyltransferase activity in the Jmj complex and the absence of domains for histone methyltransferases such as SET in Jmj suggested that the complex contains H3-K9 histone methyltransferases. Therefore, we examined whether Jmj protein interacts with known H3-K9 methyltransferases (G9a, GLP, SETDB1, and Suv39h1).

First, we examined whether endogenous H3-K9 methyltransferases bind to Jmj in NIH3T3 cells. Because the expression level of endogenous Jmj is low or cannot be detected, especially in general cell lines such as NIH3T3 cells, FLAG-Jmj expression vector was introduced into NIH3T3 cells by transfection. We found that Jmj bound to G9a and GLP, but not to SETDB1 (Fig. 3A). The binding was confirmed by negative controls (expression of only FLAG or immunoprecipitation experiments using IgG; for IgG experiments, data not shown) and reciprocal experiments. In the case of Suv39h1, endogenous Suv39h1 could not be detected, most likely because the Suv39h1 level was very low or the antibody against Suv39h1 did not work. Therefore, we examined binding
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between exogenous Suv39h1 and Jmj. However, positive signals could not be obtained (Fig. 3B). Next, we demonstrated the binding of endogenous Jmj to G9a and GLP in whole embryos at embryonic day 14.5, although the signals were weaker than those for binding between G9a and GLP (Fig. 3C). We could not detect binding between Jmj and SETDB1 in the embryos (Fig. 3D). These results showed that Jmj binds to G9a and GLP specifically among the H3-K9 methyltransferases examined.

We also examined whether G9a or GLP binds to cyclin D1 promoter and if histone H3-K9 in the cyclin D1 promoter is methylated in vivo. ChiP assay using mouse whole embryos at E10.5 indicated that signals for the binding of G9a and GLP to the cyclin D1 promoter and H3-K9 di-methylation in the cyclin D1 promoter were detected. However, these signals were not detected in the cyclin D1 exon 3 (Fig. 4A). These results are consistent with the idea that G9a and GLP methylate H3-K9 in the cyclin D1 promoter in vivo.

Next, we examined the effect of Jmj on the binding of H3-K9 methyltransferases to the cyclin D1 promoter. As shown previously, Jmj protein binds to cyclin D1 promoter in 293T cells (14) (Fig. 4B, Vector: T7-Jmj). Expression of endogenous jmj was not detected in this cell line. We could not detect any signals showing binding of H3-K9 methyltransferases (G9a, GLP, SETDB1, and Suv39h1) to cyclin D1 promoter when exogenous jmj was not expressed (Fig. 4B, upper lane, omyc and αFLAG). However, binding of G9a and GLP to cyclin D1 promoter was detected when Jmj was co-expressed (Fig. 4B, bottom lane). No

and very weak signals for SETDB1 and Suv39h1, respectively, were detected. These results show that the expression of Jmj recruited G9a and GLP to cyclin D1 promoter.

Knockdown of G9a and GLP Inhibits H3-K9 Methylation and Repression of cyclin D1 Expression by the Jmj Complex—We examined the effects of knockdown of G9a and GLP on H3-K9 methylation by Jmj in an attempt to determine the roles of G9a and GLP in the Jmj complex. First, we confirmed that Jmj expression increases H3-K9 methylation in the cyclin D1 promoter even when control siRNA oligonucleotides were transfected (Fig. 5B).

Knockdown of G9a by siRNA oligonucleotides was successfully performed (Fig. 4A, for mRNA see Fig. 5), and showed no effects except for mono-methylation in the P2 region (Fig. 5B, siRNA: G9a). Next, we knocked down both G9a and GLP. Transfection of siRNA oligonucleotides for GLP results in a marked decrease in GLP and also G9a (Fig. 5A). This result is consistent with previous finding in GLP knock-out cells (16). G9a protein decreases most likely because GLP is required for the stability of G9a protein. In fact, knockdown of GLP strongly decreased the G9a protein level, but not the mRNA level (Figs. 5A and 6). In the knockdown of G9a and GLP, all signals for H3-K9 methylation observed by jmj expression decreased in all three regions (P2-P4) (Fig. 5B, siRNA: GLP). The promoter of Amylase2.1 gene was used for a negative control experiment because the gene shows pancreatic specific expression, and was expected to be silent in the NIH3T3 cells. No apparent changes were detected among all four groups (Fig. 5B). In addition, we performed ChiP assays using an antibody that recognizes H3 regardless of modifications to exclude the possibility that the changes in histone methylation, observed above, reflect changes in histone density. No apparent changes were detected among the four groups, showing the changes did not reflect changes in histone density (Fig. 5C).

Next, we investigated the effects of knockdown of G9a/GLP on repression of cyclin D1 by Jmj. The levels of mRNA of G9a, GLP, and cyclin D1 were measured by quantitative real-time RT-PCR. Jmj expression significantly decreased the mRNA level of endogenous cyclin D1 when control siRNA oligonucleotides were transfected (Fig. 6, panel: cyclin D1, vector: FJ, siRNA: Con). However, knockdown of GLP or both G9a and GLP, but not for only G9a, increased the level significantly and up to the control levels (Fig. 6). Knockdown was confirmed by quantification of each mRNA level (Fig. 6, panels of G9a and GLP). In these knockdown experiments (Figs. 5 and 6), we used
two nonoverlapped oligonucleotides and obtained virtually the same results. These results indicated that Jmj promotes H3-K9 methylation of the cyclin D1 promoter and represses cyclin D1 expression by recruitment of GLP or both G9a and GLP.

**DISCUSSION**

Jmj binds to the cyclin D1 promoter and represses cyclin D1 transcription. The repression inhibits cell proliferation in cardiac myocytes (14). In the present study, we analyzed the mechanism of the transcriptional repression by Jmj. Our results suggest that Jmj methylates H3-K9 and represses cyclin D1 expression through G9a and GLP.

**Do G9a, GLP, and Jmj Form One Complex?**—It remains unknown whether G9a, GLP, and Jmj form one complex. G9a and GLP form a tight complex in many cells, including NIH3T3 and 293T cells, which were used in this study (16). Immunodepletion analysis of G9a or GLP resulted in depletion of both G9a and GLP, at least in ES cells (16). Loss of GLP resulted in a decrease in G9a protein but not G9a mRNA in ES and NIH3T3 cells (16) (Figs. 5A and 6), suggesting that binding of G9a to GLP is required for stabilization of G9a protein. Several transcriptional silencing complexes such as E2F6, CtBP1, and CDB/cut contain both G9a and GLP (22–24). These results suggest that the G9a and GLP complex is more predominant than their monomers and Jmj bound to the heterodimer.

**What Are the Functions of Jmj in the Complex?**—Jmj is a member of Jmj family proteins. Many members of Jmj family proteins have been demonstrated to be histone demethylases (11–13). Interestingly, Jmj shows an opposite function, that of histone methyltransferase, by forming a protein complex. Although it is unknown whether Jmj has histone demethylase activity, the likelihood is low because the sequences for binding to cofactors in jmjC are not conserved (11). Because Jmj recruits G9a and GLP to the cyclin D1 promoter (Fig. 4B), and an electrophoretic mobility shift assay showed that Jmj can bind to the cyclin D1 promoter directly,4 Jmj has a role that links G9a and GLP to the cyclin D1 promoter.

Another issue is that methylation of H3-K9 in the cyclin D1 promoter by Jmj expression could not be detected in P1 primers, which are located in the region nearest to the Jmj responsive element (−187/−52) (Fig. 2) (14). It is highly conceivable that the detection of ChIP was very difficult in the region because GC content is very high, and the titers of anti-methylated H3-K9 antibodies are not high. It is also possible that the Jmj complex binds to the Jmj responsive element, but the complex methylates H3-K9 in the upstream region and the methylation is critical for the repression of cyclin D1 expression.

**Redundant Functions of G9a and GLP**—Previous studies showed that GLP cannot compensate for the loss of G9a in ES cells. Drastic reductions in H3-K9me1 and me2 were observed in G9a-deficient ES cells, although the GLP protein level was not changed (16, 25). However, reductions in H3-K9me1 and me2 in the cyclin D1 promoter were observed in both G9a and GLP knockdown NIH3T3 cells, but not in G9a knockdown cells (Fig. 4), indicating that GLP can compensate for the loss of G9a for at least cyclin D1 gene in NIH3T3 cells. We produced cardiac myocyte-specific knock-out mice using the Cre-loxP system. G9a-deficient cardiac myocytes did not show any apparent abnormal phenotypes during development, and substantial levels of H3-K9me2 and GLP were detected in nuclei at E14.5.5 These data suggest redundant functions of G9a and GLP, and

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4 M. Toyoda et al., unpublished data.

5 T. Takeuchi, M. Inagawa, M. Tachibana, and Y. Shinkai, unpublished data.
cell type-specific or gene-specific regulation of H3-K9 methylation by these proteins. It would be interesting to determine why the function of GLP is different among cell types or target genes. It is possible that members of the complex, the enzyme activity, or localization of GLP are different.

**Functions of Jmj Family Proteins Putatively Lacking Histone Demethylase Activity**—In addition to Jmj, Hairless protein lacks conserved residues critical for histone demethylation in the JmjC domain, suggesting loss of the enzyme activity (11). Hairless is a gene responsible for the mouse mutant hairless (26). Interestingly, Hairless functions as a nuclear receptor corepressor, interacts with multiple nuclear receptors such as thyroid hormone receptor, and represses transcriptional activities in the context of these nuclear receptors (27–29). Our present data concerning Jmj suggest the possibility that Hairless is also involved in other histone modification besides histone demethylation and represses the target gene expression through these modifications. The significance and roles of JmjC of Jmj and Hairless remain unknown. However, if these JmjC domains

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**FIGURE 5.** The Jmj complex methylates H3-K9 in the cyclin D1 promoter by G9a or GLP. A, protein levels of G9a and GLP in NIH3T3 cells transfected with siRNA oligonucleotide for control, G9a or GLP were analyzed by Western blot analysis. siRNA and WB represent siRNA oligonucleotide and antibodies for Western blot analyses, respectively. B, effects of knockdown of G9a or GLP on H3-K9 methylation in the cyclin D1 promoter (P2, P3, P4). Cells were transfected with FLAG or FLAG-Jmj expression vectors and also siRNA oligonucleotide for control, G9a or GLP. For a negative control gene, the promoter of Amylase2.1 gene (Amy 2.1) was used. The transfectants for expression vectors were selected in the presence of puromycin. ChIP shows antibodies for ChIP assays. 1, 2, and 3 indicate mono-, di-, and tri-methylation, respectively. Inp, Input. P2–P4 and numbers in parentheses represent positions in the cyclin D1 promoter. C, ChIP assays were performed using the same samples and same primers (P2–P4) as in B using an antibody that recognizes H3 regardless of modifications (H3).

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**FIGURE 6.** Jmj complex represses cyclin D1 expression by G9a or GLP. NIH3T3 cells were transfected with FLAG (F) or FLAG-Jmj (FJ) expression vectors and also siRNA oligonucleotide for control, G9a or GLP. The transfectants for expression vectors were selected in the presence of puromycin. The mRNA levels of G9a, GLP, and cyclin D1 in NIH3T3 cells were measured by quantitative RT-PCR. The relative mRNA levels are presented as the means ± S.E. of four independent experiments. *, #, and † represent statistical significance with respect to the levels indicated (Fisher’s LSD test after one-way ANOVA).
can bind to methylated lysines, it is possible that the JmjC domains antagonize the functions of histone demethylases. The regulation would enable more dynamic regulation of histone methylation.

In conclusion, our studies show a novel function of Jmj family proteins in transcriptional control, and molecular mechanisms repressing cyclin D1, which has essential functions in the cell cycle machinery and is significantly involved in several cancers.

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