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Regulation of c-Myc expression by the histone demethylase JMJD1A is essential for prostate cancer cell growth and survival

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Abstract

The histone demethylase JMJD1A, which controls gene expression by epigenetic regulation of H3K9 methylation marks, functions in diverse activities, including spermatogenesis, metabolism, and stem cell self-renewal and differentiation. Here, we found that JMJD1A knockdown in prostate cancer cells antagonizes their proliferation and survival. Profiling array analyses revealed that JMJD1A-dependent genes function in cellular growth, proliferation and survival, and implicated that the c-Myc transcriptional network is de-regulated following JMJD1A inhibition. Biochemical analyses confirmed that JMJD1A enhances c-Myc transcriptional activity by upregulating c-Myc expression levels. Mechanistically, JMJD1A activity promoted recruitment of androgen receptor (AR) to the c-Myc gene enhancer and induced H3K9 demethylation, increasing AR-dependent transcription of c-Myc mRNA. In parallel, we found that JMJD1A regulated c-Myc stability, likely by inhibiting HUWE1, an E3 ubiquitin ligase known to target degradation of several substrates including c-Myc. JMJD1A (wild-type or mutant lacking histone demethylase activity) bound to HUWE1, attenuated HUWE1-dependent ubiquitination and subsequent degradation of c-Myc, increasing c-Myc protein levels. Furthermore, c-Myc knockdown in prostate cancer cells phenocopied effects of JMJD1A knockdown, and c-Myc re-expression in JMJD1A-knockdown cells partially rescued prostate cancer cell growth in vitro and in vivo. c-Myc protein levels were positively correlated with those of JMJD1A in a subset of human prostate cancer specimens. Collectively, our findings identify a critical role for JMJD1A in regulating

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proliferation and survival of prostate cancer cells by controlling c-Myc expression at transcriptional and post-translational levels.

Introduction

Histone methylation is an important epigenetic modification that determines whether a gene is transcriptionally active or inactive. Both histone methylation and demethylation are dynamically regulated by respective methyl transferases and demethylases. Methylation of histone 3 Lysine-9 (H3K9) is a repressive histone mark associated with transcriptional inactivation. JMJD1A (also known as KDM3A or JHDM2A) is a histone demethylase that removes mono- and di-methyl groups from H3K9 (specifically, from H3K9me1 or H3K9me2), enabling transcriptional activation ([1–4]). Epigenetic regulation by JMJD1A reportedly functions in biological processes as diverse as spermatogenesis, metabolism, sex determination, stem cell self-renewal and differentiation ([3–6]).

Prostate cancer is the most commonly diagnosed malignancy and second leading cause of cancer death in American men ([7]). Studies show that androgen receptor (AR), a member of the nuclear receptor superfamily, plays a key role in prostate cancer initiation, progression and resistance to androgen-deprivation therapy ([8–10]). Ligand-bound AR regulates gene expression by binding to androgen-responsive elements (AREs) of target genes and recruiting either co-activators or co-repressors. Among the former, JMJD1A reportedly serves as an AR co-activator via H3K9 demethylation at promoters or enhancers of some AR target genes ([1]). JMJD1A also functions in hypoxia-induced neuroendocrine differentiation (NED) of prostate cancer cells ([11]), an aggressive phenotype associated with metastasis and resistance to therapy ([12]). These findings suggest overall that JMJD1A may function in development and progression of prostate cancer. In addition, JMJD1A is shown to play a tumor-promoting role in several types of cancer cells such as colon carcinoma ([13]), neuroblastoma ([14]), hepatocellular carcinoma ([15]), and sarcoma ([16, 17]).

The proto-oncogene c-Myc is a master regulator for cell proliferation and transformation, and its activity underlies numerous cancers ([18]). For example, overexpression of c-Myc can lead to the transformation of primary human prostate epithelial cells in vitro ([19]). Prostate-specific overexpression of c-Myc alone promotes tumor development in mouse prostate ([20]), and c-Myc cooperates with loss of the phosphatase PTEN to drive prostate cancer progression ([21–23]). Overexpression of c-Myc is associated with prostate cancer recurrence and poor prognosis ([24, 25]). c-Myc mRNA and proteins are reportedly upregulated in human prostate cancer tissues relative to normal prostate tissue ([26, 27]). Potential mechanisms proposed to promote c-Myc upregulation include gene amplification ([28]), regulation by the long-range enhancers ([29]), and transcriptional upregulation ([30]). c-Myc is also subject to regulation by E3 ubiquitin ligases, including Fbxw7, Skp2, Pihr2 and HUWE1. HUWE1 (for HECT, UBA and WWE domain containing 1, also known as MULE) is a HECT family E3 ubiquitin ligase that regulates ubiquitination-dependent degradation of substrates including p53 ([31]), BRCA1 ([32]), Mcl-1 ([33]), TIAM1 ([34]), and Myc ([35, 36]). A recent study reveals that HUWE1 functions as a tumor suppressor by promoting c-Myc degradation in a mouse model of RAS-driven skin carcinogenesis ([36]).

Here, we identify JMJD1A as essential for proliferation of prostate cancer cells, and show that c-Myc is a key downstream effector of JMJD1A in this process. We report that JMJD1A stabilizes c-Myc protein by inhibiting HUWE1 and also increases c-Myc transcription through AR-dependent transcriptional activation. Identifying these mechanisms highlights an undisclosed JMJD1A activity underlying prostate cancer tumorigenesis and suggests that JMJD1A could be targeted for prostate cancer therapies.

Results

JMJD1A knockdown antagonizes tumorigenesis of prostate cancer cells

We previously reported that JMJD1A is upregulated in the Rv1 prostate cancer cells under hypoxia and contributes to the hypoxia-induced neuroendocrine differentiation (NED) ([11]). To further investigate JMJD1A function in prostate cancer, we knocked down JMJD1A in the androgen-independent Rv1 cells via lentiviral transduction using two different JMJD1A shRNAs. Separately, both constructs reduced expression of JMJD1A mRNA (Figure 1A) and protein (Figure 1B). However, JMJD1A knockdown alone had no effect on hypoxia-induced expression of neuron specific enolase (NSE), a neuroendocrine marker (Figure S1A). This result is consistent with our previous finding that simultaneous overexpression of three factors (Hes6, Sox9 and JMJD1A) was required for hypoxia-induced NED, whereas overexpression of just one of those factors had no effect ([11]). However, assessment of cell proliferation using an MTT assay showed that JMJD1A knockdown (JMJD1A KD) significantly inhibited Rv1 cell growth under both normoxia (Figure 1C) and hypoxia (Figure S1B). Specifically, in both normoxia and hypoxia, control Rv1 cells increased in number over three days, whereas the number of JMJD1A-KD cells remained unchanged (Figure 1C, S1B), suggesting that JMJD1A functions in prostate cancer cell proliferation and/or survival. Given that JMJD1A functions similarly in Rv1 cell growth under both normoxia and hypoxia (Figure 1C and S1B), we focused our analysis here on conditions of normoxia only.

JMJD1A knockdown by either of two different shRNAs abolished Rv1 cell colony formation in a soft agar assay (Figure 1D), a stringent indicator of anchorage-independent growth. Rv1 cells reportedly form spheres in 3D Matrigel or in suspension, conditions that foster growth of cells that express stem cell markers and exhibit higher tumorigenic activity when injected into immuno-deficient mice ([37]). Thus, we employed a sphere formation assay to assess potential cancer stem cell-like activity of Rv1 cells. JMJD1A KD abolished sphere formation by Rv1 cells in 3D Matrigel (Figure 1E, 1F) or in suspension (data not shown), supporting the idea that JMJD1A is required for proliferation or survival of prostate cancer cells with stem cell-like properties.

To further test JMJD1A function in tumorigenesis, we employed an orthotopic prostate tumor model in which Rv1 cells are injected into the prostate of nude mice. In control mice, injection of Rv1 cells resulted in formation of large prostate tumors, while comparable injection of Rv1 cells transduced with either one of two different JMJD1A shRNAs indicated that JMJD1A knockdown abolished tumorigenesis, i.e. no tumor formation by the JMJD1A-KD Rv1 cells (Figure 1G, 1H). Interestingly, JMJD1A knockdown in other prostate cancer cell lines, including the androgen-dependent LNCaP cells, AR-null PC3 or

DU145 cells, blocked the cell proliferation over 3 days in MTT assay (Figure S1C–S1F), and abolished colony formation by these cells in soft agar (Figure S1G). Overall, these results show that JMJD1A knockdown abolishes long-term proliferation and survival of prostate cancer cells in vitro and in vivo.

JMJD1A promotes proliferation and survival of prostate cancer cells

To determine if JMJD1A regulates cell proliferation, we performed a BrdU incorporation assay using JMJD1A-KD prostate cancer cells, including the androgen-sensitive Rv1 and LNCaP cells, the androgen-insensitive PC3 and DU145 cells. Equal number of control and JMJD1A-KD cells was treated with BrdU for 4 hours, and the BrdU incorporation into the DNA was measured by the BrdU immunolabeling and chromogenic readout. JMJD1A knockdown reduced BrdU incorporation by about 50% in all of the prostate cancer cell lines tested (Figure 1I), indicating that JMJD1A is required for proliferation. To determine if JMJD1A also regulates apoptosis, we stained prostate cancer cells with DAPI to visualize the nuclear fragmentation, a marker of late apoptotic events. JMJD1A knockdown resulted in an approximate 12-fold increase in the number of Rv1 cells exhibiting fragmented nuclei (Figure 1J, S1H). By contrast, JMJD1A knockdown in LNCaP, PC3 or DU145 cells had no effect on nuclear fragmentation (Figure 1J). Consistently, JMJD1A knockdown in Rv1 cells led to PARP cleavage and activation of caspase-3 (markers of early apoptotic events) based on western blot analysis (Figure 1K). JMJD1A knockdown in LNCaP, PC3 or DU145 cells had no effect on PARP cleavage or caspase-3 activation (Figure 1K). Together, these results indicate that JMJD1A promotes cell proliferation in all of the four prostate cancer cell lines tested, and it may also function in survival of specific prostate cancer cell line, such as Rv1.

JMJD1A regulates c-Myc transcriptional activity

JMJD1A regulates gene expression through H3K9 demethylation ([1], [2]). To assess global gene regulation by JMJD1A in an effort to identify how it regulates proliferation or survival of prostate cancer cells, we performed array-based gene expression analyses of Rv1 cells expressing JMJD1A shRNA. JMJD1A knockdown resulted in downregulation of 502 genes and upregulation of 211 genes (2-fold cut off; Table S1, S2). Analyses of published profiling array data based on 35 metastatic/advanced prostate cancer specimens and 58 primary prostate cancer samples ([38]) confirmed that 25% of genes downregulated by JMJD1A knockdown were highly expressed in metastatic/advanced prostate cancer specimens (Table S3), and 43% of those upregulated by JMJD1A knockdown were expressed at low levels in metastatic/advanced prostate cancer (Table S4), suggesting a role for JMJD1A-regulated genes in cancer progression. Gene function analysis using IPA software revealed cellular growth, proliferation, and survival among the top functions of JMJD1A-dependent genes (Figure 2A), which is consistent with our findings relevant to JMJD1A function in prostate cancer cell proliferation or survival (Figure 1). Gene network analysis using IPA software was performed to identify possible enrichment of transcriptional networks among JMJD1A-dependent genes. Progesterone receptor (PR), AR, p53, c-Myc and activating protein 1 (AP-1) are the top 5 transcription factors predicted to be responsive to JMJD1A inhibition (Figure 2B). AR can serve as a positive control in this analysis because of the established role of JMJD1A as an AR co-activator ([1]). The prediction of PR may be due to the alteration of AR target genes upon JMJD1A KD, as some of PR target genes defined by the IPA

software are also the AR target genes, possibly because of the overlapping of binding motifs between AR and PR in the gene regulation ([39], [40]).

To confirm whether JMJD1A regulates the activity of transcription factors predicted by IPA analysis (Figure 2B), we transfected Rv1 cells (either pLKO.1 control or shJMJD1A cells) with a luciferase reporter for AR, p53, c-Myc or AP-1. JMJD1A knockdown significantly inhibited the AR, c-Myc or AP-1 reporter but had no effect on p53 reporter activity (Figure 2C). These results suggest that JMJD1A can regulate multiple transcription factors. Because of the key role of c-Myc in prostate cancer, we determined to focus on role of c-Myc in JMJD1A-dependent proliferation of prostate cancer cells.

To promote cell proliferation and survival, c-Myc reportedly represses transcription of several CDK inhibitors and GADD45 in a cell-type and context-dependent manner ([36], [41–43]). Thus, we employed qRT-PCR to examine transcript levels of CDKN1A (p21), CDKN1B (p27), CDKN1C (p57), CDKN2B (p15), CDKN2C (p18), GADD45A, and GADD45B. Among these genes, only CDKN1A (p21) and CDKN2B (p15) were consistently upregulated in either JMJD1A-knockdown or c-Myc-knockdown cells (Figure 2D, 2E, data not shown). JMJD1A knockdown also activated the CDKN1A (p21) promoter, as reflected by increased activity of a p21 promoter-driven luciferase reporter relative to controls (Figure 2F). To determine whether JMJD1A regulates c-Myc binding to CDKN1A (p21) and CDKN2B (p15) promoters, we performed chromatin immunoprecipitation (ChIP) using control (pLKO.1) or JMJD1A-KD Rv1 cells and found that JMJD1A knockdown decreased binding of c-Myc to both promoters (CDKN1A, Figure 2G; CDKN2B, Figure 2H).

Taken together, the above results suggest that JMJD1A promotes c-Myc transcriptional activities.

c-Myc knockdown mimics knockdown of JMJD1A in prostate cancer cells

To determine if c-Myc is a downstream effector of JMJD1A, we knocked down c-Myc in prostate cancer cells (Figure S2A) and compared outcomes with those seen in JMJD1A-KD cells (Figure 1). c-Myc knockdown in prostate cancer cells (Rv1, LNCaP, PC3 or DU145) inhibited cell proliferation based on the BrdU incorporation assay (Figure S2B). c-Myc knockdown in Rv1 cells resulted in an approximate 8-fold increase in nuclear fragmentation (Figure S2C), whereas c-Myc knockdown in other prostate cancer cell lines (LNCaP, PC3 or DU145) had no effect (Figure S2C). The prostate cancer cells (Rv1, LNCaP, PC3 or DU145) with c-Myc knockdown also showed significantly inhibited colony formation (Figure S2D). Overall, c-Myc knockdown phenotypes resembled those of JMJD1A knockdown in prostate cancer cells (Rv1, LNCaP, PC3 and DU145), suggesting that c-Myc may be a downstream effector of JMJD1A.

JMJD1A promotes AR-dependent c-Myc expression

Our profiling array data showed a 2-fold decrease in c-Myc mRNA levels following JMJD1A KD in Rv1 cells (Table S1), suggesting that JMJD1A regulates c-Myc transcription. qRT-PCR analysis confirmed the reduction of c-Myc mRNA upon JMJD1A knockdown in Rv1 and LNCaP cells but not in PC3 and DU145 cells (Figure 3A).

Interestingly, western blot analysis showed decreased expression of c-Myc but not AR following JMJD1A knockdown in all four prostate cancer cell lines tested (Figure 3B), suggesting that JMJD1A regulates c-Myc expression at transcriptional and post-transcriptional levels.

We next asked how JMJD1A regulates c-Myc mRNA expression. Our IPA analysis indicated that AR is one of top transcription factors regulated by JMJD1A (Figure 2B). Our findings indicate that JMJD1A regulates c-Myc mRNA in AR-positive Rv1 and LNCaP cells, but not AR-negative cells such as PC3 and DU145 (Figure 3A). Therefore, we hypothesize that JMJD1A may regulate c-Myc transcription via the AR. To test the hypothesis, we used qRT-PCR to assess the c-Myc mRNA expression in prostate cancer cells following AR inhibition. AR knockdown in Rv1 and LNCaP cells reduced expression of c-Myc mRNA and protein (Figure 3C, 3D, S3A), but had no effect on the expression of JMJD1A (Figure S3B). To determine if that effect was AR-dependent, we knocked down JMJD1A in Rv1 or LNCaP cells stably expressing AR shRNA. Although JMJD1A knockdown reduced c-Myc mRNA in control cells that express normal levels of AR, it failed to do so in AR knockdown cells: thus the effect of JMJD1A and AR double-KD was similar to the effect of AR KD alone (Figure 3D). These results suggest that JMJD1A regulation of c-Myc mRNA is AR-dependent.

JMJD1A promotes recruitment of AR to the c-Myc gene enhancer independent of androgen

We next asked how JMJD1A increases AR-dependent c-Myc transcription. Because JMJD1A is a co-activator of AR ([1]) and the c-Myc gene enhancer contains an ARE ([44]), we performed a chromatin immunoprecipitation (ChIP) assay using a JMJD1A antibody to determine if JMJD1A associates with the ARE on the c-Myc enhancer. We observed association of JMJD1A with the c-Myc enhancer ARE and confirmed specificity of that interaction by JMJD1A knockdown, which reduced JMJD1A binding to the enhancer (Figure 3E). We observed similar results when we assessed JMJD1A binding to the PSA enhancer, which served as a positive control (Figure S3C).

Binding of JMJD1A to AREs of both the c-Myc and PSA enhancers was reduced by AR knockdown (Figure 3E, S3C), suggesting that AR is required for the binding of JMJD1A to these chromatin regions. To evaluate if JMJD1A regulates AR binding to the c-Myc enhancer, we performed an AR ChIP assay. JMJD1A knockdown reduced binding of AR to the c-Myc enhancer (Figure 3F) but not the PSA enhancer (Figure S3D). The latter result is consistent with the previous report that JMJD1A does not alter binding of AR to the PSA enhancer ([1]). Consistent with JMJD1A's role as a H3K9me2 demethylase, knockdown of either JMJD1A or AR increased levels of the H3K9me2 mark at the c-Myc (Figure 3G) or PSA (Figure S3E) enhancers. These results suggest that JMJD1A increases c-Myc transcription both by promoting AR recruitment and demethylating the c-Myc enhancer.

To determine if JMJD1A-dependent transcription of c-Myc requires the AR ligand, we maintained the prostate cancer cells (pLKO.1 or shJMJD1A) in the media supplemented with charcoal-stripped FBS (CS-FBS) that contains extremely low levels of androgen for two days, followed by treatment of cells with or without synthetic androgen R1881 for one

day. Although R1881 induced the PSA transcript slightly in Rv1 cells (Figure 3H) and strongly in LNCaP cells (Figure 3I), it failed to induce the c-Myc transcript in either Rv1 (Figure 3H) or LNCaP cells (Figure 3I), and knockdown of JMJD1A reduced the c-Myc transcript similarly with or without R1881 (Figure 3H, 3I). These results suggest that AR ligand is not required for JMJD1A-dependent transcription of c-Myc. To further confirm this conclusion, we performed the ChIP assay using AR antibody on LNCaP cells (pLKO.1 or shJMJD1A). Even in the absence of R1881, we observed the binding of AR to the c-Myc gene enhancer in the LNCaP control cells (pLKO.1), and such binding was not affected by the treatment of R1881 (Figure 3J). Knockdown of JMJD1A equally reduced the binding between AR and c-Myc enhancer in the presence or absence of R1881 (Figure 3J). In contrast, little AR was associated with PSA enhancer in the absence of R1881, and the binding of AR to PSA enhancer was highly enhanced by R1881 (Figure 3J). These results reveal a constitutive association between AR and c-Myc enhancer and the requirement of JMJD1A for such association in the presence or absence of androgen. Our result is consistent with the previous report showing that AR upregulates the expression of c-Myc in a ligand-independent manner ([44]).

To determine if histone demethylase activity is required for c-Myc expression, we knocked down endogenous JMJD1A in Rv1 cells and re-expressed JMJD1A constructs encoding either the wild-type (WT) protein or a catalytic site mutant that has no demethylase activity ([1]). Both constructs harbored mutations in the JMJD1A shRNA targeting site that made them resistant to gene silencing and enabled us to knock down endogenous JMJD1A and simultaneously re-express WT or mutant constructs. In JMJD1A-KD Rv1 cells, c-Myc mRNA and protein levels were fully rescued by WT JMJD1A (Figure 3K, 3L). By contrast, catalytically inactive JMJD1A did not rescue c-Myc mRNA expression following JMJD1A knockdown (Figure 3K) but partially rescued c-Myc protein expression, suggesting that JMJD1A histone demethylase activity is required for c-Myc transcription. Interestingly, partial rescue of c-Myc protein by catalytically inactive JMJD1A suggests transcription-independent regulation of c-Myc expression in prostate cancer cells.

JMJD1A increases c-Myc protein levels by inhibiting HUWE1-induced degradation

To investigate mechanisms underlying transcription-independent regulation of c-Myc expression by JMJD1A, we ectopically expressed Flag-tagged JMJD1A in Rv1 cells and then performed immunoprecipitation and mass spectrometry analysis in order to identify proteins interacting with JMJD1A. A top candidate protein in the analysis was HUWE1 (Table S5), an E3 ubiquitin ligase known to target Myc ([35, 36]). Therefore, we asked whether JMJD1A/HUWE1 interaction had any effect on c-Myc protein levels.

To do so, we first confirmed interaction between ectopically expressed JMJD1A and HUWE1 by co-immunoprecipitation in 293T cells (Figure S4A, S4B). We also confirmed interaction between endogenous JMJD1A and HUWE1 by co-immunoprecipitation in Rv1 cells (Figure 4A). To test whether JMJD1A/HUWE1 interaction had any effect on HUWE1-dependent c-Myc degradation, we co-expressed HUWE1 and c-Myc with JMJD1A in 293T cells. Western blot analysis showed that HUWE1 overexpression reduced c-Myc levels, an effect abolished by co-expression of JMJD1A (Figure 4B, S4C). Of note, overexpression of

the catalytically inactive form of JMJD1A also blocked HUWE1-induced c-Myc degradation (Figure 4B), indicating that of JMJD1A histone demethylase activity is not required for this activity. To determine if JMJD1A affects the HUWE1-induced degradation of c-Myc in prostate cancer cells, we overexpressed HUWE1 in PC3 cells with or without JMJD1A co-expression. Western blot analysis showed that HUWE1 overexpression reduced the endogenous c-Myc level, whereas such an effect was attenuated upon the JMJD1A co-expression (Figure S4D).

JMJD1A overexpression did not alter HUWE1-induced degradation of p53 (Figure 4C), a different HUWE1 substrate ([31]), and JMJD1A knockdown in prostate cancer cells had no effect on p53 protein levels (Figure 1K), suggesting that JMJD1A inhibits HUWE1-mediated degradation of specific substrates, such as c-Myc.

JMJD1A overexpression had no effect on HUWE1 levels (Figure 4B, 4C, S4C), suggesting that JMJD1A does not affect HUWE1 expression. HUWE1 overexpression also did not decrease JMJD1A levels (Figure 4C, S4C), suggesting that JMJD1A does not undergo HUWE1-dependent degradation. Instead, JMJD1A levels increased upon HUWE1 overexpression (Figure 4C, S4C, S4D), possibly because JMJD1A/HUWE1 interaction stabilizes JMJD1A, an issue we will address in a separate study.

To determine how JMJD1A inhibits c-Myc degradation promoted by HUWE1, we mapped interaction domains among the three proteins. To assess JMJD1A/HUWE1 interaction we co-expressed full-length HUWE1 with the N- or C-terminal fragments of JMJD1A in 293T cells. Coimmunoprecipitation and western blot analysis revealed that the JMJD1A N-terminal half interacted with HUWE1 (Figure S4E). HUWE1 consists of an N-terminal ARLD domain, followed by UBA, WWE, BH3, and acidic regions, plus a C-terminal HECT domain (Figure 4D). To determine which domain(s) interacts with JMJD1A, we engineered HUWE1 truncation mutants (Figure 4D), each containing a single domain, and co-expressed them with full-length JMJD1A in 293T cells. Coimmunoprecipitation and western blot analyses indicated that HUWE1 fragments containing either the acidic region (F5) or HECT domain (F7) interacted with JMJD1A (Figure 4E). When we asked which HUWE1 domain(s) was required for c-Myc interaction, we found that HUWE1 fragments containing the acidic region (F5) or the WWE domain (F3) interacted with c-Myc (Figure 4F). The HUWE1 acidic region (F5) interacted with both JMJD1A and c-Myc, suggesting potential competition for HUWE1 binding. To address this possibility, we co-immunoprecipitated HUWE1 and c-Myc in the presence of co-expressed JMJD1A in 293T cells. Western blot analysis showed that JMJD1A reduced binding between HUWE1 and c-Myc in a dose-dependent manner (Figure 4G). These findings suggest that JMJD1A increases c-Myc protein levels by inhibiting the HUWE1/c-Myc interaction and potentially blocking HUWE1-dependent c-Myc degradation.

To determine if JMJD1A regulates the c-Myc degradation in prostate cancer cells, we knocked JMJD1A in Rv1 or PC3 cells, and treated cells with the proteasome inhibitor MG132 before western blot analysis. The results showed that MG132 treatment attenuated the downregulation of c-Myc protein upon the JMJD1A knockdown in both prostate cancer cell lines (Figure 4H), suggesting that c-Myc undergone the proteasome-dependent

degradation in the absence of JMJD1A. To confirm if JMJD1A regulates the c-Myc stability, we performed cycloheximide chasing on the JMJD1A-KD Rv1 cells to assess the c-Myc half-life. The half-life of c-Myc in control and JMJD1A-KD Rv1 cells was about 40 minutes and 20 minutes, respectively (Figure 4I), indicating destabilization of c-Myc in the absence of JMJD1A. To determine if JMJD1A regulates HUWE1-induced c-Myc degradation in prostate cancer cells, we knocked down HUWE1 in both JMJD1A-KD Rv1 cells (shJMJD1A) and control (pLKO.1) Rv1 cells. Knockdown of HUWE1 alone did not affect c-Myc levels (Figure 4J), suggesting that HUWE1 cannot promote c-Myc degradation in the presence of JMJD1A. Indeed, HUWE1 knockdown in JMJD1A-KD Rv1 cells increased c-Myc protein levels without altering c-Myc mRNA (Figure 4J, 4K). To determine if JMJD1A regulates c-Myc ubiquitination by HUWE1, we knocked down JMJD1A in the presence or absence of HUWE1 knockdown in Rv1 cells, and treated cells with the proteasome inhibitor MG132 before immunoprecipitation of c-Myc. Western blot analysis with an ubiquitin antibody revealed increased polyubiquitination of c-Myc in JMJD1A-KD cells (Figure 4L), an effect abolished by HUWE1 knockdown (Figure 4L). These results indicate that JMJD1A inhibits polyubiquitination and degradation of c-Myc by HUWE1 in prostate cancer cells.

Notably, HUWE1 knockdown in JMJD1A-KD Rv1 cells cannot fully rescue c-Myc protein levels (Figure 4J), likely because HUWE1 knockdown has little or no effect on JMJD1A regulation of c-Myc transcription. These results further support the idea that JMJD1A regulates c-Myc at both post-translational and transcriptional levels.

c-Myc is a downstream effector of JMJD1A in prostate cancer cell tumorigenesis

IPA analysis suggested that JMJD1A regulates multiple transcriptional networks in addition to c-Myc (Figure 2B). To assess the effect of c-Myc on JMJD1A-dependent tumorigenesis, we re-expressed c-Myc in JMJD1A-KD Rv1 cells using a lentiviral vector encoding c-Myc. We have optimized the lentiviral transduction condition to restore c-Myc expression in JMJD1A-KD cells to the endogenous level as seen in the pLKO.1 control cells (Figure 5A, S5). This approach will allow us to evaluate the specific effect of c-Myc downstream of JMJD1A. c-Myc re-expression partially rescued colony formation in soft agar by JMJD1A-KD Rv1 cells (Figure 5B). Similarly, c-Myc re-expression in JMJD1A-KD PC3 or LNCaP cells partially rescued anchorage-independent growth of these cells in soft agar (Figure 5C, S5), as well as sphere formation by JMJD1A-KD Rv1 cells in 3D Matrigel (Figure 5D). The latter finding is consistent with the report that c-Myc is important to the maintenance of prostate cancer stem-like cells ([37]).

We next addressed the role of c-Myc downstream of JMJD1A in vivo using an orthotopic prostate tumor model in which Rv1 cells expressing luciferase are injected into the dorsal prostate of nude mice and tumor formation is monitored using an in vivo imaging system (IVIS). In contrast to control cells, which formed large prostate tumors, we detected no tumor formation in mice injected with the JMJD1A-KD Rv1 cells (Figure 5E, 5F). c-Myc re-expression in injected JMJD1A-KD Rv1 cells partially rescued tumor formation, although tumor size was only 30% of that of control cell-derived tumors (Figure 5E, 5F). These results support a role for c-Myc in JMJD1A-dependent tumorigenesis of prostate cancer cells and suggest potential involvement of other JMJD1A effector(s) in tumorigenesis.

High c-Myc levels correlate with JMJD1A overexpression in a subset of human prostate specimens

To confirm the relevance of our findings to human prostate cancer, we evaluated expression of JMJD1A and c-Myc in a human prostate cancer tissue microarray (TMA) containing representative samples of Gleason stage 3–4 tumors (Figure 6A, 6B). JMJD1A and c-Myc expression was primarily nuclear, but weak cytoplasmic staining of both proteins was also seen in some samples (Figure 6A). Based on respective staining intensity, we defined c-Myc samples as either c-Myc-low or -high. JMJD1A staining was likewise defined as either JMJD1A-low or -high. Analysis to evaluate JMJD1A or c-Myc staining levels (Figure 6B) showed that although low c-Myc staining was not correlated with low JMJD1A staining, c-Myc-high staining was positively correlated with JMJD1A-high samples ($p<0.05$, Fisher's exact test), suggesting a potential association of these factors in human prostate tumors.

Discussion

Our study reveals a critical role for JMJD1A in prostate cancer cell proliferation through regulation of c-Myc expression. JMJD1A knockdown in prostate cancer cells inhibited their proliferation and survival. Profiling array analysis revealed the c-Myc gene signature among the top transcriptional networks altered in response to JMJD1A inhibition. Indeed, JMJD1A knockdown inhibited transcription of c-Myc and reduced c-Myc protein levels. Knockdown of c-Myc showed similar phenotypes in prostate cancer cells as knockdown of JMJD1A. Restoration of c-Myc expression in JMJD1A-KD cells partially rescued growth of prostate cancer cells in vitro and in nude mice. We conclude that c-Myc serves as a key downstream effector of JMJD1A in driving prostate cancer cell proliferation as well as survival.

JMJD1A reportedly regulates gene expression through H3K9 demethylation ([1], [4], [13], [45]). Consistent with its established role as an AR co-activator, we found that JMJD1A can recruit AR to the c-Myc enhancer and demethylate H3K9me2 at that locus. We also found that androgen is not required for the JMJD1A-dependent binding of AR to c-Myc enhancer. The latter finding is consistent with the report that AR upregulates c-Myc mRNA independent of its ligand in prostate cancer cells, and AR/c-Myc mRNAs are strongly correlated in the tissues of castration-resistant prostate cancer ([44]). Furthermore, our rescue experiment using the catalytically inactive form of JMJD1A demonstrates that its histone demethylase activity is required for c-Myc transcription. In addition to this function, we identify a new, histone demethylase-independent role for JMJD1A in regulating c-Myc protein: JMJD1A binds to the ubiquitin ligase HUWE1 and inhibits its interaction with c-Myc, thereby increasing c-Myc protein levels by attenuating its ubiquitination and subsequent degradation. Therefore, our study reveals two mechanisms used by JMJD1A to increase c-Myc transcription and stability.

Similar to other E3 ubiquitin ligases, HUWE1 likely destabilizes several substrates ([31–34], [36]). We report that JMJD1A interacts with HUWE1 fragments containing the HECT domain or the acidic region, and that it inhibits degradation of oncogenic c-Myc but not the tumor suppressor p53, both known HUWE1 substrates. Thus, JMJD1A provides a layer of regulation for selective degradation of HUWE1 substrates such as c-Myc. These findings support a tumor suppressor function for HUWE1 in prostate cancer cells based on its

degradation of c-Myc, a concept recently advanced in a skin cancer model using the HUWE1 knockout mice ([36]).

c-Myc mRNA and proteins are upregulated in prostate cancer ([26], [27]), and c-Myc overexpression drives prostate cancer progression ([21–23]). Nonetheless, mechanisms leading to c-Myc overexpression remain unclear ([27]). Here, we find that JMJD1A can increase c-Myc expression in prostate cancer cell lines. More importantly, we observe that high levels of c-Myc are correlated with high JMJD1A levels in a prostate cancer TMA, suggesting that JMJD1A contributes to c-Myc overexpression in a subset of human prostate cancers. Although several E3 ubiquitin ligases reportedly promote c-Myc degradation, it is not known whether any ubiquitin ligase regulates c-Myc expression in prostate cancer. Regulation of c-Myc protein by the JMJD1A/HUWE1 axis is evidence that the ubiquitin-proteasome system likely contributes to c-Myc overexpression observed in human prostate cancer.

Growth of prostate cancer cells upon JMJD1A knockdown was partially rescued by c-Myc re-expression. However, that effect was moderate, particularly in the orthotopic tumor model. These observations suggest that in addition to regulation of c-Myc expression, there might be additional layers of modulation of c-Myc function by JMJD1A. Alternatively, these observations suggest that JMJD1A may also regulate other downstream effectors in prostate cancer cells. IPA analysis of profiling array data and luciferase reporter assay implicate that JMJD1A knockdown may alter the activity of transcription factor AP-1, which has been implicated in prostate cancer development and progression ([46], [47]). Future studies are needed to determine if JMJD1A regulates the transcriptional activity of c-Myc and AP-1 as co-activators, and if other transcription factors function in JMJD1A-dependent proliferation or survival of prostate cancer cells.

JMJD1A knockdown in prostate cancer cells blocked cell proliferation over days, and abolished colony formation by these cells in soft agar, supporting a function for JMJD1A in long-term cell proliferation and survival. Importantly, JMJD1A-KD Rv1 cells could not form spheres in 3D Matrigel or in suspension, methods commonly used to assess cancer stem cell (CSC) activity ([37], [48], [49]). Such spheres formed by Rv1 cells are known to enrich in highly tumorigenic CSC populations ([37]). These results strongly suggest that JMJD1A is required for both bulk tumor and cancer stem-like cell populations in prostate cancer. Notably, Jmjd1a knockout mice are viable and show variable phenotypes including spermiogenic defects ([5]), obesity ([4]), and male-to-female sex reversal ([3]). Given these relatively mild phenotypes and the strong inhibitory effect of JMJD1A knockdown on prostate cancer cell growth, JMJD1A could serve as a therapeutic target for aggressive prostate cancers. Inhibitors targeting Jmj demethylase activity have been developed, but specific JMJD1A inhibitors are not yet available. Our results demonstrating catalysis-dependent and -independent JMJD1A regulation of c-Myc expression suggest that JMJD1A motifs governing both catalytic activity and protein-protein interactions could be targeted in development of JMJD1A inhibitors.

Materials and Methods

Antibodies and reagents

Antibodies to AR (sc-816), ubiquitin (sc-9133), HA (sc-805), GFP (sc-8334 or sc-9996), myc (sc-789), tubulin (sc-8035) (all from Santa Cruz), JMJD1A (A301-539A or 538B) or HUWE1 (A300-486A) (Bethyl Laboratories), AR (06-680) or H3K9me2 (07-441) (EMD Millipore), c-Myc (9402), p53 (9282), PARP (9542), active caspase-3 (9661) (all from Cell Signaling), and Flag (F7425) and actin (A5441) (Sigma) were used according to the manufacturers' recommendations.

Cell Lines

LNCaP, PC3 and DU145 cells were purchased from American Type Culture Collection (ATCC). Rv1 cells were kindly provided by Dr. James Jacobberger. These cells are maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics, and regularly tested to ensure that they are mycoplasma-free.

Animal studies

Athymic nude mice were purchased from the Jackson Laboratory and housed in the animal facility at Sanford-Burnham Medical Research Institute or the University of Maryland School of Medicine. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC # 10-093, # 0613011) and conducted following both Institutes' animal policy in accordance with NIH guidelines.

Prostate tumor samples

A total of 38 prostate cancer specimens with a Gleason score 3–4 were obtained from the Vancouver Prostate Tissue Bank at the University of British Columbia (Clinical Research Ethics Board number: H09-01628). All specimens were from radical prostatectomy. H&E slides were reviewed and desired areas were marked. The TMA was manually constructed (Beecher Instruments, MD, USA) by punching duplicate cores of 1 mm from each sample.

Statistical analysis

The in vitro experiments were repeated at least three times. Data are presented as the mean ±s.d. Student's t test (two-tailed) was used to compare the difference between two groups of datasets with similar variance. Fisher's exact test was used to assess the relationship between IHC staining. $p < 0.05$ was considered statistically significant.

Additional methods are presented in the Supplemental Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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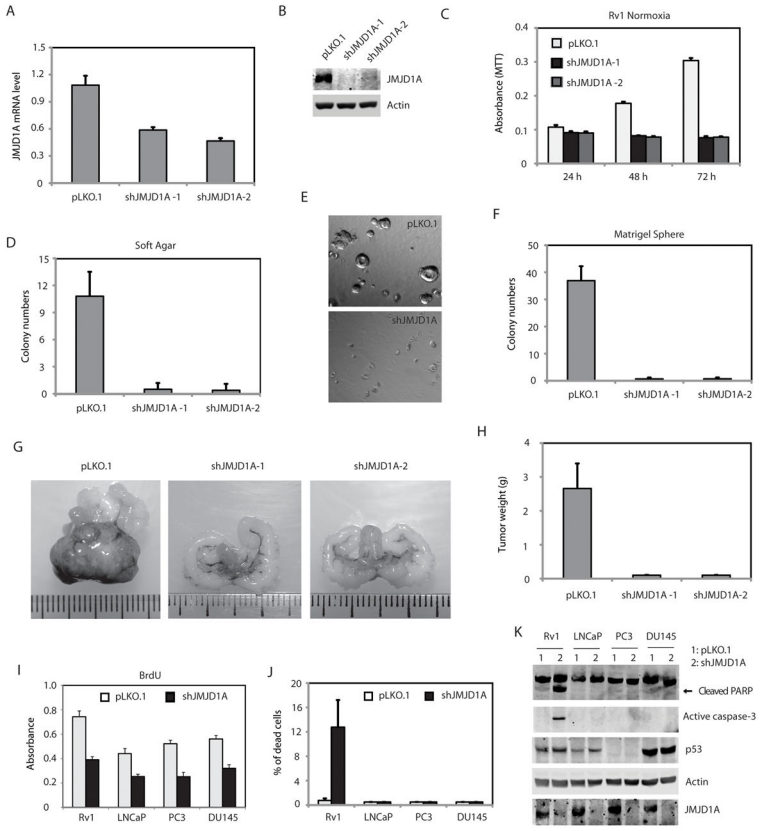

Figure 1.

A. JMJD1A knockdown (KD) in Rv1 cells. Rv1 cells transduced separately with JMJD1A shRNAs (shJMJD1A-1 or shJMJD1A-2) were analyzed for JMJD1A transcripts by qRT-PCR 48h later ($p<0.005$ for pLKO.1 vs shJMJD1A-1, $p<0.001$ for pLKO.1 vs shJMJD1A-2). **B.** KD of JMJD1A protein in Rv1 cells. Rv1 cells described in A were analyzed by western blotting using JMJD1A or actin antibodies. **C.** MTT assay on JMJD1A-KD Rv1 cells. Cells were transduced with indicated shRNAs for 24h and assayed 24, 48, or 72h later (pLKO.1 vs shJMJD1A-1 or shJMJD1A-2: $p>0.1$ at 48 h, $p<1\times10^{-5}$ at 72 h or 96 h). **D.** The effect of JMJD1A KD on colony formation by Rv1 cells. Cells were maintained in soft agar for 3 weeks, and colony number per field was determined. JMJD1A KD abolished colony formation ($p < 1\times10^{-9}$). **E** and **F**. The effect of JMJD1A KD in Rv1 cells on sphere formation in Matrigel. Cells were grown for 10 days in Matrigel and the number of spheres per field was determined. JMJD1A KD abolished sphere formation ($p<1\times10^{-15}$ for pLKO.1 vs shJMJD1A-1 or shJMJD1A-2). **G** and **H.** Effect of JMJD1A KD in Rv1 cells on prostate tumor formation in an orthotopic model. Rv1 cells (pLKO.1, shJMJD1A-1, or shJMJD1A-2) were injected into the dorsal prostates of nude mice. Three weeks later tumors were monitored and weighed ($n = 5$ for each group, $p < 1\times10^{-4}$ for pLKO.1 vs shJMJD1A-1 or shJMJD1A-2). Images in **G** depict tumor formation in the mouse genitourinary tract. **I.** Indicated prostate cancer lines transduced with either control pLKO.1 or shJMJD1A were incubated with 10 uM BrdU for 4 h and BrdU incorporation was then determined using a BrdU cell proliferation kit. The reading of absorbance at 490 nm is an indicator of BrdU levels in DNA. JMJD1A KD decreased BrdU incorporation ($p<0.005$ for Rv1, LNCaP or

PC3, $p<0.01$ for DU145). **J.** Prostate cancer cells transduced with either pLKO.1 or shJMJD1A were stained with DAPI, and the number of cells exhibiting fragmented nuclei was determined. JMJD1A KD induced nuclear fragmentation in Rv1 cells ($p<1\times10^{-14}$) but not in other prostate cancer cells ($p>0.1$). **K.** Various prostate cancer cell lines transduced with pLKO.1 control (#1) or shJMJD1A (#2) were analyzed by western blotting with indicated antibodies.

A

Molecular and Cellular Function	p-value
1. Cellular growth and proliferation	1.05E-09
2. Cellular assembly and organization	1.16E-08
3. Cellular function and maintenance	1.16E-08
4. Cell morphology	1.45E-07
5. Cell death and survival	4.52E-07

B

Transcription factor	p-value
1. PR	7.31E-11
2. AR	1.80E-09
3. TP53	1.46E-05
4. Myc	2.17E-05
5. Fos	3.22E-05

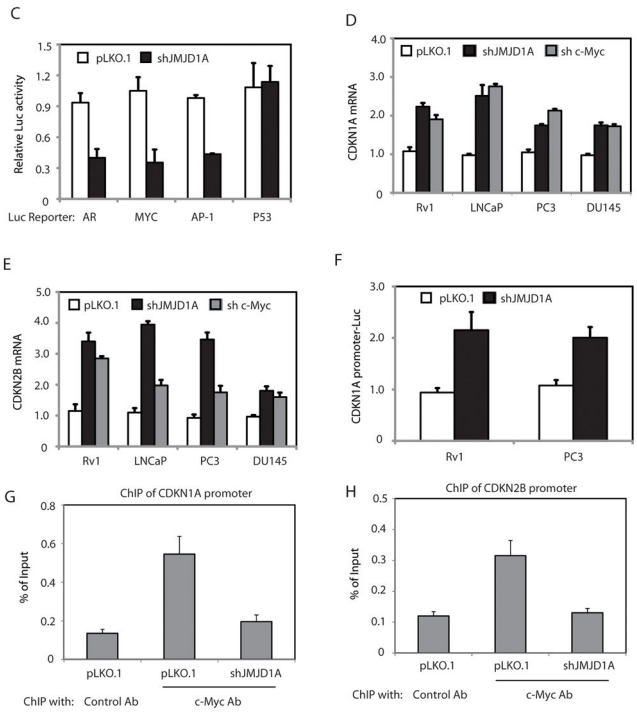

Figure 2.

A. The function of JMJD1A-dependent genes predicted by IPA analysis. **B.** Top five transcription factors whose activity might be altered by JMJD1A KD based on IPA analysis of canonical gene pathways. **C.** Rv1 cells transduced with control pLKO.1 or shJMJD1A were transfected with construct harboring a luciferase reporter for AR, c-Myc, AP-1 or p53. JMJD1A KD decreased the activity of luciferase reporter for AR ($p<0.05$), c-Myc ($p<0.005$), AP-1 ($p<0.005$), but had no effect on the p53 reporter ($p>0.1$). **D** and **E.** Indicated prostate cancer lines were transduced with either JMJD1A or c-Myc shRNA constructs. After 48 h, RNA was subjected to qRT-PCR analysis for CDKN1A (D) or CDKN2B (E) transcripts. D shows that KD of JMJD1A or c-Myc increases CDKN1A transcript levels ($p<0.005$ for Rv1 or LNCaP; $p<0.01$ for PC3 or DU145), while E shows a similar effect for CDKN2B transcripts ($p<0.05$ for all cell lines indicated). **F.** Indicated prostate cancer cell lines transduced with control pLKO.1 or shJMJD1A were transfected with a luciferase reporter driven by the CDKN1A promoter. JMJD1A KD increased promoter activity ($p<0.05$ for Rv1 or PC3 cells). **G** and **H.** Indicated Rv1 cells were subjected to a ChIP assay using control or c-Myc antibodies. JMJD1A KD reduced c-Myc binding to the promoters of CDKN1A (G, $p<0.05$) and CDKN2B (H, $p<0.05$).

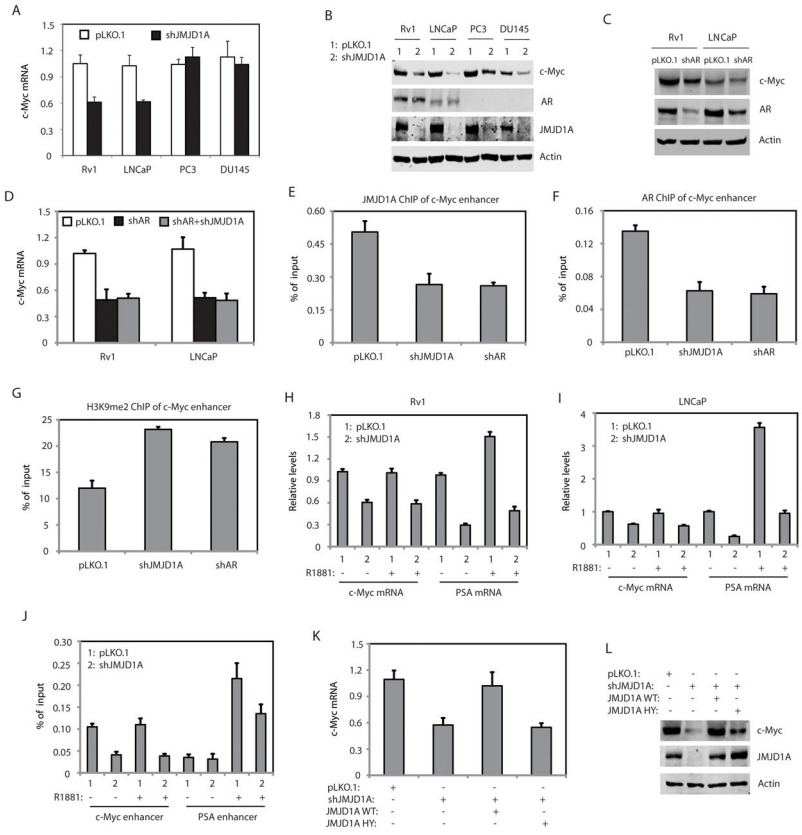

Figure 3.

A. qRT-PCR analysis showing the effect of JMJD1A KD on c-Myc transcripts. JMJD1A KD decreased c-Myc mRNA levels in Rv1 and LNCaP cells ($p<0.05$) but not in PC3 or DU145 cells ($p>0.1$). **B.** Effect of JMJD1A KD on c-Myc protein levels. Indicated prostate cancer cell lines transduced with pLKO.1 (#1) or shJMJD1A (#2) were analyzed by western blotting with c-Myc, AR and JMJD1A antibodies. **C.** Effect of AR KD on c-Myc protein levels. Rv1 or LNCaP cells with AR knockdown were analyzed by western blotting with c-Myc or AR antibodies. **D.** qRT-PCR analysis of c-Myc mRNA in indicated prostate cancer cell lines. AR KD decreased c-Myc mRNA levels ($p<0.005$ for Rv1, $p<0.05$ for LNCaP). KD of both AR and JMJD1A reduces c-Myc mRNA transcript levels similarly to KD of AR only ($p>0.1$). **E.** Rv1 cells were transduced with JMJD1A or AR shRNAs for 48 h and subjected to a ChIP assay using a JMJD1A antibody. Chromatin was analyzed by qPCR for regions of the c-Myc enhancer exhibiting an ARE. KD of either JMJD1A or AR decreased JMJD1A binding to ARE region ($p<0.05$). **F.** Rv1 cells with KD of JMJD1A or AR were subjected to ChIP analysis using an AR antibody. KD of either JMJD1A or AR reduced AR binding to the c-Myc enhancer ARE ($p<0.05$). **G.** Rv1 cells with KD of JMJD1A or AR were subjected to a ChIP assay using an H3K9me2 antibody. KD of JMJD1A ($p<0.01$) or AR ($p<0.005$) increased H3K9me2 levels at the c-Myc enhancer ARE. **H** and **I**. Effect of androgen on the c-Myc mRNA in Rv1 cells (H) or LNCaP cells (I). 1 and 2 represents pLKO.1 and shJMJD1A, respectively. Cells were treated with 1 nM of R1881 for 24 h before qRT-PCR analysis of c-Myc or PSA. R1881 induced the PSA transcript in pLKO.1 ($p<0.01$ for Rv1, $p<0.005$ for LNCaP) or JMJD1A-KD cells ($p<0.05$ for Rv1 or LNCaP),

but had no effect on the c-Myc transcript in Rv1 and LNCaP cells ($p>0.1$ for either pLKO.1 or shJMJD1A). JMJD1A KD reduced the c-Myc transcript in the presence ($p<0.05$ for Rv1 or LNCaP) or absence ($p<0.01$ for Rv1, $p<0.005$ for LNCaP) of R1881. **J.** Effect of androgen on the binding of AR to c-Myc enhancer in LNCaP cells (1: pLKO.1, 2: shJMJD1A). Cells were treated with 1 nM of R1881 for 24 h before the ChIP analysis using an AR antibody. R1881 enhanced the binding of AR to the PSA enhancer ARE ($p<0.05$ for pLKO.1 or shJMJD1A), but had no effect on the binding of AR to the c-Myc enhancer ARE ($p>0.1$ for either pLKO.1 or shJMJD1A). JMJD1A KD reduced the binding of AR to the c-Myc enhancer ARE in the presence or absence of R1881 ($p<0.05$). **K.** Wild-type (WT) or a catalytically inactive form (HY) of JMJD1A was expressed in JMJD1A-KD Rv1 cells, and cells were assayed for c-Myc transcripts by qRT-PCR. Ectopically expressed JMJD1A (either WT or HY) harbors silent mutations making it resistant to JMJD1A shRNA. $p<0.005$ for pLKO.1 vs. shJMJD1A; $p<0.05$ for pLKO.1 vs. shJMJD1A + JMJD1A HY; $p=0.53$ for pLKO.1 vs. shJMJD1A + JMJD1A WT. **L.** Rv1 cells described in K were analyzed by western blotting with c-Myc or JMJD1A antibodies.

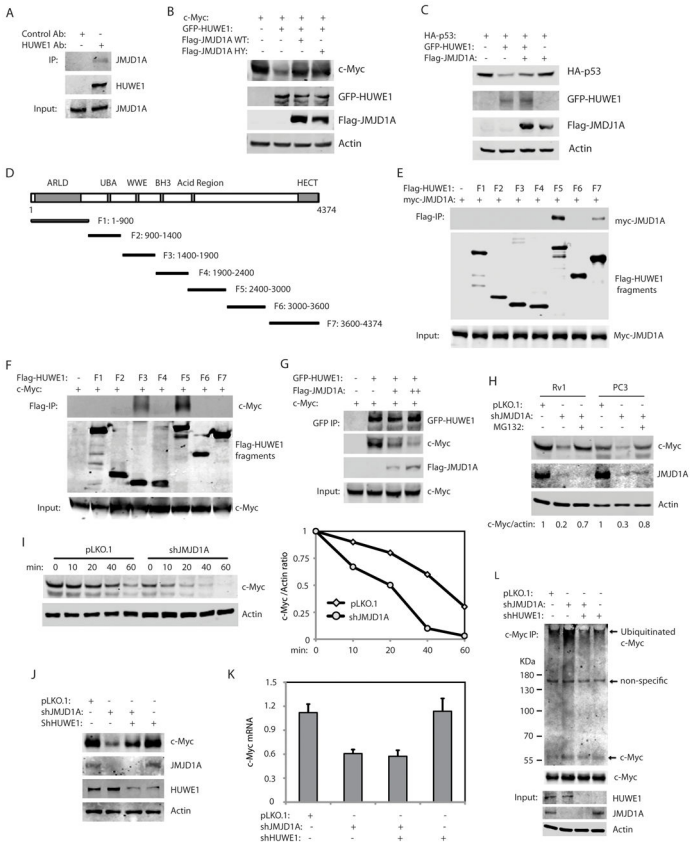

Figure 4.

A. Rv1 cells were immunoprecipitated with control or HUWE1 antibody plus protein A/G beads. Bound proteins were eluted and analyzed by western blotting with JMJD1A or HUWE1 antibodies. **B.** 293T cells were transfected with c-Myc, GFP-HUWE1 and Flag-JMJD1A (WT or the catalytically inactive HY mutant) for 24 h. Whole cell lysates were analyzed by western blotting with c-Myc, GFP or Flag antibodies. **C.** 293T cells were transfected with HA-p53, GFP-HUWE1 or Flag-JMJD1A for 24 h. Whole cell lysates were analyzed by western blotting with HA, GFP, or Flag antibodies. **D.** Diagram showing HUWE1 domains and fragments, indicated in grey. Seven HUWE1 deletion mutants were generated (F1 to F7). **E.** 293T cells were transfected with myc-tagged JMJD1A and Flag-tagged HUWE1 fragments (F1 to F7) for 24h, and lysates were subjected to immunoprecipitation with anti-Flag M2 beads. Bound proteins were analyzed by western blotting with myc or Flag antibodies. **F.** 293T cells were transfected with c-Myc and Flag-tagged HUWE1 fragments (F1 to F7) for 24 h, and lysates were immunoprecipitated with anti-Flag M2 beads. Bound proteins were analyzed by western blotting with c-Myc or Flag antibodies. **G.** 293T cells were transfected with GFP-HUWE1, c-Myc and increasing amount of Flag-JMJD1A for 24 h. Cells were treated with 10 μM MG132 for 4 h before immunoprecipitation with GFP antibody plus protein A/G beads. The bound proteins were analyzed by western blotting with GFP, Flag or c-Myc antibodies. **H.** Rv1 or PC3 cells were transduced with JMJD1A shRNA for 48 h, and treated with or without 10 μM of MG132 for 4 h. Cell lysates were analyzed by western blotting with c-Myc or JMJD1A antibodies. The relative intensity ratios between c-Myc and actin for each lane were shown at bottom of the

panel. **I.** Rv1 cells were transduced with JMJD1A shRNA for 48 h, and then treated with 50 ug/ml of cycloheximide. Cell lysates were collected every 10 or 20 minutes after the cycloheximide treatment, and analyzed by western blotting with c-Myc or actin antibodies. The relative intensity ratios between c-Myc and actin were plotted on a graph. The intensity ratios for cells not treated with cycloheximide were set as one. **J.** Rv1 cells were transduced with JMJD1A or HUWE1 shRNA constructs individually or in combination, and cell lysates were analyzed by western blotting with indicated antibodies. **K.** Rv1 cells described in J were used for qRT-PCR analysis of c-Myc transcripts. **L.** Rv1 cells described in J were treated with 10 μM of MG132 for 4 h, and cell lysates were immunoprecipitated with a c-Myc antibody plus protein A/G beads. Bound proteins were analyzed by western blotting with ubiquitin or c-Myc antibodies.

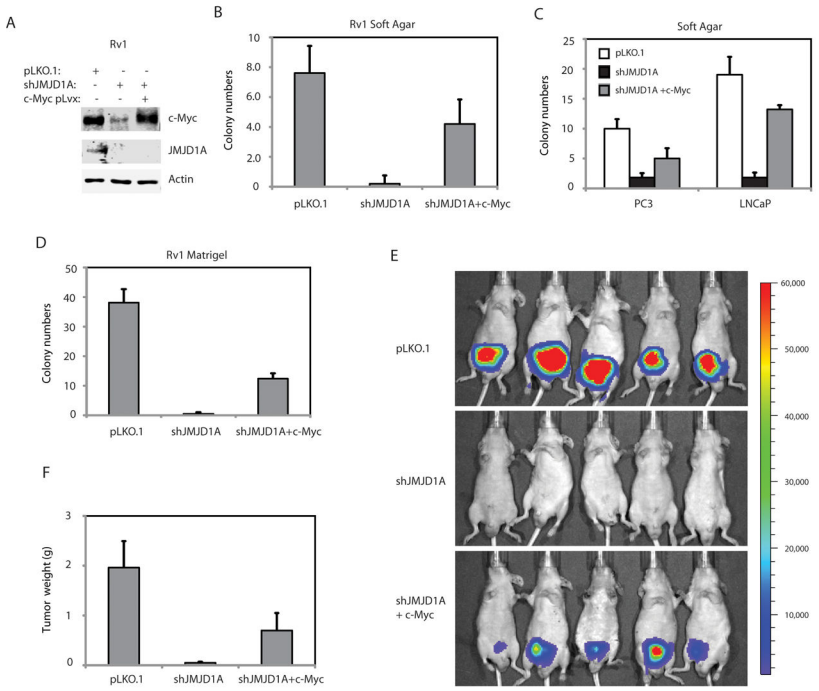

Figure 5.

A. c-Myc re-expression in JMJD1A-KD Rv1 cells via lentiviral transduction. Cells were analyzed by western blotting with c-Myc or JMJD1A antibodies. **B.** c-Myc re-expression in JMJD1A-KD Rv1 cells partially rescued colony formation in soft agar ($p<1\times10^{-10}$ for pLKO.1 vs shJMJD1A, $p<1\times0^{-8}$ for shJMJD1A vs shJMJD1A+c-Myc). **C.** c-Myc re-expression in JMJD1A-KD PC3 or LNCaP cells partially rescued colony formation. PC3: $p<1\times10^{-7}$ for pLKO.1 vs shJMJD1A, $p<1\times10^{-5}$ for shJMJD1A vs shJMJD1A+c-Myc. LNCaP: $p<5\times10^{-4}$ for pLKO.1 vs shJMJD1A, $p<5\times10^{-4}$ for shJMJD1A vs shJMJD1A+c-Myc. **D.** c-Myc re-expression in JMJD1A-KD Rv1 cells partially rescued sphere formation in 3D Matrigel ($p<1\times10^{-14}$ for pLKO.1 vs shJMJD1A, $p<1\times10^{-13}$ for shJMJD1A vs shJMJD1A+c-Myc). **E.** c-Myc re-expression in JMJD1A-KD Rv1 cells partially rescued orthotopic tumor formation in nude mice. Luciferase-expressing Rv1 cells (pLKO.1, shJMJD1A, or shJMJD1A+c-Myc) were injected into mouse prostate (n=5 for each group). After 3 weeks, mice were injected with luciferin (i.p.), and tumor formation was monitored using in vivo bioluminescence imaging. **F.** Tumor weights of mice described in E were determined.

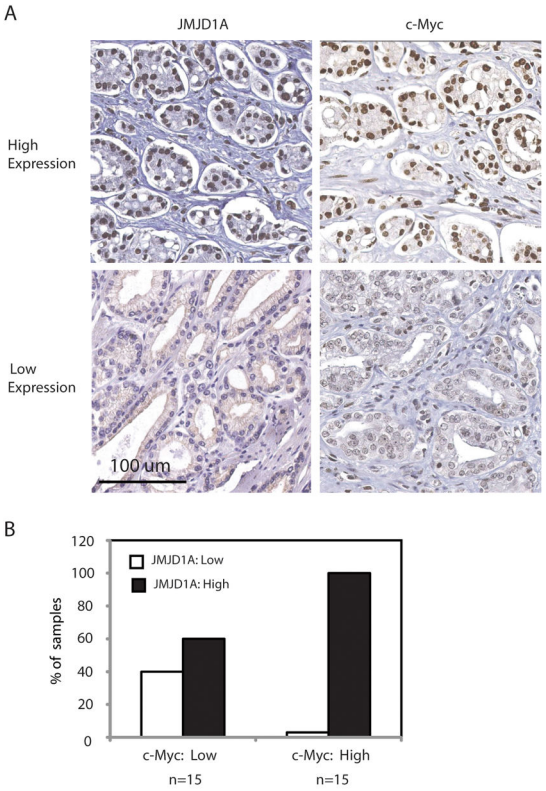

Figure 6.

A. Representative images showing c-Myc and JMJD1A immunohistochemistry (IHC) staining of human prostate cancer specimens from a prostate cancer TMA. Staining was visualized by DAB with hematoxylin counterstaining. **B.** Quantification of c-Myc or JMJD1A staining in prostate cancer TMA samples. Staining intensity of c-Myc or JMJD1A was scored as 0 to3 (0: no staining, 1: weak staining, 2: medium staining, and 3: strong staining. 0 and 1 were classified as low-expression, whereas 2 and 3 were defined as high-expression. High c-Myc expression was correlated with high JMJD1A expression ($p<0.05$, Fisher's exact test).