Heat Shock Protein 90 (Hsp90) Selectively Regulates the Stability of KDM4B/JMJD2B Histone Demethylase*5

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Background: The levels of the KDM4B histone demethylase are elevated in different types of cancer cells, and its depletion suppresses tumor formation.

Results: Pharmacological inhibition of Hsp90 promotes ubiquitin-dependent proteasomal degradation of KDM4B, hence altering the histone code.

Conclusion: KDM4B is a bona fide client protein of Hsp90.

Significance: Hsp90 inhibitors may be useful for the treatment of tumors driven by KDM4B overexpression.

The family of KDM4A-D histone demethylases selectively demethylates H3K9 and H3K36 and is implicated in key cellular processes including DNA damage response, transcription, cell cycle regulation, cellular differentiation, senescence, and carcinogenesis. Various human cancers exhibit elevated protein levels of KDM4A-D members, and their depletion impairs tumor formation, suggesting that their enhanced activity promotes carcinogenesis. However, the mechanisms regulating the KDM4 protein stability remain largely unknown. Here, we show that the molecular chaperon Hsp90 interacts with and stabilizes KDM4B protein. Pharmacological inhibition of Hsp90 with geldanamycin resulted in ubiquitin-dependent proteasomal degradation of KDM4B, but not of KDM4C, suggesting that the turnover of these demethylases is regulated by distinct mechanisms. This degradation was accompanied by increased methylation of H3K9. We further show that KDM4B is ubiquitinated on lysines 337 and 562; simultaneous substitution of these residues to arginine suppressed the geldanamycin-induced degradation of KDM4B, suggesting that the ubiquitination of Lys-337 and Lys-562 targets KDM4B for proteasomal degradation upon Hsp90 inhibition. These findings constitute a novel pathway by which Hsp90 activity alters the histone code via regulation of KDM4B stability. This pathway may prove a druggable target for the treatment of tumors driven by enhanced KDM4B activity.

Histone methylation is a reversible and dynamically regulated process. Two classes of lysine demethylases have been discovered. The first consists of lysine-specific demethylase 1 (LSD1) that demethylates lysine through an amine oxidative reaction (1). The second class includes the Jumonji C (JmjC) domain-containing proteins that demethylate lysine residues through a hydroxylation reaction. The JmjC catalytic domain forms an enzymatically active pocket that coordinates ferrous oxide and α-ketoglutarate co-factors and subsequently produces a highly reactive oxoferryl that hydroxylates the methylated lysine (2, 3). The human KDM4 family consists of four members: KDM4A, KDM4B, KDM4C, and KDM4D (also known as JMJD2A-D). These enzymes specifically catalyze the demethylation of H3K9me2/me3 and H3K36me2/me3 (4–7).

The KDM4 family is implicated in key cellular processes, including transcription, DNA damage response, cell cycle regulation, cellular differentiation, senescence, and carcinogenesis (8–10). Members of this family are up-regulated in different types of human cancer, and their depletion impairs cancer cell proliferation and tumor formation, suggesting a causative role of KDM4A-D in promoting tumorigenesis (4–7, 11–13). For instance, overactivity of KDM4B promotes gastric tumorigenesis (14–17). Consistently, inactivation of KDM4B leads to cell cycle arrest and apoptosis of gastric cell lines (18). Given that elevated levels of KDM4A-D promote carcinogenesis, it becomes essential to explore the mechanisms regulating the stability of KDM4A-D proteins. Recent studies identified several E3 ubiquitin ligases that regulate the stability of KDM4A-B proteins during cell cycle progression and in response to DNA damage (10, 19, 20).

Here, we reveal a previously unrecognized role of heat shock protein 90 (Hsp90)2 in regulating the stability of KDM4B protein. Inhibition of Hsp90 leads to ubiquitin-dependent proteasomal degradation of KDM4B protein. Moreover, we have identified two ubiquitinated lysine residues on KDM4B that are involved in the geldanamycin (GA)-induced degradation of KDM4B protein. Our results provide a new and attractive pathway for modulating KDM4B protein level in cancer cells by inhibiting the activity of Hsp90 molecular chaperone.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Establishment of Stable Cell Lines—U2OS-TetON stable cell lines that conditionally express the following fusions: EGFP-KDM4B, EGFP-KDM4C, or EGFP fused to nuclear localization signal (EGFP-NLS) were established. Briefly, fragments including the above fusions were subcloned into pTRE2-puro. The resulting pTRE2-puro-Hs-KDM4B/C-EGFP or pTRE2-puro-EGFP-NLS vectors were transfected into U2OS-TetON cells. Puromycin-resistant clones (0.6 μg/ml puromycin) were selected and tested for

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2 The abbreviations used are: Hsp90, heat shock protein 90; EGFP, enhanced green fluorescent protein; NLS, nuclear localization signal; dox, doxycycline; GA, geldanamycin; DMSO, dimethyl sulfoxide.

5 This article contains supplemental Figs. S1–S3.

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doxycycline-induced expression of EGFP using fluorescence microscopy. Clones that showed EGFP expression only after the addition of 1 μM doxycycline were selected for further characterization. All point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs used in this study were verified by restriction digestion and nucleotide sequencing.

Cell Lines and Growth Conditions—All cell lines were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. U2OS and 293T cell lines were grown in Dulbecco’s modified Eagle’s medium. The MCF-7 cell line was grown in RPMI. U2OS-TetON cells were grown in the presence of 200 μg/ml Geneticin (G418). U2OS-TetON-EGFP-KDM4B and U2OS-TetON-EGFP-NLS were grown in the presence of 200 μg/ml G418 and 0.6 μg/ml puromycin. The U2OS-TetON-EGFP-KDM4C cell line was maintained in 200 μg/ml G418 and 100 μg/ml hygromycin B.

Production of a Polyclonal Human KDM4B Antibody—KDM4B rabbit antibody (designated B2-201) was produced against KDM4B amino acids 528–727 fused to glutathione S-transferase (GST) using standard immunization protocol. The antigen was then immobilized on beads and used for affinity purification of the immune serum (Adar Biotech).

Immunofluorescence—Cells were grown on coverslips and immunostained as described previously (21). Anti-H3K9me3 (Abcam, Ab8988) primary antibody (1:1000) was visualized by Alexa Fluor 568 secondary antibody (Molecular Probes; 1:500). Images were acquired using an inverted confocal microscope (Zeiss LSM-700) with constant acquisition parameters across all the experiments.

Cell Transfections—Cell transfections with plasmid DNA or siRNA were performed using Polyjet (Bio-Consult) and Lipofectamine 2000 (Invitrogen), respectively, following the manufacturer’s instructions. siRNA used in this study include Stealth KDM4B siRNA (Invitrogen) and Stealth RNAi negative control. All constructs and siRNA sequences are available upon request.

Western Blot, Immunoprecipitation, and GFP-TRAP Pulldown—Protein lysates were prepared using two different methods. First, cells were lysed using standard Nonidet P-40 lysis buffer (22). Second, protein lysates were prepared using hot lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5). Lysate was boiled for 15 min, sonicated, treated with Benzonase for 30 min at room temperature, and centrifuged at 14,000 rpm for 20 min at 4 °C, and then supernatant was subjected to immunoblotting using the following primary antibodies: anti-KDM4B (in-house, B2-201, 1:1000), anti-H3K9me3 (Abcam, Ab8988; 1:2000), anti-H3 (Abcam, ab1791; 1:2000), anti-Hsp90 (Santa Cruz Biotechnology, sc-13119; 1:1000), anti-β-actin (Sigma, A5441; 1:20,000). The membranes were then washed and incubated with appropriate secondary antibodies anti-mouse(IgG)-HRP (Amersham Biosciences) or anti-rabbit(IgG)-HRP (Jackson ImmunoResearch Laboratories) and developed using the Quantum ECL detection kit (Advanta).

Immunoprecipitation was performed as described previously (23). In brief, whole cell extract were prepared using Nonidet P-40-lysis buffer, and 2–4 mg were precleared using protein G-Sepharose beads (GE Healthcare). The precleared extracts were rotated at 4 °C overnight with 1–4 μg of antibody and protein G-Sepharose. Then, the immunocomplexes were washed five times and immunoblotted as described above. For the GFP-TRAP assay, protein lysates were precleared with agarose beads (Pierce; catalog number 26150) and incubated for 2 h with GFP-TRAP beads (ChromoTek). Next, the immunocomplexes were washed and analyzed by Western blotting.

Preparation of Protein Samples for Mass Spectrometric Analysis—Proteins extracts were resolved in a long gel using the PROTEAN® II xi cell system (Bio-Rad). The gel was run at 4 °C, at 18 V overnight, stained using InstantBlue (BioConsult) and destained with water. The bands of interest were excised from the gel and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

RESULTS

KDM4B Histone Demethylase Interacts with Hsp90.—To identify KDM4B-interacting proteins, we established a U2OS-TetON cell line that conditionally expresses functional EGFP-KDM4B upon the addition of doxycycline (dox) (hereafter called U2OS-TetON-EGFP-KDM4B). A control cell line expressing EGFP protein fused to nuclear localization signal (hereafter called U2OS-TetON-EGFP-NLS) was also established as described under “Experimental Procedures.” To map KDM4B-interacting partners, GFP-TRAP pulldown assay was performed on dox-treated cells expressing either EGFP-KDM4B or EGFP-NLS fusion. The pulled down proteins were subjected to mass spectrometry. Results show several peptides of Hsp90α and Hsp90β isoforms that were co-purified with EGFP-KDM4B, but not with EGFP-NLS, indicating that KDM4B interacts with Hsp90 protein (Fig. 1A). KDM4B-Hsp90 interaction was further confirmed using GFP-TRAP pulldown of EGFP-KDM4B fusion (Fig. 1B).

Hsp90 Inhibition Promotes KDM4B Degradation and Alters the Methylation of H3K9.—To study the biological relevance of Hsp90-KDM4B interaction, we pharmacologically inhibited the activity of Hsp90 using GA (24). U2OS-TetON-EGFP-KDM4B and U2OS-TetON-EGFP-NLS cell lines were treated with dox to induce the expression of EGFP-KDM4B and EGFP-NLS fusions. Cells were then treated with either DMSO or 5 μM GA for 5 h, fixed, and immunostained. Results show that GA treatment leads to a severe reduction in the fluorescence intensity of EGFP-KDM4B fusion. Interestingly, this reduction is accompanied by an increase in the levels of H3K9me3 as compared with the GA-untreated cells (Fig. 1C). On the other hand, no significant changes in the fluorescence intensity of the EGFP-NLS signal and in the levels of H3K9me3 were observed in U2OS-TetON-EGFP-NLS cells (Fig. 1D). Altogether, these observations suggest that the inhibition of Hsp90 activity impairs the stability of KDM4B but not EGFP-NLS fusion.

To test whether the GA-dependent decrease in the fluorescence intensity results from the degradation of EGFP-KDM4B protein, cells expressing either EGFP-KDM4B or EGFP-NLS were treated with GA and subjected to Western blot. Results show that GA treatment leads to a rapid decrease (2–3-fold) in the levels of EGFP-KDM4B, but not in EGFP-NLS fusion. Con-
**FIGURE 1.** Pharmacological inhibition of Hsp90 molecular chaperone promotes degradation of KDM4B, but not KDM4C, histone demethylase. A, LC-MS/MS analysis shows peptide sequences of Hsp90α and Hsp90β proteins that were co-purified with EGFP-KDM4B but not with EGFP-NLS fusion. U2OS-TetON-EGFP-KDM4B and U2OS-TetON-EGFP-NLS cells were treated with dox and subjected to GFP-TRAP pulldown as described under “Experimental Procedures.” The immunoprecipitated proteins were resolved in gel and identified by LC-MS/MS. B shows that EGFP-KDM4B interacts with Hsp90 protein. As in A, cells were subjected to GFP-TRAP pulldown followed by Western blot. U2OS-TetON-EGFP-NLS cells were used as a negative control. This result is typical of two independent experiments. The intensity of the bands was quantified as in E. C and D, pharmacological inhibition of Hsp90 leads to a reduction in the fluorescence intensity of EGFP-KDM4B, but not EGFP-NLS fusion. The disappearance of EGFP-KDM4B signal is accompanied by an increase in the methylation levels of H3K9me3 mark. Expression of either EGFP-KDM4B or EGFP-NLS (green) was induced by growing U2OS-TetON-EGFP-KDM4B (C) and U2OS-TetON-EGFP-NLS cells (D) in the presence of dox for 18 h. Next, cells were treated with either 5 μM GA or DMSO for 5 h prior to fixation and immunostained with H3K9me3 antibody (red). Nuclei were then stained with DAPI (blue). These results are typical of at least five independent experiments. Graphs in the right panel show changes in the relative fluorescence intensity of EGFP-KDM4B, EGFP-NLS and H3K9me3 before and after GA treatment. Error bars indicate S.D. Scale bar, 5 μm. E, Hsp90 inhibition leads to a rapid degradation of EGFP-KDM4B, which is accompanied by an overall increase in the levels of H3K9me3. As in C, except that cells were treated with GA and protein lysates were prepared using hot lysis and immunoblotted with the indicated antibodies. Results shown are typical of at least four different experiments. The GFP and H3K9me3 signals were quantified using ImageJ software and normalized to β-actin and H3 signals, respectively. The GFP and H3K9me3 levels from untreated cells were set to 1 (time = 0 h). F, Western blot shows that the endogenous KDM4B protein in MCF-7 cells is degraded following the inhibition of Hsp90. This blot is representative of two different experiments. The levels of KDM4B signal were quantified and normalized to β-actin as in E. G, pharmacological inhibition of Hsp90 has no detectable effect on the protein levels of EGFP-KDM4C fusion. U2OS-TetON-EGFP-KDM4C cells were treated with dox for 18 h followed by GA treatment for the indicated times. Protein lysates were prepared as in E. Results shown are typical of three independent experiments. The intensity of the bands was quantified as in E.
subsequently, the degradation of EGFP-KDM4B was accompanied by more than 3-fold increase in the levels of H3K9me3. On the other hand, no significant changes in the protein levels of histone H3 and β-actin were observed (Fig. 1E). Moreover, the levels of H3K9me3 were not affected in GA-treated TetON-EGFP-NLS cells (Fig. 1E). To further characterize the effect of GA on the stability of EGFP-KDM4B protein, we determined the half-life of EGFP-KDM4B protein using a cycloheximide chase assay in untreated and GA-treated cells. Results show that GA treatment shortens the half-life of EGFP-KDM4B to 30 min as compared with a half-life of 1 h in untreated cells (supplemental Fig. S1). This result further supports our conclusion that Hsp90 activity regulates the turnover of KDM4B protein.

To assess whether Hsp90 inhibition also affects the levels of the endogenous KDM4B protein, we raised polyclonal antibody (B2-201) against 528–727 amino acids of the human KDM4B protein. The antibody was affinity-purified and assessed for its ability to recognize the denatured form of KDM4B protein. To do so, whole cell lysate and GFP-TRAP immunoprecipitates of U2OS-TetON-EGFP-NLS and U2OS-TetON-EGFP-KDM4B cells were subjected to Western blot. Results show that the B2-201 antibody recognizes a major band of ~180 kDa, corresponding to the molecular mass of EGFP-KDM4B protein. This band is absent in cell lysates prepared from U2OS cells (supplemental Fig. S2A). Moreover, Western blot analysis of the MCF-7 cell line (human breast adenocarcinoma cell line that expresses high levels of the endogenous KDM4B protein) shows that KDM4B antibody recognizes a major band of ~120 kDa, corresponding to the molecular mass of the endogenous KDM4B. The intensity of this band was severely reduced in MCF-7 cells treated with either one of three different sequences of KDM4B siRNA (supplemental Fig. S2B). These results altogether confirm that B2-201 antibody specifically recognizes both the overexpressed and the endogenous forms of KDM4B.

Following this, protein lysates were prepared from untreated and GA-treated MCF-7 cells and analyzed by Western blot. Results demonstrate that pharmacological inhibition of Hsp90 leads to degradation of the endogenous KDM4B protein, but has no detectable effect on the levels of β-actin (Fig. 1F). These observations suggest that KDM4B is a bona fide client protein of Hsp90 molecular chaperone.

**Pharmacological Inhibition of Hsp90 Has No Effect on the Protein Levels of EGFP-KDM4C**—We sought to assess whether similar to KDM4B, inhibition of Hsp90 activity affects the stability of the other members of KDM4 family. Toward this, we established a U2OS-TetON cell line that expresses functional EGFP-KDM4C fusion following the addition of dox (see “Experimental Procedures”). Interestingly, Western blot analysis shows that, unlike KDM4B, GA treatment has no significant effect on the levels of EGFP-KDM4C protein (Fig. 1G). Altogether, we concluded that Hsp90 regulates the stability of KDM4B, but not KDM4C, histone demethylase.

**Ubiquitin-dependent Proteasomal Degradation of KDM4B Protein**—To get further insights into the mechanism regulating the stability of KDM4B protein, we wanted to test whether the Hsp90-dependent degradation of KDM4B is mediated through the proteasome. Toward this, doxycycline-treated U2OS-TetON-EGFP-KDM4B cells were treated with either DMSO or proteasome inhibitor, MG132, prior to the addition of GA. As expected, chemical inhibition of Hsp90 leads to a rapid degradation of EGFP-KDM4B; however, this degradation is suppressed in MG132-treated cells (Fig. 2A). We concluded therefore that GA-dependent degradation of Hsp90 is mediated by the proteasome.

To assess whether KDM4B is ubiquitinated prior to its degradation, protein lysates from U2OS-TetON-EGFP-KDM4B cells treated or not treated with either GA, MG132, or both were subjected to GFP-TRAP pulldown and immunoblotting. Results show that GA treatment leads to the rapid degradation of EGFP-KDM4B, and consequently, the ubiquitinated forms of the protein could not be detected (Fig. 2B, lane 3). On the other hand, cells treated with MG132 show a dramatic increase in the ubiquitinated forms of EGFP-KDM4B (Fig. 2B, lane 4). Moreover, pretreatment of the GA-treated cells with MG132 significantly restored the ubiquitinated forms of EGFP-KDM4B (Fig. 2B, lane 5). Collectively, we conclude that EGFP-KDM4B undergoes polyubiquitination prior to its proteasomal degradation.

**Hsp90-dependent Degradation of KDM4B Protein Is Mediated by KDM4B Ubiquitination on Lys-337 and Lys-562**—We sought to map the ubiquitinated residues of KDM4B protein. Toward this, EGFP-KDM4B protein was purified from U2OS-TetON-EGFP-KDM4B cells using GFP-TRAP and subjected to LC-MS/MS analysis, which revealed two ubiquitinated lysine residues, Lys-337 and Lys-562 (data not shown). We substituted these two residues to arginine and assessed the demethylase activity of the observed KDM4B mutant in vivo. Results show that overexpression of the KDM4B mutant in U2OS cells leads to a dramatic decrease in the levels of the H3K9me3 mark as compared with untransfected U2OS cells. We therefore concluded that the demethylase activity of KDM4B-K337R-K562R mutant remains intact (supplemental Fig. S3). Next, we tested the ability of KDM4B mutant to undergo ubiquitination in cells. Toward this, 293T cells were transfected with an expression construct encoding FLAG-ubiquitin along with constructs expressing wild-type EGFP-KDM4B or EGFP-KDM4B-K337R-K562R mutant. Whole cell lysates were then prepared and subjected to GFP-TRAP pulldown followed by Western blot using FLAG, GFP, and ubiquitin antibodies. Results show that, in both the presence and the absence of MG132, the ubiquitination levels of KDM4B mutant are significantly lower than the wild-type protein, indicating that KDM4B is ubiquitinated on Lys-337 and Lys-562 residues (Fig. 2C). Prompted by these observations, we sought to assess whether the ubiquitination of Lys-337 and Lys-562 is implicated in GA-induced degradation of KDM4B protein. Toward this, U2OS cells expressing wild type or EGFP-KDM4B-K337R-K562R mutant were treated with either DMSO or GA and subjected to two assessments. Western blot showed no detectable changes in protein levels of EGFP-KDM4B-K337R-K562R mutant after GA treatment as compared with the substantial decrease in the levels of wild-type KDM4B protein (Fig. 2D). These observations were further confirmed by immunofluorescence analysis showing that, unlike cells expressing wild-type EGFP-KDM4B, the fluorescence intensity of cells expressing EGFP-KDM4B-K337R-K562R mutant remains unaffected following GA treatment.
 Altogether, these results strongly suggest that ubiquitination of Lys-337 and Lys-562 regulates the turnover of KDM4B protein following Hsp90 inhibition.

DISCUSSION

Here, we reveal a previously unrecognized role of Hsp90 molecular chaperone in regulating the stability of KDM4B, but not KDM4C protein. We have presented two lines of evidence suggesting that KDM4B is a client protein of Hsp90. First, KDM4B interacts with Hsp90 protein (Fig. 1, A and B), and second, inhibition of Hsp90 destabilizes KDM4B and leads to its degradation through the proteasome (Figs. 1 and 2). Moreover, we have identified two ubiquitinated lysine residues, Lys-337 and Lys-562, that mediate the Hsp90-dependent degradation of KDM4B protein through the proteasome. Dox-treated U2OS-TetON-EGFP-KDM4B cells were treated with either DMSO or 20 µM MG132 prior to the addition of 5 µM GA. Results shown are representative of two independent experiments. B shows ubiquitination of EGFP-KDM4B protein. Dox-treated U2OS-TetON-EGFP-KDM4B cells were treated with either GA for 6 h or MG132 for 8 h or pretreated with MG132 for 2 h before the addition of GA. Protein lysates were subjected to GFP-TRAP pulldown using high stringency buffer, resolved by SDS-PAGE, and immunoblotted with GFP and ubiquitin antibodies. Results shown are representative of two independent experiments. C shows that the ubiquitination efficiency of KDM4B-K337R-K562R mutant is severely impaired. 293T cells were cotransfected with an expression vector encoding FLAG-ubiquitin along with constructs encoding either EGFP-KDM4B-WT or EGFP-KDM4B-K337R-K562R mutant. Next, protein lysates were prepared from mock- and MG132-treated cells and subjected to GFP-TRAP pulldown followed by Western blot. Results are representative of two independent experiments. D shows that KDM4B-K337R-K562R mutant is resistant to Hsp90-dependent degradation. U2OS cells expressing EGFP-KDM4B-K337R-K562R were treated with GA, and protein lysates were immunoblotted using GFP and ß-actin antibodies. Quantifications of the GFP signals after normalization to ß-actin were performed as in Fig. 1E. E shows no significant changes in the fluorescence intensity of EGFP-KDM4B as compared with wild-type EGFP-KDM4B. Cells expressing either wild type or KDM4B mutant were treated with GA for the indicated times, fixed, and stained with DAPI (blue). Scale bar, 10 µm.
tion of KDM4B protein. It has been shown that Hsp90 collaborates with Hsp70 for achieving the correct folding of their client proteins (25). Consistently, we found that KDM4B also interacts with Hsp70 (data not shown). On this basis, we propose that Hsp90 and Hsp70 molecular chaperones promote the folding of the nascent KDM4B polypeptide. Following Hsp90 inhibition, KDM4B remains unfolded and undergoes polyubiquitination and degradation through the proteasome.

Several studies have implicated Hsp90 in modulating the histone code and gene expression by chaperoning various chromatin remodeling factors (26–29). Here, we show that the degradation of KDM4B protein is accompanied by an increase in the levels of H3K9me3. Thus, our results provide an additional pathway by which Hsp90 can affect the histone code through chaperoning KDM4B histone demethylase.

Several Hsp90 client proteins are known oncogenes (e.g., ErbB2, s-Src, b-Raf, and Cdk4 (30–33)). Moreover, Hsp90 regulates the stability of proteins that are involved in apoptotic pathways, such as p53 (34). Interestingly, cancer cells utilize the Hsp90 chaperone machinery to protect the mutated and the overexpressed oncoproteins from misfolding and degradation in the proteasome. For these reasons, Hsp90 is considered a promising and attractive target for treating cancer. Accordingly, several inhibitors of Hsp90 are being used in clinical trials to cure different types of human cancer (35, 36). Our data identified KDM4B as a new oncogenic client of Hsp90 and thus provided a novel pathway for destabilizing KDM4B in cancer cells. In light of this, we suggest that Hsp90 inhibitors might be effective to treat tumors driven by KDM4B overexpression through the inhibition of Hsp90 activity.

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