KDM4C Activity Modulates Cell Proliferation and Chromosome Segregation in Triple-Negative Breast Cancer

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ABSTRACT: The Jumonji-containing domain protein, KDM4C, is a histone demethylase associated with the development of several forms of human cancer. However, its specific function in the viability of tumoral lineages is yet to be determined. This work investigates the importance of KDM4C activity in cell proliferation and chromosome segregation of three triple-negative breast cancer cell lines using a specific demethylase inhibitor. Immunofluorescence assays show that KDM4C is recruited to mitotic chromosomes and that the modulation of its activity increases the number of mitotic segregation errors. However, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation assays demonstrate that the demethylase activity is required for cell viability. These results suggest that the histone demethylase activity of KDM4C is essential for breast cancer progression given its role in the maintenance of chromosomal stability and cell growth, thus highlighting it as a potential therapeutic target.

KEYWORDS: KDM4C, breast cancer, chromosome segregation, histone demethylation

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Introduction

The development of human cancer is strongly related to alterations in the cellular genetic expression profile and defects in the distribution of chromosomal information during mitosis, both factors associated with macromolecules in charge of the epigenetic regulation of cell cycle.[1,2] Of the several types of cancers, breast cancer is a heterogeneous neoplasm that is further classified depending on its biological expression profile into subtypes, which include basal like (closely related to the triple-negative breast cancer subtype), normal like, Luminal A, Luminal B, and HER2/ERBB2 enriched.[3] Of these, the basal-like/triple-negative tumors (which do not present the expression of estrogens, progesterones, and HER2 receptors) exhibit the most aggressive phenotype with a high rate of metastasis and poor prognosis.[4,5] Additionally, triple-negative breast cancer accounts for 15%–20% of the 500,000 breast cancer-associated female deaths worldwide each year and is considered as an international public health issue, thus supporting the continuous search for novel therapeutic targets.[4,6–8]

The preservation of the phenotype of eukaryotic cells depends closely on the structural conservation and the dynamic regulation of chromatin, the main nuclear DNA packaging structure.[9,10] Chromosomal information of mother cells is duplicated and segregated to its daughter cells in a highly regulated and complex process known as mitosis.[11,12] Defects in the distribution of this genetic information are associated with the development of cancer.[13–15] The transcription of genetic information is regulated by different mechanisms, including RNA interference, DNA methylation, and posttranscriptional modification (PTM) of histones.[1,16,17] Among these, histones can undergo various covalent modifications such as phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, acetylation, deamination, proline isomerization, and methylation.[18,19]

Histone methylation/demethylation is one of the most important PTMs that is directly associated with the activation or repression of genes and is involved in DNA recombination, repair, imprinting, and mitosis.[20–22] Histone methylation was considered as a stable chromatin mark until the relatively recent discovery of histone demethylases that have provided a new perspective, as alterations in these chromatin remodelers are associated with the development of several diseases, including cancer.[23–27]

Demethylation of histones at lysine is associated with two families of macromolecules known as histone lysine demethylases (KDMs). The lysine-specific demethylases (LSD/KDM1), which are flavin-dependent enzymes, can act on the di- and mono-methylated forms of histone lysines, and the JMJs present the unique ability to modify a trimethylated

residue of lysine in an Fe^{2+}- and 2-oxoglutarate-dependent mechanism.[27–29] The JMJ proteins have raised great interest due to their significance in the development of a wide number of cancer cell types.[30–32] As part of this family, the members of the subset JMJD2/KDM4 are associated with breast cancer development, tumor cell development and proliferation, as well as progression of abnormal cells to an invasive and highly metastatic form by regulating their migratory and invasive capacities.[28,33–36]

An important member of this protein family, the lysine demethylase KDM4C, is a crucial oncogene that is amplified and overexpressed in several neoplasms,[37–42] including the most aggressive forms of basal-like breast cancers,[43] and is recognized as a potential prognostic marker for the invasive phenotype of this disease.[44,45] On the other hand, KDM4C is also the only member of the KDM4/JMJD2 subfamily associated with the segregation of chromosomes during mitosis in the U2OS osteosarcoma cell line,[46] underscoring its potential relevance in the evolution of cancer associated with chromosomal instability and mitotic defects.[14,47] However, the specific mechanism and pathways associated with KDM4C activity in tumor progression remain to be determined.

This article presents the importance of KDM4C activity for cell proliferation and chromosome segregation of three triple-negative breast cancer cell lines by using a specific KDM4 inhibitor, NCDM-32b.[48] Our results show that KDM4C is important for cell proliferation and for maintaining a correct distribution of the genetic information during mitosis for these aggressive and highly invasive breast neoplasms, highlighting the importance of this histone demethylase as a potential anti-breast cancer therapeutic target.

Methods

Cell culture and activity inhibition. The HCC38, MDA-MB-436, and MDA-MB-453 triple-negative breast cancer cell lines were obtained from American Tissue Culture Collection (ATCC). HCC38 was grown in Roswell Park Memorial Institute-1640 media (ATCC modification, Gibco), while MDA-MB-436 and MDA-MB-453 were grown in Dulbecco's Modified Eagle medium media (Gibco) in the presence of 0.01 mg/mL insulin. All media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO_2. To inhibit KDM4C activity for subsequent experiments, cells were cultured up to 30%–40% confluence and synchronized by serum starvation in a serum-free medium overnight (mean efficiency 82%, data not shown), followed by release from cell cycle arrest by phosphate-buffered saline (PBS) washing and the addition of fresh medium supplied with 10% fetal bovine serum.[49] Cells were then subcultured in six-well plates (1×10^6 cells/well) up to 70% confluence and incubated with the specific inhibitor NCDM-32b (Wako Chemicals) at 50 and 100 µM/well. KDM4C inhibition was evaluated by immunofluorescence assay (IFA) or MTT methodology.[50]

Immunofluorescence assays. Triple-negative breast cancer cells were grown on coverslips and treated with NCDM-32b as previously described. After 48 hours, the samples were pre-extracted with 0.2% PBS/Triton X-100 and fixed in 3.7% formaldehyde for 15 minutes. The fixed cells were rendered permeable with 0.5% Triton X-100 in PBS and then incubated with 3% bovine serum albumin in PBS for one hour. Next, the cells were treated with anti-KDM4A (Abcam ab24545), anti-KDM4C (Abcam ab85454), or anti-trimethyl H3K9 (Abcam ab10812) antibody overnight at 4°C at 1:250 dilution, followed by three washes with PBS containing 0.01% Triton X-100 and the secondary antibody incubation (Alexa Fluor 488, 1:500 dilution, Abcam 150077) for one hour. Finally, the cells were washed twice and mounted in ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). All images were obtained on an Eclipse Ni-E microscope (Nikon) and analyzed with ImageJ software (National Institutes of Health).[51]

Proliferation assays. Treated cells were tested for cancer cell proliferation by an MTT assay.[36] Briefly, 1500 cells were seeded and cultured in 96-well plates according to previously described conditions. One day later (day 0), the cells were treated with NCDM-32b (100 µL/well) under conditions similar to that of corresponding controls without the inhibitor. Each experiment included blanks without cells, without MTT, and without treatment to overcome any background interference. The samples were then treated with 20 µL MTT (Life Technologies M6494) for four hours at 24 and 48 hours post inhibition. Next, the supernatant was removed, and 150 µL of dimethyl sulfoxide was added. Absorbance was measured at 550 nm using a microplate reader (TriStar² LB 942 Multimode Reader, Berthold Technologies).

Statistical analysis. All the experiments were performed in duplicate, and the results obtained were from at least two independent experiments. The statistical comparison of results was performed by applying analysis of variance (one way) and two means evaluation (Student's t-test) using the SPSS 10.0 (IBM Corporation) software, with a significance cutoff of $P < 0.05$.

Results

KDM4C is located in mitotic chromosomes and its NCDM-32b-specific inhibitor modulates the trimethylated form of lysine 9 on histone 3. IFAs with anti-KDM4C and anti-KDM4A proteins were performed in the triple-negative breast cancer cell lines. KDM4A was not recruited to mitotic chromosomes and presented a diffuse cellular distribution, whereas KDM4C was able to interact with mitotic chromosomes, thus highlighting its potential relevance for chromosome segregation in contrast with the other members of KDM4 subfamily (Fig. 1). To evaluate the effect of KDM4C inhibition on breast neoplasms, a KDM4C-specific inhibitor was tested in three triple-negative breast cancer cell lines. IFA analysis confirmed a significant increase in the levels of H3K9-3me when the cells were exposed to NCDM-32b (Fig. 2),
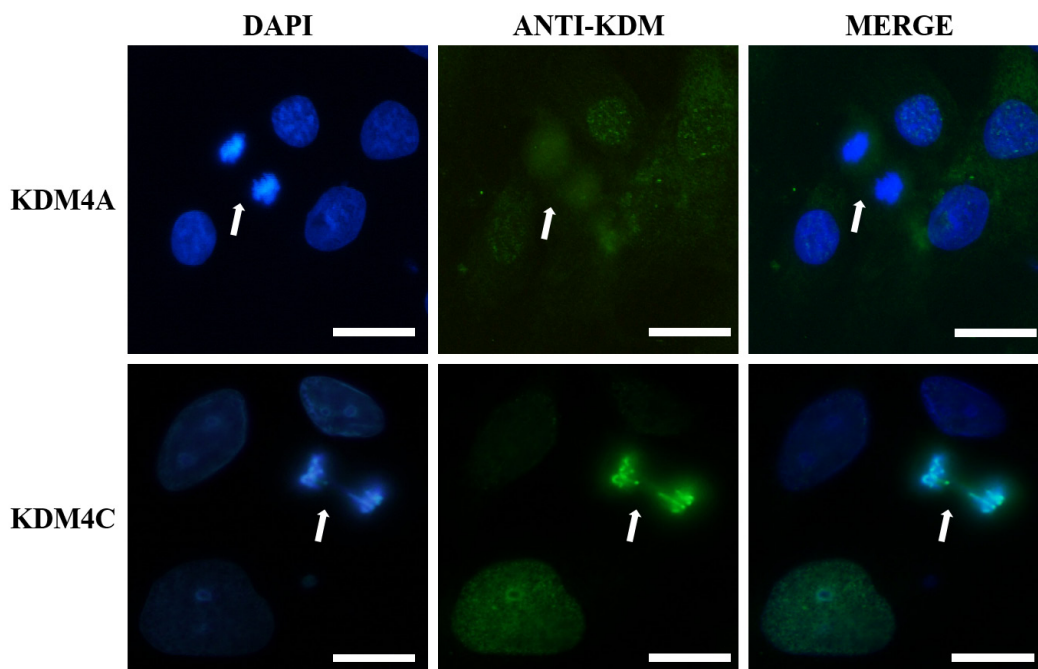
Figure 1. KDM4A and KDM4C cellular localization during mitosis. Representative immunofluorescent images of HCC38 cells in anaphase are presented (white arrows), illustrating a different distribution pattern for both proteins. Note that KDM4C colocalizes with DNA signal detected with DAPI, while KDM4A is excluded from mitotic chromosomes. The presented localization is conserved along all cell lines included in the study. White scale bar: 10 μm.
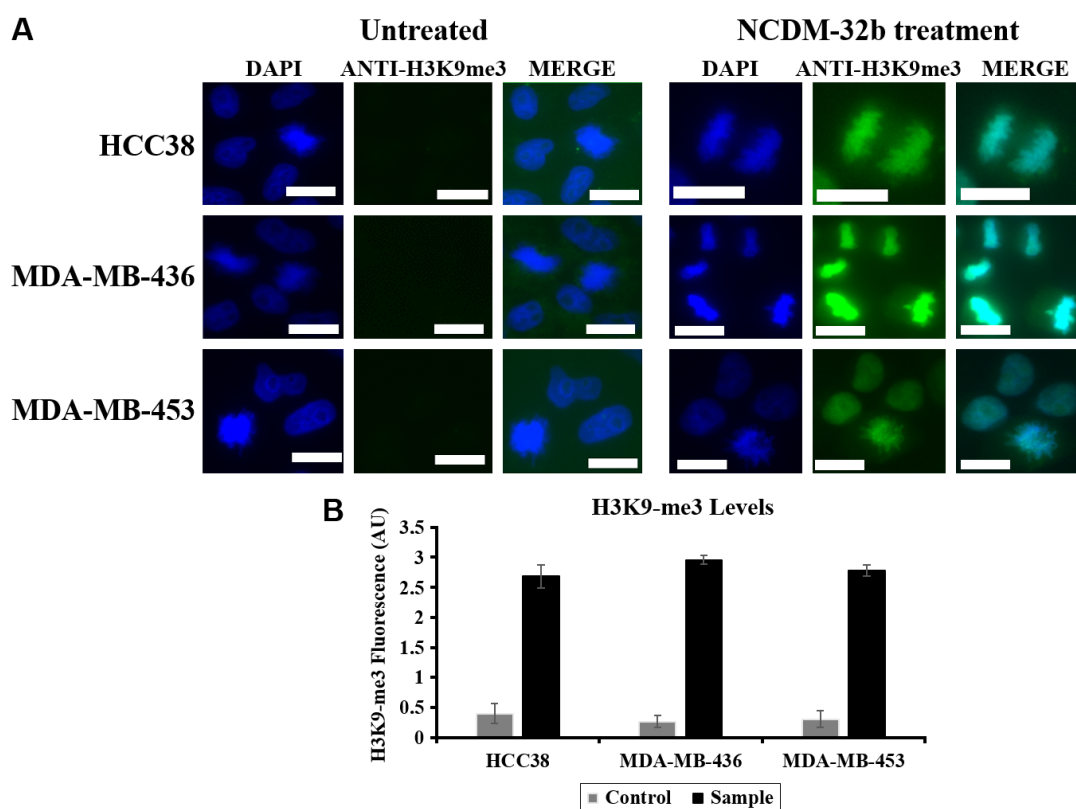
Figure 2. KDM4C inhibition with NCDM-32b affects histone demethylation levels. (**A**) Immunofluorescence of HCC38, MDA-MB-436, and MDA-MB-453 cell lines treated (right) or untreated (left) with inhibitor. Note the significant increment in fluorescence intensity for treated samples with respect to the untreated cells. All images were recorded at 80 milliseconds under the same conditions of exposition. White scale bar: 10 μm. (**B**) Quantification of histone 3 lysine 9 trimethyl levels by ImageJ software (National Institutes of Health), showing a significant increment on H3K9-3me levels up to six times with respect to the control for all treatments.

Figure 3. Effect of the activity of KDM4C on chromosomal stability. (**A**) Representative immunofluorescence at 100 times for triplicated samples analyzed as methods description for HCC38, MDA-MB-436, and MDA-MB-453 cell lines. A total of 30–40 random cells (1 × 105 cells/well) were analyzed per sample to evaluate different mitotic segregation erro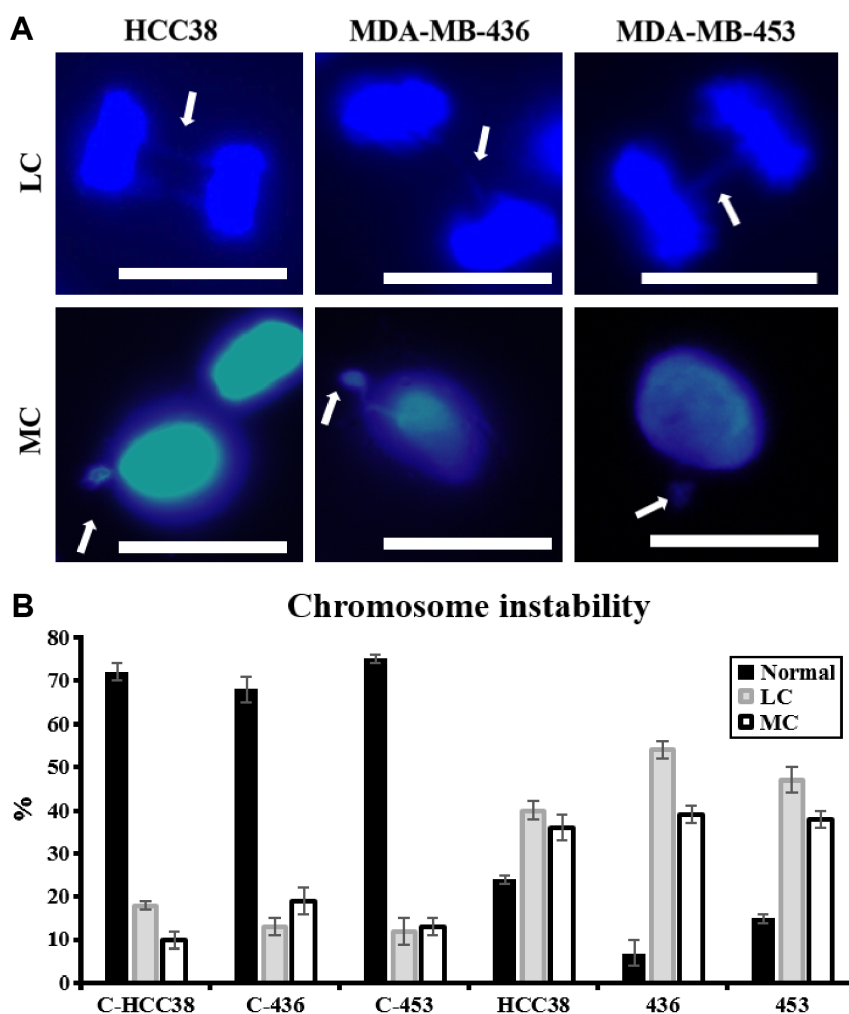rs. Upper line represents LC and lower line represents micronucleus. DNA was detected by using DAPI. White scale bar: 10 μm. (**B**) Quantification of chromosome segregation errors, determined for this experiment. Each sample was analyzed 48 hours post treatment and was compared to its corresponding control. Differences are presented as percentages.

demonstrating that NCDM-32b was capable of modulating KDM4C activity under our experimental conditions.

Inhibition of demethylase activity of KDM4C increases the number of chromosome segregation errors. The relevance of modulation of KDM4C's activity for the segregation of genetic information was analyzed by treating synchronized HCC38, MDA-MB-436, and MDA-MB-453 cell lines with NCDM-32b inhibitor (Fig. 3). Mitotic defects such as lagging chromosomes (LCs; associated with delayed movement during anaphase) increased by a mean of 38%, and the presence of micronuclei (resulting from mitotic segregation defects) increased by 20%–26% in the three breast cancer cell lines under KDM4C inhibition compared to untreated cells (Fig. 3B). Together, these observations demonstrated that KDM4C activity is highly relevant in the maintenance of proper gene distribution through mitosis.

Proliferation of triple-negative breast cancer cells is affected by KDM4C inhibition. The proliferation of NCDM-32b-treated and NCDM-32b-untreated triple-negative breast cancer cells was analyzed by an MTT assay. We observed a significant reduction in the proliferation of the three cell lines after 24 and 48 hours of KDM4C inhibition (means of 15% and 48%, respectively) compared to untreated cells (Fig. 4), indicating that KDM4C was associated with triple-negative breast cancer viability, which was consistent with previous observations with other KDM4 macromolecules.[36,52]

Discussion

This study presents the significance of inhibition of KDM4C demethylase activity in three triple-negative breast cancer cell lines. First, we show that the reduction of demethylase activity affects tumor cell growth along with an increase in the
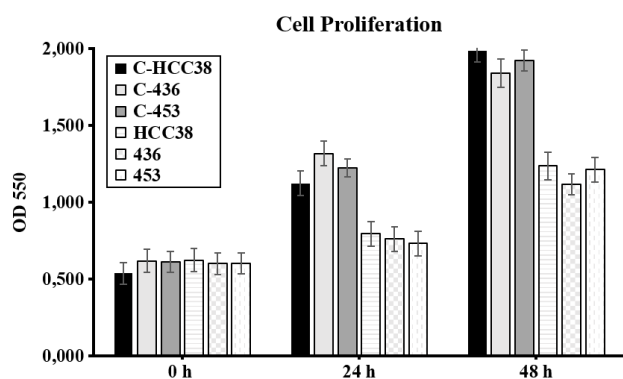
Figure 4. Cell proliferation assays. Optical density at 550 nm was evaluated at 24 and 48 hours by an MTT assay for unexposed HCC38, MDA-MB-436, and MDA-MB-453 cell lines (controls, labeled with C-) or samples exposed to NCDM-32b treatment. Note the significant reduction in the optical density of treated cells in comparison with their corresponding controls.

number of chromosome segregation errors in inhibitor-treated cells compared with untreated controls. Histone demethylases are epigenetic regulators that can remove methyl groups of specific lysine residues. Among these, the JMJ/KDM demethylases are of special interest because they can modify trimethylated lysine residues[1] and are associated with the development of several forms of cancer.[28,33–36] Accordingly, KDM4 histone demethylases are considered as promising therapeutic targets to treat some types of neoplasms.[27,53] It has also been reported that commonly observed amplicons, such as 9p24.3, in breast cancer contain the *kdm4c* gene, which encodes a histone demethylase that is widely associated with the development of several cancers, including prostate and esophageal tumors.[32,50,54]

Previous studies have shown that KDM4C has a unique cellular distribution in the U2OS osteosarcoma cell line that is functionally related to the maintenance of chromosome stability, as inhibition of KDM4C's expression promotes the occurrence of mitotic errors.[46] Our experiments in triple-negative breast cancer cells have revealed that KDM4C is recruited to mitotic chromosomes as opposed to the structurally similar KDM4A demethylase, which is completely excluded (Fig. 1). Such localization is associated with the preservation of H3K9 trimethylation levels as seen by a significant increment in the corresponding signal of tri-methylated lysine residues when compared to untreated cells (Fig. 2), indicating that KDM4C activity may be necessary for the correct distribution of genetic information in breast cancer cells.

This is consistent with the fact that an increase in mitotic errors is observed in triple-negative breast cancer cell lines that are treated with a KDM4-specific inhibitor such as NCDM-32b, similar to the use of RNA interference in other neoplastic cell lines in previous studies.[46] A higher number of LCs and micronuclei are observed in treated cells

under our experimental conditions (Fig. 3), which suggest that demethylation of H3K9-3me by KDM4C is a key process for maintaining chromosomal stability in triple-negative breast cancer. Interestingly, a recent article shows that micronuclei in some cell lines can be recovered by the daughter cells.[55] However, the evidence suggesting the association between chromosome segregation errors and chromosome instability[56–58] in conjunction with the exposition of external chromosomes in micronuclei to DNA damage that can be inherited by daughter cells[59] underscores the need for additional studies to identify the specific role of KDM4C for the proper segregation of DNA content as part of the complex linkage between mitosis defects, aneuploidy, and chromosome instability. In this regard, previous reports have shown that a sequential process involving deacetylation and methylation of H3K9 is relevant for the condensation of chromosomes,[60] as a potential epigenetic pathway associated with these relevant events.

On the other hand, we have performed similar analysis on the depletion of KDM4C by RNAi in triple-negative breast cancer cells where an increment in chromosome segregation errors is observed together with a significant reduction in cell proliferation and a differential modulation of the cell's migration and invasion capacities (Garcia, Lizcano. Role of the Jumonji-domain containing protein KDM4C in triple-negative breast cancer. Submitted to *J Breast Cancer*, unpublished results). Together, these results highlight the importance of KDM4C and its demethylase activity for mitotic segregation.

In addition, the influence of KDM4C activity on the proliferation of triple-negative breast cancer cells was analyzed in which inhibition of the demethylase activity led to a significant reduction in cell multiplication (Fig. 4). This behavior has been observed for KDM4C in tumor cells with a different expression profile, including HER2/neu (+) cells.[43,61,62] This important demethylase has been associated with regulation of cell growth in normal and tumoral cells,[63] suggesting that KDM4C or its target genes are associated with the proliferation of triple-negative breast cancer in a HER2/neu-independent pathway that remains to be determined.

In summary, these observations highlight the relevance of histone demethylase, KDM4C, as a potential therapeutic target for the most aggressive form of breast cancer and support the importance of histone demethylases belonging to the LSD family[64] and other members of the JMJ/KDM subgroup[65–67] as part of a new generation of potential therapeutic targets against breast cancer.

Conclusion

This work presents the importance of KDM4C activity for triple-negative breast cancer, which is directly associated with the growth of the most aggressive form of breast cancer, represented by three triple-negative carcinoma cell lines. Although extended analysis is required to uncover the

complete mechanism associated with this critical demethylase, our study determines that KDM4C's activity is highly required for the correct segregation of chromosomes during mitosis in these cancer cell lines, which strongly supports the potential of KDM4C as a promising therapeutic target for new inhibitors and strategies against the main cause of cancer death in women worldwide.

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Author Contributions

Wrote the first draft of the manuscript, conceived and designed the experiments, and analyzed the data: JG. Made critical revisions, jointly developed the structure and arguments for the article, and approved the final version: FL. All authors reviewed and approved the final manuscript.

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