The histone demethylase JMJD2B is critical for p53-mediated autophagy and survival in nutlin-treated cancer cells

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Running title: JMJD2B regulates p53-mediated autophagy

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ABSTRACT

Autophagy promotes cancer cell survival in response to p53 activation by the anticancer agent Nutlin-3a (Nutlin). We previously reported that Nutlin kills MDM2-amplified cancer cells and that this killing is associated with an inhibition of glucose metabolism, reduced alpha-ketoglutarate (α-KG) levels, and reduced autophagy. In the current report, using SJSA1, U2OS, A549, and MHM cells, we found that Nutlin alters histone methylation in an MDM2 proto-oncogene (MDM2)-dependent manner and that this, in turn, regulates autophagy-related gene (ATG) expression and cell death. In MDM2-amplified cells, Nutlin increased histone (H) 3 lysine (K) 9 and K36 trimethylation (me3) coincident with reduced autophagy and increased apoptosis. Blocking histone demethylation restored autophagy and rescued these cells from Nutlin-induced killing. Further experiments suggested that MDM2 amplification increases histone methylation in Nutlin-treated cells by causing depletion of the histone demethylase Jumonji domain-containing protein 2B (JMJD2B). Finally, JMJD2B knockdown or inhibition increased H3K9/K36me3 levels, decreased ATG gene expression and autophagy, and sensitized MDM2-nonamplified cells to apoptosis. Together, these results support a model in which MDM2- and JMJD2B-regulated histone methylation levels modulate ATG gene expression, autophagy, and cell fate in response to the MDM2 antagonist Nutlin-3a.

Introduction

Wild-type p53 is a transcription factor and tumor suppressor that is activated in response to stress. Activated p53 can promote either transient cell cycle arrests that contribute to survival, or permanent cell cycle arrest (senescence) or apoptosis that inhibits survival and contributes to tumor suppression. These effects are mediated by
p53 responsive gene products including the cyclin-CDK inhibitor p21 or the apoptosis inducing factors PUMA, Noxa, and Bax (1-4). The outcome of p53 activation (survival vs senescence/apoptosis) is believed to depend in part on the level of stress.

In addition to these canonical functions, p53 also has non-canonical functions that include its ability to regulate autophagy (5,6). Autophagy is a process in which organelles, mis-folded proteins, and other intracellular components are degraded in autophagolysosomes (7-9). Autophagy is a multistep process. A first step in autophagy is formation of phagophore membranes. This step is promoted by an autophagy initiating complex that includes the proteins ULK1 and ULK2. Subsequent steps are mediated in large part by the products of various autophagy-related genes (ATGs). These steps include elongation of phagophore membranes, capture of cargo (organelles, mis-folded proteins, etc) by phagophore membranes, conversion of phagophore membranes into autophagosomes, fusion of cargo-containing autophagosomes with lysosomes, and degradation of cargo in these autophagolysosomes by lysosomal enzymes. Autophagy occurs at low levels normally but is stimulated in response to metabolic stresses such as low nutrient levels. The degradation products of autophagy can be used to generate and maintain the levels of key nutrients and metabolites needed for survival. Autophagy in this case allows survival until the metabolic stress caused by low nutrient levels can be resolved. p53 has been reported to both promote and inhibit autophagy (6). p53 can promote autophagy, in part, by activating ULK1 and also by binding the promoter regions of ULK1 and various ATG genes and promoting their expression (5,10,11). In contrast, Kroemer and colleagues reported that cytoplasmic but not nuclear p53 can inhibit autophagy (5). There is some evidence that autophagy mediated by p53 increases survival. For example, treatment with the autophagy inhibitor bafilomycin A1 increased apoptosis in cells treated with the p53-activator Nutlin (12,13)

p53 can also regulate cancer cell metabolism (14,15). Cancer cells often have an altered metabolism that includes increased glucose uptake and glycolysis and reduced oxidative phosphorylation. p53 can inhibit glycolysis by repressing expression of glycolytic enzyme genes and promote oxidative phosphorylation by increasing expression of genes like SCO2 (15,16). Most but not all MDM2-amplified cells undergo apoptosis in response to Nutlin treatment whereas most MDM2 non-amplified cells undergo cell cycle arrest with minimal apoptosis. We reported in MDM2-amplified cells that Nutlin treatment inhibits glucose metabolism and reduces alpha-ketoglutarate (α-KG) levels and that this is critical for Nutlin-induced apoptosis (12,17,18). In contrast, glucose metabolism and α-KG levels were maintained in MDM2 non-amplified cells treated with Nutlin. In these cells Nutlin increases autophagy that protects cells from apoptosis (12,17). We also found the sensitivity of MDM2-amplified cells to Nutlin-induced apoptosis is due, in part, to MDM2-mediated downregulation of SPI1 and subsequent downregulation of glycolytic genes (17).

Glycolysis promotes autophagy by in some way maintaining expression of various ATG genes in Nutlin treated cells (12,18), although the underlying mechanism for this is not known. Glycolytic metabolites are linked to histone modification that can regulate gene expression. Notably, α-KG is a metabolic intermediate of glucose. Recently we found that Nutlin suppresses α-KG and autophagy in MDM2-amplified cells while increasing α-KG and autophagy in MDM2-non-amplified cells (18). Importantly, α-KG is an activating cofactor
for JMJD family histone lysine demethylases (19). These enzymes can regulate gene expression by altering the histone methylation status at gene promoters (20,21). Histone methylation can regulate autophagy at gene expression levels. For example, Artal-Martinez de Narvajas et al reported the G9a histone methyltransferase inhibits autophagy by promoting H3K9me2 in the promoters of LC3 and other autophagy genes and repressing their expression (22). Histone methylations H3K27me3, H3K9me3, and H3K4me3 are found in LC3, ATG4b and p62 gene promoters (23).

The JMJD2 (Jumonji C domain containing histone demethylase 2) family of proteins selectively demethylate H3K9me3 and H3K36me3. Among the JMJD2 family, JMJD2B is a p53 target gene (24). We envisioned that JMJD2B could be induced by Nutlin-mediated activation of p53 and then regulate histone methylation to affect ATG gene expression and autophagy. In the current report, we found JMJD2B-mediated histone demethylation promotes ATG gene expression, autophagy, and survival in MDM2 non-amplified cells treated with Nutlin. We also found that JMJD2B is depleted in MDM2-amplified cells treated with Nutlin in a manner that appears to be MDM2-dependent. The depletion of JMJD2B leads to increased histone methylation, reduced ATG gene expression and autophagy, and increased killing in MDM2-amplified cells.

Results

We previously showed glycolysis and α-KG can protect cells against Nutlin-induced apoptosis by in some way maintaining expression of ATG genes required for autophagy (12,17,18). α-KG is an intermediate metabolite of glucose and a cofactor for JMJD family histone lysine demethylases (19). Thus, we speculated JMJD histone demethylases could promote autophagy by regulating histone methylation and ATG gene expression (22). To begin to test this possibility, two MDM2-amplified cell lines (MHM and SJSA1) (25) and two MDM2 non-amplified cell lines (A549 and U2OS) were treated with Nutlin and the levels of histone H3 lysine methylation determined by immunoblotting. As shown in Fig 1A, H3K9me2/3 and H3K36me3 levels were decreased in Nutlin-treated A549 cells and unaffected in Nutlin-treated U2OS cells. In contrast, H3K9me2/3 and H3K36me3 levels were increased in Nutlin-treated MHM and SJSA1 cells. H3K4me3 levels were unaffected by Nutlin in all cells. H3K27me3 was barely detectable in U2OS, MHM, and SJSA1 cells compared with that in A549 cells. The results indicate H3K9me2/3 and H3K36me3 are increased in MDM2-amplified MHM and SJSA1 cells that are sensitive to Nutlin-induced apoptosis (17), and either decreased or maintained in MDM2 non-amplified A549 and U2OS cells that are resistant to this apoptosis (17).

Next we asked if increased histone methylation contributes to apoptosis. For this, apoptosis was monitored in MHM and SJSA1 cells co-treated with Nutlin and the pan histone methylation inhibitor DZnep or IOX-1, a pan-inhibitor of JMJD2 histone demethylases. Co-treatment with DZnep blocked the increase in histone methylation seen in Nutlin-treated MHM cells (Fig. 1B) and reduced apoptosis in Nutlin-treated MHM and SJSA1 cells (Fig. 1C). In contrast, IOX-1 caused a slight increase in apoptosis in Nutlin-treated cells (Fig. 1C). These findings suggested that increased histone methylation contributes to Nutlin-induced apoptosis. To examine this further, we treated A549 and U2OS cells with Nutlin and IOX-1 and monitored histone methylation and apoptosis. Co-treatment with Nutlin and IOX-1 increased or maintained histone methylation in Nutlin-treated A549 and U2OS cells (Fig. 1D and E) and sensitized these cells to
apoptosis (Fig. 1F). Together, the results support the idea that increasing histone methylation sensitizes cells to Nutlin-induced apoptosis.

We next wished to ask if histone methylation regulates apoptosis in Nutlin-treated cells by affecting autophagy, since previous studies showed histone methylation can repress expression of LC3 and other autophagy genes (22). To examine this, we used autophagy flux as a measure of autophagy in Nutlin-treated cells. LC3-II is an autophagy protein that is degraded in autophagolysosomes. Bafilomycin A1 (BafA1) blocks autophagic degradation, including degradation of LC3-II. Thus, the extent to which LC3-II accumulates in BafA1 treated cells indicates the rate at which autophagic degradation is occurring, or autophagy “flux”. We first monitored LC3-II accumulation in MDM2-amplified MHM and SJSA1 cells treated with BafA1. As shown in Figs 2A and C, LC3-II accumulated in BafA1 treated MHM and SJSA1 cells, indicating autophagic degradation was occurring. LC3-II accumulated to a lesser extent in MHM and SJSA1 cells treated BafA1 plus Nutlin vs cells treated with BafA1 alone (Fig. 2A and C). This is consistent with our previous study (12) and indicates Nutlin inhibits autophagy flux in these cells. Importantly, LC3-II accumulated to a greater extent in cells co-treated with BafA1 plus Nutlin and DZNep compared to cells treated with BafA1 plus DZNep only (Fig. 2A and C). This indicates Nutlin treatment promotes autophagy flux when histone methylation is inhibited by DZNep. Monodascaverine (MDC) is a fluorescent marker of intact autophago-lysosomes, and MDC-labeling is a common measure of autophagy (26). We monitored MDC labeling of autophagic vesicles in MHM and SJSA1 cells treated with Nutlin alone or Nutlin plus DZNep. As shown in Figs. 2B and D, MDC labeling was reduced in Nutlin-treated MHM and SJSA1 cells, consistent with reduced autophagy. However, Nutlin increased MDC labeling when histone methylation was inhibited by co-treatment with DZNep. Altogether, the results indicate decreasing histone methylation can restore or increase autophagy in Nutlin-treated MHM and SJSA1 cells.

We next asked if increasing histone methylation by IOX1 treatment could reduce autophagy flux and MDC labeling in MDM2 non-amplified A549 cells. As shown in Fig 2E, LC3-II accumulated to a greater extent in A549 cells co-treated with Nutlin plus BafA1 compared to cells treated with BafA1 alone (Fig. 2E). This result suggests increasing histone methylation by IOX-1 treatment reduces autophagy flux. As shown in Fig 2F, MDC labeling was increased by Nutlin treatment in A549 cells, and this effect was blocked by IOX1 (Fig. 2F). In total, these results suggest increasing histone methylation in Nutlin-treated A549 cells reduces autophagy.

The above results suggest histone methylation, specifically H3K9/K36me3, is differentially regulated in MDM2-amplified (MHM and SJSA1) cells vs. MDM2 non-amplified (A549 and U2OS) cells and plays a repressive role in autophagy. JMJD2 family proteins promote demethylation of H3K9/K36me3, and JMJD2B is a p53 target gene (24,27). We speculated JMJD2B may regulate histone methylation and autophagy in response to p53 activation by Nutlin. To test this possibility, we first monitored JMJD2B gene and protein expression in Nutlin-treated MHM, SJSA1, A549, and U2OS cells. As shown in Fig. 3A, JMJD2B mRNA levels were increased in all four cell
lines treated with Nutlin. In U2OS and A549 cells, JMJD2B protein was also increased in response to Nutlin (Fig. 3B), consistent with the mRNA increase. However, JMJD2B protein was markedly decreased Nutlin-treated MHM and SJSA1 cells (Fig 3B). Notably, MDM2 levels were highly induced in MHM/SJSA1 cells compared with that in A549/U2OS cells (Fig. 3B). Co-treatment with the proteasome inhibitor MG132 restored JMJD2B protein levels in MHM and SJSA1 cells treated with Nutlin (Fig. 3C), at least partially, but only slightly increased JMJD2B protein levels in U2OS and A549 cells (Fig. 3D). These results suggest the decrease in JMJD2B protein levels in MDM2-amplified MHM/SJSA1 cells is due to increased protein degradation.

Results in Fig 3 suggested that reduced levels of JMJD2B may cause increased histone methylation and, subsequently, reduced autophagy and increased death in MDM2-amplified cells treated with Nutlin. If this were true, we reasoned knockdown of JMJD2B would also increase histone methylation in MDM2 non-amplified cells treated with Nutlin, and this would coincide with reduced autophagy and increased cell death. To test this, A549 cells were transfected with control siRNA or JMJD2B siRNA and then treated with vehicle or Nutlin. As shown in Fig 4A, H3K9me3 and H3K36me3 levels were increased in JMJD2B knockdown cells treated with Nutlin but not in control cells. Immunoblotting confirmed JMJD2B levels were depleted by the JMJD2B siRNA. We again used LC3 accumulation in BafA1-treated cells as a measure of autophagy flux. As shown in Figs 4B and D, LC3-II accumulated to a greater extent in control A549 and U2OS cells treated with Nutlin plus BafA1 compared to BafA1 alone, indicating Nutlin promotes autophagy flux in these cells. However, this effect was lost when JMJD2B was depleted by siRNA.

Similarly, Nutlin-treatment increased MDC labeling in control A549 and U2OS cells but not in cells where JMJD2B was depleted by siRNA (Fig 4C and 4E). These results indicate that depletion of JMJD2B blocks or reduces autophagy in Nutlin treated A549 and U2OS cells. Finally, we monitored apoptosis (% sub-G1 cells) in response to Nutlin in either control A549 and U2OS cells or cells in which JMJD2B was depleted by siRNA. As shown in Fig 4C and 4E, Nutlin promoted apoptosis only in cells where JMJD2B was depleted but not in control cells. These results support a role for JMJD2B in suppressing histone methylation in order to maintain or increase autophagy and survival in MDM2 non-amplified cells treated with Nutlin.

MDM2 is an ubiquitin E3-ligase that can bind and promote the degradation of p53 and other proteins. The finding that JMJD2B was depleted in MDM2 amplified cells treated with Nutlin but not in MDM2 non-amplified cells raised the possibility that MDM2 may bind and promote JMJD2B degradation in these cells. We carried out co-immunoprecipitation studies in SJSA1 cells to ask if MDM2 and JMJD2B can bind each other. In first experiments, to avoid JMJD2B degradation, cells were treated with Nutlin for only 6 hrs. This treatment length increases MDM2 levels but does not yet cause depletion of JMJD2B. As shown in Fig 5A, JMJD2B co-immunoprecipitated with MDM2 in SJSA1 cells treated with Nutlin for 6 hrs. We carried out similar experiments in which SJSA1 cells were treated with Nutlin for 24 hrs followed by treatment with MG132 for 4 hrs. As shown in Fig 5B, JMJD2B levels were reduced with 24 hr Nutlin treatment but restored by MG132. Further, MDM2 was co-immunoprecipitated with JMJD2B in SJSA1 cells treated with Nutlin for 24hr and subsequently treated with MG132 (Fig 5B). The results demonstrate JMJD2B and MDM2 can interact. Because
MDM2 is ubiquitin ligase, we tested if MDM2 downregulates JMJD2B via ubiquitination by co-expression of MDM2 and JMJD2B in p53-null H1299 cells. However, we failed to see JMJD2B ubiquitination by MDM2 (data not shown). Thus, we did not gain any evidence for JMJD2B degradation directly regulated by MDM2. Nutlin increases MDM2 protein levels but also inhibits the ability of MDM2 to bind and degrade p53 and other proteins that interact with MDM2 in the p53 binding domain. We speculated that if Nutlin reduces JMJD2B protein levels and blocks autophagy in MDM2 amplified cells by inhibiting MDM2, then depletion/knockdown of MDM2 would have the same effect as Nutlin. To examine this, we monitored JMJD2B protein levels, histone methylation, and autophagy in SJSA1 cells that were either treated with Nutlin or where MDM2 was depleted by siRNA and/or treated with Nutlin. We choose SJSA1 for this experiment because we have found MDM2 can be more efficiently knocked down with transfection of MDM2 siRNA compared with MHM cells. As shown in Fig 5C, both knockdown of MDM2 and Nutlin treatment increased p53 levels. However, only Nutlin treatment but not MDM2 knockdown caused depletion of JMJD2B and increased histone methylation (H3K9me3). Moreover, MDM2 knockdown blocked the Nutlin-induced increase in H3K9me3 and decrease in JMJD2B. Further, Nutlin treatment reduced MDC labeling (Fig 5D) and reduced autophagy flux (reduced the accumulation of LC3 by BafA1) (Fig 5E) which was reversed by MDM2 knockdown. Finally, Nutlin treatment caused abundant apoptosis in SJSA1 cells which was also reversed by MDM2 knockdown (Fig 5F). These results indicate the depletion of JMJD2B and inhibition of autophagy in MDM2-amplified cells treated with Nutlin is MDM2-dependent.

We previously reported that ULK1, ATG3, ATG7, and ATG16L1 are downregulated by Nutlin in MHM and SJSA1 cells and this is reversed by α-KG (18). Because α-KG is a cofactor for JMJD2B, it may upregulate ATG genes via JMJD2B-mediated histone demethylation. Since JMJD2B levels are increased by Nutlin in A549 and U2OS cells, we speculated JMJD2B may promote ATG gene expression in these cells. To test this, we first compared ATG gene expression in response to Nutlin in MHM, SJSA1, A549, and U2OS cells. The results show that ULK1, ATG3, ATG5, ATG10, and ATG16L1 are downregulated by Nutlin in MHM and SJSA1 cells (Fig. 6A). However, these genes are not downregulated by Nutlin in A549 and U2OS cells and, in fact, ULK1 and ATG16L1 are upregulated by Nutlin in these cells (Fig. 6B).

The opposite effect of Nutlin on ULK1 and ATG16L1 expression in MHM/SJSA1 vs. A549/U2OS cells may be regulated by JMJD2B and histone methylation. If so, then knockdown of JMJD2B should decrease ULK1 and ATG16L1 expression in A549 and U2OS cells while inhibition of histone methylation should increase their expression in H1299 and SJSA1 cells. To test this, we first knocked down JMJD2B with siRNA in A549 and U2OS cells and monitored ULK1 and ATG16L1 expression. The results showed that ULK1 and ATG16L1 mRNA levels were increased by Nutlin in control cells. However, this Nutlin-induced increase in ULK1 and ATG16L1 mRNA was prevented by JMJD2B knockdown (Fig. 6C and E). Immunoblotting also showed that Nutlin induced an increase in ULK1 and ATG16L1 proteins that was blocked by knockdown of JMJD2B (Fig. 6D and F). These results show that JMJD2B promotes ULK1 and ATG16L1 expression in Nutlin-treated A549 and U2OS cells. Finally, we sought further evidence that histone methylation regulates ATG gene expression.
expression in MDM2 amplified cells treated with Nutlin. To this end, we inhibited histone methylation in MHM cells by DZnep treatment and analyzed expression of ULK1 and ATG16L1 in response to Nutlin. Consistent with Fig 6A and our previous report (18), Nutlin treatment decreased ULK1 and ATG16L1 expression in MHM and SJSB1 cells (Fig. 7A). Importantly, however, co-treatment with DZnep blocked the decrease caused by Nutlin. Immunoblotting showed that ULK1 and ATG16L1 proteins were decreased by Nutlin and this effect was blocked or reversed by DZnep (Fig. 7B).

Altogether, the results suggest p53-mediated upregulation of JMJD2B and subsequent histone demethylation promotes ULK1 and ATG16L1 expression and autophagy in MDM2 non-amplified cells treated with Nutlin. However, in MDM2 amplified cells treated with Nutlin, JMJD2B protein is downregulated in a MDM2-dependent manner, leading to increased histone methylation, repression of ULK1 and ATG16L1 expression, reduced autophagy, and increased apoptosis.

Discussion

MDM2 antagonists like Nutlin activate wild-type p53 and are being developed as therapeutic agents for p53 wild-type cancers. We reported that p53 activated by Nutlin treatment can either promote or inhibit autophagy dependent on the MDM2 gene amplification status in cells. Results from the current study indicate the histone demethylase JMJD2B promotes autophagy via upregulating autophagy (ATG) genes and determines cell fate in response to Nutlin. JMJD2B belongs to the JMJD2 family of proteins that removes methyl groups specifically from histone H3 lysine 9 and lysine 36 (19,20). The JMJD2B gene is also transcriptionally activated by p53 (24). We treated different cell lines with Nutlin to activate p53 and then monitored histone methylation and autophagy. In the MDM2 amplified cell lines MHM and SJSB1 Nutlin treatment increased H3K9me2/3 and H3K36me3 levels. This increased histone methylation coincided with reduced expression of ULK1 and ATG16L1, reduced autophagy flux, and apoptosis. Importantly, decreasing histone methylation by DZnep restored ULK1 and ATG16L1 expression and autophagy and rescued these cells from Nutlin-induced killing. In MDM2 non-amplified cell lines U2OS and A549, Nutlin treatment decreased H3K9me2/3 and H3K36me3 levels. This decrease in histone methylation coincided with increased autophagy and increased expression ULK1 and ATG16L1. Increasing histone methylation through knockdown of JMJD2B or use of a JMJD2 inhibitor IOX1 reduced ULK1 and ATG16L1 expression and autophagy in these cells and sensitized the cells to apoptosis by Nutlin. The results support a model in which p53-regulated autophagy and cell fate in response to Nutlin treatment is controlled by JMJD2-dependent histone demethylation.

It has been reported that p53 and MDM2 can bind and/or regulate the activity and expression of different histone methylation and demethylation enzymes, and that they have opposing effects on histone methylation. For example, Zheng et al reported p53 promotes expression of the JMJD2B histone demethylase while reducing expression of the histone methyltransferase SUV39H1 (27). The authors found that this results in reduced H3K9me3 levels, relaxed chromatin structure, and increased DNA repair. In contrast, MDM2 was reported to increase H3K9 methylation in p53 target genes, due most likely to the ability of MDM2 to bind and recruit SUV39H1 and EHMT1 (another histone methyltransferase) to p53-responsive genes (28). Tang et al
reported p53 can repress expression of the histone methyltransferase EZH2, which normally promotes H3K27 tri-methylation (29). By contrast, Wienken et al reported that MDM2 can increase H3K27me3 by binding the EZH2-containing polycomb repressor complex 2 (PRC2) (30). Importantly, p53 has also been reported to interact with SUV39H1, SETD2, and JMJD2 proteins to regulate target gene expression (31,32). Results from the current study suggest another mechanism by which p53 and MDM2 can regulate histone methylation that involves changes in JMJD2B protein levels. Specifically, we found Nutlin increased H3K9me2/3 and H3K36me3 levels in MDM2 amplified MHM and SJSA1 cells, but either did not alter or reduced the levels of these methylations in MDM2 non-amplified U2OS and A549 cells. JMJD2 family proteins remove methyl groups specifically from H3K9 and H3K36 (19,20). Given the effect of Nutlin treatment on JMJD2B levels in these different cells, we speculated changes in histone methylation could result from changes in JMJD2B protein level. Knockdown of JMJD2B or inhibition of JMJD2 by IOX1 increased histone methylation in Nutlin-treated A549 and U2OS cells. This supports the idea that reduced histone methylation in Nutlin-treated A549 and U2OS cells requires JMJD2 demethylase activity. Together, these data indicate histone methylation in Nutlin-treated cells is regulated, at least in part, by JMJD2 histone demethylases.

We found JMJD2B mRNA and protein expression in response to Nutlin is different in MDM2 amplified vs. MDM2 non-amplified cells. JMJD2B mRNA was increased by Nutlin in all the cell lines, consistent with reports that JMJD2B is transcriptionally activated by p53. JMJD2B protein was increased by Nutlin treatment in A549 and U2OS cells. However, JMJD2B protein levels were markedly decreased by Nutlin treatment in the MDM2 amplified cells MHM and SJSA1. This decrease was due to enhanced protein degradation since it was largely prevented by the proteasome inhibitor MG132. Nutlin binds MDM2 and increases MDM2 protein levels, but also inhibits MDM2 from functionally interacting with p53 and other proteins that bind the MDM2 N-terminus. We therefore asked if inhibiting MDM2 in MDM2 amplified cells (by siRNA knockdown) could mimic the effects of Nutlin. However, MDM2 knockdown did not reduce JMJD2B protein levels and also did not reduce ULK1 and ATG16L gene expression and autophagy. Moreover, knockdown of MDM2 blocked Nutlin-induced loss of JMJD2B, inhibition of autophagy, and apoptosis. These results indicate the ability of Nutlin to reduce JMJD2B levels and reduce ULK1/ATG16L expression and autophagy is not due to inhibition of MDM2 activity. Given that Nutlin binds MDM2, the results suggest the ability of Nutlin to reduce JMJD2B levels in MDM2 amplified cells and cause a subsequent reduction in ULK1/ATG16L expression and autophagy requires MDM2 protein. Because MDM2 is an E3 ubiquitin ligase, we speculated the very high MDM2 levels may bind and promote JMJD2B degradation in MDM2 amplified cells treated with Nutlin. Indeed, we observed MDM2 and JMJD2B co-immunoprecipitate with each other in MDM2 amplified cells treated with Nutlin and MG132. However, in subsequent and ongoing studies we have gained no evidence that MDM2 can promote the ubiquitination or degradation of JMJD2B in transfected cells. Further studies are required to determine how MDM2 affects JMJD2B protein levels in Nutlin-treated, MDM2 amplified cells.

Previous studies showed histone methylation can repress various genes known to participate in autophagy. For example, Artal-Martinez de Narvajas et al reported the
G9a histone methyltransferase inhibits autophagy by promoting H3K9me2 in the promoters of LC3 and other autophagy genes and repressing their expression (22). P53 has been reported to promote or inhibit autophagy dependent on its subcellular localization. For example, nuclear p53 can promote autophagy by binding the promoters of ULK1, DRAM1, and various ATG genes and promoting their expression. In contrast, cytoplasmic p53 has been reported to inhibit autophagy. The JMJD2B gene is transcriptionally activated by p53. In the current report JMJD2B mRNA and protein levels were increased in A549 and U2OS cells treated with Nutlin, most likely due to p53-mediated activation of the JMJD2B gene. Further, increased expression of JMJD2B was necessary for increased ULK1 and ATG16L expression and autophagy in these cells. These results indicate yet another way in which p53 can promote autophagy is by increasing JMJD2B expression and causing a subsequent increase in ULK1 and ATG16L. A question that arises is how JMJD2B promotes ULK1/ATG16L expression and autophagy. H3K9me3 and H3K36me3 are substrates for demethylation by JMJD2B and both are associated with condensed chromatin and repression of gene transcription. Thus, one possibility is that JMJD2B increases ULK1 and ATG16L gene expression by reducing H3K9me3 and/or H3K36me3 levels in the ULK1 and ATG16L gene promoters. It is also possible that JMJD2B modulates expression of other proteins that, in turn, increase expression of ULK1 and ATG16L.

MDM2 amplified cancer cells are, in most cases, especially sensitive to Nutlin induced apoptosis. The molecular basis for this is incompletely understood. Results from the current study indicate the heightened sensitivity of MDM2 amplified cells to Nutlin-induced apoptosis can be explained in part by reduced JMJD2B, increased histone methylation, and subsequent loss of ATG gene expression and autophagy (see proposed model in Fig. 8). Further, the results suggest JMJD2 histone demethylases could be targeted to increase cancer cell sensitivity to MDM2 antagonists like Nutlin.

Experimental procedures

Cells and Reagents
SJSA1, U2OS, A549 cells were from ATCC. MHM cells were kindly provided by Dr. Ola Myklebost, Norwegian Radium Hospital. All cell lines were grown in RPMI medium with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were plated 48h before treatment. Nutlin, monodansylcadaverine (MDC), dimethyl ketoglutarate (DMKG), BIX-01294, and BafA1 were obtained from Sigma Aldrich (St. Louis, MO, USA). DZnep was from EMD Millipore (Darmstadt, Germany). IOX1 was from Selleck Chemicals (Houston, TX, USA).

Immunoblotting
Whole cell extracts were prepared by scraping cells in RIPA buffer, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies to ATG16L1, H3K4me3, H3K9me3, H3K9me2, H3K9me3, H3K27me3, H3K36me3, H3, and OGDH were from Cell Signaling (Boston, MA, USA); LC3B and ULK1 antibodies were from Abcam (San Francisco, CA, USA). p62, β-actin, and p53 (DO-1) antibodies were from Santa Cruz (CA, USA). Primary antibodies were detected with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase was from Invitrogen (Carlsbad, CA, USA), using Clarity
chemiluminescence from BIO-RAD (Hercules, CA, USA).

**Flow Cytometry**
For cell cycle analysis, cells were harvested and fixed in 25% ethanol overnight. The cells were then stained with propidium iodide (25 µg/ml, Calbiochem). Flow cytometry analysis was performed on a Gallios™ Flow Cytometer (Beckman Coulter), analyzed with FlowJo 10 (Treestar Inc). For each sample, 10,000 events were collected.

**siRNA-mediated transient Knockdown**
JMJD2B siRNA (On-target plus smart pool) and Control siRNA (On-target plus siControl non-targeting pool) were purchased from GE Dharmacon (Lafayette, CO) and were transfected according to the manufacturer’s guidelines using DharmaFECT I reagent.

**RNA isolation and Real-time quantitative PCR analysis**
Total RNA was prepared using Total RNA Mini Kit (IBI Scientific, IA, USA); the first cDNA strand was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Manufacturers’ protocols were followed in each case. The PCR primers for ULK1, ATG3, 4a, 7, 10, 12, 16L1, LC3 and β-actin genes are previously described (12,33). SYBR green PCR kit (Midwest Scientific, St, Louis, USA) was used according to the manufacturer’s instructions. AB7300 system was used as follows: activation at 95°C; 2 minutes, 40 cycles of denaturation at 95°C; 15 seconds and annealing/extension at 60°C; 60 seconds, followed by melt analysis ramping from 60°C to 95°C. Relative gene expression was determined by the ΔΔCt method using β-Actin to normalize.

**Quantitative analysis of autophagosomes and autolysosomes**
For analysis of autophagosomes/autolysosomes, MDC sequestration was conducted as previously described (34).

**Statistical analysis**
One-way analysis of variance (ANOVA) was used to determine the statistical significance of differences among experimental groups. Student’s t-test was used to determine the statistical significance between experimental groups.

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**Abbreviations:** ATG16L1, autophagy-related 16-like 1; H3K9me3, histone 3 lysine 9 trimethylation; H3K36me3, histone 3 lysine 36 trimethylation; JMJD, Jumonji C domain-containing histone demethylase; LC3, microtubule-associated protein 1 light chain 3; MDC, monodascaverine; SETD2, SET domain containing 2; siRNA, small interfering RNA; shRNA, short-hairpin RNA; SUV39H1, suppressor of variegation 3–9 homolog 1; TCA cycle, tricarboxylic acid cycle; ULK1, unc-51-like kinase 1; ULK2, unc-51-like kinase 2;

**Figure legends**

**Figure 1. Histone methylation status decides cell fate in Nutlin-treated cells.** A. Cells were treated 24 hr with Nutlin (10 µM). Whole cell lysates were immunoblotted for the indicated proteins. B. Cells were treated 24 hr with vehicle or Nutlin (10 µM) and/or DZnep (10 µM). Whole cell lysates were immunoblotted for the indicated proteins. C. Cells were treated 72 hr with vehicle or Nutlin (10 µM) and/or DZnep (10 µM) or IOX-1 (0.1 mM) and then analyzed for sub-G1. % average sub-G1 cells (triplicate) is presented with SD indicated. The asterisk indicates there is a significant difference between Nutlin- and Nutlin plus DZnep-treated MHM (p<0.01) and SJSA1 (p=0.01)). D and E. Cells were treated 24 hr with vehicle or Nutlin (10 µM) without or with IOX-1 (0.1 mM). Whole lysates were immunoblotted for the indicated proteins. F. Cells were treated 72 hr with vehicle or Nutlin (10 µM) and/or IOX-1 (0.1 mM) and then analyzed for sub-G1. Average % sub-G1 cells (triplicate) is presented with SD indicated. The asterisk indicates there is significant difference between Nutlin and Nutlin plus IOX-1 in A549 (p<0.01) and U2OS (p<0.01) cells.

**Figure 2. Histone methylation suppresses autophagy.** A and C. MHM and SJSA1 cells were treated 24 hr with Nutlin (10 µM) and/or DZnep (10 µM) in the presence or absence of bafilomycin A1 (BafA1, 10nM), lysates were immunoblotted for the indicated proteins. B and D. MHM and SJSA1 cells were treated 24hr with Nutlin (10 µM) and/or DZnep (10 µM) and then analyzed for MDC sequestration, average relative MDC (triplicate) is presented with SD indicated. The asterisk indicates there is a significant difference between vehicle and Nutlin (p<0.05) as well as Nutlin and Nutlin plus DZnep (p<0.01) in MHM and SJSA1 cells. E. A549 cells were treated 24 hr with Nutlin (10 µM) and/or IOX-1 (0.1 mM) in the presence or absence of BafA1(10 nM), lysates were immunoblotted for the indicated proteins. F. A549 cells were treated 24hr with Nutlin (10 µM) and/or IOX-1 (0.1 mM) and then analyzed for MDC sequestration, average relative MDC (triplicate) is presented with SD indicated. The asterisk indicates there is a significant difference between vehicle and Nutlin (p<0.01) as well as Nutlin and Nutlin plus IOX-1 (p<0.01) cells.

**Figure 3. Nutlin upregulates JMJD2B proteins in U2OS and A549 but downregulates in MHM and SJSA1 cells.** A. Cells were treated 24 hr with vehicle or Nutlin (10 µM). JMJD2B mRNA were determined and presented (average from triplicate, SD is too small to show). B. Cells were treated 24 hr with vehicle or Nutlin (10 µM). Lysates were immunoblotted for the indicated proteins.
proteins. C and D. Cells were treated 24 hr with Nutlin (10 µM) and then with MG132 (10 µM) for 4 hr. Lysates were immunoblotted for the indicated proteins.

**Figure 4. Knockdown of JMJD2B suppresses autophagy and sensitizes cells to Nutlin.** A. A549 cells were transfected with control siRNA or JMJD2B siRNA and then treated 24 hr with Nutlin (10 µM), lysates were immunoblotted for the indicated proteins. B and D. A549 and U2OS cells were transfected with control siRNA or JMJD2B siRNA and then treated 24 hr with Nutlin (10 µM) and/or BafA1 (10 nM). Whole cell lysates were immunoblotted for the indicated proteins. Note: 4A and 4B are from the same experiment. In 4B, the first four lanes of the JMJD2B blot are reused from Fig 4A, in order to illustrate differences in JMJD2B expression in the absence and presence of BafA1. C. Left panel: A549 cells were transfected with control siRNA or JMJD2B siRNA and then treated 24 hr with vehicle or Nutlin (10 µM). MDC sequestration was determined and average relative MDC (triplicate) is presented with SD indicated (left panel). The asterisk indicates there is a significant difference between vehicle treated and Nutlin-treated conditions in control siRNA (p<0.01) and as well as Nutlin-treated control siRNA and JMJD2B siRNA (p<0.01) cells. Right panel: Cells were treated similarly for 72 hr and then analyzed for sub-G1. % sub-G1 cell were presented with SD indicated. There is a significant difference between Nutlin-treated control siRNA and JMJD2B siRNA (p<0.01) cells. E. Left panel: U2OS cells were transfected with control siRNA or JMJD2B siRNA and then treated 24 hr with vehicle or Nutlin (10 µM). MDC sequestration was determined and average relative MDC (triplicate) is presented with SD indicated. The asterisk indicates there is a significant difference between vehicle treated and Nutlin-treated conditions in control siRNA (p<0.05) and as well as Nutlin-treated control siRNA and JMJD2B siRNA (p<0.05) cells. Right panel: Cells were treated similarly for 72 hr and analyzed for sub-G1. % sub-G1 cell were presented with SD indicated. There is a significant difference between Nutlin-treated control siRNA and JMJD2B siRNA (p<0.01) cells.

**Figure 5. MDM2 associates with and mediates downregulation of JMJD2B.** A. SJSA1 cells were treated 6 hr with Nutlin (10 µM) and MG132 (10 µM). Lysates were immunoprecipitated with anti-MDM2 antibody and blotted for JMJD2B and MDM2. B. SJSA1 cells were treated 24 hr with Nutlin (10 µM) and then 4 hr with MG132 (10 µM). Lysates were immunoprecipitated with anti-JMJD2B antibody and blotted for MDM2 and JMJD2B. C. SJSA1 cells were transfected with control siRNA or MDM2 siRNA and then treated with vehicle or Nutlin (10 µM) for 24 hr. Whole cell lysates were immunoblotted for the indicated proteins. D. Control siRNA and MDM2 siRNA cells were treated with vehicle or Nutlin (10 µM) for 24 hr. MDC sequestration was determined and average relative MDC (triplicate) is presented with SD indicated. The asterisk indicates there is a significant difference between vehicle and Nutlin (p<0.05) in control cells and between Nutlin-treated control cells and Nutlin-treated MDM2 knockdown cells (p<0.01). E. Control siRNA and MDM2 siRNA cells were treated 24 hr with vehicle or Nutlin (10 µM) in the presence or absence of BafA1 (10 nM). Cell lysates were immunoblotted for the indicated proteins. F. Control siRNA and MDM2siRNA cells were treated with vehicle or Nutlin (10 µM) for 72 hr and then analyzed for Sub-G1. Average % Sub-G1 cells were presented with SD indicated. The asterisk indicates there is significant differences between vehicle and Nutlin cells (p<0.01) as well as Nutlin-treated control siRNA cells and Nutlin-treated MDM2 siRNA cells (p<0.01).

**Figure 6. JMJD2B promotes ULK1 and ATG16L1 expression.** MHM and SJSA1 cells (A) and A549 and U2OS cells (B). mRNA for the indicated ATG genes were determined and presented as
graphs (average from triplicate, SD is too small to show). C and E. A549 and U2OS cells were transfected with control siRNA or JMJD2B siRNA and treated with vehicle or Nutlin for 24 hr. mRNA for ULK1 and ATG16L1 were determined and presented as graphs with SD indicated. The asterisk indicates there are significant differences between vehicle and Nutlin treatment in control A549 and U2OS cells (p<0.05), and between Nutlin-treated control cells and Nutlin-treated JMJD2B knockdown cells (p<0.01). Whole cell lysates were immunoblotted for the indicated proteins (D and F).

**Figure 7. Histone methylation represses ULK1 and ATG16L1 expression.** A. MHM and SJSA1 were treated 24 hr with vehicle or Nutlin (10 µM) in the presence or absence of DZnep (10 µM). mRNA for ULK1 and ATG16L1 were determined and presented as graphs with SD indicated (triplicate). The asterisk indicates there are significant differences between vehicle and Nutlin treatment in control MHM and SJSA1 cells (p<0.05), and between Nutlin-treated and Nutlin plus DZnep-treated cells (p<0.01). B. Whole cell lysates were immunoblotted for the indicated proteins.

**Figure 8. Proposed model.** A. In MDM2 non-amplified A549 and U2OS cells, Nutlin activates p53 that upregulates JMJD2B. JMJD2B demethylates H3K9me3 and H3K36me3 that are promoted by histone methyltransferases (HMT). Histone demethylation leads to transactivation of ULK1 and ATG16L1 genes that are critical for p53-mediated autophagy. B. In MDM2 amplified MHM and SJSA1 cells, Nutlin-induced p53 increases MDM2 to very high levels. High levels of MDM2 lead to degradation of JMJD2B through an unknown mechanism. Loss of JMJD2B results in increased HMT-mediated histone methylation that represses ULK1 and ATG16L1 genes leading to suppression of p53-mediated autophagy.
Figure 1

A. A549  U2OS  MJM  SJSA1
     Nutlin:  - +   - +    - +    - +
     H3K9me3
     H3K9me2
     H3K9me3
     H3K27me3
     H3K36me3
     H3
     p53
     Actin

B. DZnep:  - - +    +    - - +
     Nutlin:  - +   - +    - +    - +
     H3K9me2
     H3K9me3
     H3K36me3
     H3
     p53

C. % Sub-G1 cells
     Nutlin:  - +   - +    - +
     DZnep:  - - +    +
     IOX1:  - - - - +    +

D. IOX1:  - - +  +    +
     Nutlin:  - - - - +    +
     H3K9me2
     H3K9me3
     H3K36me3
     H3

E. IOX1:  - - + +    +
     Nutlin:  - - - - +    +
     H3K9me3
     H3K36me3
     H3

F. % Sub-G1 cells
     Nutlin:  - +   - +
     IOX1:  - - +  +

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Figure 2

A. DZnep: - - - - + + + +
   BafA1: - - + + - - + +
   Nutlin: - + - - + + - +
   LC3-I
   LC3-II
   p53
   Actin
   MHM

B. Relative MDC
   Nutlin: - + - +
   DZnep: - - + +
   MHM

C. DZnep: - - - - + + + +
   BafA1: - - + + - - + +
   Nutlin: - + - - + + - +
   LC3-I
   LC3-II
   p53
   Actin
   SJSA1

D. Relative MDC
   Nutlin: - + - +
   DZnep: - - + +
   SJSA1

E. IOX1: - - - - + + + +
   BafA1: - - + + - - + +
   Nutlin: - + - - + + - +
   LC3-I
   LC3-II
   p53
   Actin
   A549

F. Relative MDC
   Nutlin: - + - +
   IOX1: - - + +
   A549
Figure 3

A. 

![Graph showing relative mRNA levels for A549, U2OS, MHM, and SJSA1 with Nutlin and NT markers.]

B. 

![Western blot images for MHM, SJSA1, A549, and U2OS showing relative levels of JMJD2B, MDM2, p53, and Actin with Nutlin and MG132 treatments.]

C. 

|           | MHM | SJSA1 |
|-----------|-----|-------|
| MG132     | -   | -     |
| Nutlin    | +   | +     |

D. 

|           | U2OS | A549 |
|-----------|------|------|
| MG132     | -    | -    |
| Nutlin    | +    | +    |

Lanes: 1 2 3 4 5 6 7 8
Figure 5

A. Table and image showing protein expression levels for IP: MDM2 and Lysates with MG132 and Nutlin treatments for SJSA1 cells.

B. Image showing protein expression levels for IP: JMJD2B and Lysates with MG132 and Nutlin treatments for SJSA1 cells.

C. Table and image showing protein expression levels for MDM2si and Nutlin treatments with SJSA1 cells.

D. Bar chart showing relative MDC for MDM2si and Nutlin treatments for SJSA1 cells.

E. Image showing protein expression levels for Control siRNA, MDM2siRNA, BafA1, and Nutlin treatments with SJSA1 cells.

F. Bar chart showing % Sub-G1 cells for MDM2si and Nutlin treatments for SJSA1 cells.
Figure 6

A.

![Graph showing relative mRNA levels for ULK1, ATG3, ATG4B, ATG5, ATG7, ATG10, ATG12, and ATG16L1 across different conditions.]

B.

![Graph showing relative mRNA levels for ULK1, ATG3, ATG4B, ATG5, ATG7, ATG10, ATG12, and ATG16L1 across different conditions.]

C.

![Bar graph showing relative mRNA levels for ULK1 and ATG16L1 in A549 cells with varying JMJD2Bsi and Nutlin treatments, with significance markers (* and **) indicating statistical differences.]

D.

![Western blot images for A549 cells showing ULK1, ATG16L1, JMJD2B, p53, and Actin proteins under different conditions.]

E.

![Bar graph showing relative mRNA levels for ULK1 and ATG16L1 in U2OS cells with varying JMJD2Bsi and Nutlin treatments, with significance markers (* and **) indicating statistical differences.]

F.

![Western blot images for U2OS cells showing ULK1, ATG16L1, JMJD2B, p53, and Actin proteins under different conditions.]

Legend:

- MHM NT
- MHM NUTLIN
- SJSA1 NT
- SJSA1 NUTLIN
- A549 NT
- A549 NUTLIN
- U2OS NT
- U2OS NUTLIN

Cell lines: A549, U2OS
Figure 7

A. Relative mRNA

- **Cell line: MHM**
- **Nutlin:** - + - +
- **DZNep:** - - + +

- **Cell line: SJSA1**
- **Nutlin:** - + - +
- **DZNep:** - - + +

B. Western Blot

- **MHM**
- **DZNep:** - - + +
- **Nutlin:** - + - +

- **SJSA1**
- **DZNep:** - - + +
- **Nutlin:** - - + +

- **Western Blot**
  - **ULK1**
  - **ATG16L1**
  - **p53**
  - **Actin**
Figure 8

A. A549/U2OS Cells

- Nutlin
- p53
- JMJD2B
- Autophagy
- ULK1
- ATG16L1
- HMT

B. MHM/SJSA1 Cells

- Nutlin
- MDM2
- JMJD2B
- Autophagy
- ULK1
- ATG16L1
- HMT
The histone demethylase JMJD2B is critical for p53-mediated autophagy and survival in nutlin-treated cancer cells
Lei Duan, Ricardo E. Perez, Xin Lai, Ling Chen and Carl G. Maki

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