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Short title: Role of *Jmjd5* in mouse embryos

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SUMMARY

Covalent modifications of histones play an important role in chromatin architecture and dynamics. In particular, histone lysine methylation is important for transcriptional control during diverse biological processes. A nuclear protein, Jmjd5 (also called Kdm8) is a histone lysine demethylase that contains a JmjC domain at the C-terminal region. In this study, we have generated *Jmjd5*-deficient mice (*Jmjd5$^{\Delta/\Delta}$*) to investigate the in vivo function of *Jmjd5*. *Jmjd5$^{\Delta/\Delta}$* embryos showed severe growth retardation, resulting in embryonic lethality at the midgestation stage. Mouse embryonic fibroblasts (MEFs) derived from *Jmjd5* hypomorphic embryos (*Jmjd5$^{neo/neo}$*) also showed growth defect. Quantitative PCR analysis for various cell cycle regulators indicated that only *Cdkn1a* expression was up-regulated in *Jmjd5$^{neo/neo}$* MEFs and *Jmjd5$^{\Delta/\Delta}$* embryos. Knock-down assay with *Cdkn1a*-specific small interfering RNAs revealed that the growth defect of *Jmjd5$^{neo/neo}$* MEFs was significantly rescued. In addition, a genetic study using *Jmjd5$^{\Delta/\Delta}$*; *Cdkn1a$^{\Delta/\Delta}$* double knock-out mice showed that the growth retardation of *Jmjd5$^{\Delta/\Delta}$* embryos was partially rescued by *Cdkn1a* deficiency. Chromatin immunoprecipitation analysis showed that increased di-methylated lysine 36 of histone H3 (H3K36me2) and reduced recruitment of endogenous Jmjd5 were detected in the transcribed regions of *Cdkn1a* in

Jmjd5^{neo/neo} MEFs. Taken together, these results suggest that Jmjd5 physiologically moderates

embryonic cell proliferation through the epigenetic control of *Cdkn1a* expression.

KEY WORDS: Jmjd5 (Kdm8), Cdkn1a, cell proliferation

INTRODUCTION

In the eukaryotic nuclei, genomic DNA is efficiently packed into chromatin which is constructed by numerous nucleosomes. In each nucleosome, approximately 146 base pairs of DNA are wrapped around a histone octamer containing two copies of four core histones: H2A, H2B, H3 and H4. Histone H1, the linker histone, helps further compact the nucleosomal DNA into higher-order structures. In addition, the flexible amino-termini extending from core histones (termed as histone tails) post-translationally receive various covalent modifications such as methylation, acetylation, phosphorylation, ubiquitylation and SUMOylation. These histone modifications can alter the structure of the nucleosome and may also change the assembly between chromatin and the nuclear proteins that recognize specific histone modifications (Mosammaparast and Shi, 2011).

Among these modifications, methylation of specific lysine residues on histone H3 (e.g., H3K4, H3K9, H3K27 and H3K36) is closely related to gene expression (Martin and Zhang, 2005). During transcriptional processes, methylation at H3K4 and H3K36 correlates with transcriptionally active genes, whereas methylation at H3K9 and H3K27 is linked to transcriptional repression. To add more complexity, each of the lysine residues has four distinct

methylation states that are un-, mono- (me1), di- (me2) or tri- (me3) methylated. Importantly, methylation is a reversible modification regulated by histone lysine methyltransferases (KMTs) or histone lysine demethylases (KDMs). The methylation status of histone H3 is dynamically regulated by KMTs and KDMs, and is implicated in diverse biological processes including cellular proliferation, differentiation, DNA repair and recombination.

Two classes of KDMs have been identified (Klose et al., 2006). KDM1 (also called LSD1) encodes a flavin adenine dinucleotide-dependent amine oxidase, and is known to remove mono- and di-methylated lysine modifications. The Jumonji C (JmjC)-domain family of KDMs contains proteins that are Fe (II) and α-ketoglutarate-dependent enzymes. Unlike KDM1, JmjC proteins can remove all three methylation states. Recent studies suggest that the deregulation of JmjC members is associated with human diseases such as neurological disorders and cancers (Pedersen and Helin, 2010). For instance, mutations in *JARID1C/KDM5C* were found in patients with X-linked mental retardation (XLMR, Jensen et al., 2005), and *PHF8* mutations were also observed in patients with XLMR and cleft lip/palate (Abidi et al., 2007; Koivisto et al., 2007; Laumonnier et al., 2005). Gene amplification of *JMJD2C/KDM4A* was detected in esophageal squamous carcinomas, medulloblastoma and breast cancer (Ehrbrecht et al., 2006;

Liu et al., 2009; Northcott et al., 2009; Yang et al., 2000), and elevated expression of

PLU1/KDM5B was reported in breast, prostate and lung cancers (Hayami et al., 2010; Lu et al., 1999; Xiang et al., 2007). Furthermore, inactivating somatic mutations of *UTX/KDM6A* have been reported in multiple tumor types (van Haaften et al., 2009).

Genetically modified mice for several JmjC members have previously been generated suggesting the involvement of JmjC proteins in various pathological/developmental processes. For example, deletion of *Jmjd1a/Kdm3a* showed an adult-onset obesity phenotype and down-regulation of several metabolic genes (Inagaki et al., 2009; Tateishi et al., 2009). Deletion of *Fbxl10/Kdm2b* showed an exencephalic phenotype and caused increased cell proliferation and cell death in neural progenitor cells, indicating an essential role for this gene during neural development (Fukuda et al., 2011).

Recently, *Jmjd5*, a member of the JmjC family, was reported as a demethylase for di-methylated lysine 36 of histone H3 (H3K36me2), regulating the expression of the *CCNA1* gene in MCF7 breast cancer cells (Hsia et al., 2010). In *Arabidopsis thaliana*, Jmjd5 was co-regulated with evening-phased clock components and was positively associated with clock genes expressed at dawn (Jones et al., 2010). They also showed that knock-down of *Jmjd5* in

U2OS osteosarcoma cells led to fast-running circadian oscillations, suggesting its interchangeable role in both plant and human circadian systems. However, the physiological role of *Jmjd5* in the context of a whole mammalian organism remains unknown.

In order to understand the function of *Jmjd5* in cellular and developmental processes, we have successfully generated *Jmjd5* mutant mice. We found that the deletion of *Jmjd5* allele caused severe growth retardation, resulting in embryonic lethality at the midgestation stage. MEFs derived from *Jmjd5* hypomorphic embryos also showed the defect in cell proliferation. We found the increased expression of *Cdkn1a* as one of the mechanisms leading to cell growth retardation. ChIP analysis indicated that Jmjd5 was directly recruited on the *Cdkn1a* locus, resulting in the alteration of H3K36me2 status of the *Cdkn1a* gene. These results suggest that Jmjd5 histone demethylase is involved in embryonic cell proliferation by fine-tuning the expression of *Cdkn1a*.

MATERIALS AND METHODS

Generation of *Jmjd5*-deficient mice

The targeting vector for *Jmjd5*-deficient mice was constructed by a recombineering system (Liu et al., 2003). The vector was first linearized by Pme I digestion and used for electroporation into the V6.5 clone derived from C57BL/6 and 129Sv ES cells (Eggan et al., 2001). The cells were subjected to G418 selection (150 μg/ml), and independent colonies were screened by Southern blotting. Genomic DNAs isolated from ES clones were digested with Cla I or Eco RV and hybridized with a 5'-probe (nucleotides 4641 to 5187 in NCBI Reference Sequence: NC_000073.5) and a 3'-probe (nucleotides 13709 to 14028), respectively. These probes were generated by polymerase chain reaction (PCR) using the following primers: for 5'-probe, 5'-TCCCTTACACGGCTATGGTC-3' and 5'-AGCTGAGAGAATTCAGGGCA-3'; for 3'-probe, 5'-GGGTGAGTCAGAATAGTGTA-3' and 5'-TCATTGGCCAAGGATTCAGA-3'.

One derivative of each ES cell line containing the floxed *Jmjd5* allele was injected into the blastocoel cavity of 3.5-day C57BL/6 blastocysts. Chimeric mice were generated at UNITECH Co., Inc. (Japan) and mated with C57BL/6 females. The offspring (*Jmjd5$^{+/neo}$*) was screened for germline transmission by PCR. F1 mice were backcrossed with C57BL/6 mice.

CAG-Flp transgenic mice (Ando et al., 2000), *Pgk2-Cre* transgenic mice (Kanki et al., 2006), and *Cdkn1a*-deficient mice (Deng et al., 1995) were used for the generation of *Jmjd5$^{flox/flox}$* mice, *Jmjd5$^{\Delta/\Delta}$* mice and *Jmjd5$^{\Delta/\Delta}$*; *Cdkn1a$^{\Delta/\Delta}$* mice, respectively. All animal experiments were approved by the Animal Care and Use Committee of Kanazawa University.

Genotyping for *Jmjd5*-deficient mice

For the genotyping of adult mice, the tails were lysed in lysis buffer (20 mM Tris-HCl pH 8.0, 5 mM EDTA, 400 mM NaCl, 0.3% SDS and 0.2 mg/ml Proteinase K) for DNA extraction. Genomic DNA was used for PCR using Ex-Taq DNA polymerase (Takara) or Southern blotting. For the genotyping of embryos, a part of the yolk sac was lysed and the lysate was used for direct PCR using MightyAmp DNA polymerase (Takara). The primers used for genotyping are listed in Table S1. For Southern blotting, genomic DNA was digested with Stu I or Kpn I and hybridized with a *Jmjd5*-specific probe (nucleotides 10833 to 11233 in NCBI Reference Sequence: NC_000073.5). Signals were visualized with the Gene Image Random-Prime Labeling and Detection System (Amersham). The probe was amplified using the primers: 5'-TTCTGACCTCCACATATGACACAG-3' and 5'-CCAACCTGACTCAACCTACTCC-3'.

Preparation of *Jmjd5* hypomorphic MEFs

MEFs were established from wild-type and *Jmjd5^neo/neo^* embryos using standard protocols (Nagy

et al., 2003) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal

bovine serum, 2 mM glutamine and penicillin/streptomycin at 37˚C in 5% CO_2. For cell growth

assay, 3 x 10^4 cells were plated on 12-well plates, and the cell number was counted every 24

hours. For retroviral infection, 1.5 x 10^5 cells were plated on 6-well plates, and the cell number

was counted at 48 hours after infection.

Preparation of plasmids and anti-Jmjd5 antibody

For whole mount *in situ* hybridization, a fragment of *Jmjd5* cDNA (nucleotides 73 to 1502 in

NCBI Reference Sequence: NM_029842.5) or *Cdkn1a* cDNA (nucleotides 104 to 587 in NCBI

Reference Sequence: NM_007669.4) was amplified and cloned into pSTBlue-1 vector

(Novagen). For luciferase assay, pTAL-Luc (Clontech) was used as a control vector (tk-luc). To

prepare p53-responsive luciferase vector (p53BS), complementary oligonucleotides,

5'-GAGAACATGTCCCAACATGTTG-3' and 5'-TCCAACATGTTGGGACATGTTC-3',

corresponding to the p53-binding site from human *Cdkn1a* locus (El-Deiry et al., 1993), were

synthesized. Six annealed oligonucleotides were tandemly ligated and inserted into pTAL-Luc.

Mouse *Jmjd5* cDNA was tagged with FLAG-His$_6$-tag and cloned into pDON-5 Neo

plasmid (Takara) to produce retroviruses. A Jmjd5 H319A mutant, which corresponds to the

catalytically inactive JMJD5 mutant H321A in humans (Hsia et al., 2010), was generated by

using a PCR-based Mutagenesis kit (Takara). The shRNA-expressing retroviruses were

constructed and produced as described previously (Yoshida et al., 2011). Oligonucleotides for

shRNA production are listed in Table S2.

For the preparation of anti-Jmjd5 rabbit polyclonal antibody, histidine-tagged

C-terminal Jmjd5 (D175 to Y396) proteins purified from genetically engineered *Escherichia*

coli were used.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted with Trizol (Invitrogen) and synthesized to cDNA using SuperScript

VILO cDNA synthesis kit (Invitrogen). qRT-PCR analysis was performed with FastStart

Universal SYBR Green Master (Roche) using 7900HT Fast Real-Time PCR System (Applied

Biosystems). PCR data were normalized with the expression of *Gapdh*. Primers designed for the

qPCR are listed in Table S1.

Chromatin immunoprecipitation (ChIP) analysis.

ChIP experiments were performed as described previously (Kimura et al., 2008). The complexes

that were immunopreciptated with anti-K36me2 mouse monoclonal antibody or anti-Jmjd5

antibody were collected by Dynabeads Protein A or G (Invitrogen). The precipitates were used

for the detection of *Cdkn1a* genomic regions by qPCR. Primers designed for ChIP are listed in

Table S1.

Knock-down assay.

Each Stealth RNAi for *Cdkn1a* (Invitrogen) was introduced into the cells using Lipofectamine

RNAiMAX (Invitrogen) by a reverse transfection procedure. The cells were collected for RNA

extraction after 48 hours, or the cell number was counted after 72 hours. Stealth RNAi Negative

Control Kit (Invitrogen) was used for control. The siRNAs used for this study are listed in Table

S2.

Immunoblotting.

MEFs were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA supplemented with protease inhibitors). The lysates were separated on SuperSep Ace 5-20% running gel (Wako). Anti-p21 (SX118, BD Biosciences), anti-p53 (FL393, Santa Cruz) and anti-GAPDH (6C5, Millipore) antibodies were used. As a positive control for the detection of endogenous p21 and p53, wild-type MEFs were treated with 0.03 μg/ml adriamycin for 24 hours.

Senescence-associated β-galactosidase (SA-β-gal) staining and Immunofluorescence.

SA-β-gal staining was performed according to the protocol described in the reference (Kitajima et al., 2010). As a positive control for the detection of senescent cells, MEFs (1 x 10^4 cells) were sparsely seeded on 6-well plates and cultured for 15 days. For immunofluorescence, MEFs were fixed with 4% paraformaldehyde in phosphate-buffered saline. The specimens were incubated with anti-single stranded DNA (ssDNA) antibody (Dako) and treated with Alexa546-conjugated anti-rabbit IgG antibody (Invitrogen). Nuclei were visualized with

4',6-diamidino-2-phenylindole (DAPI). As a positive control for the detection of apoptotic cells,

wild-type MEFs were treated with 0.06 µg/ml adriamycin for 24 hours.

Whole mount *in situ* hybridization (WISH) analysis.

WISH of mouse embryos was performed as described previously (Yamaguchi et al., 1993) with

the exception that hybridization was performed at 70°C. Digoxygenin (DIG)-labeled RNA

probes were synthesized with DIG RNA Labeling Mix (Roche).

Luciferase assay.

Reporter vector was introduced into wild-type MEFs or *Jmjd5$^{neo/neo}$* MEFs by Fugene HD

Transfection Reagent (Roche). The cells were collected after 48 hours, and the luciferase

activities were determined with Dual-Luciferase Reporter Assay System (Promega).

RESULTS

***Jmjd5*-deficient mice are embryonic lethal at the midgestation stage.**

In order to investigate the in vivo function of *Jmjd5*, we generated a conditional *Jmjd5* knock-out mice (*Jmjd5$^{flox/flox}$*) by the targeted disruption of exon IV, which is where the JmjC domain starts to get encoded (Fig. 1A). The recombination of the *Jmjd5^{flox}* allele by Cre recombinase causes a frame-shift mutation, resulting in the lack of production for Jmjd5 protein with JmjC domain. We mated *Jmjd5$^{flox/flox}$* female mice with spermatocytes specific Cre-expressing transgenic male mice (*Pgk2-Cre*, Ando et al., 2000), and mutant mice with a *Jmjd5* deletion allele (*Jmjd5$^{\Delta}$*) were established. *Jmjd5$^{+/\Delta}$* heterozygous mice were viable and fertile, and no morphological changes were observed compared with wild-type mice. To generate *Jmjd5* null mice (*Jmjd5$^{\Delta/\Delta}$*), *Jmjd5$^{+/\Delta}$* mice were intercrossed, and the genotype for each mouse was determined by Southern blot analysis. For Southern blot, genomic DNAs isolated from wild-type or *Jmjd5$^{+/\Delta}$* mice tails were digested with Stu I or Kpn I, and were hybridized with the *Jmjd5*-specific probe. As shown in Fig. 1B, this probe was able to recognize the 2.3 kb wild-type and the 1.4 kb mutant fragments from the Stu I digested genomic DNA as well as the 1.6 kb wild-type and the 0.7 kb mutant fragments from the Kpn I digested genomic DNA.

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However, we could not find $Jmjd5^{\Delta/\Delta}$ homozygous mice in the offspring (Table S3), suggesting

that $Jmjd5^{\Delta/\Delta}$ mice were embryonic lethal. Thus we next performed further genotyping analyses

at various embryonic stages. The genotype for each embryo was determined by polymerase

chain reaction (PCR) analysis, which could detect a 1.5 kb band for the wild-type allele and a

0.5 kb band for the mutant allele, respectively (Fig. 1C). $Jmjd5^{\Delta/\Delta}$ embryos could be detected

from embryonic day (E) 6.5 to E 11.5 (Fig. 1C and Table S3), but were almost absorbed at E

11.5 (5 of 5), suggesting that they died around E 11.0.

At the same time, we analyzed the temporal and spatial expression pattern of

endogenous $Jmjd5$ during early embryogenesis. Quantitative reverse transcription (qRT)-PCR

analysis showed that $Jmjd5$ was expressed at all embryonic stages examined (Fig. 1D), and the

expression of $Jmjd5$ was maintained at and just beyond the lethal stage (E11.0) of embryonic

development. Whole mount in situ hybridization (WISH) analysis using the anti-sense RNA

probe for $Jmjd5$ indicated that endogenous $Jmjd5$ was ubiquitously expressed in the whole

embryo at E 8.5 (5 of 5, Fig. 1E left panel), while the complementary sense RNA probe detected

no signal (2 of 2, Fig. 1E right panel). Thus these results suggested an important role of $Jmjd5$

during embryogenesis at least after the post-implantation stages.

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Next, we mated $Jmjd5^{+/\Delta}$ mice and dissected the pregnant mice to observe the

phenotype of $Jmjd5^{\Delta/\Delta}$ embryos and the littermates from E 6.5 to E 10.5 (Fig. 2). Although the

results showed that there was no phenotypic difference between $Jmjd5^{\Delta/\Delta}$ embryos and the

control littermates at E 6.5 (8 of 8, Fig. 2A,A'), the growth of $Jmjd5^{\Delta/\Delta}$ embryos at E 9.0 was

obviously delayed compared with that of control embryos (8 of 8, Fig. 2B,B'). The $Jmjd5^{\Delta/\Delta}$

embryos at E 9.0 looked similar with the wild-type embryo at E 8.5. At this stage, we were not

able to observe other morphological defects in the appearance of $Jmjd5^{\Delta/\Delta}$ embryos. At E 10.5,

$Jmjd5^{\Delta/\Delta}$ embryos showed the severe growth retardation including the incomplete embryonic

turning. We also observed typical angiogenesis defects in $Jmjd5^{\Delta/\Delta}$ embryos that contained

avascular yolk sacs (8 of 8, Fig. 2C') and the clumps of red blood cells (7 of 8, Fig. 2D'). This

suggested the possibility that the abnormal vascular development might lead to the insufficient

circulatory system in $Jmjd5^{\Delta/\Delta}$ embryos, resulting in the observed embryonic growth retardation.

However, a slight growth retardation of $Jmjd5^{\Delta/\Delta}$ embryos has already been confirmed at E 7.5

when endothelial precursors (angioblasts) start developing in the mouse embryo (data not

shown). Thus we concluded that Jmjd5 may be a critical factor for embryonic survival,

especially for embryonic cell proliferation after the post-implantation stages.

Jmjd5 regulates the cell proliferation of mouse embryonic fibroblasts (MEFs).

To examine whether Jmjd5 could intrinsically regulate the embryonic cell proliferation, we attempted to prepare *Jmjd5*-deficient MEFs to see the effect of Jmjd5 on the cell growth. However, it was difficult to establish *Jmjd5$^{\Delta/\Delta}$* MEFs, because *Jmjd5$^{\Delta/\Delta}$* embryos did not survive to E 14.5, one of the most suitable stages for the preparation of MEFs. Interestingly, we found the *Jmjd5$^{neo/neo}$* homozygous mice were also embryonic lethal without any excision of *Jmjd5* allele (See Fig. 1 and Table S4). At E 10.5, whereas the majority of *Jmjd5$^{neo/neo}$* embryos (25 of 37) showed severe growth retardation as observed for *Jmjd5$^{\Delta/\Delta}$* embryos (Fig. S1, panel B), the 12 remaining *Jmjd5$^{neo/neo}$* embryos seemed to be normal except for the smaller embryonic body (Fig. S1, panel C) and survived until E 14.5. This result indicated that *Jmjd5$^{neo/neo}$* embryos displayed a hypomorphic phenotype. Furthermore, RT-PCR analysis indicated that the endogenous *Jmjd5* expression was significantly reduced in *Jmjd5$^{neo/neo}$* embryos (Fig. S2). Since *Jmjd5$^{flox/flox}$* mice, in which *Pgk-Neo* cassette has been already removed by mating with *CAG-Flp* mice, were viable and looked normal (data not shown), it was suggested that exogenous *Pgk-Neo* cassette from our targeting vector could be interfering with the expression

of endogenous *Jmjd5*, resulting in the hypomorphic phenotype of *Jmjd5^{neo/neo}* mice. Thus we used these *Jmjd5^{neo/neo}* embryos to establish *Jmjd5* hypomorphic MEFs for further analyses.

Pregnant mice were dissected at 14.5 days post coitum (p.c.), and MEFs were prepared from each embryo. Genotypes were determined by PCR analysis, and a single band of 0.5 kb corresponding to *Jmjd5^{neo}* allele was detected for *Jmjd5^{neo/neo}* MEF (Fig. 3A). Approximately a 75% reduction of endogenous *Jmjd5* expression was observed by qRT-PCR analysis in the prepared MEFs (Fig. 3B), and immunoblotting with anti-Jmjd5 antibody also showed that the expression of Jmjd5 was significantly lower than wild-type (Fig. 3C). Next, we compared the cell growth between wild-type and *Jmjd5^{neo/neo}* MEFs (Fig. 3D). The wild-type MEFs proliferated exponentially and became confluent after 72 hours culture (n = 3). However, the growth of *Jmjd5^{neo/neo}* MEFs was significantly reduced compared with wild-type within 48 hours after passage (n = 3, p < 0.01), and the cell number was decreased to approximately 50% of control cells at 72 hours after passage (Fig. 3D). This result suggested that Jmjd5 played an important role in embryonic cell proliferation.

To examine whether the decreased cell number of *Jmjd5^{neo/neo}* MEFs might be due to the induced premature senescence, we carried out senescence-associated β-galactosidase

staining. The β-galactosidase positive cells were not detected in both *Jmjd5*$^{neo/neo}$ MEFs and

wild-type MEFs (Fig. 3E, middle and upper panels), although β-galactosidase positive cells

could clearly be detected in the control sparsely seeded MEFs (Fig. 3E, lower panel). Next, we

performed immunofluorescence assay with anti-single stranded DNA (ssDNA) antibody to

examine whether the cells undergo apoptosis at a higher rate. Although many cells were stained

with anti-ssDNA antibody in the positive controls (adriamycin-treated MEFs, Fig. 3F, lower

panels), we did not observe the elevated rate of the ssDNA positive cells in *Jmjd5*$^{neo/neo}$ MEFs

compared with wild-type MEFs (Fig. 3F, middle and upper panels). These results indicated that

down-regulation of Jmjd5 did not increase premature senescence and apoptosis, but rather

decreased embryonic cell proliferation.

Next we examined whether the growth retardation of *Jmjd5*$^{neo/neo}$ MEFs was

dependent on Jmjd5 demethylase activity. The *Jmjd5*$^{neo/neo}$ MEFs were infected with control

retrovirus, the retrovirus expressing wild-type Jmjd5 or the catalytically inactive mutant H319A.

The expression of *Jmjd5* was increased at the same level when MEFs were infected with

wild-type or inactive Jmjd5 expressing virus (Fig. S3). The cell number of the infected MEFs

was counted at 48 hours after infection, and the cell growth defect of *Jmjd5*$^{neo/neo}$ MEFs was

significantly rescued by re-expressing wild-type Jmjd5 (Fig. 3G). No significant change was

observed with the expression of inactive H319A mutant compared to the control. These results

suggested that the demethylase activity of Jmjd5 was required for normal embryonic cell

proliferation.

The expression of *Cdkn1a/p21* is up-regulated in *Jmjd5^{neo/neo}* MEFs and *Jmjd5^{Δ/Δ}* embryos.

A recent report showed that Jmjd5 activated the expression of the *CCNA1* gene in MCF7 breast

cancer cells by demethylating H3K36me2 on the *CCNA1* locus, resulting in cell cycle

progression (Hsia et al., 2010). Thus we first examined whether loss of Jmjd5 caused the

down-regulation of *Ccna1* in mouse embryonic cells. We extracted total RNA from *Jmjd5^{neo/neo}*

MEFs or *Jmjd5^{Δ/Δ}* embryos to synthesize cDNA, and performed qRT-PCR analysis using

Ccna1-specific primers. Unexpectedly, there was no change in the expression of *Ccna1* in

Jmjd5^{neo/neo} MEFs or *Jmjd5^{Δ/Δ}* embryos compared with wild-type (Fig. 4A,D). This indicated

that there might be a different regulatory function of Jmjd5 in mouse embryonic cells and

human cancer cell lines.

Various genetic studies using knock-out mice have previously shown that a number

of cell cycle regulators are involved in normal mouse development (Ciemerych and Sicinski, 2005). Therefore, we tried to examine whether the loss of Jmjd5 affected the expression of representative cell cycle regulators, including eleven *Cyclins*, ten *CDKs*, eight *CDK* inhibitors and *Rb* family, for mouse embryogenesis. Comparing the expression of cell cycle regulators between wild-type MEFs and *Jmjd5$^{neo/neo}$* MEFs, the expressions of *Ccnd2*, *Cdk6* and *Cdk7* were 1.57-fold, 1.44-fold and 1.43-fold higher respectively in *Jmjd5$^{neo/neo}$* MEFs (Fig. 4A,B). In addition, the expressions of *Cdkn1a* and *Cdkn2a/p16Ink4a* were 1.93-fold and 1.69-fold higher than those of wild-type, respectively (Fig. 4C). On the other hand, the expressions of other *Cyclins*, *CDKs*, *CDK* inhibitors and *Rb* family were not significantly changed in *Jmjd5$^{neo/neo}$* MEFs (Fig. 4A-C, S4). Next, we examined the expression of *Ccnd2*, *Cdk6*, *Cdk7*, *Cdkn1a* and *Cdkn2a/p16Ink4a* in wild-type embryos at the early somite stage (E 8.25, n = 3) and in the stage-matched *Jmjd5$^{\Delta/\Delta}$* embryos (n = 7) by qRT-PCR analysis. In agreement with our data from *Jmjd5$^{neo/neo}$* MEFs, we detected a significant increase of *Cdkn1a* transcripts (4.17-fold, p < 0.01) in *Jmjd5$^{\Delta/\Delta}$* embryos (Fig. 4D). However, unlike the up-regulation in *Jmjd5$^{neo/neo}$* MEFs, the expression of other candidates was not altered in *Jmjd5$^{\Delta/\Delta}$* embryos, and the expression of *Ccnd2* showed a 1.52-fold reduction (p < 0.01). Since *Cdkn1a* expression was commonly

elevated in both *Jmjd5^{neo/neo}* MEFs and *Jmjd5^{Δ/Δ}* embryos, we picked up *Cdkn1a* as one of the

most probable transcriptional targets for Jmjd5.

To evaluate the spatial alteration of *Cdkn1a* expression in *Jmjd5^{Δ/Δ}* embryos, we also

performed WISH analysis (Fig. 4E). The elevation of *Cdkn1a* was detected in the posterior half

of the *Jmjd5^{Δ/Δ}* embryo including the primitive streak where endogenous *Cdkn1a* was highly

expressed in wild-type embryo. The ectopic expression of *Cdkn1a* was also observed in the

anterior half of the *Jmjd5^{Δ/Δ}* embryo (7 of 10). A complementary sense probe for *Cdkn1a* was

not able to detect any signals (4 of 4). These results suggested that endogenous Jmjd5 could be

responsible for the spatial expression pattern of *Cdkn1a* in whole embryos.

Next we examined whether the knock-down of *Jmjd5* expression could induce the

up-regulation of *Cdkn1a*. We used the retroviruses expressing two different small hairpin RNAs

(shRNAs) for *Jmjd5* (*Jmjd5* sh#1 and sh#2). MEFs were infected with the control retrovirus or

the retrovirus expressing each *Jmjd5* shRNA. qRT-PCR indicated that *Jmjd5* transcripts were

significantly reduced with the infection of both *Jmjd5* shRNA expressing retroviruses, but not

with the control retrovirus (Fig. 4F, left). We then examined the effect of *Jmjd5* knock-down in

the expression of *Cdkn1a*. Both *Jmjd5*-specific shRNAs led to the significant increase of

Cdkn1a transcripts (1.59-fold and 1.65-fold) (Fig. 4F, right). These observations suggested that

Jmjd5 might be directly involved in the regulation of *Cdkn1a* expression in MEFs.

Jmjd5 regulates the expression of *Cdkn1a* intrinsically without the stimulation of p53-p21 pathway.

According to the results shown in Fig. 3 and 4, we speculated that *Cdkn1a* up-regulation caused

by the reduction in *Jmjd5* levels might be a major reason for the observed growth retardation.

To test our hypothesis, we performed a knock-down assay using *Cdkn1a*-specific siRNAs and

investigated whether the reduction of *Cdkn1a* could rescue the inhibition of cell proliferation in

Jmjd5$^{neo/neo}$ MEFs. Two *Cdkn1a*-specific siRNAs (siRNA#1 and #2) were designed, and the

knock-down efficiency of each siRNA was shown to be approximately 80 % (Fig. 5A). *Cdkn1a*

siRNA#1, #2 or control unrelated siRNA was transfected into *Jmjd5$^{neo/neo}$* MEFs, and the growth

of the transfected MEFs was compared. Knock-down of *Cdkn1a* in *Jmjd5$^{neo/neo}$* MEFs caused a

significant increase of cell proliferation, compared with the proliferation of *Jmjd5$^{neo/neo}$* MEFs

treated with the control siRNA as well as untreated *Jmjd5$^{neo/neo}$* MEFs (n = 3, p < 0.01, Fig. 5B).

The cell number of *Jmjd5$^{neo/neo}$* MEFs treated with the *Cdkn1a*-specific siRNAs was still less

than that of wild-type MEFs, indicating a partial rescue of cell growth by *Cdkn1a* knock-down.

These results suggested that the regulation of *Cdkn1a* expression by Jmjd5 played an important but not exclusive role in embryonic proliferation, although the incomplete knock-down of *Cdkn1a* may be responsible for the partial rescue phenotype.

Since it is known that *Cdkn1a* is a major direct target for p53 pathway (Wood and Shilatifard, 2006), we examined whether the elevation of *Cdkn1a* was dependent on the induction of endogenous p53 transcripts (*Trp53*) in *Jmjd5*-deficient cells. qRT-PCR showed that there was no difference in the expression of *Trp53* in *Jmjd5$^{neo/neo}$* MEFs and *Jmjd5$^{Δ/Δ}$* embryos compared with control cells (Fig. 5C). Immunoblotting also indicated that there was no alteration in the level of p53 protein between wild-type and *Jmjd5$^{neo/neo}$* MEFs, despite the enrichment of Cdkn1a/p21 protein in *Jmjd5$^{neo/neo}$* MEFs (Fig. 5D). We next measured the transcriptional activity of p53 in wild-type and *Jmjd5$^{neo/neo}$* MEFs by luciferase assay. We constructed a *p53BS-tk*-luciferase reporter (p53BS) containing p53 response elements derived from human *CDKN1A* (El-Deiry et al, 1993), and transfected the reporter plasmid or control plasmid (tk-luc) with a *Renilla* luciferase reference plasmid into wild-type or *Jmjd5$^{neo/neo}$* MEFs. The result showed that there was no significant difference in the luciferase activity (n = 4, Fig.

5E), indicating that the alteration of p53 transcriptional activity did not occur in $Jmjd5^{neo/neo}$ MEFs.

We investigated whether the growth retardation caused by the loss of Jmjd5 was closely associated with the deregulation of *Cdkn1a* in the embryos. *Jmjd5*-deficient mice were intercrossed with *Cdkn1a*-deficient mice ($Cdkn1a^{\Delta/\Delta}$), which were morphologically normal at E 10.5 (5 of 5, Fig 6B; Deng et al., 1995), to generate *Jmjd5* and *Cdkn1a* double knock-out mice ($Jmjd5^{\Delta/\Delta}$; $Cdkn1a^{\Delta/\Delta}$). The phenotypes were compared between the double knock-out mice and *Jmjd5*-deficient mice. The $Jmjd5^{\Delta/\Delta}$; $Cdkn1a^{\Delta/\Delta}$ mice were embryonic lethal as well as $Jmjd5^{\Delta/\Delta}$ mice (data not shown), and showed the severe growth retardation (4 of 4, Fig. 6D) compared with wild-type embryos (Fig. 6A). However, the whole sizes of $Jmjd5^{\Delta/\Delta}$; $Cdkn1a^{\Delta/\Delta}$ embryos were slightly bigger than $Jmjd5^{\Delta/\Delta}$ littermates (Fig. 6C). Other abnormal phenotypes such as the incomplete turning and the angiogenesis defect seemed not to be rescued in the double knock-out embryos. These results suggested that the deficiency of *Cdkn1a* could partially rescue the phenotype of $Jmjd5^{\Delta/\Delta}$ embryos, similarly to our result from the knock-down experiment (Fig. 5B). We demonstrated that a genetic interaction between *Jmjd5* and *Cdkn1a* was at least important for embryonic cell proliferation but also proposed that other factors regulated by

Jmjd5 would contribute to normal embryonic cell growth.

Jmjd5 is involved in the maintenance of H3K36me2 in *Cdkn1a* locus.

Previous studies suggested that Jmjd5 was an H3K36me2 histone demethylase (Hsia et al., 2010), and that methylated K36 in histone H3 was a transcriptional active mark (Li et al., 2007).

Thus we assumed that the loss of Jmjd5 caused an elevation of H3K36me2 in *Cdkn1a* locus, resulting in the activation of *Cdkn1a* in *Jmjd5*-deficient cells. To test our hypothesis, we designed primers to amplify several genomic regions of *Cdkn1a* for chromatin immunoprecipitation (ChIP) analysis (Fig. 7A). The crosslinked chromatin from wild-type or *Jmjd5*[neo/neo] MEFs was immunopreciptated with anti-K36me2 or anti-Jmjd5 antibody. The precipitates were used for the detection of *Cdkn1a* genomic regions by qPCR. The enrichment of H3K36me2 was observed in intron II (regions a and b) and exon III (region c containing the open reading frames of *Cdkn1a*) but not intron III (region d) of *Cdkn1a* (n > 9, p < 0.01) in *Jmjd5*[neo/neo] MEFs (Fig. 7B). On the other hand, the recruitment of Jmjd5 into the *Cdkn1a* locus was significantly reduced in regions a, b and c, while no significant change was observed in region d (n > 9, p < 0.01, Fig. 7C). These results suggested that reduced recruitment of Jmjd5

into several regions of the *Cdkn1a* locus induced the increased H3K36me2 in the corresponding regions of *Cdkn1a*. Based on the previous report showing the correlation between enriched H3K36me2 and transcriptional active phase (Li et al., 2007), the increased H3K36me2 was thought to induce *Cdkn1a* expression in *Jmjd5^{neo/neo}* MEFs. We also performed ChIP analysis with anti-H3K4me3 antibody to investigate whether another epigenetic mark, H3K4me3, was altered in regions a, b and c of *Cdkn1a*. The result showed that the enrichment of H3K4me3 was observed only in region a, and that significant changes were not detected in the other regions (Fig. S5). Since H3K4me3 is a transcriptional active mark (Martin and Zhang, 2005), this alteration of H3K4me3 in Jmjd5-independent manner might reflect the enhanced transcriptional activation of *Cdkn1a* in *Jmjd5^{neo/neo}* MEFs. Taken together, our data demonstrates that *Cdkn1a* is one of the downstream targets for Jmjd5 histone demethylase, and the maintenance of H3K36me2 in *Cdkn1a* locus regulated by Jmjd5 may be required for normal embryonic proliferation.

Discussion

In this study, we generated *Jmjd5*-deficient mice and observed severe growth retardation in *Jmjd5*$^{\Delta/\Delta}$ embryos, leading to embryonic lethality around E 11.0. In *Jmjd5*$^{\Delta/\Delta}$ embryos, significant up-regulation of *Cdkn1a* was detected. Similarly, growth retardation as well as an elevated expression of *Cdkn1a* was observed in *Jmjd5*$^{neo/neo}$ MEFs. ChIP analysis indicated that the detection of H3K36me2 was significantly increased in several regions of the *Cdkn1a* locus derived from *Jmjd5*$^{neo/neo}$ MEFs, where the recruitment of endogenous Jmjd5 was reduced. Thus we demonstrate for the first time that Jmjd5 moderates the expression of *Cdkn1a* during embryogenesis through the histone demethylase activity.

Currently, there are more than 30 known mammalian JmjC domain-encoding genes, and some have been reported to be involved in cell proliferation by regulating the expression of cell cycle inhibitors. Our recent study demonstrated that Utx/KDM6A directly demethylated H3K27me3 in *Rb* and *Rbl2* increasing their expression levels, resulting in the growth repression of MEFs (Terashima et al., 2010). Jmjd3/KDM6B, an H3K27 demethylase, activated the *INK4A-ARF* locus in response to oncogenic stress, and the overexpression of Jmjd3 led to the inhibition of cell proliferation (Agger et al., 2009; Barradas et al., 2009). Furthermore,

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Fbxl10/KDM2b, an H3K36 demethylase, regulated cell proliferation through the modulation of

Cdkn2b (He et al., 2008). These results strongly suggest an essential role played by the JmjC

family in cell growth signaling. Our analysis with *Jmjd5*-deficient mice indicated a novel

association of *Jmjd5* and *Cdkn1a* expression, which is critical for cell proliferation during

normal mouse development.

Genome-wide profiling of methylated histone H3 from yeast to humans showed that

H3K36 methylation tended to be enriched across the transcribed regions of active genes rather

than promoter regions (Li et al., 2007). In *Saccharomyces cerevisiae*, methylation of histone H3

by Set2, an H3K36 KMT, is associated with transcriptional elongation by RNA polymerase II

(Krogan et al., 2003; Schaft et al., 2003). However, there is still not much evidence for the

physiological function of H3K36me2 in mammals. Recently, knock-down of *Fbxl10* in MEFs

was shown to induce the activation of *Cdkn2b* by the increased H3K36me2 in the *Cdkn2b* locus

(He et al., 2008). Our study demonstrated that the disruption of *Jmjd5* in mouse cells induced

the activation of *Cdkn1a* transcripts and the elevation of H3K36me2 at *Cdkn1a* transcribed

regions, supporting the current model between transcriptional activation and methylated

H3K36.

We observed the induced expression of *Cdkn1a* in *Jmjd5$^{\Delta/\Delta}$* embryos and *Jmjd5$^{neo/neo}$*

MEFs. Although *Cdkn1a* is a p53 target gene, we did not detect any significant changes of p53

expression at both the transcriptional and the translational levels. Reporter assay showed that

the transcriptional activity of p53 was not changed in *Jmjd5$^{neo/neo}$* MEFs, and

immunofluorescence and immunoprecipitation studies indicated no alteration of the subcellular

localization of p53 and no direct interaction between Jmjd5 and p53 (data not shown). These

results suggested that the aberrant induction of *Cdkn1a* seemed to occur without p53 induction.

However, we cannot exclude the possibility that p53 signaling is altered, since p53 protein

receives various post-translational modifications such as phosphorylation, methylation,

acetylation and ubiquitylation, which are related to its stability, the specificity of target genes

and the affinity to cofactors (Carter and Vousden, 2009). Therefore, more detailed analyses

would be necessary to prove whether there is a potential connection between Jmjd5 and p53 or

not.

In all of the *Jmjd5$^{\Delta/\Delta}$* embryos examined, severe growth retardation was observed,

and the expression of *Cdkn1a* was more than four-fold higher compared to wild-type embryos.

However, growth retardation is sometimes detected indirectly by abnormal placental

development in genetically modified mice (Han and Carter, 2001). In fact, endogenous *Jmjd5* expression was detected in ectoplacental cone, a precursor of placenta, at E 8.5. The allantois need to fuse with the chorion for normal placental development, but this was not observed in some *Jmjd5*$^{\Delta/\Delta}$ embryos at E 10.5 (3 of 8) (data not shown). Thus we could not exclude the possibility that abnormal placental development might be a reason for the growth defect in *Jmjd5*$^{\Delta/\Delta}$ embryos. However, it was notable that high expression of endogenous *Jmjd5* was observed in not only the ectoplacental cone but also the entire embryonic fraction. We also demonstrated that although embryonic cells purified from *Jmjd5*$^{neo/neo}$ embryos are not under placental control, these cells still showed the defect in proliferation and the elevated expression of *Cdkn1a*. Furthermore, knock-down of endogenous *Cdkn1a* was able to rescue the growth delay significantly in *Jmjd5*$^{neo/neo}$ MEFs. The growth retardation in *Jmjd5*$^{\Delta/\Delta}$ embryos was also partially rescued under the *Cdkn1a*$^{\Delta/\Delta}$ genetic background. These results indicated that the regulation of *Cdkn1a* by Jmjd5 played an important role in cell proliferation and embryonic development. Since the rescue effect was partial, it was also suggested that additional factors regulated by Jmjd5 would contribute to embryonic development. We are now trying to identify other target genes regulated by Jmjd5 by the experiments including microarray and

ChIP-sequence, and hope to demonstrate the detailed mechanism of Jmjd5 function in embryonic cell proliferation in the near future.

Similarly to the phenotype observed in *Jmjd5*$^{\Delta/\Delta}$ embryos, the up-regulation of endogenous *Cdkn1a* has been reported in several mutant mice, resulting in the severe growth retardation (Ruland et al., 2001; Rantakari et al., 2010; Suzuki et al., 1997). Notably, the elevation of *Cdkn1a* in *Brca1*- or *Mdm4*-deficient mice has been observed (Hakem et al., 1996; Migliorini et al., 2002), and importantly, intercrossing with *Cdkn1a*-deficient mice partially rescued the phenotype, suggesting that the aberrant expression of *Cdkn1a* is a major, but not exclusive, reason for the embryonic death (Hakem et al., 1997; Steinman et al., 2004). Interestingly, the phenotype of *Histone deacetylase 1* (*Hdac1*)–deficient mice was similar, although more severe, with *Jmjd5*$^{\Delta/\Delta}$ mice. Disruption of *Hdac1* resulted in embryonic lethality before E 10.5 due to the severe growth retardation, and the expression of *Cdkn1a* and *Cdkn1b* was up-regulated (Laggeret al., 2002). This result suggested that several epigenetic controls, including histone methylation and acetylation, were closely associated with normal expression of *Cdkn1a* during embryogenesis. Therefore, the clarification of the relationship between Jmjd5 and other histone modifying enzymes would be helpful for understanding the detailed

mechanism of *Cdkn1a* regulation by Jmjd5.

Acknowledgment

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Figure legends

Fig. 1. Generation of *Jmjd5*-deficient mice and the expression of *Jmjd5* during early embryogenesis. (A) Exon-intron structure of *Jmjd5* allele (*Jmjd5⁺*) and targeting strategy to generate *Jmjd5*-deficient mice. *Jmjd5⁺* allele contains eight exons (black boxes), and exon IV contains the 5' region of the JmjC domain (blue boxes). The targeting vector contains *PGK-Neo* cassette (white box), a pair of loxP sequences (white arrowheads) and FRT sequences (orange arrowheads). In *Jmjd5ⁿᵉᵒ* allele, the targeting vector is inserted in the *Jmjd5⁺* allele. The *PGK-Neo* cassette is removed by mating with *CAG-Flp* mice to generate the *Jmjd5ᶠˡᵒˣ* allele. *Jmjd5ᶠˡᵒˣ/ᶠˡᵒˣ* mice are mated with *Pgk2-Cre* mice to establish mutant mice with *Jmjd5* deletion (*Jmjd5ᐞ*). (B) Southern blotting of the tail DNA to distinguish wild-type and *Jmjd5⁺/ᐞ* mice. (C) Genotyping PCR using genomic DNA from E 10.5 littermates of *Jmjd5⁺/ᐞ* mice intercrosses. (D) qRT-PCR analysis of endogenous *Jmjd5* expression during embryogenesis using *Jmjd5*-specific primers. (E) WISH analysis of E 8.5 embryo with the sense (right) or the complementary anti-sense (left) probe for *Jmjd5*. Scale bars: 400 μm.

Fig. 2. The phenotypic observation of *Jmjd5ᐞ/ᐞ* embryos. (A-D) Phenotypes of control

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embryos from E 6.5 to E 10.5. (A'-D') Phenotypes of $Jmjd5^{\Delta/\Delta}$ embryos from E 6.5 to E 10.5.

Scale bars: 400 μm.

Fig. 3. $Jmjd5^{neo/neo}$ **hypomorphic MEFs proliferate more slowly than wild-type MEFs.** (A) Genotyping PCR using genomic DNA from $Jmjd5^{+/+}$ or $Jmjd5^{neo/neo}$ MEFs. (B) qRT-PCR analysis of $Jmjd5$ expression in $Jmjd5^{+/+}$(grey bar) or $Jmjd5^{neo/neo}$ MEFs (black bar). (C) Immunoblotting with anti-Jmjd5 antibody showed significant reduction of endogenous Jmjd5 protein in $Jmjd5^{neo/neo}$ MEFs. *non-specific band. (D) Growth curve for $Jmjd5^{+/+}$ (grey) or $Jmjd5^{neo/neo}$ MEFs (black). The mean ± SD from at least three experiments are shown, and the statistical difference is determined by Student's t-test. **$p < 0.01$. (E) Senescence-associated β-galactosidase assays for $Jmjd5^{+/+}$ (upper panel) or $Jmjd5^{neo/neo}$ MEFs (middle panel). As a positive control for the detection of senescent cells, sparsely seeded MEFs were stained (lower panel). Scale bars: 200 μm. (F) Immunofluorescence assay with anti-ssDNA antibody for $Jmjd5^{+/+}$ (upper panels) or $Jmjd5^{neo/neo}$ MEFs (middle panels). The cells were treated with anti-ssDNA antibody (left panels) and counterstained with DAPI (right panels). As a positive control, MEFs treated with adriamycin were stained (lower panels). Scale bars: 50 μm. (G) The

growth defect of *Jmjd5^{neo/neo}* MEFs was rescued by the re-expression of wild-type Jmjd5. The

Jmjd5^{neo/neo} or *Jmjd5^{+/+}* MEFs were infected with the retrovirus expressing either without insert

(pDON-5) or with wild-type Jmjd5 or H319A mutant. The cell number was counted at 48 hours

after infection. **$p < 0.01$.

Fig. 4. The expression of *Cdkn1a* is up-regulated in *Jmjd5*-deficient cells. (A-C) The

expression of *Cyclins* (A), *CDKs* (B) and *CDK* inhibitors (C) in *Jmjd5^{+/+}* MEFs (grey bar) and

Jmjd5^{neo/neo} MEFs (black bar) detected by qRT-PCR. (D) The expression of *Ccna1, Ccnd2, Cdk6,*

Cdk7, Cdkn1a and *Cdkn2a/p16* in *Jmjd5^{+/+}* embryos at E 8.25 (grey bar) and stage-matched

Jmjd5^{Δ/Δ} embryos (red bar). The mean ± SD from at least three experiments are shown. **$p <$

0.01. (E) WISH analysis of *Jmjd5^{+/+}* and *Jmjd5^{Δ/Δ}* embryo with the sense or the anti-sense probe

for *Cdkn1a*. Arrowheads show the primitive streak, and the arrow shows the ectopic expression

of *Cdkn1a* in the anterior half. Scale bars: 400 μm. (F) qRT-PCR analysis of *Jmjd5* (left panel)

and *Cdkn1a* (right panel) transcripts in MEFs after knock-down of *Jmjd5*. (-) shows uninfected

MEFs.

Fig. 5.   Jmjd5 intrinsically regulates the expression of *Cdkn1a* without the transcriptional activation of p53. (A) qRT-PCR analysis was used for the validation of *Cdkn1a*-specific siRNAs. (B) Comparative growth assay between control and *Cdkn1a* knock-down $Jmjd5^{neo/neo}$ MEFs. Each cell number for $Jmjd5^{+/+}$ (grey bar), $Jmjd5^{neo/neo}$ (black bar), control siRNA-treated (green bar) or *Cdkn1a* specific siRNAs-treated $Jmjd5^{neo/neo}$ MEFs (blue bar) was counted at 72 hours after passage. The mean ± SD from at least three experiments are shown. **$p < 0.01$. (C) qRT-PCR analysis for the detection of *Trp53* in MEFs (left) and E 8.25 embryos (right). (D) Immunoblotting with anti-p53 antibody (left panel) or anti-Cdkn1a/p21 (right panel). The lysate of adriamycin-treated wild-type MEFs was used as a positive control for the induction of p53 (a quarter amount of the samples) or p21 protein (the same amount). Anti-GAPDH antibody was used for loading controls. (E) Luciferase assay with *tk* promoter luciferase vector (tk-luc) or p53-responsive luciferase vector (p53BS) showed no significant change in wild-type (grey bar) and $Jmjd5^{neo/neo}$ MEFs (black bar).

Fig. 6.   Phenotypic analysis of $Jmjd5^{\Delta/\Delta}$; $Cdkn1a^{\Delta/\Delta}$ double knock-out embryos. $Jmjd5^{+/\Delta}$; $Cdkn1a^{+/\Delta}$ double heterozygous mice were intercrossed. (A) Wild-type embryos, (B)

Cdkn1a-deficient embryos, (C) *Jmjd5*-deficient embryos and (D) *Jmjd5$^{\Delta/\Delta}$*; *Cdkn1a$^{\Delta/\Delta}$* embryos

were observed at E 10.5. Scale bars: 500 μm.

Fig. 7.   Elevated level of H3K36me2 and decreased recruitment of Jmjd5 in *Cdkn1a* locus

from *Jmjd5$^{neo/neo}$* MEFs. (A) Schematic structure of the *Cdkn1a* locus and the distribution of

the amplified regions (a to d) for ChIP analysis. The coding region is indicated by black boxes.

(B) ChIP analysis with anti-H3K36me2 antibody to determine the sites of H3K36me2

enrichment in four regions at the *Cdkn1* locus in *Jmjd5$^{neo/neo}$* MEFs (black bar) compared with

wild-type MEFs (grey bar). (C) ChIP analysis with anti-Jmjd5 antibody to confirm the

recruitment of endogenous Jmjd5. The mean ± SD from at least nine experiments are shown.

**p < 0.01.