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The JMJD2A Demethylase Regulates Apoptosis and Proliferation in Colon Cancer Cells

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ABSTRACT
JMJD2A is a transcriptional cofactor and enzyme that catalyzes demethylation of histone H3 lysines 9 and 36 and is overexpressed in human tumors, but its role in oncogenesis remains unclear. Here, we show that JMJD2A interacts with the tumor suppressor p53 both in vitro and in HCT116 colon cancer cells. Chromatin immunoprecipitation assays demonstrated that JMJD2A was recruited together with p53 to the promoter of the p21 cell cycle inhibitor upon stimulation with the DNA damaging agent, adriamycin. Downregulation of JMJD2A resulted in increased expression of p21 and of the pro-apoptotic Puma protein, whereas levels of the anti-apoptotic Bcl-2 protein were decreased. Furthermore, JMJD2A knock-down led to reduced HCT116, DLD-1 and HT-29 colon cancer cell proliferation, while overexpression of JMJD2A enhanced HCT116 proliferation in low serum media. Finally, JMJD2A depletion induced apoptosis in HCT116 cells and this effect was less pronounced in the absence of p53. Collectively, these data indicate that JMJD2A is a novel promoter of colon cancer cell proliferation and survival, which mediates its effects in p53-dependent and -independent ways. JMJD2A may therefore be a valid target to sensitize tumor cells to chemotherapy-induced cell death and growth suppression. J. Cell. Biochem. 113: 1368–1376, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: COLON CANCER; GENE TRANSCRIPTION; HISTONE DEMETHYLASE; JMJD2A; p53 TUMOR SUPPRESSOR

The tumor suppressor p53 is a DNA-binding transcriptional regulator that plays a central role in tumor suppression by directing cellular responses to diverse stresses. It is subject to various post-translational modifications that may result into activation or repression of p53 activity [Murray-Zmijewski et al., 2008]. Recently, methylation of p53 on several lysine residues was found to affect its activity. For instance, monomethylation of lysine 372 stimulates p53 function by increasing its stability and promoting its nuclear localization. Interestingly, the methylation status on one particular lysine residue can result in opposite effects: while monomethylation of lysine 370 represses p53, dimethylated lysine 370 has an activating effect [Chuikov et al., 2004; Huang et al., 2006a, 2007; Shi et al., 2007].

Several enzymes that methylate p53 have been identified, but the opposing demethylases remain unknown with the exception of lysine-specific demethylase 1 (LSD1), which is capable of demethylating lysine 370 [Huang et al., 2007]. We wondered if members of the jumonji C domain-containing (JMJD) family of histone demethylases may target p53, which prompted us to study the relationship between p53 and JMJD2A, also known as histone demethylase 4A. JMJD2A is able to demethylate histone H3 lysine 9 (H3K9), H3K36 and H1.4K26 [Klose et al., 2006; Whetstine et al., 2006; Shin and Janknecht, 2007c; Trojer et al., 2009]. In particular, since trimethylated H3K9 is associated with heterochromatin and methylated H1.4K26 with repression of transcription, reversal of these marks by JMJD2A is predicted to stimulate gene transcription in general [Cloos et al., 2008]. Consistently, wild-type JMJD2A, but not a catalytically inactive mutant, is capable of efficiently stimulating androgen receptor-mediated transcription [Shin and Janknecht, 2007a].

Abnormal histone lysine methylation is observed in many different tumors and may contribute to the neoplastic transformation of cells [Chi et al., 2010]. Notably, JMJD2A gene expression is upregulated in human prostate tumors, suggesting that JMJD2A may exert tumor-promoting functions [Cloos et al., 2006]. Here, we have explored whether JMJD2A stimulates proliferation and survival of colon cancer cells in a p53-dependent manner.

MATERIALS AND METHODS

CELL CULTURE
Cells were grown in DMEM media supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 10% CO_2 [Rossow and Janknecht, 2001]. Where indicated, cells were treated with 1 μM adriamycin (diluted from a 4 mM stock solution dissolved in DMSO) or with DMSO alone for specified time periods.

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COIMMUNOPRECIPITATION

Human embryonal kidney 293T cells were transiently transfected by the calcium phosphate coprecipitation method [Kim et al., 2008] with indicated expression plasmids. Cells were lysed 36 h after transfection in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 0.25 mM Na_3VO_4, 0.2 mM DTT, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and immunoprecipitations performed with anti-Flag M2 (Sigma F3165) or anti-p53 DO-1 (Santa Cruz sc-126) monoclonal antibodies essentially as described before [Papoutsopoulou and Janknecht, 2000]. Coimmunoprecipitated proteins were then detected by Western blotting utilizing enhanced chemiluminescence [Goel and Janknecht, 2003]. Similarly, endogenous JMJD2A in HCT116 cells was immunoprecipitated with anti-JMJD2A antibodies (Bethyl A300-861A) and coprecipitated p53 revealed by immunoblotting with anti-p53 DO-1 antibodies.

GST PULL-DOWN ASSAY

Fusions of p53 amino acids to glutathione-*S* transferase (GST) were produced and purified according to standard procedures [Knebel et al., 2006]. GST-p53 fusion proteins were then bound to glutathione agarose [Goel and Janknecht, 2004], incubated with an extract [Janknecht, 2003] of 293T cells transfected with Flag-tagged JMJD2A, and bound JMJD2A revealed by anti-Flag immunoblotting [Mooney et al., 2010b].

ChIP ASSAY

Chromatin immunoprecipitation (ChIP) assays were performed as described [Goueli and Janknecht, 2003]. Briefly, HCT116 cells were cross-linked with 1% formaldehyde for 10 min and the reaction stopped by the addition of 100 mM glycine. Cells were then sonicated and DNA-protein adducts immunoprecipitated with indicated antibodies. After a series of washings, DNA-protein cross-links were reversed and DNA extracted for subsequent PCR [Shin et al., 2009]. The following primers were utilized to amplify a 220 bp Puma promoter fragment: 5′-GCGAGACTGTGGCCTTGTGT-3′ and 5′-GGCCTAGCCCAAGGCAAGGA-3′. For amplification of a 218 bp p21 promoter fragment, the following primers were employed: 5′-GTGGCTCTGATTGGCTTTCTG-3′ and 5′-CCAGCCCT-GTCGCAAGGATC-3′. Amplified DNA fragments were visualized on ethidium bromide-stained agarose gels [Shin and Janknecht, 2007b].

LUCIFERASE ASSAY

293T cells were grown to 25% confluence in 12-wells coated with poly-L-lysine [Kim et al., 2010] and then transiently transfected by the calcium phosphate coprecipitation method [Goueli and Janknecht, 2004]. Two-hundred nanograms luciferase reporter plasmid (−2324 to +100 of human p21 promoter cloned into pGL2-Basic), 50 ng pcDNA3, or pcDNA3-p53, and 300 ng Flag-JMJD2 expression vectors or empty vector pEV3S were employed together with 1.8 μg Bluescript KS^+ as carrier DNA. Cells were lysed 36 h after transfection [Dowdy et al., 2003] and luciferase activity was measured in a Berthold LB9507 luminometer [Wu and Janknecht, 2002].

KNOCK-DOWN

To downregulate JMJD2A, shRNAs were cloned into the retroviral expression vector pSIREN-RetroQ (Clontech). The following sequences within the human JMJD2A open reading frame were targeted: GATAGCCAATAGCGATAAG (#2) and GTTGAG-GATGGTCTTACCT (#3). To generate respective retrovirus, 293T cells were cotransfected with the shRNA vectors and necessary packaging plasmids [Shin et al., 2007]. Supernatants were collected 48 h and 72 h after transfection, passed through a 0.45 μm filter, and concentrated by precipitation with poly(ethylene glycol)-8000. Colon cancer cells were then infected thrice with virus during a 24 h timespan in the presence of 5 μg/ml polybrene. Cells were subsequently selected for 2 days in 1 μg/ml puromycin. Then, cell extracts were prepared by boiling in Laemmli buffer and probed on Western blots [Bosc et al., 2001] with antibodies against JMJD2A (Bethyl A300-861A), p53 (Santa Cruz sc-126), p21 (Santa Cruz sc-756), Puma (Upstate 07-669), Bcl-2 (Abcam ab15182), $H3K9me_3$ (Upstate 07-442), $H3K9me_2$ (Upstate 07-441), $H3K9me_1$ (Upstate 07-450), histone H3 (Santa Cruz sc-10809) or actin (Sigma A2066).

INDUCIBLE JMJD2A HCT116 CELLS

A Flag-tagged JMJD2A cDNA fragment was amplified using Flag-JMJD2A [Shin and Janknecht, 2007a] as a template with the following primers: 5′-GCCACCATGGCTTCGTGGGGT-3′ and 5′-CTACTCCATGGCCCG-3′. Taq polymerase was added to generate 3′-terminal A overhangs. The PCR product was cloned into pcr8-GW-TopoD with the help of topoisomerase. The resultant pENTR-Flag-JMJD2A vector was verified by DNA sequencing and the insert transferred into pLenti CMVtight Puro DEST (kind gift from Dr. Eric Campeau) using Clonase II (Invitrogen) to generate pLenti CMVtight Flag-JMJD2A. HCT116 cells were infected with lentivirus derived from pLenti CMV rtTA3 Blast (generous gift from Dr. Eric Campeau) and selected for 10 days with 5 μg/ml blasticidin. Pooled clones were then infected with lentivirus derived from pLenti CMVtight Flag-JMJD2A and selected with 2.5 μg/ml puromycin for 6 days. Cells were then incubated in DMEM supplemented with 10% Tet-system approved fetal bovine serum (Clontech) without or with 0.5 μg/ml doxycycline (DOX). 72 h later, cells were analyzed as described in the text.

CELL PROLIFERATION ASSAYS

Cells were seeded in 96-well plates (2,000 cells/well) for MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay and into 6-cm dishes (50,000 cells/dish) for counting. The TACS MTT kit (Trevigen) was utilized according to the manufacturer's instructions. For counting, cells were harvested, stained with trypan blue and live cells totaled in an automated cell counter (Countess; Invitrogen). Results from triplicate experiments (average with standard error) are shown.

FLOW CYTOMETRY

Cells were grown in 6-cm dishes, harvested, washed in phosphate-buffered saline, fixed in 63% ethanol overnight at 4°C, and then incubated with 40 μg/ml propidium iodide in the presence of RNase A (50 μg/ml) for 30 min at 37°C [Mooney et al., 2010a]. Cells were then injected into a FACSCalibur (BD Biosciences) and cell cycle

profiles analyzed with Cell Quest (BD Biosciences). 20,000 events per sample were acquired for data analysis. Averages (with standard errors) of triplicate experiments are presented.

RESULTS

INTERACTION OF p53 WITH JMJD2A

In our quest to identify enzymes other than LSD1 that demethylate p53, we evaluated several JMJD demethylases, including JMJD2A. Unfortunately, we did not find evidence that JMJD2A can demethylate p53 peptides in vitro or suppress p53 methylation in vivo (not shown). However, we also analyzed during these efforts if JMJD2A may form complexes with p53. To this end, we coexpressed Flag-tagged JMJD proteins with or without p53 in human 293T cells, immunoprecipitated p53 and probed whether JMJD proteins would coprecipitate. We observed that JMJD2A, but not two other JMJD proteins (JMJD1A and HSPBAP1) formed complexes with p53 (Fig. 1A). Please note that the low amount of endogenous p53 in 293T cells was not sufficient to coimmunoprecipitate JMJD2A, possibly because these low amounts of p53 are sequestered by the viral SV40 large T antigen in 293T cells. Next, we performed reverse-order coimmunoprecipitation experiments and confirmed that p53 is indeed in a complex with JMJD2A (Fig. 1B). In addition, the homologous JMJD2C protein also interacted with p53, whereas JMJD1A again did not (Fig. 1B). Finally, we employed p53-positive human HCT116 colon cancer cells to assess if endogenous p53 binds to endogenous JMJD2A. This is the case, since JMJD2A antibodies, but not Rcl control antibodies immunoprecipitated p53 (Fig. 1C). Altogether, these results demonstrate that JMJD2A interacts with p53 in vivo.

Next, we wished to define domains in JMJD2A and p53 that mediate their interaction. To this end, we split JMJD2A into three parts and explored which of them would still coimmunoprecipitate p53. Whereas the N-terminal 350 amino acids, which encompass the catalytic domain of JMJD2A, did not interact with p53, both JMJD2A amino acids 301–703 and 704–1064 formed complexes with p53 (Fig. 2A). Similarly, we observed that the middle and C-terminal portions of JMJD2C interacted with p53 (Fig. 2A).

Conversely, we divided p53 into three parts, produced and purified respective GST fusion proteins and assessed whether JMJD2A would bind any of those in vitro. In addition to full-length p53, the middle portion of p53 encompassing its DNA-binding domain was capable of binding to both JMJD2A and JMJD2C in vitro (Fig. 2B, top panels). Equal amounts of GST fusion proteins were utilized as evidenced by a respective Coomassie-stained protein gel (Fig. 2B, bottom panel). In conclusion, JMJD2A may utilize two regions to bind to the DNA-binding domain of p53, although it remains to be determined whether JMJD2A amino acids 301–703 and 703–1064 target the same or different epitopes of the p53 DNA-binding domain.

JMJD2A MODULATES p53-DEPENDENT TRANSCRIPTION

The fact that JMJD2A forms complexes with p53 implicates that JMJD2A regulates p53 transcriptional activity. Two prominent target genes of p53 are the cell cycle inhibitor p21 and the pro-

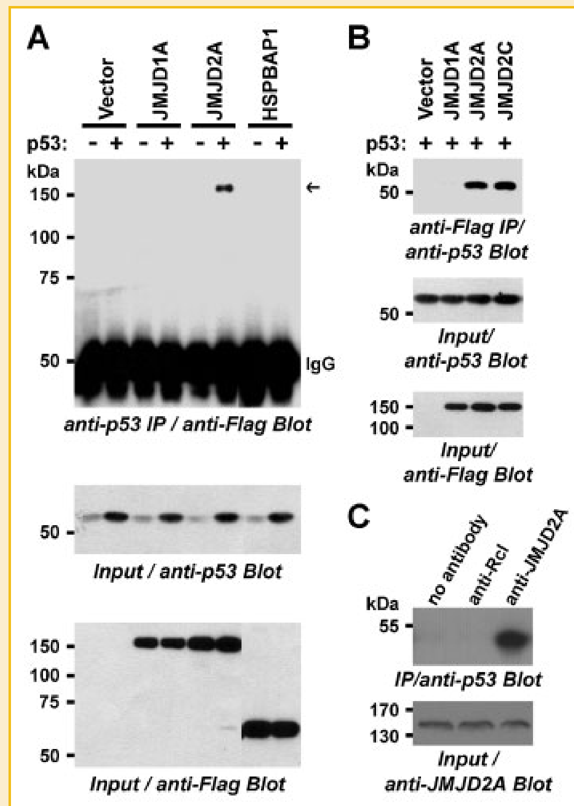

Fig. 1. Complex formation between JMJD2A and p53. A: Coimmunoprecipitation of JMJD2A with p53. Flag-tagged JMJD proteins (JMJD1A, JMJD2A and HSPBAP1) were expressed with or without p53 in 293T cells. After immunoprecipitation with p53 antibodies, coprecipitated JMJD proteins were revealed by anti-Flag Western blotting (top panel). The bottom panels show input levels of p53 and Flag-tagged proteins, respectively. B: Similarly, Flag-tagged JMJD proteins were coexpressed with p53 in 293T cells, precipitated with anti-Flag antibodies and coprecipitated p53 revealed by immunoblotting. C: Complex formation of endogenous JMJD2A and p53 in HCT116 colon cancer cells. Cell extracts were subjected to immunoprecipitation with indicated antibodies and coprecipitated p53 revealed by immunoblotting (top panel). The bottom panel is a loading control showing equal levels of JMJD2A in all lanes.

apoptotic protein Puma [Riley et al., 2008]. Thus, we interrogated whether JMJD2A would bind to the p21 and Puma promoters and if this binding would be enhanced upon treating cells with adriamycin, a DNA damaging agent that induces p53 activity. As expected, p53 binding to both the p21 and Puma promoter was enhanced upon treatment with adriamycin (Fig. 3A). In contrast, only binding of JMJD2A to the p21 promoter was increased upon adriamycin treatment. Consistent with enhanced binding of the histone demethylase JMJD2A, trimethylated H3K9 was slightly reduced upon adriamycin treatment at the p21 promoter; also consistently, H3K9 trimethylation was unaffected at the Puma promoter that showed no adriamycin-inducible binding of JMJD2A (Fig. 3A).

To explore whether JMJD2A activates or represses p53-dependent transcription, we employed a p21 promoter luciferase construct. As expected, cotransfection of p53 stimulated this reporter construct (Fig. 3B). This p53-mediated stimulation was

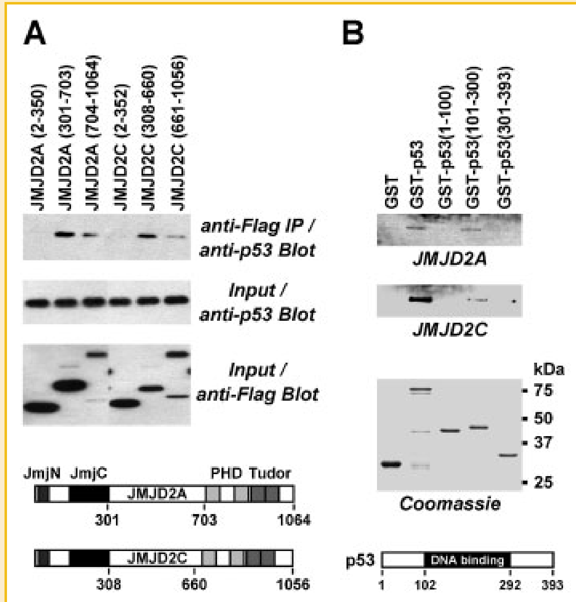

Fig. 2. Protein domains involved in JMJD2A-p53 binding. A: Flag-tagged fragments of JMJD2A and JMJD2C were coexpressed with p53 in 293T cells. After anti-Flag immunoprecipitation, coprecipitated p53 was revealed by immunoblotting (top panel). The bottom panels show input levels of p53 and Flag-tagged proteins, respectively. Schemes of JMJD2A and JMJD2C with known protein domains are depicted underneath. B: Full-length p53 or fragments thereof were fused to GST and bound to glutathione agarose. Binding of Flag-tagged JMJD2A or JMJD2C to p53 amino acids was assessed by anti-Flag immunoblotting (top panels). The bottom panel shows a Coomassie-stained protein gel indicating that comparable amounts of GST fusion proteins were employed.

blunted by overexpression of JMJD2A or JMJD2C (Fig. 3B), suggesting that JMJD2A and JMJD2C inhibit p53 function.

To further validate this conclusion, we downregulated JMJD2A in HCT116 cells with two different shRNAs. Both shRNAs caused reduction of endogenous JMJD2A levels (Fig. 4). Notably, JMJD2A depletion led to enhanced global levels of trimethylated H3K9 (Fig. 4A), consistent with JMJD2A being able to especially demethylate trimethylated H3K9 [Klose and Zhang, 2007; Cloos et al., 2008]. In contrast, global levels of di- and monomethylated H3K9 were essentially unaffected.

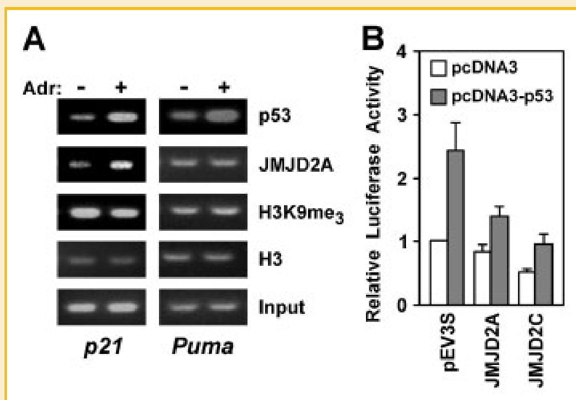

Fig. 3. JMJD2A interaction with the p21 promoter. A: ChIP assays were performed on HCT116 cells treated with or without adriamycin (Adr) for 12 h. Shown is the binding of p53, JMJD2A, trimethylated H3K9 and histone H3 to p21 or Puma promoter DNA encompassing a p53 binding site. B: Activity of a p21 luciferase reporter construct in 293T cells upon ectopic expression of p53 and JMJD2A (or JMJD2C).

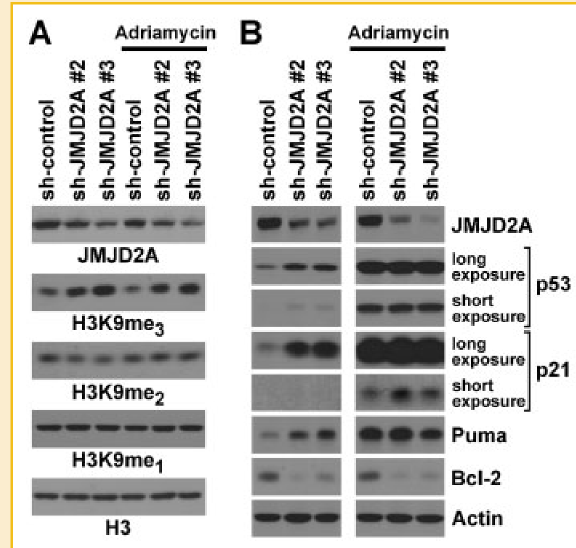

Fig. 4. Regulation of endogenous p53 target genes by JMJD2A. A: Down-regulation of JMJD2A with two different shRNAs in HCT116 cells treated with or without adriamycin for 72 h increases global trimethylated H3K9 levels. Shown are Western blots of cell extracts. B: Analogous, Western blots revealing indicated proteins after JMJD2A knock-down in HCT116 cells.

Then, we assessed by Western blotting how JMJD2A would affect expression of p21. In the absence of adriamycin, JMJD2A depletion led to strongly enhanced levels of p21 (Fig. 4B). However, JMJD2A had less effect on p21 expression upon adriamycin treatment. Together with our p21 luciferase reporter results (Fig. 3B), these data indicate that JMJD2A is a repressor of p53-dependent p21 transcription.

Similarly, Puma levels were moderately increased upon JMJD2A depletion in the absence of adriamycin, but no change was observed in the presence of adriamycin (Fig. 4B). Interestingly, a similar effect on p53 expression itself was observable. On the other hand, transcription of the survival molecule Bcl-2 is, directly or indirectly, suppressed by p53 [Miyashita et al., 1994; Budhram-Mahadeo et al., 1999]. We found that Bcl-2 expression is reduced upon JMJD2A depletion (Fig. 4B), and surprisingly to a similar extent both in the presence and absence of adriamycin. This result may suggest that JMJD2A might also be able to incapacitate the repressive activity of p53 on gene transcription.

JMJD2A STIMULATES COLON CANCER CELL GROWTH

Since JMJD2A modulates p53-dependent transcription and appears to repress especially the expression of the cell cycle inhibitor p21, we evaluated whether JMJD2A could affect HCT116 cell proliferation. As shown above, we downregulated JMJD2A expression with two different shRNAs and then monitored cell growth with the help of an

MTT assay over 72 h. Both JMJD2A shRNAs reduced cell growth compared to control shRNA (Fig. 5A). Treatment of HCT116 cells with adriamycin resulted in severe impairment of cell growth, and this was even more so upon JMJD2A depletion (Fig. 5A). To confirm these data, we also counted cells. Downregulation of JMJD2A caused decreased cell numbers in the absence and presence of adriamycin (Fig. 5B), clearly showing that JMJD2A normally stimulates HCT116 cell proliferation. Furthermore, we downregulated JMJD2A in two other human colon cancer cell lines, DLD-1 and HT-29, both of which are mutated in p53 rendering it inactive. Again, we observed that JMJD2A downregulation significantly reduced cell proliferation (Fig. 5C,D). Since DLD-1 and HT-29 cells do not possess functional p53, this shows that JMJD2A can affect colon cancer cell proliferation in a p53-independent manner.

Conversely, we investigated if JMJD2A overexpression would promote cell proliferation. To this end, we generated HCT116 cells that stably express DOX-inducible JMJD2A. DOX addition led to a more than fourfold increase of JMJD2A levels, but had no impact on cell proliferation in media containing 10% fetal bovine serum (Fig. 5E). We reasoned that the cornucopia of growth factors present in 10% serum is maximally stimulating cell proliferation, so that

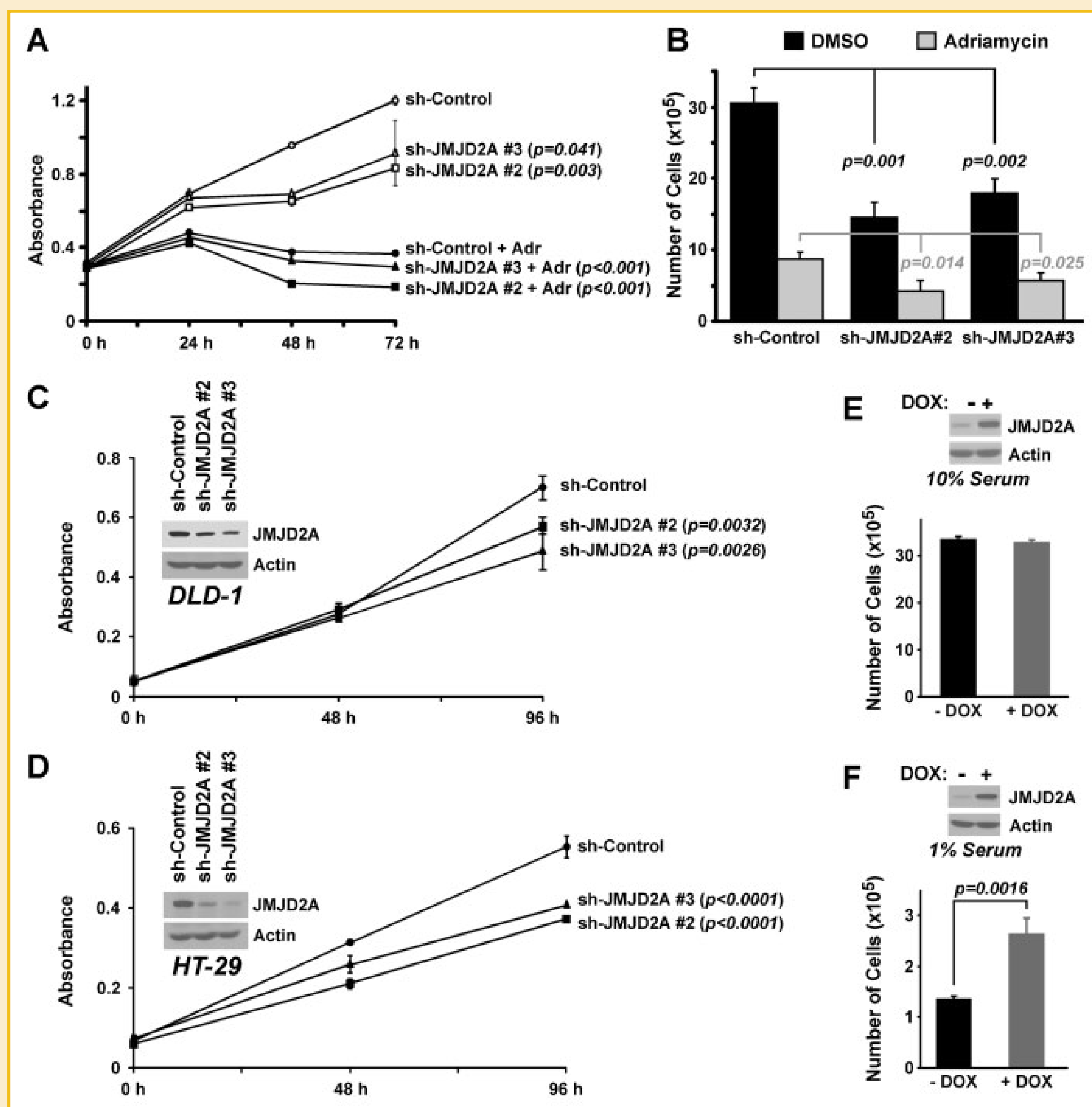

Fig. 5. Stimulation of cell proliferation by JMJD2A. HCT116 cells expressing control or two different JMJD2A shRNAs were treated with or without adriamycin (Adr). Cell proliferation was monitored utilizing an MTT assay (A) or by cell counting after 72 h (B). Statistical significance (unpaired, two-tailed t-test) of differences in growth of sh-JMJD2A#2 or #3 compared to sh-Control after 72 h DMSO treatment is indicated. Similarly, the statistical significance of different growth between sh-Control and sh-JMJD2A#2 or #3 in the presence of adriamycin is indicated. C: Cell proliferation monitored with MTT assay in DLD-1 colon cancer cells upon JMJD2A downregulation. The inset shows levels of JMJD2A and actin at 48 h as determined by Western blotting. D: The same in HT-29 colon cancer cells. E: HCT116 cells were engineered to overexpress JMJD2A upon DOX induction. 72 h thereafter, JMJD2A levels were determined by Western blotting (inset) and the number of cells counted. Cells were grown during these 72 h in media containing 10% fetal bovine serum. F: The same, but cells were grown in media supplemented with only 1% fetal bovine serum.

additional JMJD2A would have no effect. Thus, we performed the same experiment in media containing 1% serum and therefore very low levels of growth factors causing slower cell proliferation. Under this condition, overexpression of JMJD2A did stimulate cell proliferation (Fig. 5F), further proving that JMJD2A is a pro-proliferative protein in HCT116 cells.

JMJD2A AFFECTS CELL CYCLING AND PROTECTS FROM APOPTOSIS

The fact that JMJD2A stimulates cell proliferation suggested that it may influence the cell cycle. Therefore, we performed flow cytometry and determined how JMJD2A affects cell cycle distribution. There were no considerable changes in G1/G0 and S phase distribution, but the G2/M proportion of HCT116 cells was roughly twofold increased upon JMJD2A downregulation (Fig. 6A, top panel). We also utilized matching $p53^{-/-}$ HCT116 cells [Bunz et al., 1998], but did not detect any difference compared to wild-type HCT116 cells (Fig. 6A, bottom panel).

However, when cells were treated with adriamycin that induces a G2/M cell cycle arrest, JMJD2A depletion led to a significantly reduced G2/M arrest and more S phase in wild-type HCT116 cells (Fig. 6A, top panel). In $p53^{-/-}$ HCT116 cells, adriamycin not only led to more G2/M arrest, but also to an increase of cells in S phase and a drastic reduction of cells in G1/G0. Similar to wild-type cells, JMJD2A shRNAs caused an increase of S phase cell number and a decrease of G2/M arrest upon adriamycin treatment in $p53^{-/-}$ HCT116 cells; in addition, there were considerably more cells in G1/G0 compared to control shRNA (Fig. 6A, bottom panel). These data indicate that JMJD2A affects the cell cycle in both $p53^{+/+}$ and $p53^{-/-}$ cells. Since changes induced by JMJD2A depletion were

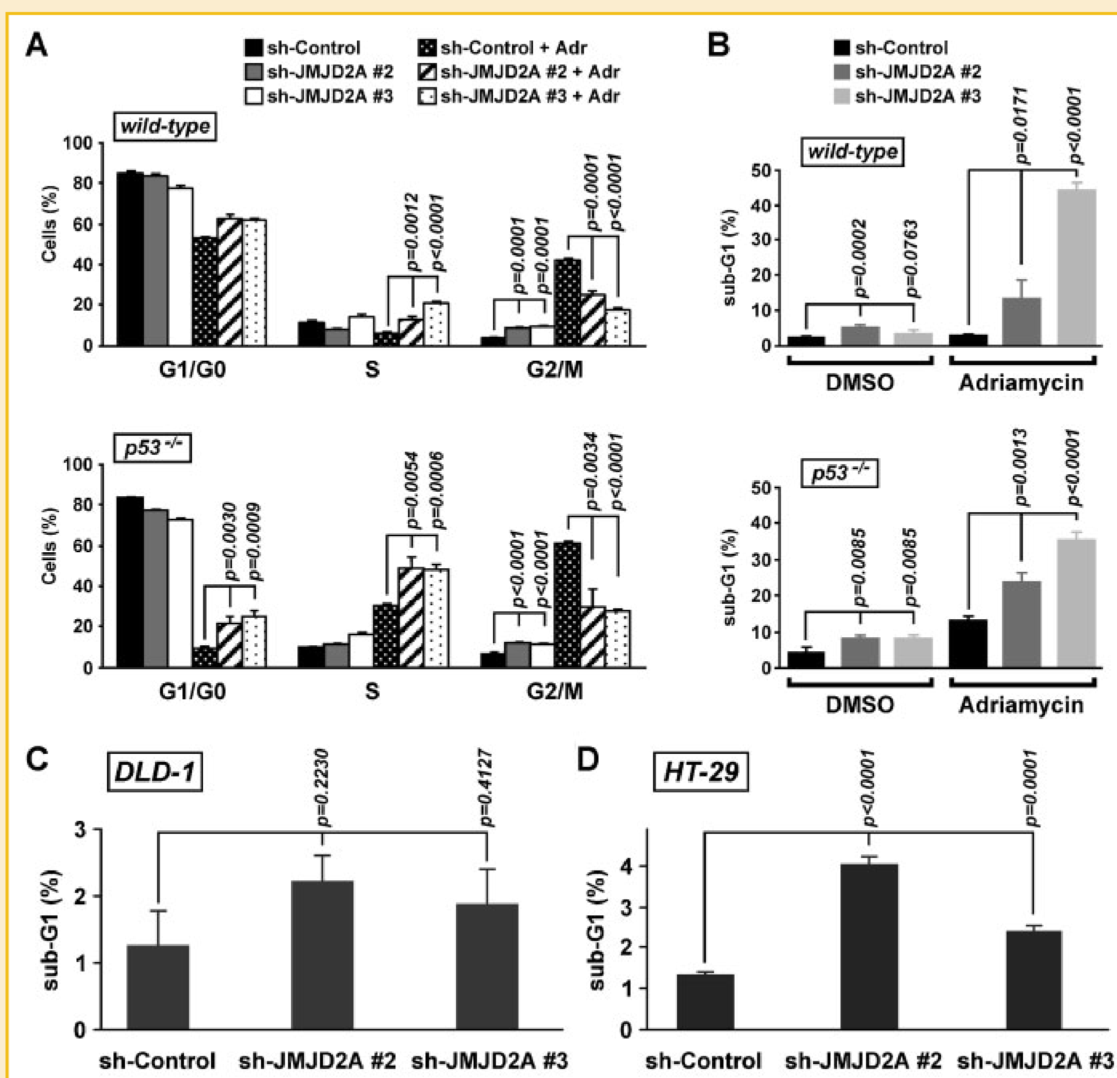

Fig. 6. Impact of JMJD2A on the cell cycle and apoptosis. A: HCT116 cells (wild-type or $p53^{-/-}$) that expressed control or JMJD2A shRNA were treated with or without adriamycin (Adr) for 72 h. Cell cycle profiles were then determined by flow cytometry. Statistical significance (unpaired, two-tailed *t*-test) is indicated. B: Analogous, levels of HCT116 cells with a sub-G1 DNA content were measured by flow cytometry. C: Levels of DLD-1 colon cancer cells with sub-G1 DNA content upon knock-down of JMJD2A after 72 h in the absence of adriamycin. D: The same in HT-29 colon cancer cells.

largely the same in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells, JMJD2A exerts its effects on cell cycling mainly in a p53-independent manner.

Another parameter that was determined by flow cytometry were cells that have a sub-G1 DNA content, which is indicative of cell death. Compared to cells containing control shRNA, both wild-type and $p53^{-/-}$ HCT116 cells treated with JMJD2A shRNAs had a roughly twofold enhanced level of sub-G1 cells (Fig. 6B). Furthermore, adriamycin treatment of wild-type HCT116 cells drastically enhanced this apoptotic effect of JMJD2A shRNAs (Fig. 6B, top panel). In $p53^{-/-}$ HCT116 cells, adriamycin led to more cell death than in wild-type cells, which would be consistent with the notion that p53 arrests cells upon DNA damage and thereby provides them time to repair the damage rather than to undergo apoptosis. But again, JMJD2A shRNAs significantly increased the level of sub-G1 cells in $p53^{-/-}$ cells (Fig. 6B, bottom panel), although this increase (1.8- to 2.6-fold) was less pronounced than in wild-type HCT116 cells (4.4- to 14.2-fold). Finally, we corroborated that JMJD2A also affects survival in cell lines other than HCT116. There was a trend for JMJD2A depletion resulting in more apoptosis in DLD-1 colon cancer cells (Fig. 6C) and a statistically significant enhancement of apoptosis in HT-29 colon cancer cells upon JMJD2A knock-down (Fig. 6D). Taken together, these results indicate that JMJD2A protects cells from apoptosis, in particular upon DNA damage.

DISCUSSION

The tumor suppressor p53 is mutated in many tumors and even haploinsufficiency causes cancer, indicating that p53 activity has to be tightly controlled to safeguard against neoplastic transformation [Rivlin et al., 2011]. Despite three decades of intense research, the regulation of p53 activity is still not fully understood. This study has identified a novel p53 interaction partner and regulator, the JMJD2A histone demethylase, thereby furthering our knowledge about the pivotal p53 tumor suppressor.

Our data show that JMJD2A forms complexes with p53 in vivo and binds to it in vitro. The N-terminus of JMJD2A, which contains its catalytic jmjC domain and the jmjN domain that modulates jmjC function [Shi and Whetstine, 2007], is not required for this interaction with p53. But JMJD2A amino acids 301–703 and amino acids 704–1064 are individually capable of binding to p53. Whereas the former amino acids do not contain known protein motifs, the C-terminal JMJD2A amino acids contain double PHD and Tudor domains that are implicated in binding to methylated histones; in particular, the Tudor domains of JMJD2A interact with trimethylated H3K4 and H4K20 [Huang et al., 2006b; Lee et al., 2008]. It remains to be studied if the binding to p53 would interfere with the ability of the JMJD2A Tudor domains to recognize trimethylated histone lysine residues.

Similar to JMJD2A, the homologous JMJD2C protein interacted with p53 in vitro and in vivo. Another close homolog of JMJD2A is JMJD2B [Cloos et al., 2008], and we predict that it likewise interacts with p53. However, JMJD2B has much lower enzymatic activity than JMJD2A and JMJD2C and also seems to be more associated with heterochromatin [Fodor et al., 2006; Whetstine et al., 2006]. Thus, it is conceivable that JMJD2B would not, or differently, modulate p53 compared to JMJD2A and JMJD2C.

The tumor suppressor p53 is upregulated upon DNA damage [Kruse and Gu, 2009] and accordingly adriamycin treatment led to enhanced binding of p53 to its target genes, the p21 cell cycle inhibitor and the pro-apoptotic Puma protein. Interestingly, adriamycin caused increased recruitment of JMJD2A to the p21 promoter, but not to the Puma promoter, suggesting that JMJD2A may differentially affect p53 target genes. Consistently, we observed that trimethylation of H3K9 was reduced at the p21, but not at the Puma promoter upon adriamycin treatment. Both p21 luciferase reporter assays as well as JMJD2A knock-down experiments indicated that JMJD2A represses p21 expression, suggesting that JMJD2A is inhibiting p53-mediated p21 transcription. This is in contrast to JMJD2A's role as a coactivator of the androgen receptor [Shin and Janknecht, 2007a], but in line with previous reports showing that JMJD2A represses transcription of E2F-regulated genes and the achaete scute-like homolog 2 gene [Gray et al., 2005; Zhang et al., 2005]. Thus, JMJD2A may act either as a corepressor or coactivator, which might depend on which transcription factor recruits JMJD2A to gene regulatory elements.

Interestingly, knock-down of JMJD2A increased p53 levels roughly twofold in the absence of adriamycin. This may explain how JMJD2A can indirectly regulate p53 target genes by decreasing p53 protein levels. In fact, this may account for how JMJD2A depletion increases levels of the pro-apoptotic Puma protein, whose expression was much less responsive to JMJD2A depletion compared to p21 and whose gene promoter did not show enhanced JMJD2A binding upon adriamycin treatment.

Bcl-2 is a pro-survival protein, whose transcription can be directly suppressed by p53 [Miyashita et al., 1994; Budhram-Mahadeo et al., 1999]. The fact that JMJD2A depletion caused reduced Bcl-2 levels suggests that JMJD2A can decrease the ability of p53 to repress Bcl-2 transcription. If so, JMJD2A would not only negatively interfere with the ability of p53 to activate transcription as in case of the p21 promoter, but also with p53's potency to repress promoter activity. Regardless, the fact that JMJD2A represses p21 and Puma expression and stimulates that of Bcl-2 strongly suggests that JMJD2A is a pro-proliferative and pro-survival factor.

Indeed, depletion of JMJD2A led to reduced proliferation of three different colon cancer cell lines, furthering the notion that JMJD2A is a pro-proliferative molecule in colon cancer cells. However, this appears to be a cell type-specific effect, since JMJD2A down-regulation or overexpression in other cell lines (293T, HeLa) did not affect proliferation [Black et al., 2010]. Furthermore, JMJD2A overexpression in HCT116 cells led to a growth advantage in media containing 1% serum, but not in 10% serum. Therefore, JMJD2A overexpression may especially be advantageous in tumors, since they are often surrounded by stroma and extracellular matrix that limit diffusion of growth factors thus resembling a low serum environment.

The pro-proliferative effect of JMJD2A in colon cancer cells is consistent with the view that JMJD2A promotes tumorigenesis. Similarly, it was shown that JMJD2B and JMJD2C stimulate growth and transformation of breast cells [Liu et al., 2009; Yang et al., 2010] and overexpression of JMJD2B and JMJD2C may also contribute to

the development of medulloblastomas [Northcott et al., 2009]. Thus, JMJD2 proteins possess features of oncoproteins and may even be such proteins, but animal models are needed to test this hypothesis in the future.

Adriamycin induces p53 activity and thereby p21 expression, which should lead to a G1 arrest of cells [Kruse and Gu, 2009]. However, it was reported before that adriamycin instead causes a predominant G2/M arrest [Siu et al., 1999; Bilim et al., 2000] and we consistently observed that the percentage of HCT116 cells in G2/M drastically increased upon adriamycin treatment. This increase was reduced upon JMJD2A knock-down both in wild-type and $p53^{-/-}$ HCT116 cells, indicating that JMJD2A helps to arrest the cell cycle at G2/M in a p53-independent manner. In addition, JMJD2A depletion furthered HCT116 and HT-29 cell death. In case of HCT116 cells, lack of p53 led to a less pronounced effect of JMJD2A knock-down upon adriamycin treatment, indicating that JMJD2A affects apoptosis in a p53-dependent and -independent manner. Similar to our results, downregulation of the only JMJD2A homolog in *Caenorhabditis elegans* led to more apoptosis in a p53-dependent manner [Whetstine et al., 2006; Black et al., 2010]. Thus, JMJD2A is not only required for maximal proliferation of colon cancer cells, but can also promote their survival.

In conclusion, the ability of JMJD2A to repress the tumor suppressor p53 and to stimulate colon cancer cell proliferation and survival strongly indicates that JMJD2A may play an important role during tumorigenesis. Accordingly, its inhibition, potentially by small molecule drugs targeting its catalytic center, could be useful in cancer therapy. In particular, JMJD2A inhibition may sensitize cells to chemotherapeutics such as adriamycin that induce cell death and suppress tumor growth.

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