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14. ABSTRACT
Amplification of 8p11-12 occurs in approximately 15% of human breast cancer (HBC), and this region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients. Earlier, we identified Wolf-Hirschhorn syndrome candidate 1-like 1 (WHSC1L1) as a candidate oncogene of 8p11-12 amplified region, based on statistical analysis of copy number increase and overexpression. In this study, we demonstrated that WHSC1L1 acts as a transforming gene: stable WHSC1L1 overexpression in non-tumorigenic MCF10A cells induces transformed phenotypes, whereas WHSC1L1 knockdown in 8p12 amplified, ER-positive breast cancer cells inhibits proliferation in vitro. We also found that overexpression of WHSC1L1 likely induces the acquisition of stem cell-like properties in vitro. WHSC1L1 family proteins have been shown to bind to methylated histones, specifically H3K36 methylation marks. We determined global methylation levels in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. We identified several WHSC1L1 target genes, one of which is the Iroquois homeobox 3 gene, a member of the Iroquois homeobox transcription factor family. In summary, our findings provide a strong foundation for further mechanistic research and therapeutic options using WHSC1L inhibitors to treat breast cancer.

15. SUBJECT TERMS
Gene amplification, PWWP-domain, histone modification

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Introduction

Breast cancer is the most common cancer among women worldwide, with 1.3 million women diagnosed each year and about 500,000 women dead per year from the disease. Distinct subtypes of breast carcinomas that are associated with different clinical outcomes have been identified by expression analysis using microarray-based technology (1, 2). Five intrinsic molecular subtypes of human breast cancer include Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2/ERBB2)-positive, basal-like, and normal-like breast cancer (2, 3). Both Luminal A and Luminal B breast cancers are estrogen receptor (ER) positive, but Luminal B cancers have poorer outcomes (4). Basal-like breast cancer is especially aggressive as it includes tumors that lack ER, progesterone receptor (PR), and HER2 expression (5, 6). These characteristics render conventional therapies ineffective and lead to poor prognosis. By understanding the genetic and epigenetic abnormalities that are associated with the different types of breast cancer, we can identify new subtype-specific targets for therapy.

Amplification of 8p11-12 occurs in approximately 15% of human breast cancer, and this region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients (7-11). Earlier, we used genomic analysis of copy number and gene expression to perform a detailed analysis of the 8p11-12 amplicon to identify candidate oncogenes in breast cancer (10). We identified *Wolf-Hirschhorn syndrome candidate 1-like 1* (*WHSC1L1*) as a candidate oncogene based on statistical analysis of copy number increase and overexpression (10). The WHSC1L1 gene encodes a PWWP domain protein that regulates gene transcription and differentiated function of cells through regulation of histone methylation (12, 13). In this proposal, we hypothesized that WHSC1L1 is the major driving oncogene in the 8p11 amplicon that is found in aggressive forms of ER positive, Luminal B breast cancers. Furthermore, we hypothesized that genetic deregulation of WHSC1L1 induces alterations in the epigenetic histone code resulting in the acquisition of cancer stem cell phenotypes. Based on this hypothesis, we predict that WHSC1L1 will be a good therapeutic target in breast cancer, particularly for those ER positive breast cancers that are, or become, refractory to endocrine therapy.

Body

1. Specific Aims

This project consists of 3 specific aims:
Aim 1: To investigate the molecular mechanism, including the structural details, of WHSC1L1 that is involved in the transforming function through the alteration of the epigenetic histone code in human breast cancer cells.
Aim 2: To determine whether the histone modulation function of WHSC1L1 is linked to cancer stem cell phenotypes.
Aim 3: To examine the potential of WHSC1L1 as a therapeutic target in aggressive, ER-positive breast cancers that harbor the 8p11 amplicon.

2. Studies and Results

***Task 1*. To investigate the molecular mechanism, including the structural details, of WHSC1L1 that is involved in transforming function through the alteration of the epigenetic histone code in human breast cancer cells**.

Previously, our group identified 21 candidate oncogenes within 8p11-12 amplicon in breast cancer based on statistical analysis of copy number increase and gene overexpression (10). Using gain- and loss-of- function approaches, we found that WHSC1L1 is the most potently transforming oncogene we tested from the 8p11-12 region (14). Very recently, we searched the Cancer Genome Atlas database that contains 744 breast invasive carcinomas. We found DNA or mRNA alterations of WHSC1L1 in 212 of 744 (28%) breast invasive carcinomas, where the major samples are gene-amplified and/or over-expressed (Figure 1). We also found that WHSC1L1 mRNA expression levels are associated with DNA copy number changes in breast cancer. This new data further supports that WHSC1L1 plays an important role in breast cancer progression.

Expression of the WHSC1L1 gene results in two alternatively spliced variants, a long isoform and a short isoform that are derived from alternative splicing of exon 10. The WHSC1L1 long isoform encodes a 1437 amino acid protein containing 2 PWWP domains, 2 PHD-type zinc finger motifs, a TANG2 domain, an AWS domain, and a SET domain. The short isoform encodes a 645 amino acid protein containing a PWWP domain only. Our western blot assays demonstrates that both SUM-44 and SUM-52 cells have amplifications of the full-length gene, but at the protein level, expression of the short isoform predominates (14). Interestingly, we identified one primary breast cancer specimen (10173A) with the 8p11-12 amplicon in which array CGH demonstrated genomic loss of the C-terminal region of the WHSC1L1 long isoform but with amplification of exons 1-10 (14). We validated this finding in that particular breast cancer specimen by genomic PCR using primers specific for the short isoform exon 10 (S-10) and the long isoform exon 20 (L-20). This result provides evidence for the importance of the short isoform of WHSC1L1 that contains only PWWP domain in cell transformation when overexpressed.

Because WHSC1L1 encodes a PWWP domain nuclear protein, it has been postulated that it can promote malignant transformation by altering the histone code, and subsequently the expression, of specific target genes. To identify genes that may be altered in their expression by overexpression of the short isoform of WHSC1L1, we performed expression profiling of MCF10A cells, MCF10A-WHSC1L1 cells, and SUM-44 cells. To identify genes most likely to be regulated by overexpression of WHSC1L1 and relevant to human breast cancer, we determined which genes are differentially expressed in MCF10A-WHSC1L1 cells relative to parental MCF10A cells, and then determined which of those genes are also differentially expressed in SUM-44 cells compared to MCF10A cells. This orthogonal analysis resulted in the identification of 184 genes differentially expressed in both SUM-44 cells and MCF10A-WHSC1L1 cells, relative to MCF10A cells. Of the 184 differentially expressed genes, 36 are coordinately up-regulated in MCF10A-WHSC1L1 cells and SUM-44 cells. Specifically, we found four up-regulated genes (TBL1X, IRX3, RAG1AP1, and RAPGEF3) and two down-regulated genes (TFBI and SFRP1) in both SUM-44 cells and MCF10A-WHSC1L1 cells, relative to MCF10A cells. To directly validate some of these array-based observations, we chose three up-regulated genes and one down-regulated gene to examine by Q-RT-PCR (14). We validated that IRX3, RAPGEF3, and TBLX1 are significantly overexpressed at the mRNA level in SUM-44 cells compared to MCF10A cells. Furthermore, knock-down of WHSC1L1 in SUM-44 cells using the shRNA constructs described in Task 3 resulted in significant down-regulation of these three putative target genes. These results support the array-based analysis and indicate that WHSC1L1 regulates the expression of these target genes.

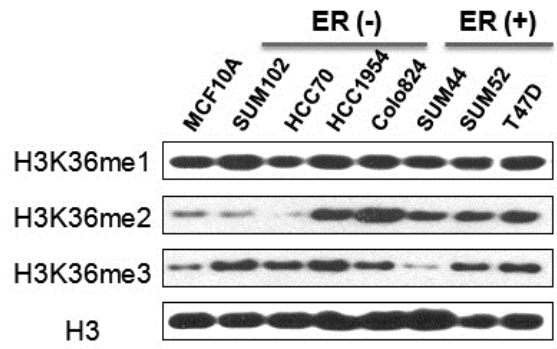

Figure 2. Global H3K36 me1, me2, and me3 levels in a panel of breast cancer cell lines. Total H3 was used as a loading control. ER: Estrogen receptor.

Very recently, WHSC1L1 family proteins have been shown to bind and modulate methylated histones, specifically H3K36 methylation marks (15). Therefore, we assessed global methylation (H3K4, H3K9, H3K27, and H3K36) levels by using western blotting in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. Our preliminary data indicated that global levels of H3K36me2 and me3 marks vary among different breast cancer cell lines (Figure 2). Histone methylation levels are mediated by a large number of enzymes and regulators, including methyltransferases, demethylases, and histone binding proteins. The breast cancer cell lines with defined histone methylation levels will provide a useful model for investigating biological and functional roles of these histone-modifying regulators in breast cancer, and for developing novel anticancer epidrugs.

Task 2. **To determine whether the histone modulation function of WHSC1L1 is linked to cancer stem cell phenotypes**.

The cancer stem cell (CSC) hypothesis suggests that only a subset of tumor cells with stem-cell-like properties is primarily responsible for the growth, progression, and recurrence of cancer (16-18). Two *in vitro* clonogenic assays have been used as methods to evaluate stem-cell-like properties. In the colony formation assay, soft agar or methylcellulose is used as the semisolid support media to prevent the migration of cells, which also leads to the formation of spatially distinct colonies (19). In the sphere formation assay, cells are plated at a clonal density so that individual cells will form spatially distinct spheres (16, 20). To determine whether overexpression of WHSC1L1 enhances the colony-formation *in vitro*, we seeded MCF10A cells stably expressing the WHSC1L1 and control cells in soft agar plates. As shown in Figure3, MCF10A cells overexpressing WHSC1L1 grew into robust colonies in soft agar, a property not observed in the parental MCF10A cells or in MCF10A cells containing the control vector. In addition, we performed mammosphere formation assays in MCF10A cells and MCF10A-WHSC1L1 cells. We found MCF10A-WHSC1L1 cells have higher capacities to generate mammospheres rather than MCF10A control cells after 10–12 days in the mammosphere cultures. These data suggest that WHSC1L1 is likely linked to the phenotypes of cancer stem cells. To determine whether WHSC1L1 also enhances self-renewal capacity *in vitro*, the first generation of the MCF10A-WHSC1L1 cell-derived mammospheres were collected and replated in the mammosphere culture condition. However, we did not detect the mammosphere formation in the replated culture. More recently, measuring the expression of aldehyde dehydrogenase (ALDH), an enzyme previously found to be expressed in hematopoietic and neuronal stem cells, has been established as a new tool to detect normal and malignant human mammary stem cells (21, 22). ALDH can be assessed by the Aldefluor assay to detect cells displaying aldehyde dehydrogenase activity (Stem Cell Technologies, Inc). However, ALDH assays did not show direct evidence that overexpression of WHSC1L1 in MCF10A cells results in expansion of cell pools with the stem cell ALDH marker. In summary, overexpression of the WHSC1L1, at least in part, induces the acquisition of stem cell-like properties *in vitro,* but unlikely influences the self-renewal potential of breast cancer stem cells.

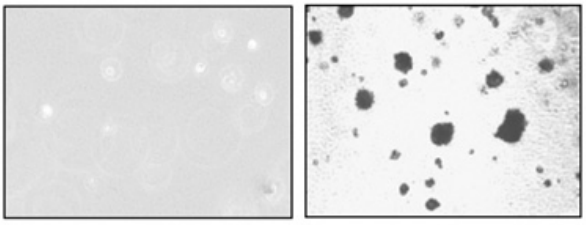

Figure 3. Representative pictures of MCF10A cells that stably overexpress WHSC1L1 and control cell soft agar colonies. Cells were grown for 3 weeks in soft agar and stained with the vital dye p-iodonitrotetrazolium violet.

***Task 3*. To examine the potential of WHSC1L1 as a therapeutic target in aggressive, ER-positive breast cancers that harbor the 8p11 amplicon.** To directly assess the contribution of endogenous WHSC1L1 overexpression on the transformation of human breast cancer, we examined the effects of knock-down of WHSC1L1 in SUM-44 and SUM-52 cells where WHSC1L1 is amplified and overexpressed, and in the control cell line MCF10A. To perform RNAi knock-down experiments, we obtained eight pGIPZ-WHSC1L1 shRNA expression constructs from OpenBiosystems (http://www.openbiosystems.com/). In this vector, TurboGFP and shRNA are part of a bicistronic transcript allowing the visual marking of the shRNA expressing cells. SUM-44, SUM-52, and control MCF10A cells were infected with these 8 shRNA lentivirus supernatants pooled or separated to determine which gave the best knock-down of WHSC1L1. Non-silencing shRNAmir lentiviral control, at the same titer as WHSC1L1 shRNA, was used in parallel as the negative control. First, the consequence of knock-down of WHSC1L1 using all eight shRNAs combined on colony formation was evaluated in all three cell lines. WHSC1L1 knock-down suppressed proliferation of SUM-44 and SUM-52 cells, while WHSC1L1 shRNAs had no effect on the growth of MCF10A cells. Next, we identified the two most efficient shRNAs with respect to knock-down of WHSC1L1 expression levels in SUM-44 and SUM-52 cells. Q-RT-PCR and western blot data revealed that the WHSC1L1-shRNAs #2 and #6 resulted in decreases in mRNA and protein levels to approximately 20-30% of the level seen in the non-silencing control-infected cells. As shown in Figure 4B and C, WHSC1L1 knock-down with both shRNA constructs slowed cell growth of SUM-44 and SUM-52 cells. The results were most striking for SUM-44 cells, in which WHSC1L1 knock-down inhibited cell proliferation by ~90% (Figure 4C). WHSC1L1 knock-down with these shRNA#2 and #6 had an undetectable effect on the cell growth of MCF10A cells. Thus, knockdown of WHSC1L1 inhibits cell proliferation in breast cancer cells with WHSC1L1 gene amplification.

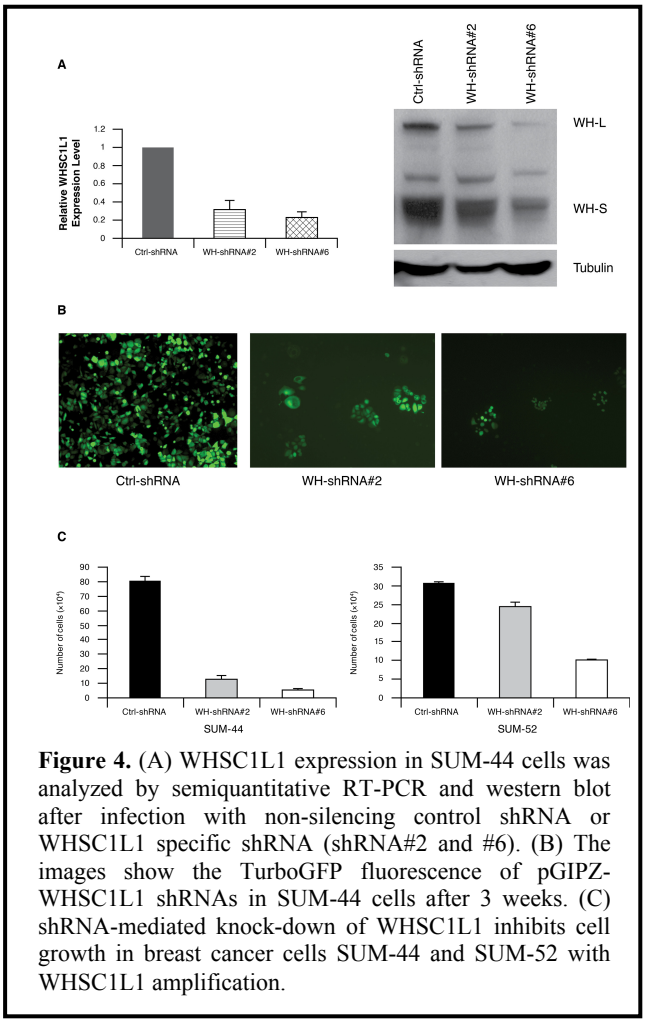

Figure 4. (A) WHSC1L1 expression in SUM-44 cells was analyzed by semiquantitative RT-PCR and western blot after infection with non-silencing control shRNA or WHSC1L1 specific shRNA (shRNA#2 and #6). (B) The images show the TurboGFP fluorescence of pGIPZ-WHSC1L1 shRNAs in SUM-44 cells after 3 weeks. (C) shRNA-mediated knock-down of WHSC1L1 inhibits cell growth in breast cancer cells SUM-44 and SUM-52 with WHSC1L1 amplification.

Key Research Accomplishments

We systematically investigated the transforming properties of the newly identified 8p11-12 candidate oncogene WHSC1L1 *in vitro*. We demonstrated that WHSC1L1 acts as a transforming gene: stable WHSC1L1 overexpression in non-tumorigenic MCF10A cells induces transformed phenotypes, whereas WHSC1L1 knockdown in 8p12 amplified, ER-positive breast cancers cells inhibits proliferation *in vitro*. We also revealed that overexpression of WHSC1L1 likely induces the acquisition of stem cell-like properties *in vitro.* We analyzed the Cancer Genome Atlas database and revealed that DNA or mRNA alterations of WHSC1L1 exist in 212 of 744 (28%) breast invasive carcinomas, where the major samples are gene amplified and/or overexpressed. We also assessed global methylation levels in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. Our data indicated that global levels of H3K36me2 and me3 marks vary among different breast cancer cell lines. Our results suggest that WHSC1L1 can act as a driver of oncogenic processing and have critical roles in breast cancer initiation and progression.

Reportable Outcomes

Manuscript (See Appendices):

1. Yang Z-Q, Liu G, Bollig-Fischer A, Giroux CN, Ethier SP. Transforming properties of 8p11-12 amplified genes in human breast cancer. Cancer Research. 70:8487-97. 2010
2. Wu J, Liu S, Liu G, Dombkowski Al, Abrams J, Martin-Trevino R, Wicha M, Ethier S and Yang Z-Q. Identification and functional analysis of 9p24 amplified genes in human breast cancer. Oncogene. 31:333-41.2012
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Presentations:

1. Zhang L, Hou JL, Holowatyj A and Yang Z-Q. The role of histone demethylase GASC1 in promoting prostate cancer progression. 104th American Association for Cancer Research Annual Meeting in Washington, DC, Apr 4-10, 2013
2. Hou JL, Wu J, Dombkowski Al, Boerner JL and Yang Z-Q. Genomic amplification and drug-resistance roles of the KDM5A histone demethylase gene in breast cancer. 103rd American Association for Cancer Research Annual Meeting in Chicago, IL, Mar 31-Apr 4, 2012

3. Yang Z-Q, Liu G and Ethier S. Oncogenic role of PWWP-domain protein WHSC1L1 in breast cancer. DOD/BCRP Era of Hope Conference in Orlando, Florida, August 2-5, 2011
4. Wu J, Liu S, Liu G, Dombkowski Al, Abrams J, Martin-Trevino R, Wicha M, Ethier S and Yang Z-Q. Identification and functional analysis of 9p24 amplified genes in human breast cancer. 102nd American Association for Cancer Research Annual Meeting in Orlando, Florida, April 2-6, 2011 (Selected for oral presentation)
5. Liu G, Yang Z-Q and Ethier S. PPAPDC1B isoform 2, a new candidate oncogene within the 8p11-12 amplicon induces a proliferative advantage in breast cancer. 102nd American Association for Cancer Research Annual Meeting in Orlando, Florida, April 2-6, 2011
6. Yang Z-Q. Histone demethylase GASC1 in cancer. The 3rd World Cancer Congress 2010 at Singapore, June 22-25 (Section Chair and Oral presentation)
7. Liu G, Yang Z-Q and Ethier S. Oncogenic PWWP-domain protein WHSC1L1 links the homeobox transcription factor IRX3 in breast cancer. 101st American Association for Cancer Research Annual Meeting in Washington, DC, April 17-21, 2010

Personnel Receiving Support

Zeng-Quan Yang (PI): Associate Professor, Wayne State University
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Conclusion

We performed comprehensive genomic and functional analyses of WHSC1L1 in a panel of breast cancer cell lines and in primary breast cancer samples. Our findings include the following: (1) we identified WHSC1L1 with the highest frequency of high-level amplification in breast cancer; (2) WHSC1L1 had the higher correlation coefficient between gene expression and copy number in breast cancer; (3) gain- and loss-of-function approaches provided strong evidence that WHSC1L1 possesses transforming properties, and likely plays a critical role in a subset of 8p11-12 amplified, aggressive breast cancer; (4) the WHSC1L1 protein is involved in histone code modification and epigenetic regulation of gene expression; (5) we have assessed global methylation levels in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines; (6) we identified several WHSC1L1 target genes, one of which is Iroquois homeobox 3 gene , a member of the Iroquois homeobox transcription factor family; and (7) our findings provide a strong foundation for further mechanistic research and therapeutic options using WHSC1L inhibitors to treat breast cancer.

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Appendices:

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Cancer Research

Transforming Properties of 8p11-12 Amplified Genes in Human Breast Cancer

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Abstract

Amplification of the 8p11-12 region has been found in about 15% of human breast cancers and is associated with poor prognosis. Earlier, we used genomic analysis of copy number and gene expression to perform a detailed analysis of the 8p11-12 amplicon to identify candidate oncogenes in breast cancer. We identified 21 candidate genes and provided evidence that three genes, namely, *LSM-1, TC-1*, and *BAG4*, have transforming properties when overexpressed. In the present study, we systematically investigated the transforming properties of 13 newly identified 8p11-12 candidate oncogenes *in vitro*. *WHSC1L1, DDHD2*, and *ERLIN2* were most potently transforming oncogenes based on the number of altered phenotypes expressed by the cells. WHSC1L1 contains a PWWP-domain that is a methyl-lysine recognition motif involved in histone code modification and epigenetic regulation of gene expression. Knockdown of *WHSC1L1* in 8p11-12–amplified breast cancer cells resulted in profound loss of growth and survival of these cells. Further, we identified several WHSC1L1 target genes, one of which is iroquois homeobox 3 gene (*IRX3*), a member of the Iroquois homeobox transcription factor family. *Cancer Res; 70(21); 8487–97. ©2010 AACR.*

Introduction

An important mechanism for the activation of oncogenes in human cancers is gene amplification, which results in gene overexpression at both the message and the protein levels (1, 2). Oncogenes, such as *ERBB2* at 17q12, *CCND1* at 11q13, and *C-MYC* at 8p24, have previously been identified as amplification targets linked to the development, progression, or metastasis of human cancers, including breast, prostate, lung, and other cancers (2, 3). *ERBB2* is the most frequently amplified oncogene in breast cancer, and its overexpression is associated with poor clinical outcomes. The prognostic and predictive values of ERBB2 amplification and overexpression have been used to guide treatment decisions for patients with both lymph node–positive and -negative diseases. More significantly, recognition of the mechanistic roles of ERBB2 in breast cancer has led to the development of ERBB2-targeting drugs such as trastuzumab to treat breast cancer (4–6).

Amplification of 8p11-12 occurs in approximately 15% of human breast cancers, and this region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients (7–11). Chin et al. performed an analysis of the association of gene amplification and disease-free survival and distant relapse in human breast cancer specimens (12). They identified 23 genes from the 8p11-12 region as being correlated with progression. Recently, our laboratory published results of a detailed analysis of copy number and gene expression in the 8p11-12 region in a panel of breast cancer cell lines and primary human breast cancers (10). We identified 21 genes that are overexpressed when their copy number is increased (10). Furthermore, we directly tested the transforming function of eight 8p11-12 amplified genes in human mammary epithelial cells. From these experiments, we identified several genes, including *LSM1, BAG4*, and *C8orf4* (*TC-1*), as having the transforming properties *in vitro* (10, 13, 14). Accumulating evidence suggests that the 8p11-12 amplicon contains multiple candidate oncogenes that could play a role in breast cancer development (7–11).

Recent extensive genomic analyses and siRNA knockdown studies have identified the Wolf-Hirschhorn syndrome candidate 1-like 1 gene (*WHSC1L1*, also known as *NSD3*) as one of the major candidate oncogenes of the 8p11-12 amplicon in breast cancer (7–11). *WHSC1L1* is the third member of a gene family that includes *NSD1* and *WHSC1* (*NSD2*; refs. 15, 16). *De novo* translocation of *NSD1* genes causes the childhood overgrowth syndrome, Sotos syndrome, that is associated with elevated risks of cancer, whereas *de novo* deletion of *NSD2/WHSC1* causes the Wolf-Hirschhorn syndrome that displays growth retardation (17, 18). WHSC1L1/NSD3, NSD1, and WHSC1/NSD2 show strong sequence similarity and share multiple functional domains (15). WHSC1L1 has two isoforms that are derived from alternative splicing of exon 10, and both WHSC1L1 protein isoforms contain a PWWP

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domain. The PWWP domain belongs to the royal superfamily that includes chromodomain, tudor, malignant brain tumor, and plant agent motifs, and these domains exist in multiple histone modifying proteins. The NH_2-terminal half of the PWWP domain exhibits a β-barrel structure that resembles a SAND domain, whereas the COOH-terminal portion is made up of a 5-helix bundle. Both the crystal and nuclear magnetic resonance (NMR) solution structures of the superfamily complexes show that the β-barrel structure recognizes and binds the histone lysine pocket (19, 20). A study on PWWP function in the DNA methyltransferase DNMT3B showed that the PWWP domain binds methylated DNA (21). Recently, Wang et al. showed that a PWWP domain protein binds to histone lysine *in vitro* and *in vivo*, and regulates Set9-mediated H4K20 methylation (22). Their results showed that the PWWP domain is a methyl-lysine recognition motif that plays important roles in epigenetic regulation.

In the present study, we systematically investigated the transforming properties of 13 newly identified 8p11-12 candidate oncogenes *in vitro*. We found that *WHSC1L1, DDHD2*, and *ERLIN2* are the most potently transforming oncogenes we tested from the 8p11-12 region based on the number of altered phenotypes expressed by the cells. Knockdown of *WHSC1L1* in 8p11-12–amplified breast cancer cells resulted in profound loss of growth and survival of these cells. Further, we identified several WHSC1L1 target genes, one of which is iroquois homeobox 3 gene (*IRX3*), a member of the Iroquois homeobox transcription factor family.

Materials and Methods

Genomic array comparative genomic hybridization (CGH)

The isolation and culture of the SUM series of human breast cancer cell lines and MCF10A cells have been described in the Supplementary Materials and Methods (10, 23). Genomic array CGH experiments were performed using the Agilent 44K human genome CGH microarray chip (Agilent Technologies). Agilent's CGH Analytics software was used to calculate various measurement parameters, including log2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe.

Semiquantitative reverse transcriptase-PCR reactions

Total RNA was prepared from human breast cancer cell lines and the MCF10A cell line by standard methods (10, 24). For reverse transcriptase-PCR (RT-PCR) reactions, RNA was converted into cDNA via a reverse transcription reaction using random hexamer primers. Primers were ordered from Invitrogen, and all the relevant primer sequences are available on request. A GAPDH primer set was used as a control. Semiquantitative RT-PCR was done using the iQSYBR Green Supermix (Bio-Rad).

Lentivirus construction and transduction of cells

The lentiviral expression constructs containing the 13 genes tested in the present experiments, listed in Table 1, were established as previously described (10). Briefly, we first created entry clones from cDNA of SUM-44 cells using the pENTR directional TOPO cloning kit and then performed the LR recombination reaction to transfer the gene into the Gateway destination vector, pLenti6/V5-DEST. Specifically, the pLenti-WHSC1L1 construct was established from the full-length short isoform, which only contained the PWWP domain. The lentivirus for each construct was generated and used to infect immortalized, nontransformed mammary epithelial MCF10A cells. Control infections with pLenti-LacZ virus were performed in parallel. Selection began 48 hours after infection in growth medium with 10 μg/mL blasticidin in the absence of insulin. Upon confluence, selected cells were passaged and serially cultured.

Growth in soft agar and Matrigel

Soft agar assays were performed as previously described (10). For three-dimensional (3D) morphogenesis assays in Matrigel, cells grown in monolayer culture were detached by trypsin/EDTA treatment and seeded in Matrigel (BD Biosciences) precoated 8-well chamber slides. The appropriate volume of medium was added and maintained in culture for 10 to 18 days. Phase-contrast images and immunostained images were photographed with bright-field and confocal microscopy (25).

Lentivirus-mediated short hairpin RNA (shRNA) knockdown of gene expression

We knocked down the expression of the human *WHSC1L1* gene in the breast cancer cell lines SUM-44 and SUM-52 and in the MCF10A cell line using the Expression Arrest GIPZ lentiviral shRNAmir system (OpenBiosystems). Lentivirus was produced by transfecting 293FT cells with the combination of the lentiviral expression plasmid DNA and Trans-Lentiviral packaging mix (OpenBiosystems). For cell infection, viral supernatants were supplemented with 6 μg/mL polybrene and incubated with cells for 24 hours. Cells expressing shRNA were selected with puromycin for 2 to 3 weeks for functional studies (cell proliferation and colony formation assays) and for 4 to 10 days after infection for RNA extraction.

Results

The effect of different 8p11-12 genes on growth factor–independent proliferation

Recently, our group identified 21 candidate oncogenes within the 8p11-12 amplicon in breast cancer based on statistical analysis of copy number increase and gene overexpression. We tested 8 of the 21 candidate oncogenes for transforming function *in vitro* and identified three genes, namely, *LSM1, BAG4*, and *C8orf4* (*TC-1*), that could induce transformed phenotypes (10). In the present report, we expanded our analysis to the remaining 13 candidate oncogenes. Table 1 shows the original 21 gene list, with the 13 genes tested in the present experiments highlighted with an asterisk. Details on the origins and sequence validations of each clone are given in Materials and Methods and in Supplementary Data.

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Table 1. List of the 21 candidate genes of the 8p11-12 region

Gene	Description
ZNF703	Zinc finger protein 703
*ERLIN2**	ER lipid raft associated 2
*PROSC**	Proline synthetase cotranscribed homolog (bacterial)
*BRF2**	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like
RAB11FIP1	Rab coupling protein = RCP
EIF4EBP1	Elongation factor 4 binding protein 1
*ASH2L**	Ash2 (absent, small, or homeotic)-like (Drosophila)
LSM1	LSM1 homolog, U6 small nuclear RNA associated (S. cerevisiae)
BAG4	BCL2-associated athanogene 4
*DDHD2**	DDHD domain containing 2
*PPAPDC1B**	Phosphatidic acid phosphatase type 2 domain containing 1B
*WHSC1L1**	Wolf-Hirschhorn syndrome candidate 1-like 1
*LETM2**	Leucine zipper-EF-hand containing transmembrane protein 2
FGFR1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)
TACC1	Transforming acidic coiled-coil
*TM2D2**	TM2 domain containing 2
C8orf4	Chromosome 8 open reading frame 4 = TC-1
*AP3M2**	Adaptor-related protein complex 3, μ 2 subunit
*POLB**	Polymerase (DNA directed), β
*VDAC3**	Voltage-dependent anion channel 3
*HOOK3**	Hook homolog 3 (Drosophila)

*Tested in the current study.

To systematically investigate the transforming properties of 13 8p11-12 candidate breast cancer oncogenes, we transduced MCF10A cells, which are highly growth factor dependent, with individual lentiviral expression vectors for each gene. Growth factor–independent proliferation of MCF10A cells transduced with each candidate gene was investigated. RT-PCR was performed to confirm the expression of the gene using primers specific for the gene and for the vector. Overexpression of WHSC1L1 protein in MCF10A-WHSC1L1 cells was further confirmed by Western blot (Supplementary Fig. S1). As shown in Fig. 1A, MCF10A cells expressing six genes, namely, *WHSC1L1, DDHD2, PROSC, BRF2, ASH2L*, and *ERLIN2*, formed expanding colonies in insulin-free medium, and then grew continuously in the absence of insulin-like growth factors. Colony formation assays in MCF10A cells with equalized viral titer of the tested genes indicated that overexpression of *WHSC1L1* and *DDHD2* resulted in the highest number of insulin-independent colonies. Growth curves of MCF10A cells overexpressing the five genes (*WHSC1L1, DDHD2, PROSC, BRF2*, and *ERLIN2*) were performed within two passages of isolation in insulin-free medium. Data in Fig. 1B show that overexpression of *WHSC1L1* not only resulted in the largest number of colonies emerging in insulin-free medium, but also gave rise to cells with the most rapid proliferation rate under these conditions. These results extend our previous findings and indicate that a total of nine genes from the 8p11-12 have the ability to induce insulin-like growth factor–independent proliferation when overexpressed in MCF10A cells.

Transforming properties of 8p11-12 candidate genes

To assess the expression of other transformed phenotypes of MCF10A cells overexpressing the newly identified candidate oncogenes, we evaluated each of them for their ability to form colonies in soft agar and for altered morphogenesis in Matrigel. Figure 2A shows that after three weeks in culture, MCF10A cells overexpressing *WHSC1L1, DDHD2*, and *ERLIN2* formed colonies in soft agar. MCF10A cells overexpressing *WHSC1L1* and *DDHD2* had the highest soft agar colony-forming efficiency (Fig. 2B). By contrast, MCF10A cells overexpressing *ASH2, BRF2*, and *PROSC* did not form soft agar colonies. We also examined whether these six genes affect the growth or morphology of colonies in 3D Matrigel culture, as aberrant behavior in this environment is frequently associated with transformation and/or tumor progression (26). In 3D basement membrane cultures, the immortalized, nontransformed mammary epithelial cells, MCF10A, formed acinar-like structures consisting of a single cell layer of polarized, growth-arrested mammary epithelial cells surrounding a hollow lumen (Fig. 2A). As shown in Fig. 2A, MCF10A cells overexpressing *WHSC1L1* formed strikingly abnormal acini that were enlarged and disorganized, and contained filled lumens. In contrast, MCF10A cells overexpressing *DDHD2* formed disorganized, small abnormal acini. MCF10A cells overexpressing *ERLIN2* also formed large, highly proliferative colonies, whereas insulin-independent MCF10A cells overexpressing the other three candidate oncogenes formed polarized, growth-arrested acinar structures with hollow lumens

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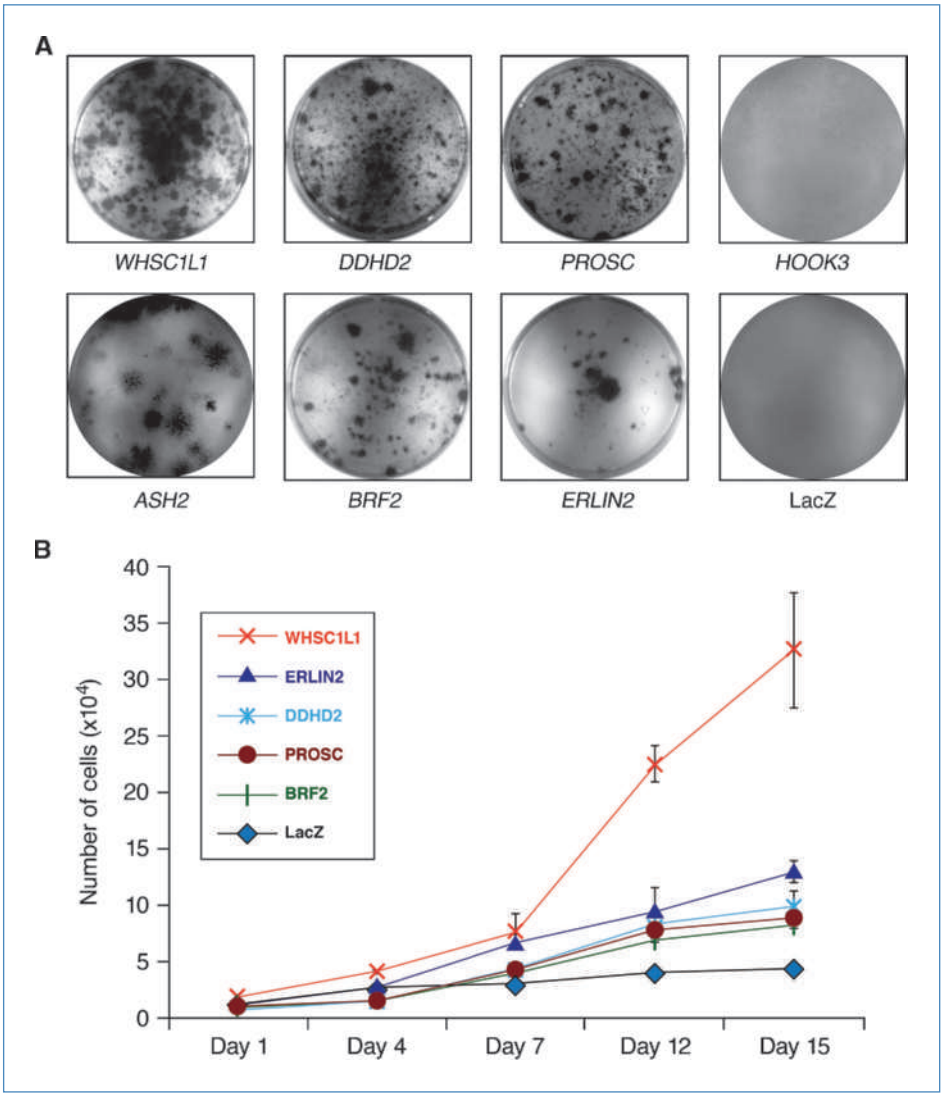

Figure 1. A, MCF10A cells expressing six genes, namely, *WHSC1L1, BRF2, DDHD2, PROSC, ERLIN2*, and *ASH2L*, formed expanding colonies in insulin-free medium, whereas MCF10A cells expressing *HOOK3* and control LacZ did not form colonies. B, *in vitro* growth rate of the MCF10A cells that stably overexpress the five genes (*ERLIN2, WHSC1L1, DDHD2, PROSC*, and *BRF2*) relative to MCF10A-LacZ control cells in insulin-deficient media. Cells were seeded into 35-mm culture wells and grown in the absence of insulin-like growth factors.

similar to MCF10A parental cells (data not shown). These experiments show that three of the transforming genes, i.e., *PROSC, ASH2L*, and *BRF2*, induced insulin-independent growth and no other altered phenotypes. By contrast, *WHSC1L1, DDHD2*, and *ERLIN2* were the most transforming oncogenes based on the number of altered phenotypes expressed by the cells.

Amplification of WHSC1L1 isoforms in breast cancer

In our analysis of the transforming properties of the 8p11 candidate oncogenes, we were surprised by the potency of *WHSC1L1* for transforming MCF-10A cells. As described above, *WHSC1L1*-overexpressing MCF-10A cells exhibited the highest transforming efficiency. The cells had the highest growth rate in insulin-free medium, and the cells grew with high efficiency in soft agar, while forming very abnormal colonies in Matrigel. Because of the extraordinary transforming potency of *WHSC1L1*, the role of this gene as a driver oncogene in breast cancer cell lines and specimens with the amplicon was examined further.

Expression of the *WHSC1L1* gene results in two alternatively spliced variants: a long isoform and a short isoform that are derived from alternative splicing of exon 10. The WHSC1L1 long isoform encodes a 1437-amino-acid protein containing two PWWP domains, two PHD-type zinc finger motifs, a TANG2 domain, an AWS domain, and a SET domain. The short isoform encodes a 645-amino-acid protein containing a PWWP domain only (Supplementary Fig. S2). The data shown in Fig. 3 show that both SUM-44 and SUM-52 cells have amplifications of the full-length gene, but at the protein level expression of the short isoform predominates. The transformation data for *WHSC1L1*-overexpressing MCF10A cells shown above were obtained using an expression construct coding for the short isoform, and similar results were obtained when we transduced MCF10A cells with a vector coding for full-length WHSC1L1 (data not shown).

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Alternative splicing in cancer is an important mechanism for gene regulation and for generating proteomic diversity. Interestingly, we identified one primary breast cancer specimen (10173A) with the 8p11-12 amplicon in which array CGH showed genomic loss of the COOH-terminal region of the WHSC1L1 long isoform but with amplification of exons 1 to 10. We validated this finding in that particular breast cancer specimen by genomic PCR using primers specific for the short isoform exon 10 (S-10) and the long isoform exon 20 (L-20) as shown in Fig. 3C and Supplementary Fig. S2. To further determine whether the WHSC1L1 short isoform protein, which only contains a PWWP domain, is also localized in the nucleus, we generated expression constructs containing the short isoform WHSC1L1 coding sequences fused to the EGFP epitope at the COOH-terminus. The constructs were transfected into MCF10A and HEK293 cells, and localization of the proteins was examined by fluorescence microscopy. The WHSC1L1 short isoform was localized to the nucleus as expected (Supplementary Fig. S3). These results indicate that both WHSC1L1 protein isoforms are localized to the nucleus, and may act as transforming oncoproteins in breast cancer cells bearing the 8p11p12 amplicon.

Knockdown of *WHSC1L1* inhibits cell proliferation in breast cancer cells

To directly assess the contribution of endogenous *WHSC1L1* overexpression on the transformation of human breast cancer, we examined the effects of knockdown of *WHSC1L1* in SUM-44 and SUM-52 cells where *WHSC1L1* is amplified and overexpressed, in SUM-149 cells that do not have the amplicon, and in the control cell line MCF10A. To perform shRNA knockdown experiments, we obtained eight pGIPZ-WHSC1L1

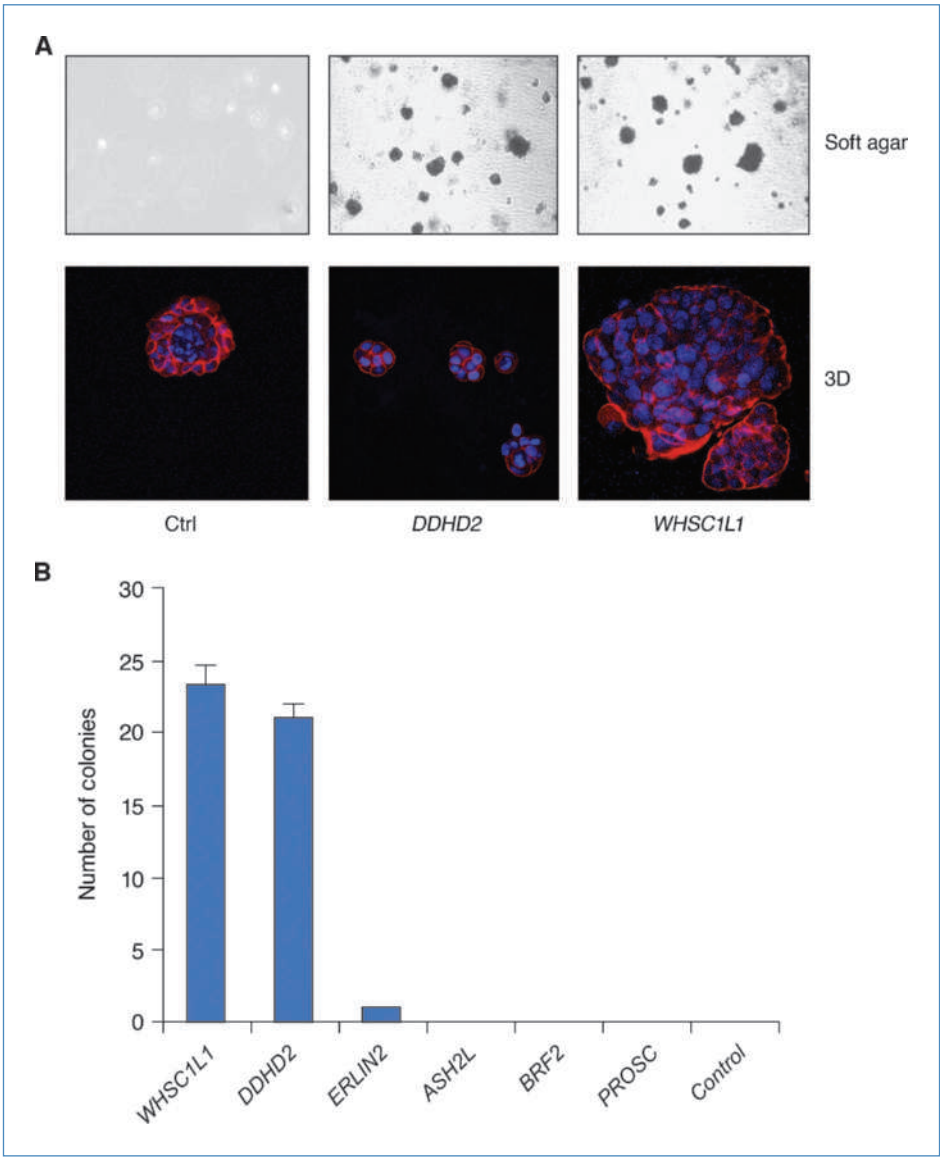

Figure 2. A, top, representative pictures of MCF10A cells that stably overexpress *DDHD2* and *WHSC1L1* genes and control (Ctrl) cell soft agar colonies. Cells were grown for three weeks in soft agar and stained with the vital dye p-iodonitrotetrazolium violet. Bottom, effects of *DDHD2* and *WHSC1L1* on mammary acinar morphogenesis. MCF10A-DDHD2, WHSC1L1, and control cells were cultured on a bed of Matrigel as described in Materials and Methods. Representative images of structures with staining for actin with phalloidin conjugated to Alexa Fluor-568 (red), and 4′, 6-diamidino-2-phenylindole (DAPI) as a marker of nuclei (blue). B, soft agar colony-forming efficiency in MCF10A cells stably overexpressing the six genes (*WHSC1L1, BRF2, DDHD2, PROSC, ERLIN2*, and *ASH2L*) and control cell soft agar colonies. Data are the mean number of colonies per low power field three weeks after seeding 10^5 cells per well.

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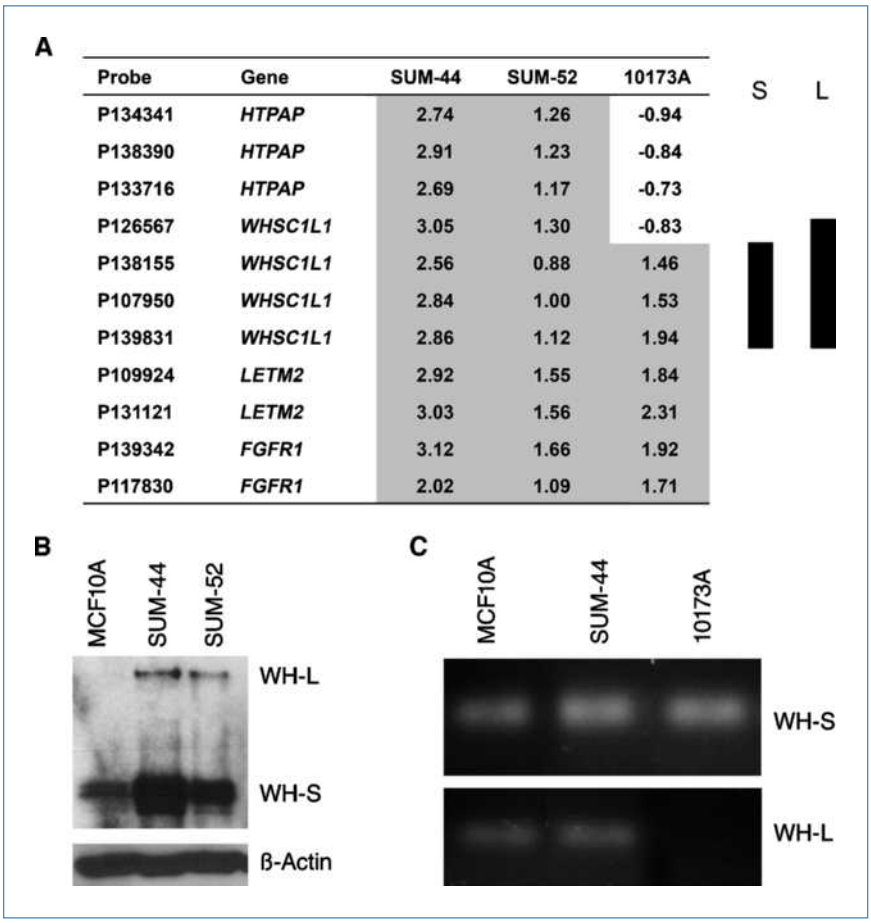

Figure 3. A, genomic copy number profiles of the WHSC1L1 region analyzed on the Agilent oligonucleotide array CGH in two SUM breast cancer cell lines (SUM-44 and SUM-52) and one primary breast cancer specimen (10173A). Array probes and genes are displayed horizontally by genome position. Log2 ratio in each sample is relative to normal female DNA. S, WHSC1L1 short isoform; L, long isoform. B, WHSC1L1 protein levels were analyzed by Western blot in two breast cancer cell lines, SUM-44 and SUM-52, and control MCF10A line. C, genomic PCR using primers specific for the short isoform exon 10 (S-10) and the long isoform exon 20 (L-20) of WHSC1L1 were used to validate array-CGH data in breast cancer specimen 10173A.

shRNA expression constructs from OpenBiosystems. (http://www.openbiosystems.com/). In this vector, TurboGFP and shRNA are part of a bicistronic transcript allowing the visual marking of the shRNA-expressing cells. SUM-44, SUM-52, and control MCF10A cells were infected with these 8-shRNA lentivirus supernatants, pooled or individually, to determine which gave the best knockdown of *WHSC1L1*. Nonsilencing shRNAmir lentiviral control, at the same titer as *WHSC1L1* shRNA, was used in parallel as the negative control. First, the consequence of knockdown of *WHSC1L1* on colony formation using all eight shRNAs was evaluated in all three cell lines. *WHSC1L1* knockdown suppressed proliferation of SUM-44 and SUM-52 cells, whereas *WHSC1L1* shRNAs had no effect on the growth of SUM-149 cells or MCF10A cells (Supplementary Fig. S4). Next, we identified the two most efficient shRNAs with respect to knockdown of *WHSC1L1* expression levels in SUM-44 and SUM-52 cells. Quantitative RT-PCR and Western blot data revealed that the WHSC1L1-shRNAs 2 and 6 resulted in decreases in mRNA and protein levels to approximately 20% to 30% of the level seen in the nonsilencing control-infected cells (Fig. 4A). As shown in Fig. 4B and C, *WHSC1L1* knockdown with both shRNA constructs slowed cell growth of SUM-44 and SUM-52 cells. The results were most striking for SUM-44 cells in which *WHSC1L1* knockdown inhibited cell proliferation by ~90% (Fig. 4C). *WHSC1L1* knockdown with shRNA 2 and 6 had an undetectable effect on the cell growth of MCF10A cells (data not shown). Thus, knockdown of *WHSC1L1* inhibits cell proliferation in breast cancer cells with *WHSC1L1* gene amplification.

IRX3 is a novel target gene of WHSC1L1

Because *WHSC1L1* encodes a PWWP domain nuclear protein that has histone methyl transferase activity, it has been postulated that it can promote malignant transformation by altering the histone code and hence expression of specific target genes. To identify genes that may be altered in their expression by overexpression of the short isoform of WHSC1L1, we performed expression profiling of MCF10A cells, MCF10A-WHSC1L1 cells, and SUM-44 cells. To identify genes most likely to be regulated by overexpression of *WHSC1L1* and relevant to human breast cancer, we determined which genes are differentially expressed in MCF10A-WHSC1L1 cells relative to parental MCF10A cells, and then determined which of those genes are also differentially expressed in SUM-44 cells compared with MCF10A cells. This orthogonal analysis resulted in the identification of 148 genes

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differentially expressed in both SUM-44 cells and MCF10A-WHSC1L1 cells, relative to MCF10A cells (Supplementary Table). Of the 148 differentially expressed genes, 36 are coordinately upregulated in MCF10A-WHSC1L1 cells and SUM-44 cells. Figure 5A shows the four upregulated genes (*TBL1X, IRX3, RAG1AP1*, and *RAPGEF3*), and two downregulated genes (*TFBI* and *SFRP1*) in both SUM-44 cells and MCF10A-WHSC1L1 cells, relative to MCF10A cells. To validate these array-based observations, we examined expression of these genes by quantitative RT-PCR in SUM-44 and MCF-10 cells, and in SUM-44 cells following knockdown of *WHSC1L1* (Fig. 5B). Figure 5 shows that *IRX3, RAG1AP1, RAPGEF3*, and *TBL1X* are significantly overexpressed at the mRNA level in SUM-44 cells compared with MCF10A cells. Furthermore, knockdown of *WHSC1L1* in SUM-44 cells using the shRNA constructs described previously resulted in significant downregulation of these four putative target genes. These results support the array-based analysis and

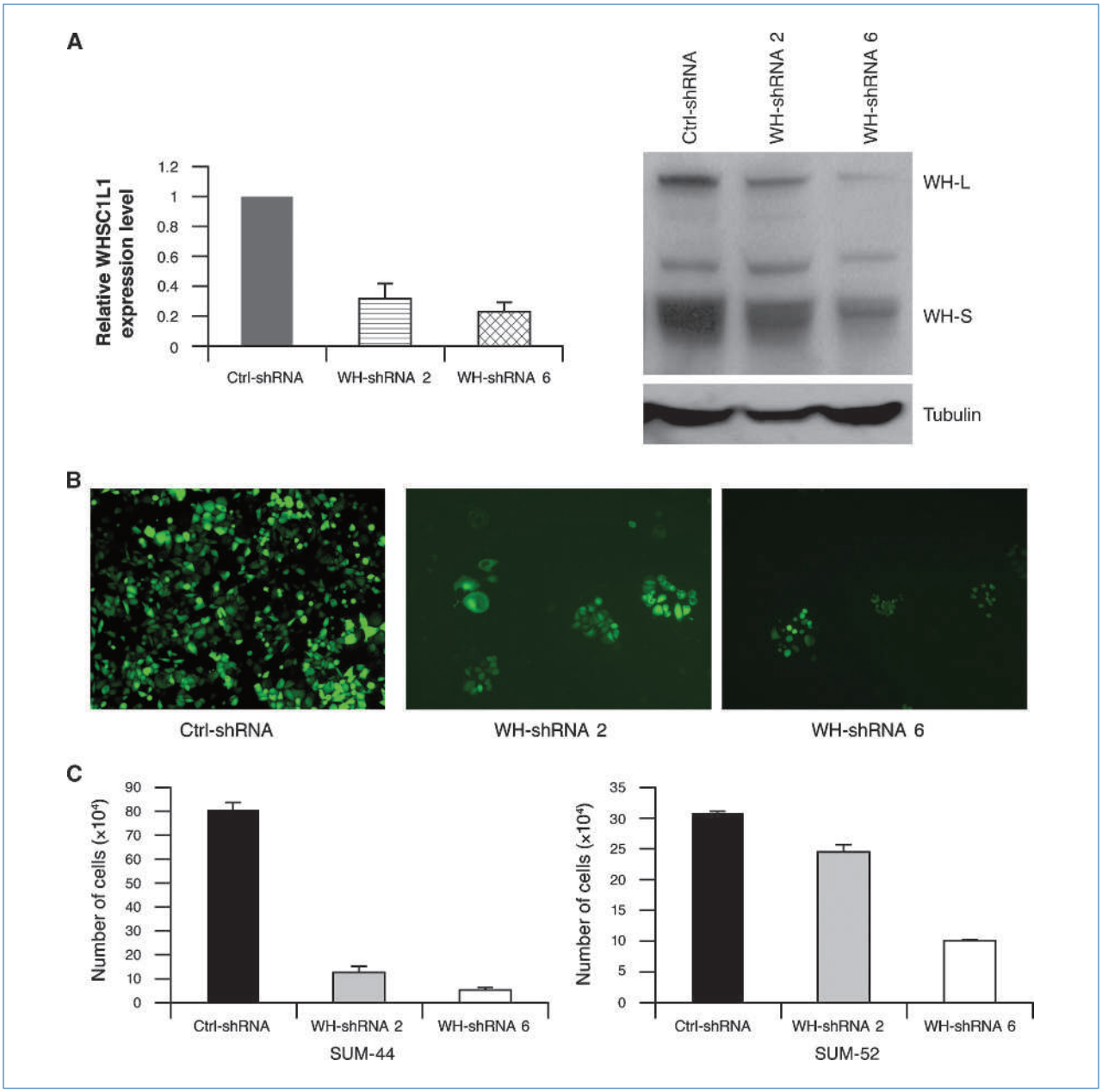

Figure 4. A, WHSC1L1 expression in SUM-44 cells was analyzed by semiquantitative RT-PCR and Western blot after infection with nonsilencing control shRNA or WHSC1L1-specific shRNA (shRNA 2 and 6). B, TurboGFP fluorescence of pGIPZ-WHSC1L1 shRNAs in SUM-44 cells after three weeks. C, shRNA-mediated knockdown of WHSC1L1 inhibits cell growth in breast cancer cells SUM-44 and SUM-52 with WHSC1L1 amplification.

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A

Gene	SUM-44	MCF10A-WH
TBL1X	15.15	3.63
IRX3	10.58	2.27
RAG1AP1	5.35	2.03
RAPGEF3	2.54	2.71
TGFBI	-119.93	-3.34
SFRP1	-40.52	-3.34

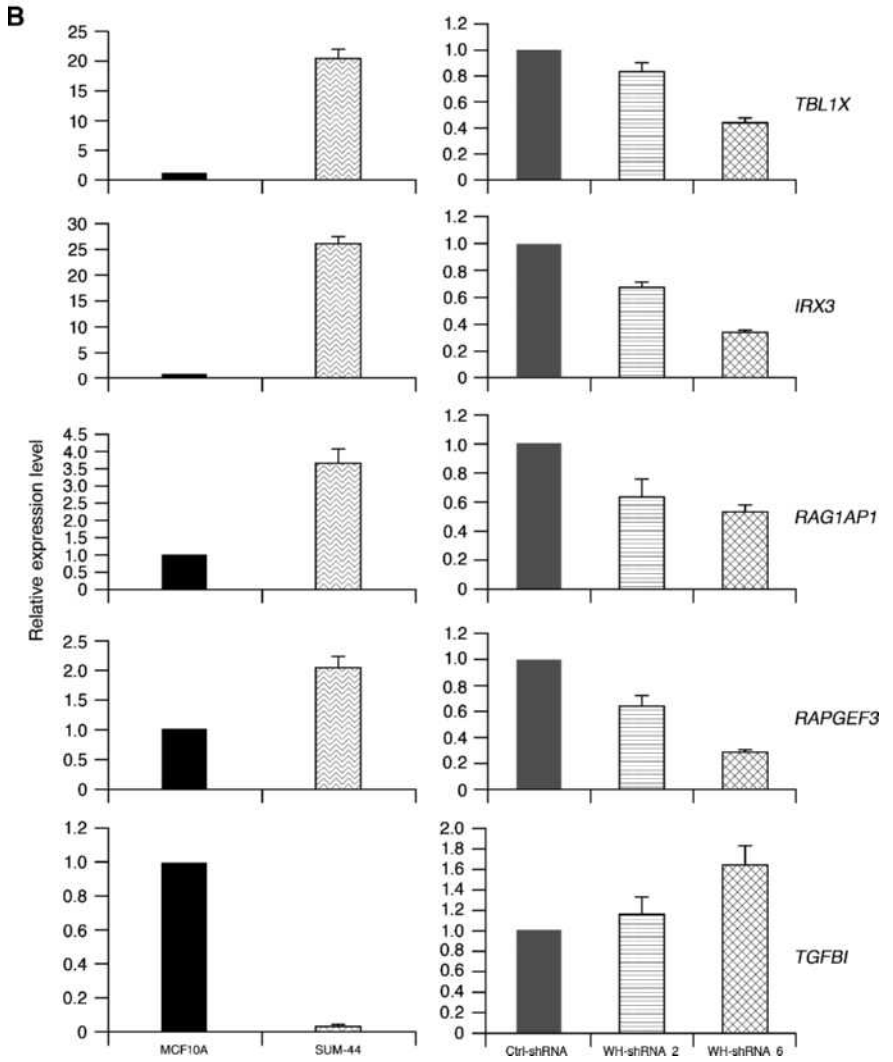

Figure 5. A, six genes differentially expressed in both SUM-44 cells and MCF10A-WHSC1L1 cells, relative to MCF10A cells with the Illumina expression Beadarray. B, *TBL1X, IRX3, RAG1AP1, RAPGEF3*, and *TGFBI* expression level was measured by semiquantitative RT-PCR in MCF10A, SUM-44 cell (left), and WHSC1L1 knockdown SUM-44 cells (right).

indicate that *WHSC1L1* regulates the expression of these target genes. Similarly, examination of one downregulated gene, *TGFBI*, also confirmed reduced expression in SUM-44 compared with MCF10A cells, and the expression of this gene was increased in SUM-44 cells bearing the *WHSC1L1* shRNA constructs.

Because *WHSC1L1* regulates the expression of *IRX3* in SUM-44 cells and MCF10A-WHSC1L1 cells, we examined the genomic state of this potentially important target gene. Interestingly, we found that SUM-44 cells have an amplification at the IRX3 locus of chromosome 16q12. In addition, SUM-225 cells, which also have the 8p11-12 amplicon, have

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an overlapping region of copy number increase in chromosome 16 (Supplementary Fig. S5). We performed fluorescence *in situ* hybridization analysis using an IRX3-specific probe prepared from BAC clone RP11-1061L23 and confirmed the presence of an independent *IRX3* amplification in SUM-44 and SUM-225 cells (Supplementary Fig. S6). Thus, these findings indicate that in SUM-44 cells, overexpression of an amplified oncogene on chromosome 8p11-12 drives the expression of another amplified gene on a different chromosome. This genetic interaction explains the very high level of expression of *IRX3* in SUM-44 cells compared with MCF10A-WHSC1L1 cells.

With respect to downregulated genes, the finding that overexpression of *WHSC1L1* resulted in downregulation of the negative regulator of WNT signaling, SFRP1, in MCF10A cells is intriguing. We have recently shown that although the *SFRP1* gene is part of the 8p11-12 amplicon and is increased in copy number in SUM-44 cells, it is highly methylated and not expressed in these cells (27). Another downregulated gene is *TGFBI*, which encodes a secreted protein induced by transforming growth factor-β (Fig. 5). Recent studies with *TGFBI*-null mice showed that *TGFBI* loss promotes cell proliferation and predisposes mice to spontaneous tumor development (28). Thus, PWWP-protein WHSC1L1 may regulate a subset of genes involved in various functional pathways in breast cancer.

Discussion

The 8p11-12 amplicon has been the subject of a number of studies using high-resolution genomic analysis of copy number and gene expression in human breast cancer (7–11, 29). Our first studies in this area showed that the 8p11-12 amplicon has a complex genomic structure and the size of the amplicon is variable in three human breast cancer lines: SUM-44, SUM-52, and SUM-225 (9, 30). In that work, we showed that *FGFR1* was only one of several candidate oncogenes in the amplicon, and we provided evidence that *FGFR1* is not the driving oncogene in every breast cancer with the 8p11 amplicon (30). In addition, our correlative evidence suggested that other genes in the region, including *LSM-1, C8orf4* (*TC-1*), *RAB11FIP1, WHSC1L1*, and *ERLIN2* were good candidate oncogenes based on their overexpression associated with gene amplification (10). Our findings are consistent with those of other laboratories. Gelsi-Boyer et al. performed a comprehensive study combining genomic, expression, and chromosome break analyses of the 8p11-12 region in 37 human breast cancer lines and 134 primary breast cancer specimens (8). They identified four overlapping amplicon cores at 8p11-12 and 14 candidate oncogenes that are significantly overexpressed in relation to amplification. In subsequent work, Bernard-Pierrot et al. carried out BAC-array CGH on 21 human breast cancer lines and 152 ductal breast carcinomas and identified five genes (*LSM1, BAG4, DDHD2, PPAPDC1B*, and *WHSC1L1*) within the 8p11-12 amplified region as consistently overexpressed due to an increased gene copy number. Finally, Chin et al. published an analysis of the association of 8p11-12 gene amplification and disease-free survival and distant relapse in human breast cancer specimens and identified 23 genes from the 8p11-12 region as being correlated with progression, all of which have been named already (12). Thus, several groups have performed extensive analyses of the 8p11-12 genomic region in human breast cancer and there is substantial agreement on the candidate oncogenes present in this region. The candidate oncogenes consistently identified by all groups include *FGFR1, WHSC1L1, RAB11FIP1, LSM1, BAG4*, and *ERLIN2*.

There are now several studies in the literature reporting experimental analysis of the transforming function of the candidate oncogenes from the 8p11-12 region. We reported that *BAG4, LSM1*, and *C8orf4* (*TC-1*) are transforming when overexpressed in MCF10A cells (10, 13, 14). In the present report, we provide evidence that three additional genes, namely, *WHSC1L*1, *ERLIN2*, and *DDHD2*, are transforming based on their ability to induce growth factor–independent proliferation, anchorage-independent growth, and altered morphogenesis in Matrigel cultures. As reported in this article, we find *WHSC1L1* to be the most potently transforming of all the 8p11 oncogenes we have tested. Our results are consistent with those reported earlier by Bernard-Pierrot et al. who performed RNAi experiments to knock down the expression of candidate genes in two cell lines (CAMA-1 and ZR-75-1) with 8p11-12 amplification (31). Their results suggest that *PPAPDC1B* and *WHSC1L1* are two driving oncogenes from this amplicon. Knockdown of *WHSC1L1* was found to inhibit the proliferation of ZR-75-1 and CAMA-1 cells, but had no effect on MCF-7 cells that lack the 8p11-12 amplicon. Further, inhibition of *WHSC1L1* increased the number of apoptotic cells as assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay in ZR-75-1 and CAMA-1 cells (31). Recently, Zhang and colleagues applied a novel algorithm, termed TRIAGE (triangulating oncogenes through clinico-genomic intersects), to a collection of microarray expression profiles of primary human breast cancers in an effort to identify candidate genes in amplicons that could contribute to patient outcome (32). They identified *RAP11FIP1* and also identified *WHSC1L1* as being strongly associated with breast cancer subtype and outcome. They selected *RAP11FIP1* for further transfection and knockdown studies and found that *RAP11FIP1* is not sufficient to transform naive cells. However, overexpression of *RAP11FIP1* in breast cancer cell lines caused decreased growth factor dependence, increased survival under anoikis conditions, and increased motility and invasion. Furthermore, *RAP11FIP1* overexpression caused an epithelial-mesenchymal transition *in vitro* and increased tumor growth *in vivo* (32). In other studies, Luscher-Firzlaff et al. reported that *ASH2L* encodes the trithorax protein and cooperates with H-Ras to transform primary rat embryo fibroblasts (33).

Based on findings from several laboratories, *WHSC1L1* is clearly emerging as an important transforming gene within the 8p11-12 amplicon in breast and other cancer types. *WHSC1L1* is involved in a chromosomal translocation, t(8;11)(p11.2;p15), in acute myeloid leukemia (34). Recent published database of the Affymetrix 250K Sty array in a collection of 244 copy-number profiles of breast cancer samples showed that *WHSC1L1* amplification occurred in ~15% samples (Supplementary Fig. S7; ref. 35). With GISTIC (Genomic

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Identification of significant Targets in Cancer), a CGH analysis program, *WHSC1L1* was identified at the peak of amplification in lung and esophageal squamous cell carcinoma (36). siRNA-mediated knockdown of *WHSC1L1* resulted in a 50% reduction in the number of soft agar colonies in a lung cancer cell line (H1703) with *WHSC1L1* gene amplification and overexpression (37). Furthermore, deep sequencing of a primary human breast cancer identified a deletion within the *WHSC1L1* gene (38). In this study, we identified one primary breast cancer specimen with the 8p11-12 amplicon in which genomic analysis showed a loss of the COOH-terminal region of the WHSC1L1 long isoform but amplification of exons 1 to 10 that coded for the short isoform. At the protein level, WHSC1L1 exists as two isoforms in breast cancer cells with 8p11-12 amplification. Alternative splicing of WHSC1L1 in breast cancer cells can be regulated at different steps of the spliceosome assembly by different splicing factors, and by many different mechanisms that rely on *cis*-acting elements (39). Future investigations are required to more precisely address the role and mechanism of action of WHSC1L1 isoforms in breast cancer.

A finding of particular interest from our study is that *IRX3*, a member of the homeobox gene family, and *TBL1X* are target genes of WHSC1L1. Interestingly, *IRX3* is also amplified in SUM-44 cells and in SUM-225 cells. This is of interest because in embryonic stem cells *IRX3* and *TBL1X* are linked in a gene expression network that regulates WNT signaling (40). In addition, we have previously shown that in breast cancers with the 8p11-12 amplicon, SFRP1, a negative regulator of WNT signaling, is silenced by promoter methylation, despite being present on the 8p11 amplicon and increased in copy number (27). These results suggest that overexpression of *WHSC1L1* and the silencing of *SFRP1* result in potent activation of a transcriptional network linked to WNT signaling and expression of stem cell phenotypes.

FGFR1 has long been considered an important candidate breast cancer oncogene from the 8p11-12 region. However, we have consistently failed to find evidence for a direct role of *FGFR1* in transformation in mammary epithelial cells. Recently, Turner et al. provided evidence for a functional role of *FGFR1* in 8p11-12–amplified breast cancers (41). Many of the results reported by Turner et al. are consistent with our previously published negative results. However, they did show that overexpression of *FGFR1* increases the sensitivity and responsiveness of cells to fibroblast growth factor ligands, which influences the response of the cells to 4-OH tamoxifen. These results suggest that *FGFR1* overexpression can play a role in endocrine therapy resistance, which may explain the consistent presence of *FGFR1* in the amplicon.

In the past, focal amplicons found in cancer specimens were considered to harbor a single driving oncogene that was responsible for the maintenance of the amplicon in the tumor, with the *ERBB2* oncogene in the 17q12 amplicon being a prime example. In some cases, amplicons have been thought to harbor more than one driver oncogene that act independently, such as the *CCND1* and *EMS1* genes present in the 11q12 amplicon. It is possible that the 8p11-p12 amplicon does not follow such a simple paradigm. Indeed, we have proposed that the 8p11-12 amplicon, rather than having a single driving oncogene, can act as an oncogenic unit consisting of multiple interacting transforming genes. This hypothesis is based on the consistent coexpression of several candidate oncogenes with transforming function when the amplicon is present in breast cancers. Within this oncogenic unit are two genes that can regulate the histone code (*WHSC1L1, ASH2L*), two genes that regulate RNA metabolism (*LSM1, BRF2*), a receptor tyrosine kinase (*FGFR1*), a gene that regulates the endoplasmic reticulum stress pathway (*ERLIN2*), and a gene that influences receptor trafficking (*RAB11FIP1*). Although it remains possible that each of these genes act independently, and function as driver oncogenes in different tumors with the same amplicon, the possibility that the genes cooperate in mediating neoplastic transformation must now be considered.

Despite the significant and exciting progress in the understanding of the 8p11-12 genomic amplification in breast cancer, we are still in the early stages of functional studies for each 8p11-12 candidate oncogene and its role in breast cancer development. Understanding how the genes in this region influence fundamental cancer processes such as progression, metastasis, and drug resistance will provide potential new avenues for therapeutic development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

Identification and functional analysis of 9p24 amplified genes in human breast cancer

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Previously, our group identified a novel amplicon at chromosome 9p24 in human esophageal and breast cancers, and cloned the novel gene, *GASC1* (*gene amplified in squamous cell carcinoma 1*, also known as *JMJD2C*/*KDM4C*), from this amplicon. GASC1 is a histone demethylase involved in the deregulation of histone methylation in cancer cells. In the current study, we aimed to comprehensively characterize the genes in the 9p24 amplicon in human breast cancer. We performed extensive genomic analyses on a panel of cancer cell lines and narrowed the shortest region of overlap to approximately 2 Mb. Based on statistical analysis of copy number increase and overexpression, the 9p24 amplicon contains six candidate oncogenes. Among these, four genes (*GASC1 UHRF2*, *KIAA1432* and *C9orf123*) are overexpressed only in the context of gene amplification while two genes (*ERMP1* and *IL33*) are overexpressed independent of the copy number increase. We then focused our studies on the *UHRF2* gene, which has a potential involvement in both DNA methylation and histone modification. Knocking down UHRF2 expression inhibited the growth of breast cancer cells specifically with 9p24 amplification. Conversely, ectopic overexpression of UHRF2 in non-tumorigenic MCF10A cells promoted cell proliferation. Furthermore, we demonstrated that UHRF2 has the ability to suppress the expression of key cell-cycle inhibitors, such as $p16^{INK4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Taken together, our studies support the notion that the 9p24 amplicon contains multiple oncogenes that may integrate genetic and epigenetic codes and have important roles in human tumorigenesis.

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Keywords: chromosome 9p24; GASC1; UHRF2; gene amplification

Introduction

Cancer results from an accumulation of genetic and epigenetic aberrations. Genetic aberrations include chromosome number changes and translocations, gene amplifications, mutations and deletions (Vogelstein and Kinzler, 2004). Epigenetic abnormalities involve both altered patterns of histone modifications as well as losses or gains of specific DNA methylation (Esteller, 2007; Jones and Baylin, 2007). Genetic and epigenetic alterations in cancer cells interact directly and indirectly. For example, a genetic alteration in the gene encoding an 'epigenetic regulator' can lead to changes within the histone code and DNA methylation, which are subsequently involved in tumorigenesis in multiple tumor types. Identification and characterization of genetic and epigenetic aberrations, as well as their interconnections, will provide important insights into the pathogenesis of cancer.

Gene amplification, which can affect gene expression by increasing gene dosage, is a well-known oncogene-activating mechanism (Albertson *et al*., 2003; Albertson, 2006). Canonical oncogenes, such as *ERBB2*, *CCND1* and *MYC*, have previously been identified as amplification targets linked to the development, progression or metastasis of human cancers, including breast, prostate, lung and other cancers (Albertson *et al*., 2003; Vogelstein and Kinzler, 2004). Previously, mapping of the 9p24 amplicon in esophageal cancer cell lines led us to the positional cloning of the *gene amplified in squamous cell carcinoma 1* (*GASC1* also known as *JMJD2C*/*KDM4C*) gene. More recently, we identified *GASC1* as one of the amplified genes at the 9p24 region in breast cancer, particularly in basal-like subtypes. Our *in vitro* assays demonstrated that GASC1 can induce transformed phenotypes when overexpressed in immortalized, non-transformed mammary epithelial MCF10A cells (Liu *et al*., 2009).

In the past, focal amplicons found in cancer specimens were considered to harbor a single driving oncogene, such as the *ERBB2* oncogene in the 17q12 amplicon (Fukushige *et al*., 1986). However, recent extensive genomic analysis and functional studies provide evidence to suggest that common amplicons in cancer cells contain multiple oncogenes that can act independently or cooperatively in mediating neoplastic

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transformation. For example, we and several other laboratories have demonstrated that the 8p11-12 amplicon harbors several driving oncogenes with transforming function when the amplicon is present in breast cancers, particularly in luminal subtypes (Yang *et al*., 2004, 2006, 2010; Garcia *et al*., 2005; Gelsi-Boyer *et al*., 2005; Pole *et al*., 2006). In the current study, we aimed to comprehensively characterize the 9p24-amplified genes for potential roles in human breast cancer. Results obtained from our studies support the notion that the 9p24 amplicon contains multiple candidate genes, including *GASC1* and *ubiquitin-like with* plant homeodomain *and ring finger domains 2* (*UHRF2*), that may integrate genetic and epigenetic codes and thus have important roles in human tumorigenesis.

Results

High-resolution array comparative genomic hybridization (CGH) narrowed a focal chromosomal amplification at 9p24 in cancer

Previously, our group identified an amplicon at the 9p24 chromosomal region in human esophageal cancer and identified the novel oncogene *GASC1* from this amplicon (Yang *et al*., 2000). Later studies showed a gain/amplification of the *GASC1* region in 7 of 50 breast cancer cell lines, including HCC1954, Colo824, SUM-149, HCC70, HCC38, HCC2157 and MDA-MB-436 (Neve *et al*., 2006; Liu *et al*., 2009). To further demonstrate that the 9p24 region is amplified in various tumor specimens, we searched the recently published array CGH database in a collection of 3131 copy-number profiles across multiple cancer types (Beroukhim *et al*., 2010). Copy number increases at the 9p24 region mostly occurred in small-cell lung, breast and esophageal squamous cancers. In 243 breast cancer samples, approximately 15% contained 9p24 gains, and 4.53% of cases had high-level amplification based on Genomic Identification of significant Targets in Cancer (GISTIC) analysis (Supplementary Figure S1) (Beroukhim *et al*., 2010).

The frequent occurrence of the 9p24 amplicon in various human tumors underscores its importance in tumorigenesis. For the purpose of further characterization of the 9p24 amplicon, we carried out high-resolution array CGH (Agilent 244 K chip, Santa Clara, CA, USA) analysis of four cancer cell lines, including three breast cancer cell lines, Colo824, HCC1954 and HCC70, and one esophageal cancer cell line, KYSE150 (Shimada *et al*., 1992; Yang *et al*., 2000; Liu *et al*., 2009). In our array CGH study, high-level copy number gain (amplification) was defined by a log2 ratio $\geqslant 1$ and low-level copy number gain by a log2 ratio between 0.3 and 1. The 244 K array CGH confirmed our previous findings that all four cell lines contain 9p24 amplification, and provided the amplicon boundaries at high resolution (Figure 1a and Supplementary Table 1A) (Yang *et al*., 2000; Liu *et al*., 2009). Of note, we found that the centromeric boundary of the 9p24 amplicon in KYSE150 cells is located within the coding region of a *protein tyrosine phosphatase, receptor type, D* (*PTPRD*) gene, resulting in amplification of the C-terminal region, but deletion of the N-terminal region of the gene (Supplementary Table 1A and Supplementary Figure S2). The *PTPRD* gene spans approximately 2.3 Mb, from 8.30 to 10.60 Mb, and is represented by 201 probes in Agilent 244 k CGH arrays (Supplementary Table 1A). We validated our CGH results by real-time PCR using primers specific for the PTPRD's intron 7–exon 8 and intron 8–exon 9 sequences (Supplementary Figure S3). As shown in supplementary Figure S4, compared with the control cells that do not have 9p24 amplification, KYSE150 cells had an elevated copy number of *PTPRD* intron 8–exon 9, whereas the copy number of *PTPRD* intron 7–exon 8 in KYSE150 was lower than that of the control, implying that the amplification/deletion break point is located in this region. Interestingly, recent published genomic data indicated that the centromeric boundaries of the 9p24 gain/amplification region in basal-like primary breast tumor ($\sim$8.28 Mb), brain metastasis ($\sim$8.88 Mb) and xenograft samples ($\sim$7.78 Mb) are also adjacent to or located at PTPRD genome region (Supplementary Figure S5) (Ding *et al*., 2010).

In order to define the minimal common region of gain/amplification, we also analyzed our previous 44 k array CGH data obtained from the SUM-149 breast cancer cell line (Supplementary Table 1B) (Liu *et al*., 2009). Compared with HCC1954 and Colo824 cells, SUM-149 cells exhibited low-level copy number gain ($0.3 \leqslant \log_2$ ratio < 1.0) at the 9p24 region. In agreement with this data, we demonstrated in our previous fluorescence *in situ* hybridization study that 10–14 copies of the GASC1 BAC probe were observed in the interphase nuclei of HCC1954 cells, while only 5–7 copies of the probe were observed in the SUM-149 cells (Liu *et al*., 2009). Furthermore, our array CGH revealed the distal boundary of 9p24 gain in SUM-149 cells maps to 5.53–5.76-Mb site (Supplementary Table 1B). Combination of our array CGH data with that published by other groups allowed us to narrow down the commonly gained/amplified 9p24 region to approximately 2 Mb, from 5.53 to 7.78Mb (Figure 1b, Supplementary Table 1 and Supplementary Figure S5).

The 9p24 amplicon contains multiple candidate oncogenes

Accumulated evidence suggests that the common amplicons occurring in breast and other cancers contain multiple oncogenes that could have a role in cancer initiation and progression. As mentioned above, the shortest region of overlap of the 9p24 amplicon spans approximately 2 Mb, and excluding pseudogenes, contains 11 genes (Figure 1b and Table 1). We carried out real-time RT–PCR to measure the expression level of these genes in a panel of cancer cell lines with or without 9p24 amplification (Figure 2a and Table 1). We then used Kendall's tau, a measure of association, to assess if the association between copy number and expression for each gene is statistically significant. Using $P = 0.01$ as a cut-off for a statistically significant association, we confirmed that *GASC1* is a target of the amplicon.

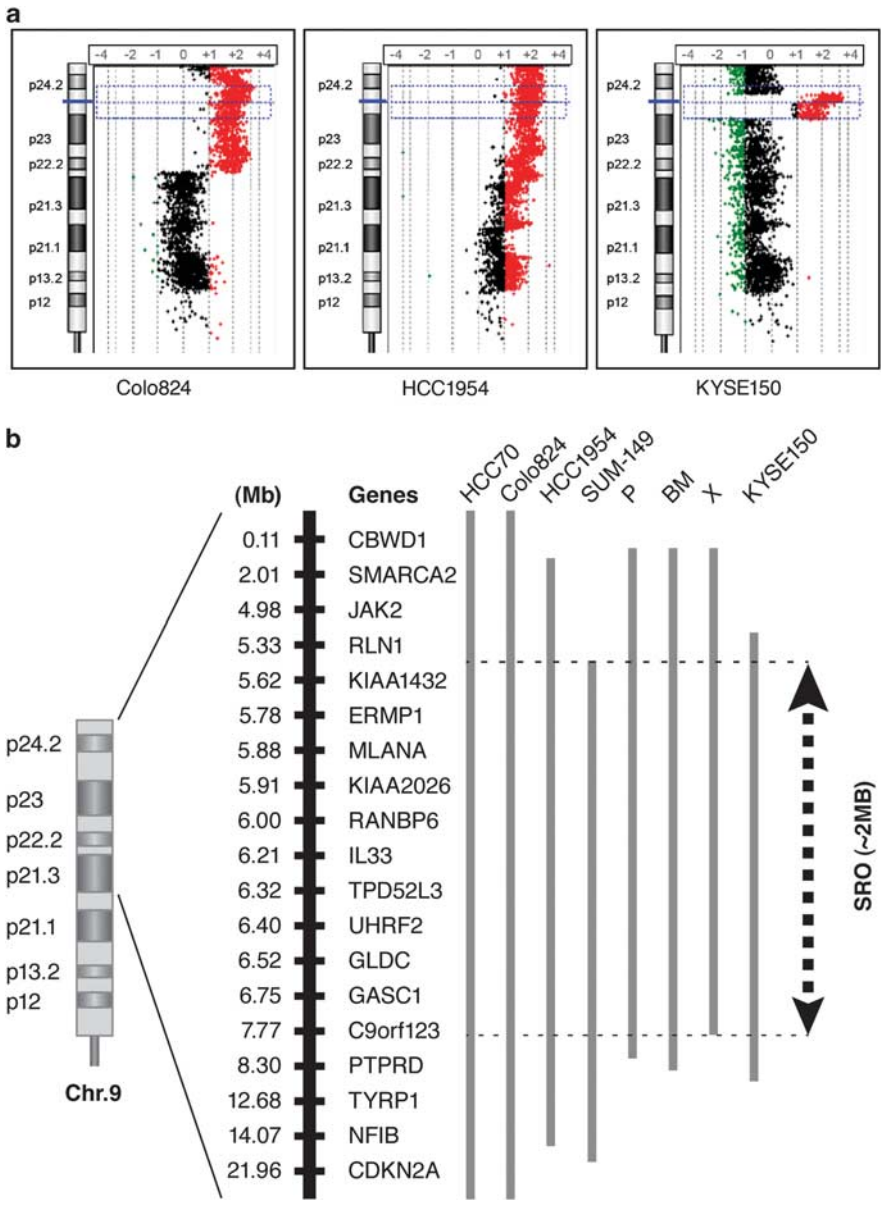

Figure 1 Genomic analysis of the 9p24 region in human cancer cell lines. (**a**) Genome view of chromosome 9p analyzed on the Agilent oligonucleotide array (Agilent Technology) in Colo824, HCC1954 and KYSE150 cells. (**b**) Schematic representation of the 9p24-amplified region in four breast cancer cell lines (HCC70, HCC1954, Colo824 and SUM-149), one esophageal cancer cell line (KYSE150) and the recently published genomic data of basal-like primary breast tumor (P), brain metastasis (BM) and xenograft (X) samples (Ding *et al*., 2010). Localization of the 9p21-24 genes is shown to the right of the chromosome 9 ideogram. The lines at far right represent the amplified region of each sample based on our array CGH data and Ding *et al*.'s published data. SRO, shortest region of overlap.

In addition, we identified three new potential targets, *UHRF2, KIAA1432* and *C9orf123* (Table 1). In contrast, the elevated expression of two genes, *ERMP1* and *IL33*, is independent of their copy number status in human cancer cells (Figure 2a and Table 1). However, *ERMP1* and *IL23* are also potential oncogene candidates because of their frequent overexpression. We measured protein levels of GASC1 and UHRF2 by western blot analysis in a panel of breast cancer cell lines. These experiments demonstrate that Colo824, HCC1954, HCC70 and SUM-149 cells expressed higher levels of GASC1 and UHRF2 than breast cancer cell lines without gene amplification (Figure 2b). Thus, we propose that the 9p24 amplicon contains five candidate oncogenes in addition to *GASC1*, including *UHRF2, KIAA1432, C9orf123, ERMP1 and IL33*, all of which could have a role in tumorigenesis.

UHRF2 gene amplification and overexpression promotes cell proliferation

The UHRF2 is a nuclear protein involved in cell-cycle regulation (Mori *et al*., 2002; Bronner *et al*., 2007). We therefore sought to examine the biological effect of

Table 1 Statistical analysis of association between copy number and expression of genes within the 9p24 amplicon

Gene	*Description*	*Kendall's tau*	P-*value*
KIAA1432	KIAA1432	0.78	<0.01
ERMP1	Endoplasmic reticulum metallopeptidase 1	0.67	0.02
MLANA	Melan-A	0.44	0.12
KIAA2026	KIAA2026	0.61	0.03
RANBP6	RAN-binding protein 6	0.61	0.03
IL33	Interleukin 33	0.33	0.25
TPD52L3	Tumor protein D52-like 3	0.56	0.05
UHRF2	Ubiquitin-like with PHD and ring finger domains 2	0.78	<0.01
GLDC	Glycine dehydrogenase (decarboxylating)	0.28	0.35
GASC1	Lysine (K)-specific demethylase 4C	0.78	<0.01
C9orf123	Chromosome 9 open-reading frame 123	0.83	<0.01

Abbreviation: PHD, plant homeodomain.

UHRF2 knockdown on the proliferation of breast cancer cells with 9p24 amplification. To perform knockdown experiments, we obtained two pGIPZ-UHRF2 short hair pin RNA (shRNA) expression constructs from OpenBiosystems (http://www.openbiosystems.com/). In this pGIPZ vector, TurboGFP and shRNA are part of a single transcript allowing the visual marking of the shRNA-expressing cells. HCC1954 cells were transduced with the pGIPZ-UHRF2 shRNA, and a non-silencing shRNA lentivirus at a similar titer was used in parallel as the negative control. We selected cells with puromycin 48 h after infection. Pooled cell clones were monitored for TurboGFP expression by fluorescence microscopy. UHRF2 expression levels were measured by real-time RT–PCR, which revealed that the UHRF2-shRNA cell clones showed downregulation of UHRF2 expression to 30–45% of the level seen in the non-silencing shRNA-infected cell clones (Figure 3a). UHRF2 shRNA#2 more effectively knocked down expression than shRNA#1, and thus we used it in five cell lines: HCC1954 and HCC70 with UHRF2 gene amplification, SUM-52 and SUM-102 without the amplification as well as the non-tumorigenic MCF10A cells, which also lack the amplification. Subsequently, the effect of decreased UHRF2 expression on cell proliferation was examined. Knocking down UHRF2 inhibited the growth of HCC1954 and HCC70 cells by approximate 50%, but had only a minor effect on SUM-52, SUM-102 and MCF10A cells ($P<0.01$) (Figures 3b and c). The inhibition of HCC1954 cell growth by knockdown of UHRF2 was reproduced with the UHRF2 shRNA#1 (data no shown). Thus, UHRF2 knockdown has a more profound growth inhibition effect on cells with *UHRF2* gene amplification than in cells without the amplification.

The effects of UHRF2 on cell growth and transformation were further examined by ectopic overexpression in the non-tumorigenic MCF10A cells. Lentivirus carrying either the control vector or a UHRF2 expres-

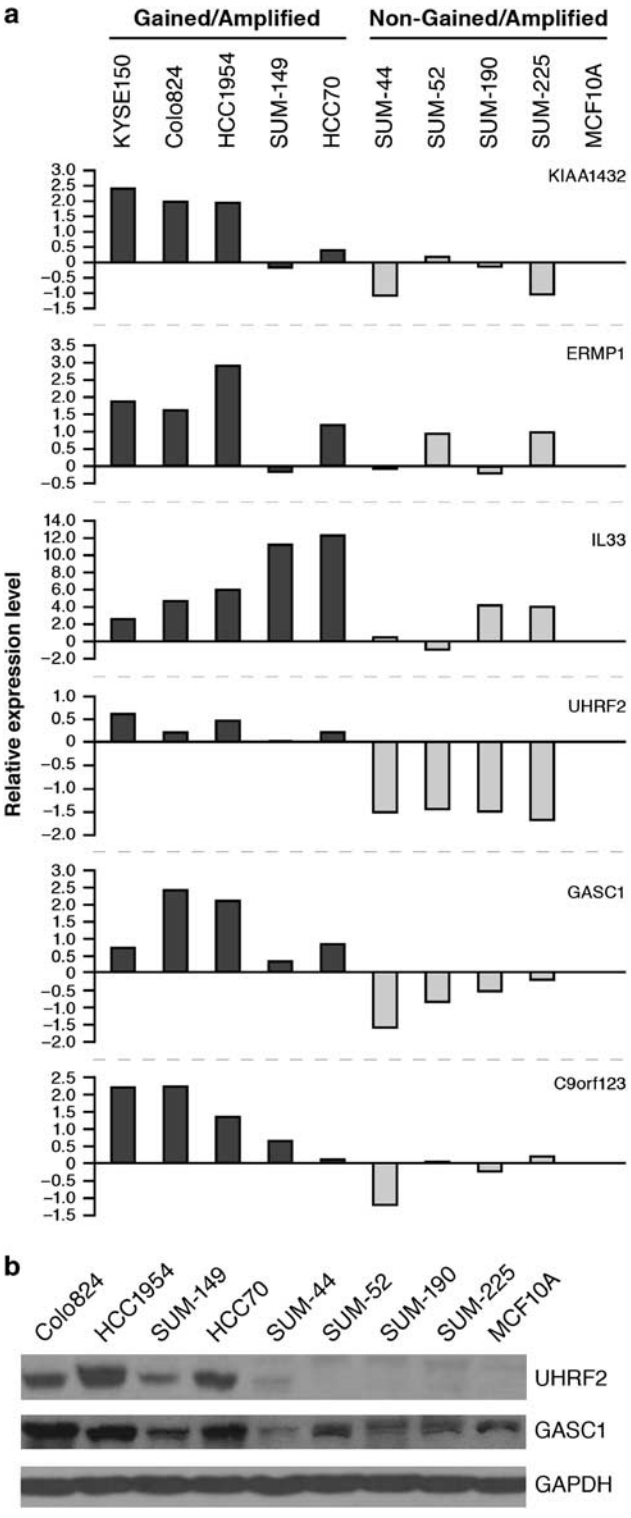

Figure 2 (**a**) Expression level of six genes in the 9p24 amplicon. Gene expression was examined in cancer cells with 9p24 gain/amplification (KYSE150, Colo824, HCC1954, SUM-149 and HCC70) or without the gain/amplification (SUM-44, SUM-52, SUM-190 and SUM-225). mRNA expression levels in the MCF10A cells, an immortalized but non-tumorigenic breast epithelial cell line without 9p24 gain/amplification, were arbitrarily set as 0. Relative expression levels were shown as $\log_2$ values. (**b**) UHRF2 and GASC1 protein levels were analyzed by western blot in eight breast cancer cell lines with or without 9p24 amplification, as well as in MCF10A control cells.

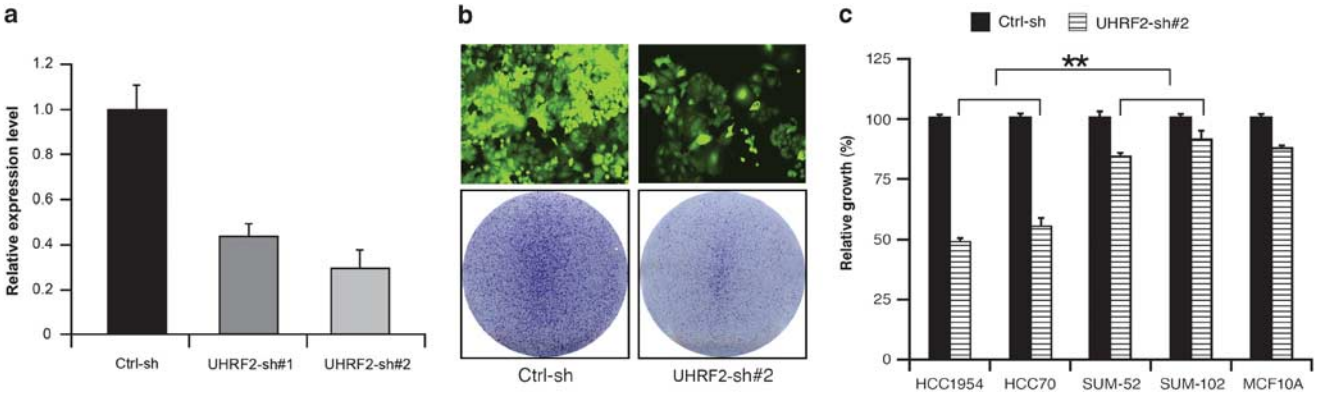

Figure 3 Effect of UHRF2 knockdown on cancer cell growth. (**a**) Knockdown of UHRF2 mRNA in HCC1954 cells with two different shRNAs was confirmed by real-time RT–PCR. The real-time RT–PCR data were normalized with a GAPDH control and is shown as the mean ± s.d. of triplicate determinations from two independent experiments. The baseline for the cells infected with control shRNA was arbitrarily set as 1. (**b**) Top panel shows TurboGFP images of HCC1954 cells after viral infection with control shRNA and UHRF2 shRNA#2. After seeding the same number of HCC1954 cells with or without UHRF2 knockdown, cells were stained with crystal violet at day 7 (bottom panel). (**c**) Relative cell growth after knocking down UHRF2 in five cell lines: HCC1954 and HCC70 with 9p24 amplification, SUM-52 and SUM-102 without the amplification as well as non-tumorigenic MCF10A cells. The same number of cells were seeded and allowed to grow for 7 days after attachment. Relative growth is shown as the mean ± s.d. of triplicate determinations (**$P<0.01$).

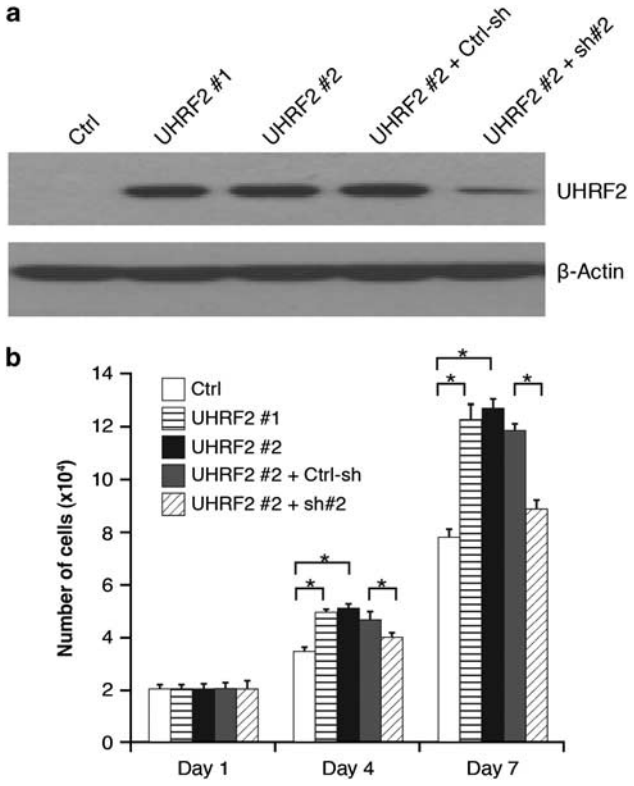

Figure 4 (**a**) Stable overexpression of UHRF2 in MCF10A cells with the pLenti6/V5-UHRF2 construct (MCF10A-UHRF2). Overexpression of UHRF2 protein in two cell clones (UHRF2#1 and UHRF2#2), and knockdown of UHRF2 in clone #2 cells were confirmed by western blot. (**b**) Ectopic overexpression of UHRF2 confers a growth advantage to MCF10A cells, which can be reversed by UHRF2 shRNA (*$P<0.05$).

sion construct was transduced into MCF10A cells and stable, independent clones were isolated. Western blot confirmed the overexpression of UHRF2 protein in these clones (Figure 4a). Compared with the control, MCF10A cells overexpressing UHRF2 grew more rapidly than control cells ($P<0.05$), and this growth advantage was reversed by UHRF2 shRNA (Figure 4b). However, MCF10A-UHRF2 cells retained the parental cells' characteristics of anchorage- and growth factor-dependent growth (data no shown). Taken together with the UHRF2 knockdown results, our data indicate that UHRF2 has a role in cell proliferation in breast cancer cells with the 9p24 amplification.

UHRF2 mediates tumor suppressor gene inactivation in breast cancer

UHRF family members, including UHRF1 and UHRF2, are multi-domain proteins that participate in methylation-dependent transcriptional regulation (Bronner *et al*., 2007; Unoki *et al*., 2009; Rottach *et al*., 2010). Recent studies revealed that UHRF1 functions as a transcriptional co-repressor and participates in transcriptional regulation of $p21^{Waf1/Cip1}$ by recruitment of DNA and histone methyltransferases (Kim *et al*., 2009; Unoki *et al*., 2009). Knocking down UHRF2 affects the expression level of $p21^{Waf1/Cip1}$ in lung cancer cells (He *et al*., 2009). To determine whether UHRF2 affects $p21^{Waf1/Cip1}$ expression in human breast cancer cells, we examined $p21^{Waf1/Cip1}$ mRNA and protein levels after UHRF2 knockdown in HCC1954 cells. As shown in Figures 5a and b, UHRF2 knockdown resulted in increased expression of $p21^{Waf1/Cip1}$ at both the mRNA and protein levels. $p21^{Waf1/Cip1}$ is a well-known target gene of p53-mediated transcriptional regulation (el-Deiry *et al*., 1993). However, HCC1954 cells harbor an inactivating mutation (Tyr163→Cys163) in the p53 gene (Sjoblom *et al*., 2006). As expected, our western blot demonstrated that the expression level of p53 was not affected by UHRF2 knockdown in HCC1954 cells (Supplementary Figure S6), indicating

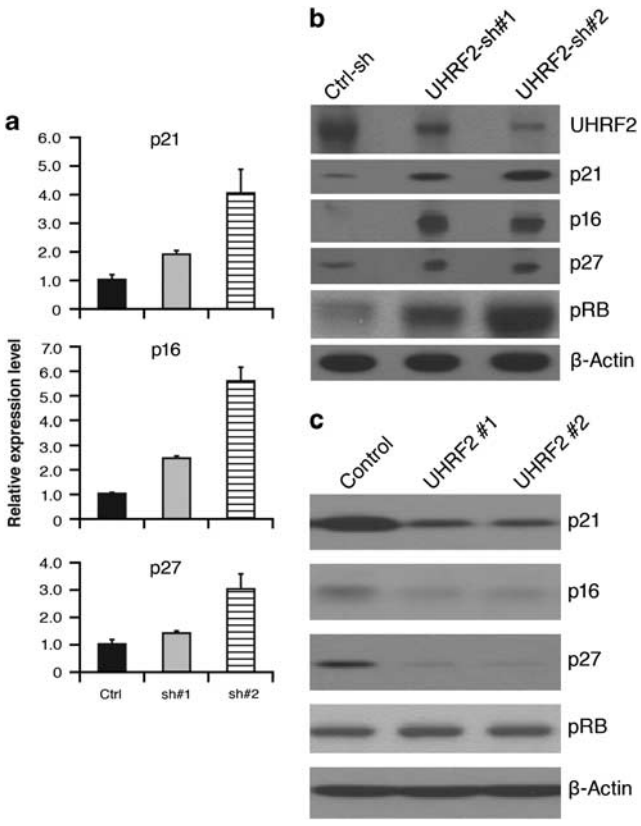

Figure 5 UHRF2 influences expression of $p16^{INK4a}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and pRB. (**a**) mRNA levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$ and $p27^{Kip1}$ were examined by real-time RT–PCR after knocking down UHRF2 in HCC1954 cells. The baseline for the cells infected with control shRNA was arbitrarily set as 1. (**b**) Protein levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$, $p27^{Kip1}$ and pRB in HCC1954 cells stably expressing control shRNA, UHRF2 shRNA#1 or shRNA#2 were analyzed by western blot. The migration control for the hypophosporylated (p) form of RB protein is shown in Supplentmentary Figure S7. (**c**) Overexpression of UHRF2 in MCF10A cells results in reduced protein levels of $p16^{INK4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, but not of pRB as determined by western blot.

that the increased expression of $p21^{Waf1/Cip1}$ in UHRF2 knockdown cells was achieved through a p53-independent pathway.

To determine whether UHRF2 affects the expression of other cell-cycle inhibitors and/or classical tumor suppressors, we examined the expression levels of $p16^{INK4a}$, $p27^{KIP1}$ and pRB after UHRF2 knockdown in HCC1954 cells. As shown in Figures 5a and b, when the expression of UHRF2 was decreased, there was a concomitant increase in the expression levels of $p16^{INK4a}$, $p27^{Kip1}$ and pRB. We further examined the expression levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$, $p27^{KIP1}$ and pRB in MCF10A-UHRF2 clones. As shown in Figure 5c, overexpression of UHRF2 in MCF10A cells led to reduced expression of $p21^{Waf1/Cip1}$, $p16^{INK4a}$ and $p27^{KIP1}$. However, the level of pRB protein was not affected in MCF10A-UHRF2 cells (Figure 5c). These data suggest that amplification and overexpression of UHRF2 suppresses the expression of tumor suppressor genes in cancer cells, which may explain its growth-promoting capability.

Discussion

Recent studies have demonstrated that regions of amplification such as 8p11-12, 11q13, 17q22-23 and 20q12-13 can be complex and frequently contain multiple genes that can work individually and/or in combination to influence the transformed phenotype in human cancer cells (Santarius *et al*., 2010). Previous studies revealed the existence of 9p24 amplification in various tumor types (Italiano *et al*., 2006; Han *et al*., 2008; Vinatzer *et al*., 2008; Liu *et al*., 2009; Natrajan *et al*., 2009; Northcott *et al*., 2009). In human breast cancer, it had been determined that 9p24 amplification more frequently occurs in the basal-like subtype, which is clinically characterized as highly aggressive and is usually associated with a poor prognosis (Han *et al*., 2008; Liu *et al*., 2009). In the present studies, we extended our previous work on the 9p24 amplicon and examined 9p24 genes in a thorough and systemic way (Yang *et al*., 2000; Liu *et al*., 2009). Our array CGH analyses at a higher resolution enabled us to narrow the amplicon to approximately 2 Mb, which contains 11 genes. We identified four genes, *GASC1*, *UHRF2*, *KIAA1432* and *C9orf123*, that were overexpressed in association with copy number increase at the $P<0.01$ level (see Table 1). In addition, two genes, *ERMP1* and *IL33*, were found to be overexpressed in breast cancer cells both with and without copy number increases. Thus, like other focal amplicons found in cancer, the 9p24 amplicon also contains multiple candidate oncogenes.

Based on the known biological functions of the six candidate oncogenes, GASC1 and UHRF2 appear to have a role in the regulation of gene expression by acting as epigenetic regulators. The *KIAA1432* gene encodes a binding partner of a gap junction protein (GJA1, also known CX43). The association with KIAA1423 protein is important for GJA1 to have a role as a gap junctional channel (Akiyama *et al*., 2005). The *C9orf123* gene encodes a putative transmembrane protein, and its biological function is currently unknown. The ERMP1 is an endoplasmic reticulum-bound peptidase and required for normal ovarian histogenesis (Garcia-Rudaz *et al*., 2007). As a cytokine, interleukin-33 may function as an alarm in that it is released upon endothelial or epithelial cell damage (Kurowska-Stolarska *et al*., 2011). By contrast, the *PTPRD* gene, likely inactivated by partial deletion and/or rearrangement, is increasingly thought to be a tumor suppressor gene. Recent studies indicate that inactivation of *PTPRD* by gene deletion or mutation contributes to the pathogenesis of a wide range of human cancers, including colon, lung, glioblastoma and melanoma (Ostman *et al*., 2006; Solomon *et al*., 2008; Veeriah *et al*., 2009; Kohno *et al*., 2010; Giefing *et al*., 2011). In breast cancer cells, it has been reported that *PTPRD* can also be inactivated at the transcriptional level by DNA hypermethylation (Chan *et al*., 2008). Future investigations are required to more precisely address the role of each candidate gene in cancer development.

Using esophageal cancer lines, we originally identified and cloned the *GASC1* gene from an amplified region at 9p24 (Yang *et al*., 2000). Based on the presence of a

bipartite nuclear location sequence and two plant homeodomain fingers, we had initially predicted a role in transcriptional regulation for GASC1 (Yang *et al*., 2000). Indeed, subsequent studies identified GASC1 as a member of the JMJD2 (jumonji domain containing 2), subfamily of jumonji genes that alter chromatin architecture through histone lysine demethylase activity (Katoh, 2004; Cloos *et al*., 2006; Tsukada *et al*., 2006; Whetstine *et al*., 2006). Specifically, GASC1 can activate transcription by removing the repressive tri- and dimethylated histone H3 lysine 9 marks (H3K9me3/me2) at specific genomic loci (Chen *et al*., 2006; Cloos *et al*., 2006; Whetstine *et al*., 2006; Klose and Zhang, 2007; Shi and Whetstine, 2007). We and several other laboratories showed that GASC1 regulates the expression of several classical oncogenes, including *MYC*, *NOTCH1*, *SOX2* and *MDM2* in normal and cancer cells (Loh *et al*., 2007; Ishimura *et al*., 2009; Liu *et al*., 2009; Wang *et al*., 2010). Importantly, stable overexpression of GASC1 in the non-tumorigenic breast cell line MCF10A induces transformed phenotypes, whereas knockdown in tumor cells inhibits proliferation, consistent with *GASC1* as a member of a new class of oncogenes that are involved in the deregulation of histone methylation in cancer cells.

A finding of particular interest from our current study is that the newly identified candidate *UHRF2* also has a potential involvement in methylation-dependent transcriptional regulation. UHRF2, and its close homolog UHRF1, contain similar functional domains. These domains include an ubiquitin-like domain, a plant homeodomain domain, a tudor domain, a SRA domain and a RING domain (Hopfner *et al*., 2000; Mori *et al*., 2002; Bronner *et al*., 2007; Rottach *et al*., 2010). Recent studies demonstrated that UHRF1 has the ability to bind hemi-methylated DNA and methylated H3K9 through its SRA domain and tudor domain, respectively (Bronner *et al*., 2007; Qian *et al*., 2008; Rottach *et al*., 2010). UHRF1 can repress transcription of tumor suppressor genes including $p16^{INK4a}$ and $p21^{Waf1/Cip1}$ via recruitment of DNA methyltransferases (DNMT1 and DNMT3A/B), H3K9 methyltransferases (G9a), and HDAC1, interconnecting DNA methylation and histone modification pathways (Kim *et al*., 2009; Unoki *et al*., 2009). Interestingly, an unbiased proteomic screen for binding proteins to modified lysines on histone H3 also determined that UHRF2 interacts with dimethylated H3K9 peptide (Chan *et al*., 2009). In the current study, we demonstrated that UHRF2 has the ability to repress transcription of key cell-cycle inhibitors and tumor suppressors, including $p16^{INK4a}$, p21Cip1 and $p27^{Kip1}$. Thus, we speculate that UHRF2 may have an oncogenic role by mediating tumor suppressor gene inactivation via both DNA methylation and histone modification pathways.

During the review of this manuscript, Rui *et al*. (2010) published their studies on 9p24 amplification in primary mediastinal B-cell lymphoma (PMBL) and Hodgkin lymphoma. They identified a 9p24 amplicon, which largely overlaps with the 9p24 amplicon described in this report. They revealed that within an approximately 3.5-Mb minimal common region of copy number gain, 10 genes (*JAK2*, *C9orf46*, *CD274*, *PDCD1LG2*, *KIAA1432*, *KIAA2026*, *RANBP6*, *UHRF2*, *GLDC* and *GASC1*) were upregulated in expression in association with gene amplification. Further, they demonstrated that two genes, *JAK2* and *GASC1*, cooperate to modify the epigenome of 9p24-amplified lymphomas, thereby promoting proliferation and survival. Their data and our studies share in common the observation that three genes, *GASC1, UHRF2* and *KIAA1432*, are upregulated via gene copy number gains, and that *GASC1* is an important gene for the proliferation and survival of cancer cells with 9p24 amplification (Liu *et al*., 2009; Rui *et al*., 2010). Notably, our array CGH and previous fluorescence *in situ* hybridization analysis found that JAK2 is not gained/amplified in KYSE150 esophageal cancer cells or SUM-149 breast cancer cells (Yang *et al*., 2000; Liu *et al*., 2009). Our new finding suggests that the epigenetic regulator UHRF2 likely contributes to cell proliferation in a subset of breast cancer with 9p24 amplification. It will be important to further investigate whether the two 9p24 co-amplified genes, *GASC1* and *UHRF2*, promote tumor growth co-operatively or independently.

Materials and methods

Cell culture

The culture of cancer cells KYSE150, Colo824, HCC70, HCC1954, SUM-44, SUM-52, SUM-149, SUM-190, SUM-225, and the immortalized non-tumorigenic MCF10A cells is described in the Supplementary Materials and methods.

Array CGH

Genomic array CGH experiments were done using the Agilent 244 K human genome CGH microarray chip (Agilent Technologies, Palo Alto, CA, USA) as described previously (Yang *et al*., 2006). Briefly, for each array, female DNA (Promega, Madison, WI, USA) was used as a reference sample and labeled with Cy-3. The samples of interest were each labeled with Cy-5. Agilent's CGH Analytics software was used to calculate various measurement parameters, including log2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe. Array data have been posted at the NCBI GEO database (GEO accession: GSE28989, GSM718287, GSM718288, GSM718289, GSM718290).

Real-time RT–PCR

Total RNAs were prepared from cells by using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and were converted into complementary DNAs with the qScript complementary DNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Primers were purchased from Invitrogen (Carlsbad, CA, USA). Real-time RT–PCR was performed using the iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

Lentivirus-mediated UHRF2 shRNA knockdown or overexpression

UHRF2 knockdown was achieved by using the Expression Arrest GIPZ lentiviral shRNAmir system (OpenBiosystems). The lentiviral expression construct expressing the *UHRF2* gene (pLenti-UHRF2-V5) was established as described previously (Yang *et al*., 2006). Lentivirus was produced by transfecting

293FT cells with the combination of the lentiviral expression plasmid DNA and viral packaging mix (OpenBiosystems). Cells were infected with the virus by incubating with the mixture of growth medium and virus-containing supernatant (1:1 ratio), supplemented with polybrene at a final concentration of 5 μg/ml. An equal volume of fresh growth medium was added after 24 h and selection of stable cells was started after 48 h.

Examination of cell growth

Cell growth was assessed by using a Coulter counter or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). For MTT assay, cells were seeded in six-well plates at a density of 2×10^4 cells per well and allowed to attach overnight. At designated time points, thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St Louis, MO, USA) was added to each well of cells (final 0.5 mg/ml) and incubated for 3–5 h at 37 °C. After removing the growth medium, dimethyl sulfoxide was added to solubilize the blue MTT-formazan product, and the samples were incubated for a further 30 min at room temperature. Absorbance of the solution was read at a test wavelength of 570 nm against a reference wavelength of 650 nm.

Immnuoblotting and antibodies

Whole cell lysates were prepared by scraping cells from the dishes into cold radioimmuno precipitation assay lysis buffer and sonicating for 10 s. After centrifugation at high speed in the cold, protein content was estimated with the Bradford method. A total of 20–100 μg of total cell lysate was resolved by SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Antibodies used in the study were as follows: anti-UHRF2 (Abcam ab28673, Cambridge, MA, USA), anti-GASC1 (Bethyl Laboratories A300-885A, Montgomery, TX, USA), anti- $p21^{Waf1/Cip1}$ (Cell Signaling 2947, Danvers, MA, USA), anti-p53 (Calbiochem Ab-2 OP09, Gibbstown, NJ, USA), anti-$p27^{Kip1}$ (Oncogene NA35, Cambridge, MA, USA), anti-$p16^{INK4a}$ (Oncogene NA29), anti-RB (Proteintech Group 10048-2-Ig, Chicago, IL, USA), anti-GAPDH (Cell Signaling 2118, Danvers, MA, USA) and anti-β-actin (Sigma-Aldrich A5441).

Statistical analysis

Kendall's tau was used to assess the statistical significance of the association between copy number and expression for each gene. Holm's step-down procedure was used to adjust significance level for the large number of estimates to reduce the likelihood of false positive results. We used $P = 0.01$ as a cut-off for a statistically significant association between copy number and expression. For analyzing the results of cell growth, a two-tailed independent Student's *t*-test was performed. A value of $P < 0.05$ was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Original Article

Genomic amplification and a role in drug-resistance for the KDM5A histone demethylase in breast cancer

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Abstract: Lysine-specific demethylase 5A (KDM5A), an enzyme that removes activating H3K4 di- and trimethylation marks, plays critical roles in controlling transcription and chromatin architecture, yet its biological functions largely remain uncharacterized, particularly in the context of human cancer. In the present study, we found that the *KDM5A* gene was significantly amplified and over-expressed in various human tumors, including breast cancer. Reducing the expression of KDM5A by shRNA knockdown inhibited proliferation of KDM5A-amplified breast cancer cells. More importantly, we demonstrated that KDM5A over-expression was associated with breast cancer drug resistance. Furthermore, knockdown of KDM5A gene expression altered H3K4 methylation and induced upregulation of CDK inhibitors as well as genes mediating apoptotic cell death. Taken together, our study strongly links KDM5A histone demethylase activity to breast cancer proliferation and drug resistance, and suggests KDM5A is a potential target for breast cancer therapy.

Keywords: KDM5A, histone demethylases, gene amplification, drug-resistance

Introduction

Cancer has been traditionally viewed as a genetic disorder. However, it is increasingly apparent that epigenetic alterations, including histone modifications, DNA methylation, and microRNA dysregulation, play fundamental roles in cancer initiation and progression. Specifically, the use of systematic genome-wide discovery efforts has unexpectedly revealed a high frequency of cancer-specific alterations in genes involved in epigenetic histone modification in multiple tumor types [1-3]. The identification of these epigenetic modifier genes has raised important questions regarding the mechanisms by which they contribute to malignant transformation and progression. Furthermore, a better understanding of the intertwined relationship between genetic and epigenetic alterations in tumorigenesis is indisputably important for the development of new prognostic markers and therapeutic targets.

The epigenetic modifier gene *KDM5A* (*Lysine-specific demethylase 5A*, also known as *RBP2* and *JARID1A*), encodes a lysine-specific histone 3 demethylase [4-6]. Histone lysine methylation is a principal chromatin-regulatory mechanism that influences fundamental nuclear processes [7]. Lysine (K) residues on the tails of histone H3 can accept up to three methyl groups to form mono-, di-, and trimethylated derivatives (me1, me2, and me3, respectively). Depending on the site and degree of methylation, lysine methylation can have different transcriptional and biological outcomes. Specifically, KDM5A can function as a transcriptional repressor through the demethylation of tri- and dimethylated histone H3 at lysine 4 (H3K4) active marks [4-6]. KDM5A has been shown to regulate the expression of multiple genes and has also been shown to be required for normal development [4-6]. Indeed, KDM5A was originally identified as the retinoblastoma-binding protein and was implicated in regulation of retinoblastoma target genes [8]. Mutations in the *Drosophila* KDM5A homolog *lid* result in severe defects in cell growth and differentiation and are homozygous lethal [9]. More recently, several studies have shown that dysregulation of

KDM5A is associated with human cancer. KDM5A is over expressed in gastric cancer, and its inhibition triggers cellular senescence of gastric cancer cells [10]. In acute myeloid leukemia (AML), KDM5A has been shown to form a fusion protein with a nucleoporin 98 gene (NUP98), and overexpression of this fusion protein alone is sufficient to induce AML in murine models. Furthermore, genetic ablation of KDM5A decreases tumor formation and prolongs survival in pRB-defective mice [11]. Very recently, KDM5A was found to be a critical epigenetic factor for the development of drug resistance in lung cancer cells [12]. However, the role played by KDM5A in breast cancer remains poorly understood. In this study, we observed a significant amplification and over-expression of the *KDM5A* gene in various tumors, including breast cancer. We found that breast cancer cells with *KDM5A* gene amplification had intrinsic drug resistance properties and knocking down KDM5A with shRNAs improved the efficacy of epidermal growth factor receptor (EGFR) inhibitors against these breast cancer cells. Furthermore, increasing the expression of KDM5A in breast cancer led to global histone methylation level changes and altered the expression of a subset of key genes, including tumor suppressor p21 and apoptosis effector BAK1. Our findings suggest that genetic alteration of KDM5A may play a critical role in the pathogenesis of breast cancer.

Material and methods

Genomic array CGH

The isolation and culture of the SUM series of human breast cancer cell lines, Colo824, HCC1937, HCC1428, ZR-75-1 and non-tumorigenic mammary epithelial MCF10A cells have been described in detail previously [13, 14]. Genomic array CGH experiments were done using the Agilent 244K human genome CGH microarray chip (Agilent Technologies, Palo Alto, CA, USA) as described previously [13]. Briefly, for each array, female DNA (Promega, Madison, WI) was used as a reference sample and labelled with Cy-3. The samples of interest were each labelled with Cy-5. Agilent's CGH Analytics software was used to calculate various measurement parameters, including log2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe. Array data have been posted at the NCBI GEO database (GEO accession: GSE28989, GSM718287, GSM718288, GSM718289, GSM718290).

Lentivirus-mediated KDM5A shRNA knockdown

KDM5A knockdown was achieved by using the Expression Arrest GIPZ lentiviral shRNAmir system (OpenBiosystems). Lentivirus was produced by transfecting 293FT cells with the combination of the lentiviral expression plasmid DNA and viral packaging mix (OpenBiosystems). Cells were infected with the virus by incubating with the mixture of growth medium and virus-containing supernatant (1:1 ratio), supplemented with polybrene at a final concentration of 5μg/ml. An equal volume of fresh growth medium was added after 24 hours and selection of stable cells was started after 48 hours. Cells expressing shRNA were selected with puromycin for 2-4 weeks for functional studies (cell proliferation and colony formation assays) and for 4 to 10 days after infection for protein and RNA extraction.

Examination of cell growth

Cell growth was assessed by using a Coulter counter or the MTT assay [15]. For the MTT assay, cells were seeded in 6-well plates at a density of 2×10^4 cells per well and allowed to attach overnight. At designated time points, thiazolyl blue tetrazolium bromide (MTT, Sigma Aldrich) was added to each well of cells (final 0.5 mg/ml) and incubated for 3-5 hours at 37° C. After removing the growth medium, DMSO was added to solubilize the blue MTT-formazan product and the samples were incubated for an additional 30 minutes at room temperature. Absorbance of the solution was read at a test wavelength of 570nm against a reference wavelength of 650nm.

Cell growth in soft agar

Soft agar assays were performed as previously described [13]. Briefly, dishes were coated with a 1:1 mix of the appropriate 2x medium for the cell line being studied and 1% Bactoagar. ZR-75-1, HCC1937 and SUM149 cells transduced with a control (Ctrl-sh) or with KDM5A shRNAs (sh#4 and sh#5) were plated at $1x10^5$ cells/well in a 1:1 mixture of appropriate 2x medium and 0.3% Bactoagar. Cells were fed 3 times/week for 3-4 weeks, stained with 500μg/ml *p*-iodonitrotetrazolium violet (Sigma-Aldrich, St

Louis, MO, USA) overnight, photographed (left panel), and counted with an automated mammalian cell colony counter (Oxford Optronix GELCOUNT, Oxford, United Kingdom).

Immunoblotting and antibodies

Whole cell lysates were prepared by scraping cells from the dishes into cold RIPA lysis buffer and sonicating for 10 seconds. After centrifugation at high speed in the cold, protein content was estimated with the Bradford method. A total of 20-100μg of total cell lysate was resolved by SDS-PAGE and transferred onto PVDF membrane. Antibodies used in the study included anti-KDM5A (Bethyl Laboratories A300-897A, Montgomery, TX, USA) and anti-β-Actin (Sigma-Aldrich A5441, St Louis, MO, USA), anti-phospho-EGFR (Tyr1068) antibody (Cell Signaling #2234, Danvers, MA, USA), anti-EGFR antibody (Cell Signaling #D38B1), anti-H3K4me3 (Abcam ab8580, Cambridge, MA, USA), p21 (Cell Signaling #3814) and BAK1 Cell Signaling #2947) antibodies.

Statistical analysis

Kendall's tau was used to assess the statistical significance of the association between copy number and expression for each gene. Holm's step-down procedure was used to adjust significance level for the large number of estimates to reduce the likelihood of false positive results. We used $P = 0.01$ as a cut-off for a statistically significant association between copy number and expression. For analyzing the results of cell growth, a two-tailed independent Student's *t*-test was performed. A value of $P < 0.05$ was considered statistically significant.

Results

KDM5A is significantly amplified and over expressed in human tumors

To identify genomic aberrations in human breast cancer, we first performed genomic PCR and Agilent oligonucleotide array-based comparative genomic hybridization (CGH) on a panel of breast cancer cell lines and 50 primary human breast cancers. We observed that the *KDM5A* gene is located within a focal peak region (12p13.3) of gain/amplification in approximately 15% of breast cancers (**Figure 1A**). Of the fifty-one breast cancer lines examined, nine also showed KDM5A gain/amplification:

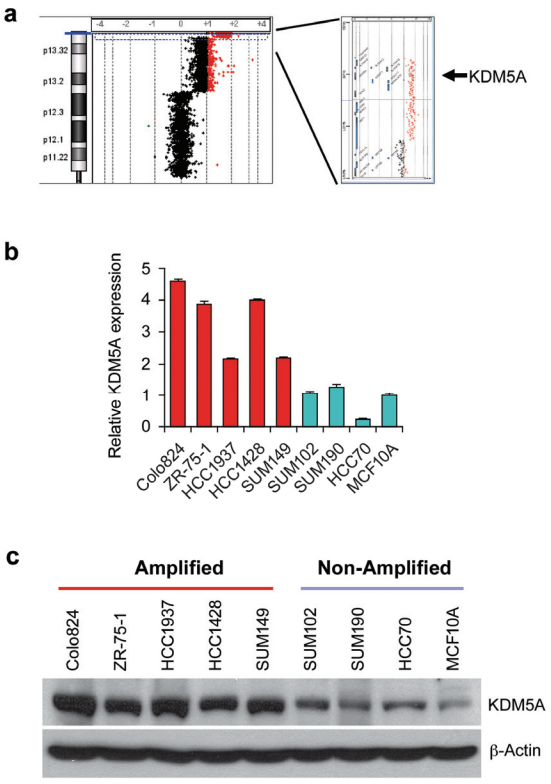

Figure 1. KDM5A is amplified and over-expressed in human breast cancer. (A) The representative array-CGH image showing chromosome 12p and KDM5A amplification in one breast cancer sample. (B) The mRNA expression level of KDM5A was examined by qRT-PCR assays in breast cancer cells with KDM5A gain/amplification (Colo824, ZR-75-1, HCC1937, HCC1428 and SUM149) or without the gain/amplification (SUM102, SUM190 and HCC70). mRNA expression level in the MCF10A cells was arbitrarily set as 1. Significance was set as $P<0.05$ by the student's t-test ($P<0.05$). (C) KDM5A protein levels were analyzed by Western blot in eight breast cancer cell lines with or without gene amplification, as well as in MCF10A control cells.

Colo824, ZR-75-1, HCC1937, HCC1428, SUM-149, HCC3153, HCC2185, HCC1187 and HBL100. To obtain further support for the involvement of KDM5A amplification in human tumors, we searched the published array-CGH database that contains a collection of 3131 copy-number profiles across different solid and liquid cancers. Using the CGH analysis program, Genomic Identification of Significant Targets in Cancer (GISTIC), we saw a significant gain/amplification (~23%) of KDM5A across the entire data set of 3131 tumors [16]. Thus, KDM5A is significantly amplified in various tu-

mors, including breast cancer. To measure expression levels of KDM5A, we performed quantitative RT-PCR (qRT-PCR) and Western blot assays in our panel of breast cancer cell lines. As expected, cell lines with KDM5A gene gain/amplification, Colo824, ZR-75-1, HCC1937, HCC1428 and SUM149 cells, showed higher mRNA and protein levels of KDM5A than the ones without the gene amplification ($P<0.001$) (**Figure 1B** and **C**). Thus, KDM5A gene amplification correlates with increased expression at both mRNA and protein levels in a subset of breast cancer cells.

Knockdown of KDM5A inhibits proliferation of KDM5A amplified breast cancer cells

To assess the contribution of endogenous KDM5A to breast cancer transformation, we knocked down KDM5A using a shRNA approach in breast cancer cells with or without KDM5A amplification. We obtained five pGIPZ-KDM5A shRNA expression constructs, and identified the two that most effectively knocked down KDM5A expression in ZR-75-1, HCC1937, SUM149 and SUM102 cells (**Figure 2A**). KDM5A knockdown caused significant growth inhibition of ZR-75-1, HCC1937 and SUM149 cells, all of which harbor KDM5A amplification ($p<0.05$). In contrast, there was no significant inhibition on the growth of SUM102 cells or the non-tumorigenic human mammary epithelial cells MCF10A, neither of which harbors the KDM5A gene amplification (**Figure 2B**). Furthermore, knockdown of KDM5A suppressed anchorage-independent growth of ZR-75-1, HCC1937 and SUM-149 cells (**Figure 2C**). Taken together, these data suggest that KDM5A may play an important role in the *in vitro* proliferation and maintenance of transformed phenotypes of breast cancer cells with KDM5A gene amplificaiton.

KDM5A is strongly associated with breast cancer drug resistance

KDM5A was recently identified as an important factor that is positively associated with EGFR inhibitor (erlotinib)-resistant phenotypes in lung cancer [12]. EGFR is over expressed in approximately 60% of basal breast cancers and correlates with poor prognosis, but has yet to emerge as a good therapeutic target in basal breast cancer [17]. We therefore sought to examine the EGFR family drug sensitivities of basal breast cancer cell lines with or without KDM5A gene amplification and overexpression. We found that HCC1937 and SUM149 cells (with KDM5A amplification) exhibited significantly higher EGFR inhibitor IC_{50} values as compared to SUM102 and MCF10A cells (without KDM5A amplification), although all cell lines expressed high-levels of EGFR protein (**Figure 3A** and data not shown). Next, we treated HCC1937, SUM149 and SUM102 breast cancer cell lines as well as the MCF10A line with 2μM or 4μM erlotinib for six, nine, twelve, and thirty days. Western blot with anti-phospho-EGFR (P-1068) antibody showed that erlotinib suppressed EGFR kinase activity in all of the treated cell lines (**Figure 3A**). As seen in **Figure 3B**, a subpopulation in the three cancer cell lines survived the drug treatments, even beyond thirty days. As expected based on the IC_{50} values of the EGFR inhibitors, HCC1937 and SUM149 cells had more drug-tolerant cells than SUM102 cells, whereas no drug-tolerant MCF10A cells were detected after treatment for thirty days (**Figure 3B**). These data suggest that breast cancer cells with KDM5A gene amplification are intrinsically more resistant to EGFR inhibitors than cells without KDM5A amplification.

To determine whether the drug-tolerant subpopulation has increased KDM5A expression, we treated SUM149 and SUM102 cells with erlotinib for six, nine and thirty days and then isolated total RNA and protein. qRT-PCR and immunoblotting experiments revealed that both mRNA and protein expression of KDM5A were increased in drug-tolerant cells as compared to parental control cells (**Figure 3C**). Thus, similar to the study done in lung cancer cells, KDM5A expression underwent up-regulation in the drug-tolerant subpopulations of breast cancer cells [12]. Next, to determine whether suppressing KDM5A in breast cancer cells circumvents erlotinib resistance, we challenged stable KDM5A -knockdown HCC1937 and SUM149 cell lines with erlotinib for thirty days. KDM5A knockdown significantly reduced the number of drug-tolerant cells in both cancer cell lines (**Figure 3D**). Taken together, our data reveal a strong association between KDM5A expression and breast cancer drug resistance.

Knockdown of KDM5A alters H3K4 methylation and induces up-regulation of CDK inhibitors and genes mediating apoptotic cell death

Because KDM5A is the key histone demethy-

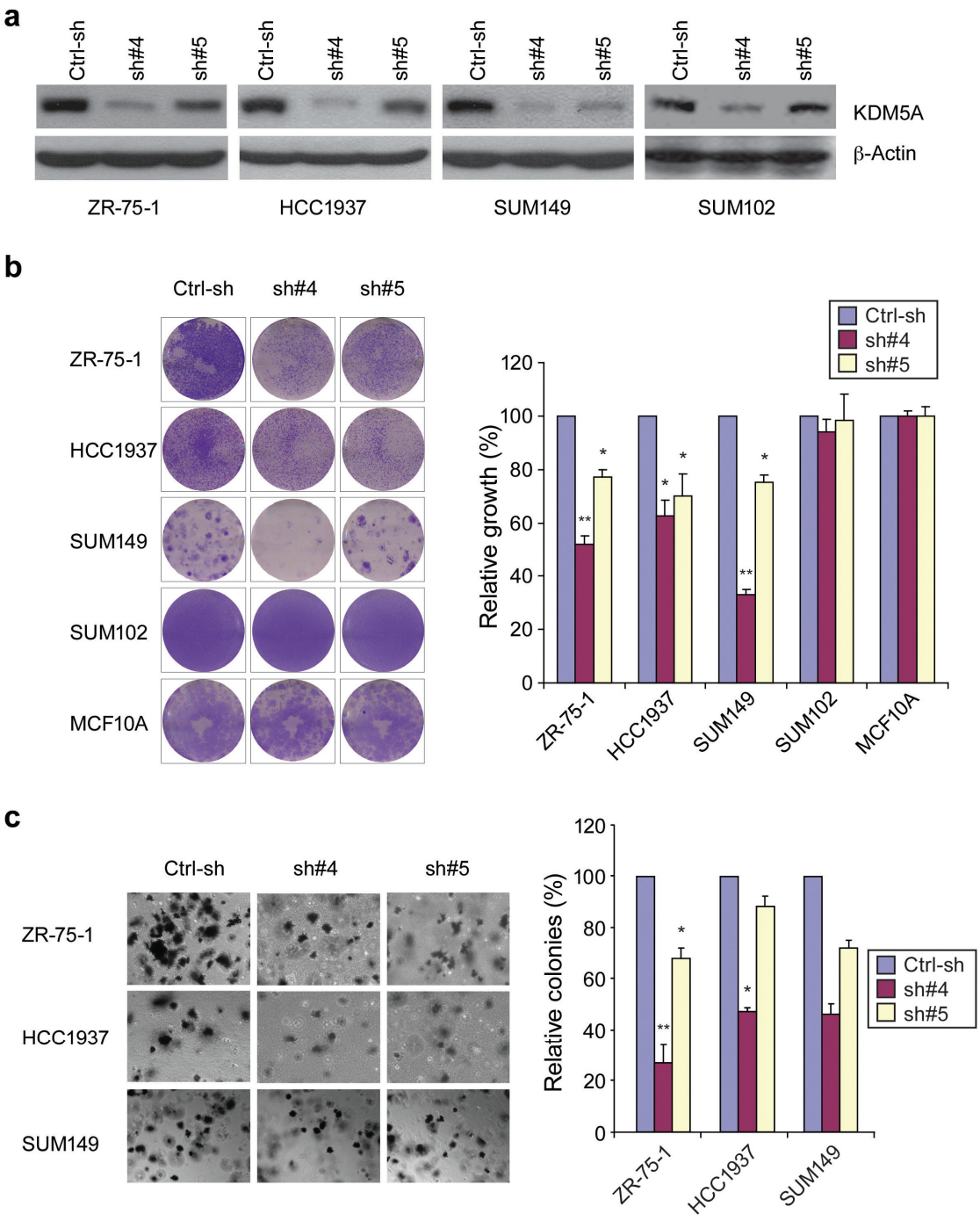

Figure 2. Reducing KDM5A expression by shRNA knockdown resulted in decreased cell proliferation and colony formation in soft agar. (A) Knockdown of KDM5A in four breast cancer cell lines with two different shRNAs was confirmed by Western blot assays. (B) shRNA-mediated knockdown of KDM5A inhibits cell growth in breast cancer cells with gene amplification. Cells (ZR-75-1, HCC1937 and SUM149 with KDM5A amplification, SUM102 without the amplification, as well as non-tumorigenic MCF10A) infected with control (Ctrl-sh) or KDM5A shRNAs (sh#4 and sh#5) were plated at equal density and selected with puromycin for 4 weeks. Surviving cells were stained with Crystal Violet (left panel) or counted (right panel). Relative growth was shown as the mean ± SD of triplicate determinations (*P<0.05 and ** P<0.01, Student's t test). (C) Knockdown of KDM5A impeded the anchorage-independent growth of breast cancer cells. Relative colony number (right panel) was shown as the mean ± SD of triplicate determinations (*P<0.05 and ** P<0.01, Student's t test).

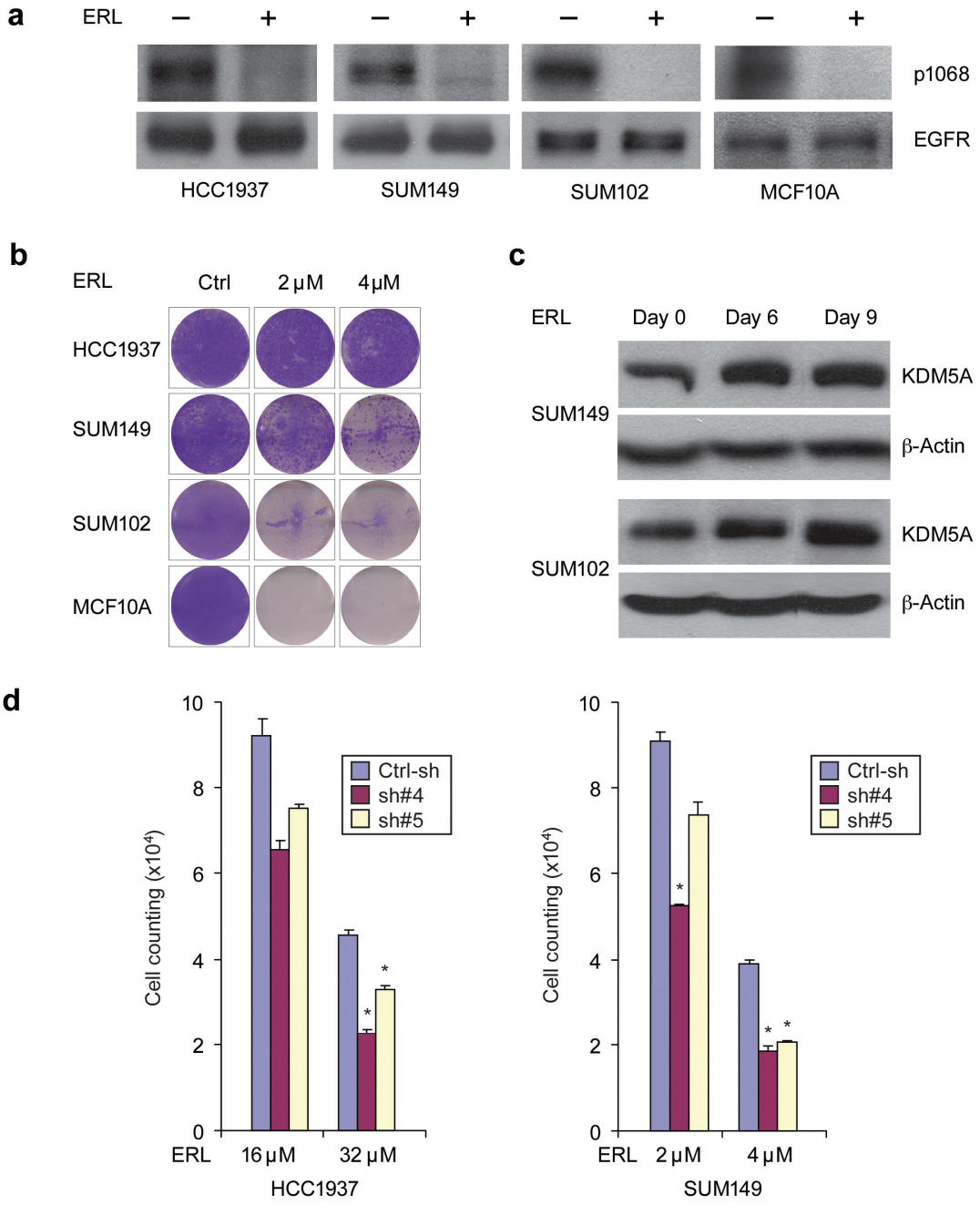

Figure 3. KDM5A is associated with breast cancer drug resistance. (A) EGFR inhibitor erlotinib (ERL) suppressed EGFR kinase activity in HCC1937, SUM149 and SUM102 breast cancer cell lines and MCF10A control line. Cells were treated with 4 μM erlotinib or vehicle for 1 hour. Protein extracts were immunoblotted with anti-phospho-EGFR (Tyr1068) and anti-EGFR antibodies. (B) Breast cancer cell lines HCC1937, SUM149 and SUM102 as well as the control MCF10A line were plated and left either untreated (Ctrl) or treated with 2 and 4 μM erlotinib for 30 days. Cells were fixed and stained with Crystal Violet or counted. Each experiment was performed in triplicate, and a representative image is presented. (C) Drug-tolerant subpopulation of SUM149 and SUM102 cells had increased KDM5A expression. Cells were plated and treated with 4 μM erlotinib for 6, 9 and 30 days with media/drug changes every two days and then isolated total RNA and protein. Protein extracts were immunoblotted with a KDM5A antibody. (D) KDM5A knockdown reduced the number of drug-tolerant cells in SUM149 and HCC1937. Stable KDM5A-knockdown and control HCC1937 and SUM149 cells were treated with the indicated concentration of erlotinib for 30 days. Cell counting was shown as the mean ± SD of triplicate determinations (*P<0.05 and ** P<0.01, Student's t test).

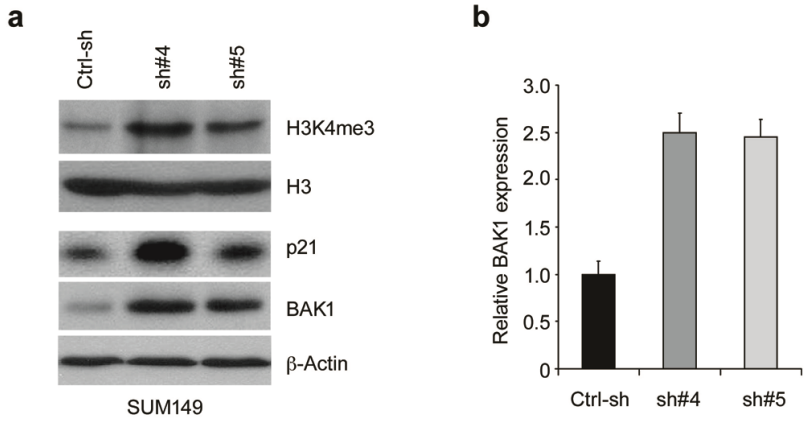

Figure 4. Knockdown of KDM5A altered H3K4 methylation and induced up-regulation of CDK inhibitors and genes mediating apoptotic cell death. (A) KDM5A was knocked down in SUM149 cells and the whole lysate was harvested for Western blot analysis. (B) mRNA levels of BAK1 were examined by real-time RT-PCR after knocking down KDM5A in SUM149 cells. The baseline for the cells infected with control shRNA was arbitrarily set as 1.

lase that specifically targets H3K4me3 and me2 active marks, the possibility exists that knocking down KDM5A in breast cancer cells would result in increased H3K4me3/me2 levels, and consequently the up-regulation of a specific set of genes. Thus, we first sought to examine the global H3K4me3 methylation status in KDM5A-knockdown SUM149 cells. As expected, shRNA-mediated inhibition of KDM5A expression in SUM149 cells resulted in increased H3K4me3 levels (**Figure 4**). Next, to identify genes with altered expression upon KDM5A knockdown, we performed a genome-wide expression profiling analysis. Knockdown of KDM5A in SUM149 cells yielded 208 up-regulated genes and 188 down-regulated genes with at least a two-fold change relative to control (data not shown). Previous studies demonstrated that KDM5A can inhibit the expression of cyclin-dependent kinase inhibitor p21 via its H3K4 demethylase activity in gastric cancer cells [10]. Our expression profiling analysis and Western blot experiments (**Figure 4A**), which showed p21 up-regulation with KDM5A knockdown, corroborate this finding and suggest that p21 is a KDM5A target gene in breast cancer cells.

Bioinformatic analyses of the results obtained from the genome-wide expression profiling study were performed with the Pathway-Express (PE) and Onto-Express (OE) programs [18]. In SUM149 cells, the pathways most affected by KDM5A knockdown included those involved in the regulation of transcription, organismal development, oxidation reduction and apoptosis (data not shown). Of particular interest is the apparent inverse relationship in expression between KDM5A and BAK1 (BCL2-antagonist/killer 1). BAK1 plays a key role in trigging apoptosis and its altered expression may help explain the drug resistance phenotypes associated with KDM5A amplification and over-expression [19]. To validate these array-based observations, we examined the expression of BAK1 by quantitative RT-PCR and Western blot in SUM149 cells following KDM5A knockdown (**Figure 4**). Depletion of KDM5A in SUM149 cells resulted in up-regulation of BAK1, indicating that KDM5A regulates the expression of this target gene. Thus, KDM5A may regulate a subset of genes involved in various functional pathways in breast cancer.

Discussion

In the present study, we demonstrated that the H3K4 demethylase KDM5A is amplified and over expressed in various tumors, including breast cancer. Knockdown of KDM5A with shRNAs inhibited the growth of breast cancer cells harboring the KDM5A amplification. Furthermore, breast cancer cells with *KDM5A* gene amplification have intrinsic drug resistance properties and knocking down KDM5A improves the efficacy of EGFR inhibitors against these breast cancer cells. Our finding that KDM5A up-regulation alters H3K4 methylation status, and thus may repress the expression of a set of key genes including CDK inhibitors as well as genes mediating apoptotic cell death, provides a potential mechanism for KDM5A mediated drug resistance. Our study points to an important role for the histone demethylase KDM5A in human breast cancer, and this protein represents a potential target for the devel-

opment of novel anticancer drugs.

A growing body of evidence indicates that amplification, translocation or mutation of histone methyltransferases and demethylases is linked to the development of many human cancers. For example, we originally identified and cloned the histone demethylase *GASC1* gene from an amplified region at 9p24 in esophageal cancer [20]. Later studies showed *GASC1* amplification in other tumor types, including lymphoma, medulloblastoma, lung and breast cancers [21-23]. We subsequently demonstrated that stable over-expression of GASC1 in the non-tumorigenic MCF10A cell line induces transformed phenotypes whereas knockdown in tumor cells inhibits proliferation, supporting a role for GASC1 as a transforming oncogene [13]. Houvras et al. revealed that the histone methyltransferase SETDB1 is recurrently amplified in melanoma and cooperates with oncogenic BRAF in accelerating oncogenesis [24]. Amplification and translocation of NSD1, 2 and 3 methyltransferase genes has been found in breast and lung cancers, and leukemia [25-29]. Very recently, Kuo et al. demonstrated that NSD2, via H3K36me2 catalysis, promotes transcription and cell transformation [30]. Here, we identified and investigated a frequently amplified region of DNA located on chromosome 12p13.3. Integration of copy number and gene expression data revealed the KDM5A gene as a candidate oncogene responsible for driving recurrent 12p13.3 amplification (data not shown). Furthermore, we validated the biologic effect of KDM5A upregulation by showing that KDM5A suppression impedes cell proliferation and anchorage-independent growth in breast cancer cell lines with KDM5A amplification. Our studies, together with others, indicate that genetic alteration in components of the histone modification machinery plays a central role in cancer initiation and progression.

Histone lysine methylation is a key regulator of gene transcription and chromatin architecture. In the case of H3K4 methylation, this mark is generally associated with active transcription [31]. KDM5A is capable of removing the H3K4me3/me2 mark from histones, which makes it a potential player in the downregulation of tumor suppressors. Indeed, previous studies revealed that KDM5A can inhibit the expression of p16, p21, and p27 via its H3K4 demethylase activity in gastric cancer cells [10]. In this study, we demonstated that upregulation of KDM5A alters H3K4 methylation status and may regulate a subset of genes, including p21 and BAK1, a protein that effects apoptosis-triggering cues [19]. Apoptosis is a predominant mechanism by which targeted or chemotherapeutic agents kill cancer cells. Genetic or epigenetic perturbations resulting in a defective execution of an apoptotic response could potentially result in drug-tolerant tumor cells [32]. Thus, although we cannot rule out the possibility that other target genes regulated by KDM5A are involved in drug resistance, our findings suggest that BAK1 might be an important downstream mediator of this phenotype. Furthermore, targeting histone demethylases is currently an active frontier in novel epigenetic drug development [33, 34]. Given that KDM5A is amplified and over expressed in various tumors, and plays a critical role in mediating transforming and drug resistance phenotypes, KDM5A may represent a potentially excellent target for the development of novel anticancer drugs.

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Genetic alterations of histone lysine methyltransferases and their significance in breast cancer

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Key words: breast cancer, histone lysine methyltransferase, gene amplification, deletion, mutation.

Abstract

Histone lysine methyltransferases (HMTs), a large class of enzymes that catalyze site-specific methylation of lysine residues on histones and other proteins, play critical roles in controlling transcription, chromatin architecture, and cellular differentiation. However, the genomic landscape and clinical significance of HMTs in breast cancer remain poorly characterized. Here, we conducted a meta-analysis of approximately 50 HMTs in breast cancer and identified associations among recurrent copy number alterations, mutations, gene expression, and clinical outcome. We identified 12 HMTs with the highest frequency of genetic alterations, including 8 with high-level amplification, 2 with putative homozygous deletion, and 2 with somatic mutation. Different subtypes of breast cancer have different patterns of copy number and expression for each HMT gene. In addition, chromosome 1q contains four HMTs that are concurrently or independently amplified or overexpressed in breast cancer. Copy number or mRNA expression of several HMTs was significantly associated with basal-type breast cancer and shorter patient survival. Integrative analysis identified 8 HMTs (WHSC1L1, SETDB1, SETDB2, ASH1L, SMYD2, SMYD3, SUV420H1, and KMT2C) that are dysregulated by genetic alterations, classifying them as candidate therapeutic targets. Together, our findings provide a strong foundation for further mechanistic research and therapeutic options using HMTs to treat breast cancer.

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The Role of Histone Demethylase GASC1 in Cancer and its Therapeutic Potential

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Abstract: Interdependent genetic and epigenetic events control the initiation and progression of tumors. Genetic amplification and overexpression of the GASC1 (*gene amplified in squamous cell carcinoma 1*) gene has been found in various tumor types and this upregulation correlates with a poor prognosis for cancer patients. Gain- and loss-of –function approaches demonstrate the importance of GASC1 for the maintenance of cancer phenotypes. The GASC1 gene encodes a Jumonji C domain-containing protein, a newly identified histone lysine demethylase, that mainly catalyzes demethylation of tri- and di-methylated forms of histone H3 lysine 9 (H3K9me3/me2) epigenetic repressive marks. Recent studies indicated that over-production of GASC1 may induce alterations in epigenetic histone methylation and affects the expression of key genes that are implicated in carcinogenesis and stem cell properties in human cancer. Furthermore, histone demethylases, such as GASC1, represent highly promising anti-cancer therapeutic targets; a number of GASC1 inhibitors have been identified and reported. This review provides an overview of the current findings on genetic alterations and the biological function of GASC1 in cancer, together with a summary of recent advances in GASC1 inhibitor discovery.

Key Words: Breast cancer, esophageal cancer, GASC1, gene amplification, histone demethylase, histone methylation, therapeutic target.

INTRODUCTION

Cancer arises through the accumulation of genetic and epigenetic alterations [1]. Genetic alterations include chromosome number changes and translocations, gene amplification, deletion, and mutations; epigenetic alterations involve histone modifications, DNA methylation, and microRNA dysregulation. It is speculated that genetic and epigenetic alterations operate interdependently in the initiation and progression of cancer, e.g. epigenetic alterations can be derived from genetic alterations that dictate abnormal chromatin regulation. Recently, the use of systematic genome-wide discovery efforts has revealed the genetic alteration of histone-modifying enzymes, including histone demethylases, at a high frequency in multiple tumor types [2-6]. An imbalance between histone methylation and demethylation is believed to be implicated in tumorigenesis [7-9]. These findings highlight the central role of dysregulation of histone-modifying enzymes in tumorigenesis. Furthermore, a better understanding of the intertwined relationship between genetic and epigenetic alterations in tumorigenesis is indisputably important for the development of new prognostic markers and therapeutic targets.

In 2000, Yang *et al.* identified and cloned a novel cancer gene, called *GASC1* (*gene amplified in squamous cell carcinoma 1*), from an amplified region at 9p24 in esophageal cancer cells [2]. Later studies showed that GASC1 amplification/overexpression occurs in various tumor types, and this upregulation correlates with a poor prognosis for cancer patients [3,4,10-16]. Recently, the GASC1 protein has been identified as a member of the JMJD2 (jumonji domain containing 2) subfamily of jumonji proteins, a set of newly identified transcriptional regulators that function as histone lysine demethylases [14,17-19]. Histone demethylases play essential roles in regulating gene expression and chromatin architecture, and are thus implicated in developmental processes, aging, DNA repair, stem cell biology, and tumorigenesis [7,20-22]. Furthermore, histone demethylases, such as GASC1, represent highly promising anti-cancer therapeutic targets, not only because of their potential oncogenic roles in cancer, but also because of their druggable enzyme activities [23-26]. Here, we will review the current findings on genetic alterations of histone demethylase GASC1 (also referred to as JMJD2C or KDM4C) in multiple tumor types and discuss the potential mechanism by which GASC1 mediates epigenetic histone modifications and promotes tumorigenesis. We also highlight the recently identified GASC1 inhibitors, and discuss the potential and caveats of targeting the GASC1 demethylase for the treatment of cancer.

Identification of the GASC1 Gene from an Amplified Region at 9p24 in Esophageal Cancer

An important mechanism for the activation of oncogenes in human cancers is gene amplification, which results in gene overexpression at both the RNA and protein levels [27,28]. Yang *et al.* originally became interested in the 9p24 (GASC1) amplified region in human cancer cells after comparative genomic hybridization (CGH) analysis of esophageal cancer cell lines. Of the 29 esophageal cancer cell lines examined, 5 (17.2%) were identified that had an increase in

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Table 1. GASC1 Amplification in Various Tumor Types

Cancer Subset	Frequency of Amplification			Total Cancer Samples	Cell Lines
	Overall	Focal	High-level		
All cancers	0.115	0.0287	0.0105	3131	611
Breast	0.1564	0.0453	0.0453	243	50
Lung	0.1344	0.0478	0.0065	774	129
Esophageal squamous	0.2045	0.0227	0.0	44	12
Ovarian	0.1942	0.068	0.0194	103	7
Colorectal	0.1925	0.0062	0.0124	161	33
Glioma	0.122	0.0	0.0	41	13
Medulloblastoma	0.1328	0.0078	0.0078	128	9
Hepatocellular	0.0826	0.0165	0.0	121	11
Prostate	0.1087	0.0217	0.0	92	9
Renal	0.0238	0.0159	0.0	126	27

Note: Data was obtained from the array CGH database of 3131 cancer samples, including 611 cancer cell lines. (http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf)

line KYSE150, established from a poorly differentiated, aggressive esophageal squamous cell carcinoma in a 49-year-old patient, exhibited a high level of amplification at the 9p23-24 region [2,29]. Because amplified regions often harbor oncogenes and/or other tumor-associated genes, and because 9p23-24 amplification has been reported in various other types of cancers, fluorescence *in situ* hybridization (FISH) and Southern blot analysis were used to map the 9p23-24 amplicon. Northern blotting was implemented to detect target genes/transcripts present within this amplicon, and one EST clone, R24542, was found showing overexpression in cell lines that exhibited amplification at 9p23-24. Two different cDNA libraries were screened using the R24542 clone as a probe. With this strategy, a novel gene, *GASC1*, was successfully cloned [2].

Amplification and Overexpression of GASC1 in Tumors

Recent studies clearly established that GASC1 is amplified and overexpressed in various tumor types, including lymphoma, medulloblastoma, lung, prostate and breast cancers [3,4,10-15]. Yang and colleagues performed extensive genomic analyses on a panel of breast cancer cell lines and primary samples, and found that the GASC1 region was amplified in 7 of 50 breast cancer cell lines, including HCC1954, Colo824, SUM149, HCC70, HCC38, HCC2157, and MDA-MB-436 cells; and in approximately 15% of primary breast cancers [3,15]. Based on the molecular signature, all seven GASC1-amplified lines belonged to basal-type breast cancer, an aggressive subtype of breast cancer with a poor prognosis [30]. Furthermore, by analyzing the breast cancer gene expression dataset, the level of GASC1 transcript expression was found to be significantly higher in the 116 basal-type tumors than in the 83 non-basal-type tumors (Kruskal-Wallis test $P<0.001$) [3,31]. Gain and/or amplification of the GASC1 region was also detected in approximately 35%–45% of primary mediastinal B cell lymphoma (PMBL) and approximately 33% of Hodgkin lymphoma (HL) [13]. Likewise, it is revealed that amplification of GASC1 occurs in 7.3% of medulloblastoma cases [4,32]. In another case, by combining cytogenetic, FISH, and CGH analyses of a metastatic case of lung sarcomatoid carcinoma, Italiano *et al.* detected an amplification of the GASC1 region and showed that this amplification was a significant element for pathogenesis of this tumor because it was detected in two different metastases as well as in the primary tumor [11].

To further demonstrate that the GASC1 gene is amplified in various tumor specimens, we queried the array CGH database: a collection of 3131 copy-number profiles across multiple cancer types [33]. In these 3131 tumor samples, there are 11.5% cases containing GASC1 amplification, where the GASC1 gene is also in the focal amplification peak in 2.87 % cases, particularly in breast and lung cancers (Table **1**). In 243 breast cancer samples, there exist 15.64% cases containing GASC1 amplification, in which 4.53% cases have the high-level amplification based on the GISTIC (Genomic Identification of Significant Targets in Cancer) analysis. In 774 lung cancer samples, there are 13.44% cases containing GASC1 amplification. In esophageal squamous cancer samples there are 20.45% exhibiting amplification, for ovarian cancer 19.42%, and for colorectal cancer 19.25% (Table **1**). It has also been reported that the expression of GASC1 is significantly increased in prostate cancers relative to normal tissue [14,16]. In summary, GASC1 is amplified and overexpressed in multiple tumor types.

Transforming Properties of GASC1

Since the discovery of the *GASC1* gene, studies have shown its transforming properties in various cell models. To test whether GASC1 is potently transforming in human mammary epithelial cells, wild-type GASC1 was cloned into a lentiviral vector and transduced into human nontumorigenic mammary epithelial MCF10A cells. Over expression of GASC1 in MCF10A cells resulted in the acquisition of phenotypes that are hallmarks of neoplastic transformation, including growth factor-independent proliferation and anchorage-independent growth in soft agar [3]. To further examine the effects of GASC1 activity in a context that more closely resembles *in vivo* mammary architecture, Yang and

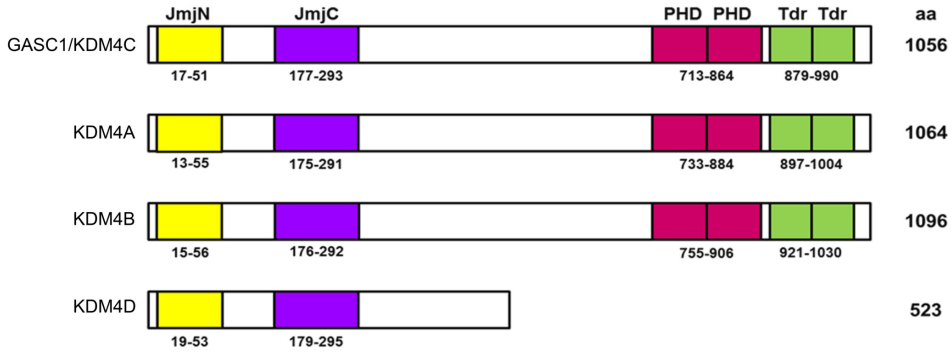


Fig. (1). Domain structure of GASC1 and its homologues KDM4A, B and D. The location and length of each domain is based on the data from the National Center for Biotechnology Information (NCBI).

pression on three dimensional morphogenesis in Matrigel. Whereas MCF10A cells formed polarized, growth-arrested acinar structures with hollow lumens similar to the glandular architecture *in vivo*, MCF10A-GASC1 cells formed abnormal acini at a high frequency that were grossly disorganized, and contained filled lumens [3]. These results indicate that GASC1 over expression disrupts epithelial cell architecture, which occurs frequently during the early stages of cancer formation.

The importance of GASC1 for the maintenance of cancer phenotypes has also been shown in breast, esophageal, prostate cancers and lymphoma with shRNA knockdown approaches. The Expression Arrest GIPZ lentiviral shRNAmir system was used to stably knock down GASC1 expression in three GASC1 amplified breast cancer cell lines, HCC1954, Colo824 and SUM149, demonstrating that GASC1 inhibition significantly slowed cell growth and inhibited colony formation of GASC1-amplified breast cancer, while it had only a slight effect on the cell growth of MCF10A control cells [3]. Inhibition of GASC1 expression caused a significant reduction of proliferation in the KYSE150 esophageal cancer line [14]. In prostate cancer, it was reported that the GASC1 protein interacts with the androgen receptor (AR) and functions as a co-activator of AR-induced transcription; reduction of GASC1 with shRNAs inhibited androgen-dependent proliferation of prostate cancer cells [34]. As mentioned above, the 9p24 region is frequently amplified in lymphomas, specifically PMBL and HL. To identify oncogenes in this amplicon, Rui *et al.* employed an unbiased approach using RNA interference genetic screening to discover the functionally critical genes in the 9p24 amplicon in PMBL and HL. They found that two genes, GASC1 and JAK2, cooperate to sustain the proliferation and survival of these lymphomas [13]. In summary, evidence has accumulated indicating the oncogenic roles of GASC1 in several types of cancer cells.

GASC1 as a Histone Demethylase

Chromatin modification has emerged in the last few years as an important mechanism of epigenetic regulation; it is clear that aberrant regulation of histone modification is relevant to the initiation and progression of cancer [1,9]. The basic unit of chromatin is the nucleosome that consists of 147 base pairs of DNA wrapped around a repetitive nucleosome core composed of four couples of histones H2A, H2B, H3 and H4. These histones are predominantly globular except for their N-terminal tails, which contain a plethora of posttranslational modifications. Histone tail modifications include methylation, acetylation, phosphorylation, ubiquitination and isomerization, resulting in a combination of histone marks referred to as the histone code [35-37]. Histone lysine methylation, governed by the opposing activities of histone methyltransferases and demethylases, serves as the principal chromatin-regulatory mechanism that influences fundamental nuclear processes and has a central role in transcriptional regulation [21,22,35]. Different transcriptional and biological outcomes result from methylation at different lysine residues, degree of methylation at the same lysine residues, and the location of the methylated histone within a specific gene locus. Lysine methylation at five sites on histone H3 (K4, K9, K27, K36, and K79) has shown an effect on gene transcription [21,22,35]. In general, methylation of H3K4, H3K36 and H3K79 are associated with the activation of transcription, whereas tri- and di-methylated forms of H3K9 (H3K9me3/me2) and H3K27 (H3K27me3/me2) are associated with repression of transcription [38,39].

When GASC1 was originally cloned in 2000, it was predicted that GASC1 is likely a nuclear protein involved in chromatin-mediated transcriptional regulation; however, at that time, the role and mechanism of this protein that regulates cellular processes, including transcriptional regulation in normal and cancer cells, was unknown. In 2004, Katoh *et al.* determined that GASC1 belongs to the JMJD2 (Jumonji domain containing 2) subfamily of the Jumonji family [40]. This group designated GASC1 as JMJD2C which contains one Jumonji (Jmj)C domain, one JmjN domain, two Plant Homeo Domain (PHD)-type zinc fingers and two Tudor domains (Fig. **1**). In 2006, there was a breakthrough in the understanding of how chromatin is regulated with the identification of JmjC domain-containing proteins, including GASC1, as a new class of histone demethylases [14,18,41]. In 2007, the new name KDM4C (lysine-specific demethylase 4C) was given to the GASC1 protein [42]. On the basis of homology, the JmjC family consists of 30 members, and thus far 18 of these have been identified to possess histone demethylase activity, and were further classified into seven subfamilies (KDM2-8) [21]. There are six members (KDM4A-F) of the human KDM4 (JMJD2) subfamily, of which two, KDM4E/F, are likely to be pseudogenes [40]. The KDM4A, B and C (GASC1) proteins, that share more than 50% percent of sequence identity, contain JmjN, JmjC,

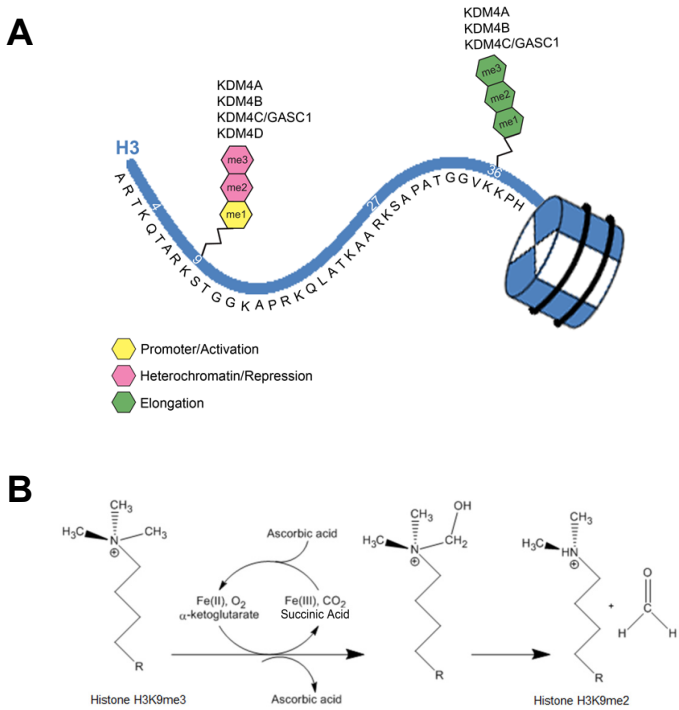


Fig. (2). GASC1 functions as a histone demethylase. (A) GASC1 catalyzes demethylation of H3K9me3/me2 and H3K36me3/me2. In general, H3K9me3/me2 is associated with repression of transcription while methylation of H3K36 is associated with transcriptional elongation. (B) GASC1 catalyzes the hydroxylation of methyllysine residues in a Fe(II) and α-ketoglutarate-dependent manner, releasing succinate and CO_2. In a second, nonenzymatic step, formaldehyde is spontaneously released after the decomposition of the N-hydroxymethyl moiety. For simplicity, only trimethylated H3K9 is illustrated. The same mechanism can be applied for demethylation of di-, or trimethylated H3K9 and H3K36.

PHD and Tudor domains, while the KDM4D protein lacks C-terminal PHD and Tudor domains (Fig. **1**).

The N-terminal Jumonji domain of GASC1 is the histone demethylase catalytic core [14,18,41]. Jumonji, which is Japanese for "cruciform", was the name given to the transcription factor whose ablation in mice resulted in neural plate deformation that resembles a cross [43]. Two conserved sequences have been noted in Jumonji and were referred to as JmjN and JmjC based on their relative locations to each other within the protein [44-46]. The JmjC domain is the catalytic domain of GASC1 for histone demethylation; the JmjN domain has been found to provide structural integrity and to form extensive interactions with the catalytic JmjC domain [17,19,41]. Both JmjC and JmjN are essential for the demethylase activity of JmjC-containing-proteins including GASC1 [17,47]. The demethylase reaction catalyzed by the Jumonji domain of GASC1 is a dioxygenase reaction that depends on two cofactors, Fe(II) and α-ketoglutarate (α-KG) (Fig. **2**). Early studies demonstrated that the Jumonji domain of GASC1 catalyzes demethylation of H3K9me3/me2 and H3K36me3/me2 *in vitro* and in cells [14,17-19]. Recently, combined structural, biochemical, and cellular studies demonstrated that GASC1 catalyzes the demethylation of H3K9me3/me2, and less efficiently (4-5 fold less than H3K9me3/me2), H3K36me3/me2 substrates [48]. In addition, competitive experiments employing H3K9me3 and H3K9me2 peptides revealed a clear preference for the tri- over the dimethylated state for the GASC1 substrate [48]. The crystal structure of the GASC1 catalytic domain (1-347 aa) is available at the Protein Data Bank (PDB) database (2XML). *In vitro* biochemical and cellular assays revealed that residues H190, E192 and H288 within the JmjC domain of GASC1 form an essential part of the Fe(II)-binding groove, and mutating H190 and E192 is sufficient to abrogate its H3K9 demethylation activity [14]. Thus, multiple studies indicate that the Jumonji domain of GASC1 predominantly demethylates H3K9me3. On the other hand, C-terminal PHD and Tudor domains likely contribute to efficient nuclear localization of GASC1 and mediate GASC1 associating with histone and other proteins [49]. However, the exact roles of the GASC1 PHD and Tudor domains in GASC1-dependent chromatin regulation remain unclear.

Molecular Mechanisms and Potential Functions of GASC1 in Tumorigenesis

The importance of H3K9 and H3K36 di- and tri-methyl marks in transcription and other processes that GASC1 regulates implies that the regulation of GASC1 is necessary and important for normal cellular function. Thus, it is speculated that deregulation of GASC1 can lead to imbalances in histone methylation pathways that affect many chromatin-regulated processes, of which transcription regulation, DNA repair, and chromosome stability are the most relevant for the pathogenesis of human cancers. Several recent studies provide clues on the mechanisms by which GASC1 contributes to tumorigenesis via its histone demethylation function. In general, H3K9me3/me2 marks are associated with the promoter of silenced euchromatic genes; its removal by

GASC1 will result in transcriptional activation [38,39]. Indeed, studies have shown that GASC1 enhances the expression of important genes, such as classical oncogenes MDM2 and MYC, as well as key stem transcription factor NANOG, through its H3K9 demethylation function [3,13,50-52]. In order to uncover the function of GASC1 in oncogenesis, Ishimura *et al.* searched for the downstream target genes regulated by GASC1 using mouse embryonic fibroblasts (MEFs) [50]. Exogenous overexpressing GASC1 in MEF cells increases the expression of the MDM2 oncogene at the mRNA and protein levels. A chromatin immunoprecipitation (ChIP) assay showed that GASC1 was recruited to the P2 promoter region of the MDM2 gene, resulting in demethylation of H3K9me3/me2. However, there was no detectable change of the H3K36me3 level at the P2 promoter of MDM2 with GASC1 overexpression. Furthermore, siRNA-mediated knockdown of GASC1 caused reduction of MDM2 expression in the cells. Wissmann *et al.* identified GASC1 as the first histone tri-demethylase regulating AR function [34]. GASC1 interacts with the AR *in vitro* and *in vivo*; assembly of ligand-bound AR and GASC1 on the promoter of AR-target genes results in demethylation of H3K9me3 and stimulation of androgen receptor-dependent transcription. Conversely, knockdown of GASC1 inhibits androgen-induced removal of H3K9me3 and transcriptional activation. Rui *et al.* demonstrated that knockdown of GASC1 by shRNA inhibits MYC expression by directly altering the H3K9me3 mark at the promoter and intron 1 regions of MYC in lymphoma cells.

The cancer stem cell (CSC) hypothesis suggests that a subset of tumor cells with stem-cell-like properties is primarily responsible for the growth, progression and recurrence of cancer [53-55]. H3K9 methylation status in pluripotent embryonic stem cells (ESCs) is maintained both globally and locally by an intricate interplay between the activities of pluripotency factors and histone demethylases [52,56,57]. Functionally, key pluripotent factors, including OCT4 (POU5F1), NANOG and SOX2, form a robust autoregulatory circuit that maintains ESCs in a self-renewing state [57,58]. Interestingly, GASC1 is preferentially expressed in undifferentiated ES cells [59]. In 2007, Loh *et al.* identified GASC1 as a *bona fide* target of the OCT4 in mouse ESCs [52]. More significantly, they identified NANOG as a target of GASC1 and confirmed the recruitment of GASC1 to the NANOG promoter [52]. They demonstrated that GASC1 is required to reverse H3K9me3 repressive marks at the NANOG promoter region in ESCs. Loss-of-function approaches illuminated that GASC1 is critical for the maintenance of the self-renewal state of ES cells [52]. Thus, GASC1 is a component of the ESC transcription circuitry designed to maintain ESC properties. Notably, introduction of GASC1 in MCF10A cells could increase higher capacity to generate mammospheres, a phenotype of cancer stem cells [3]. Because of its regulation of important ESC factors, GASC1 could provide a link between stem cell function and cancer initiation and progression when it functions as an oncogene. The effects of GASC1 demethylase function on cancer stem cells are still under intense investigation, and many questions remain largely unanswered. Nevertheless, recent studies support the notion that amplification and subsequent over-production of GASC1 induces alterations in epigenetic histone methylation epigenetic histone methylation and affects the expression of a set of key genes that are implicated in carcinogenesis and stem cell properties in human cancer.

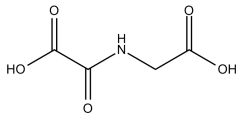
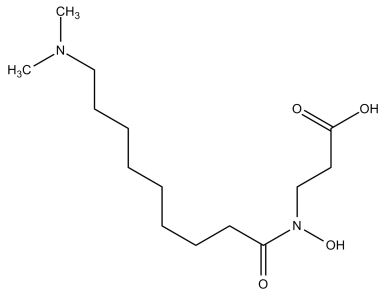
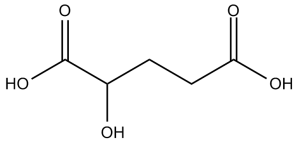
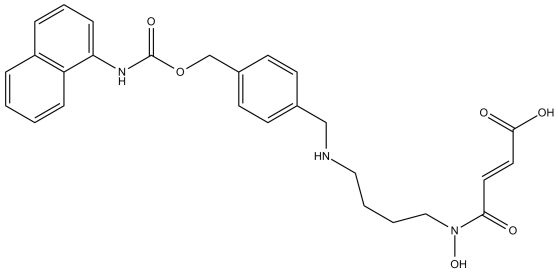
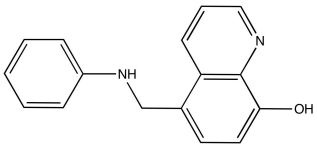
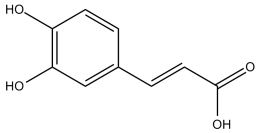
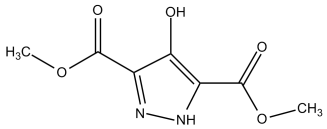
GASC1 as a Potential Therapeutic Target

The discovery that histone demethylases, including GASC1, play critical roles in tumorigenesis by controlling epigenetic oncogenic programming provides a unique opportunity to develop demethylase inhibitors as a novel class of anti-cancer drugs [23,24,60,61]. On the basis of the three–dimensional structure and catalytic mechanism of the GASC1/JMJD2 family of histone demethylases mentioned above, a number of JMJD2 inhibitors have been identified and reported. Here, we focus on the reported inhibitors of GASC1 (Table **2**).

The GASC1 demethylase is an Fe(II)- and α-KG-dependent enzyme that oxygenates methylated histone lysine residues, which in turn leads to their demethylation (Fig. **2**). The efforts to target cofactors essential for the activity of GASC1 has provided the first promising results. N-oxalylglycine (NOG), first tested by Cloos *et al.* in 2006, was found to weakly inhibit the GASC1 demethylation of H3K9me3 [14]. As an α-KG analogue, it is speculated that NOG displaces α-KG from the iron-binding residues of GASC1, inhibiting GASC1 activity. Based on the crystal structure model of the GASC1 Jumonji domain complexed with α-KG, Hamada *et al.* designed and synthesized a series of GASC1 small molecule inhibitors. Compound 8 (later named NCDM-32), was found to be the most selective and potent GASC1 inhibitor [26,62]. Compound 8, with eight methylene chains, showed a low micromolar IC_{50} value against GASC1 as compared to NOG (Table **2**). The noted interactions between GASC1 and Compound 8 indicate the importance in potency for the tertiary amino group as well as the linker length of the inhibitor for interaction. Overall, Compound 8 showed 500-fold greater GASC1-inhibitory activity than NOG [62]. By using biochemical, structural and cellular assays, Chowdhury *et al.* found that 2-hydroxyglutarate (2HG) inhibits α-KG-dependent oxygenases, including GASC1 [63]. Methylstat, a cell-active selective histone demethylase inhibitor, inhibits the subfamily of trimethyl lysine demethylases. This small molecule inhibitor contains a (methyllysine) substrate mimic, an (α-KG) cofactor mimic, and a linker to attach them (Table **2**). Importantly, Methylstat inhibited cell growth of GASC1 amplified KYSE150 esophageal cancer with a half maximal growth inhibitory concentration (GI_{50}) at approximately 5.1 μM.

A high-throughput RapidFire mass spectrometry assay was used to screen more than 100,000 compounds to identify GASC1 inhibitor candidates. This assay employs a short amino acid peptide substrate, corresponding to the first 15 amino acid residues of histone H3, and monitors the direct formation of the dimethylated-Lys9 product from the trimethylated-Lys9 peptide substrate [64]. With this assay, 1126 compounds have been found with IC_{50} values less than 100μM. For example, Compound 5, that contains the core structure of 8-hydroxyquinolines (8HQs), displayed strong potential (IC_{50} : 2.1 μM) to inhibit GASC1 demethylase activity. Another study demonstrated that 8HQs inhibit KDM4

Table 2. Summary of GASC1 Small Molecular Inhibitor

Name	Structure	Activity (IC_{50})	References	PubMed ID Numbers
N-oxalylglycine (NOG)		500 μM	Cloos *et al.* (2006)	16732293
Compound 8 (NCDM-32)		1.0 μM	Hamada *et al.* (2010) Suzuki *et al.* (2011)	20684604 21955276
2-hydroxyglutarate (2HG)		(*R*)-2HG = 79 μM (*S*)-2HG = 97 μM	Chowdhury *et al.* (2011)	21460794
Methylstat		3.4 μM	Luo *et al.* (2011)	21585201
Compound 5		2.1μM	Hutchinson *et al.* (2012)	21859681
Compound 1 (Caffeic Acid)		13.7 μM	Nielsen *et al.* (2012)	22575654
Compound 2		147 μM	Leurs *et al.* (2012)	22917519

subfamily demethylases via binding to the active-site Fe(II) and display activity against KDM4A in cell-based studies [65]. Upon screening a 640 member natural product library for inhibitors of GASC1, Nielsen *et al.* tested a subset of 21 compounds in the formaldehyde dehydrogenase assay and discovered Compound 1 (Caffeic Acid) as a GASC1 inhibitor (Table **2**). Compound 1 is a known anti-oxidant shown to inhibit cancer cell proliferation through oxidative processes [66]. Very recently, a heterocyclic ring system library was screened against GASC1 in the search for novel inhibitory scaffolds. A 4-hydroxypyrazole scaffold (Compound 2) was identified as a new inhibitor of KDM4C (Table **2**) [67].

PERSPECTIVES AND CONCLUSION

Cancer is traditionally viewed as a genetic disorder; however, accumulated evidence shows that epigenetic disruption plays a critical role at every stage of tumorigenesis and holds a significant impact on tumorigenic mechanisms and the development of cancer therapies. While epigenetics and genetics can cooperate in cancer initiation and progression, the interconnectedness between these two processes is becoming increasingly apparent with the realization that epigenetic modifiers are genetically altered at a high frequency in multiple tumor types. Notably, the GASC1 gene was originally discovered and cloned from the genetic amplified region in esophageal cancer. Lately, it has been identified as the key epigenetic histone modifier, histone lysine demethylase, which plays an important role in epigenetic histone modification. Recent studies revealed that GASC1 is amplified and over-expressed in various aggressive tumors, and is implicated in the transforming phenotypes in several *in vitro* models. However, many vital questions remain regarding the molecular mechanisms by which GASC1-dependent chromatin regulation translates into oncogenicity and contributes to cancer initiation and progression. For example, to better understand how GASC1 affects chromatin organization and transcription, it will be critical to determine the genome-wide targets of GASC1, as well as the effect of GASC1 deletion and overexpression on transcription and histone modification patterns. It is important to investigate whether GASC1 targets different genes in different types of cancer. It also should be noted that GASC1 has been reported to demethylate non-histone substrates *in vitro* and *in vivo*, and the identified substrates share sequence similarity to H3K9 [68-70]. Very recently, it is revealed that GASC1 can demethylate the K191me2 of the chromobox homolog 4 (CBX4, also known as polycomb 2 protein: Pc2), which plays an important role in cell cycle and growth control [70]. However, the interplay between histone and non-histone methylations regulated by GASC1 has not been addressed. Thus, increasing an understanding of this exciting biology and the mechanisms of GASC1 demethylation function is a significant component of further studies and research.

Given the critical roles of GASC1 in cancers, it is very likely that inhibitors of GASC1 will move forward into clinical trials. However, one must keep in mind the caveat that most GASC1 inhibitor scaffolds derive from other structurally or mechanistically related enzymes and these compounds are, therefore, oftentimes also active against other enzyme families. In addition, most inhibitors are cofactors and/or substrate mimics and so far have only very limited or undetermined specificity for GASC1. It will, thus, be an utmost objective for the near future to discover more potent and, especially important, more selective inhibitors with the ability to specifically target GASC1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Review Article

Histone lysine demethylase (KDM) subfamily 4: structures, functions and therapeutic potential

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Abstract: KDM4 histone demethylases catalyze the removal of methyl marks from histone lysine residues to epigenetically regulate chromatin structure and gene expression. KDM4 expression is tightly regulated to insure proper function in diverse biological processes, such as cellular differentiation. Mounting evidence has shown that disrupting KDM4 expression is implicated in the establishment and progression of multiple diseases including cancer. In particular, genomic regions encoding the *KDM4A*, *B* and *C* genes are often amplified, disrupting normal cellular proliferation. Furthermore, KDM4 demethylases are promising druggable targets. In this review, we highlight the latest advances in characterizing the structures and regulatory mechanisms of KDM4 proteins, as well as our current understanding of their alterations and roles in tumorigenesis. We also review the reported KDM4 inhibitors and discuss their potential as therapeutic agents.

Keywords: Histone lysine demethylase, KDM4, JmjC domain, cancer

Introduction

Cell proliferation and cell fate are dynamically controlled through posttranslational histone modifications, including methylation, which is established and tightly regulated by histone methyltransferases and demethylases. These modification marks, which primarily localize to the flexible histone tails, but also to core histone residues, function to alter DNA compaction and to recruit transcription factors and transcriptional machinery [1, 2]. Methylation of lysine and arginine histone side chains and core domains serves to modulate the epigenetic landscape with significance in transcriptional control during embryonic development, genomic imprinting and X chromosomal inactivation [2-4]. The accumulated evidence also links improper histone methylation to the dysregulation of cellular processes underlying several human diseases. For instance, it is now clear that members of the histone lysine demethylase (KDM) subfamily 4 are commonly overexpressed in human cancers, where they have been found to disrupt normal cellular proliferative balance [5, 6]. Here, we aim to review our current understanding of the structures, functions and therapeutic potential of this subfamily of proteins.

Histone lysine demethylase families

Histone methylation is known to occur on the lysine residues of histones 3 and 4 (H3, H4), and the linker histone H1, isotype 4 (H1.4). On H3, four N-terminal lysine residues (K4, K9, K27, K36) and two structural residues (K56, K79) are able to be methylated [1, 7-10]. The linker histone H1.4, which is associated with intergenic regions of the genome, can also be methylated at lysine 26 (H1.4K26) [11, 12]. At these histone lysine residues, methyltransferases and demethylases can, respectively, add or remove mono- (me1), di- (me2), or trimethyl (me3) marks, the degree of which alters chromatin compaction and gene expression. Methylation of H3K4, H3K36 and H3K79 is generally associated with gene activation, while methylation of H3K9, H3K27, H3K56, H4K20 and H1.4K26 is linked to transcriptional repression [1, 13].

Structurally, the histone lysine demethylases are a diverse group of proteins which can be

broadly categorized under two functional enzymatic families. The first family includes the lysine specific demethylase (LSD1, also known as KDM1A), which, along with the structurally similar KDM1B (LSD2), consist of the flavin adenine dinucleotide (FAD)-dependent amine oxidases, which can remove mono- and dimethyl histone lysine marks [14-16]. These amine oxidases, however, are unable to demethylate trimethyl lysine residues since they require a lone pair of electrons only present on mono- and dimethyl lysine histone residues. The second family of histone demethylases consists of the Jumonji C (JmjC)-domain containing proteins which employ an oxygenase mechanism to demethylate specific histone mono-, di- and trimethyllysine residues. The enzymatic function of the JmjC domain relies on α-ketoglutarate (α-KG), Fe(II), and molecular oxygen as cofactors in the demethylation reaction [13]. An analysis of public protein-domain databases has revealed that humans encode 32 such JmjC-domain containing genes, 24 of which have documented biochemical demethylase activity (**Table 1**). The function of these diverse JmjC-domain containing proteins is further distinguished by combinations of other conserved domains including the PHD, Tudor, CXXC, FBOX, ARID, LRR, as well as JmjN domains. Based on sequence homologies and structural similarities, these 24 JmjC-domain containing demethylases can be categorized into seven functionally divergent protein subfamilies (**Table 1**) [17, 18].

Genomic and protein structures of KDM4 demethylases

Within the family of JmjC-domain containing demethylases is the large KDM4 subfamily. In the human genome are five functional KDM4 member genes (*KDM4A-E*). Those encoding *KDM4A*, *B* and *C* localize to human chromosomes 1p34.1, 19p13.3, and 9p24.1, respectively. *KDM4D* localizes to human chromosome 11q21, and forms a cluster with two additional intronless *KDM4* genes, *KDM4E* and *KDM4F* [19]. Previously, *KDM4E* and *F* were considered pseudogenes, however *KDM4E* expression has recently been observed, suggesting its role as a functional gene [1, 20, 21]. The KDM4 subfamily is highly conserved, with orthologs of KDM4A, B, and C found among all vertebrates, and orthologs of KDM4D found in placental mammals [21].

The KDM4A, B and C proteins, which share more than 50% sequence identity, each contain JmjN, JmjC, two plant homeodomains (PHD) and two Tudor domains. KDM4D and KDM4E, in contrast, are considerably shorter proteins which lack the C-terminal region, including the PHD and Tudor domains (**Table 1**). As with all JmjC-domain containing demethylases, the KDM4 JmjC domain bears catalytic function while the JmjN domain interacts extensively with JmjC and provides structural integrity [5, 22]. Recent biochemical studies indicate that KDM4A-C catalyze the removal of H3K9 and H3K36 di- and trimethyl marks, while KDM4D can only demethylate H3K9me3/me2. KDM4E meanwhile, catalyzes the removal of two methyl groups from H3K9me3 and H3K56me3 [23]. Interestingly, the H3K56me3 heterochromatic mark is highly conserved, found also in *C. elegans*, where it regulates DNA replication [23].

Beyond the catalytic core of KDM4A-C, the C-terminal PHD and Tudor domains bear important histone reader functions. Structural and biochemical studies have demonstrated that the Tudor domains of KDM4A can recognize and bind two unrelated histone marks, H3K4me3/me2 and H4K20me3/me2, by means of distinct binding mechanisms. Three aromatic residues in the KDM4A-Tudor domains, F932, W967, and Y973, can form an open cage that accommodates H3K4me3 binding [24]. In contrast, KDM4A binding to H4K20me3 requires the Tudor domains to adopt opposite relative orientations, using the same three aromatic residues which contact different surfaces [25]. In addition, the PHD domains in other histone regulatory proteins have been demonstrated to bind unmodified, methylated, and/or acetylated histone residues on one or more histone tails, offering flexibility in directing epigenetic modifications [26, 27]. However, as of yet, no functional studies or three-dimensional structure of the KDM4A-C PHD domains have been reported, highlighting the need to clarify the molecular function of these domains.

Expression and physiological functions of KDM4 demethylases

Previous studies have indicated that KDM4A and C are broadly expressed in mouse and/or human tissues, while KDM4D and E are pre-

Table 1. Functional classification and histone substrates of the histone lysine demethylases

Official Symbol	Other Aliases	Gene Location	Gene ID	Protein Domains	Histone Substrates
KDM1A	LSD1, AOF2, BHC110, KDM1	1p36.12	23028		H3K4me1/me2, H3K9me1/me2
KDM1B	LSD2, AOF1, C6orf193,	6p22.3	221656		H3K4me1/me2
KDM2A	JHDM1A, FBXL11, CXXC8, FBL11, FBL7, LILINA	11q13.2	22992		H3K36me1/me2
KDM2B	JHDM1B,CXXC2, FBXL10, Fbl10, PCCX2	12q24.31	84678		H3K4me3, H3K36me1/me2
KDM3A	JHDM2A, JHMD2A, JMJD1, JMJD1A, TSGA	2p11.2	55818		H3K9me1/me2
KDM3B	JMJD1B, 5qNCA, C5orf7, NET22	5q31	51780		H3K9me1/me2
JMJD1C	KDM3C, TRIP8	10q21.3	221037		H3K9me1/me2
KDM4A	JMJD2A, JHDM3A, JMJD2, TDRD14A	1p34.1	9682		H3K9me2/me3, H3K36me2/me3, H1.4K26/me2/me3
KDM4B	JMJD2B, TDRD14B	19p13.3	23030		H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3
KDM4C	GASC1, JMJD2C, JHDM3C, TDRD14C	9p24.1	23081		H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3
KDM4D	JMJD2D	11q21	55693		H3K9me2/me3, H1.4K26me2/me3
KDM4E	JMJD2E, KDM4DL	11q21	390245		H3K9me2/me3, H3K56me3
KDM5A	JARID1A, RBBP-2, RBBP2, RBP2	12p11	5927		H3K4me2/me3
KDM5B	JARID1B, CT31, PLU-1, PUT1, RBBP2H1A	1q32.1	10765		H3K4me2/me3
KDM5C	JARID1C, MRXJ, MRXSCJ, MRXSJ, SMCX, XE169	Xp11.22-p11.21	8242		H3K4me2/me3
KDM5D	JARID1D, HY, HYA, SMCY	Yq11	8284		H3K4me2/me3
KDM6A	UTX, KABUK2,	Xp11.2	7403		H3K27me2/me3
KDM6B	JMJD3	17p13.1	23135		H3K27me2/me3
JHDM1D	KDM7A	7q34	80853		H3K9me1/me2, H3K27me1/me2
PHF8	KDM7B, JHDM1F, MRXSSD, ZNF422	Xp11.22	23133		H3K9me1/me2, H4K20me1
PHF2	KDM7C, CENP-35, GRC5, JHDM1E	9q22.31	5253		H3K9me2
MINA	MINA53, MDIG, FLJ14393, ROX, NO52	3q11.2	84864		H3K9me3
NO66	ROX, NO66, MAPJD	14q24.3	79697		H3K4me1/me3, H3K36me2
KDM8	JMJD5	16p12.1	79831		H3K36me2

Note: SWIRM: Swi3p, Rsc8p, and Moira domain (pink); Amine Oxidase domain (olive green); Spacer region (light green); CW-type zinc-finger domain (fuchia); JmjC domain (red); CXXC zinc-finger domain (purple); PHD: plant homeodomain (green); FBOX: F-box domain (black); LRR: Leu-rich repeat domain (brown); JmjN domain (blue); Tudor domain (yellow); ARID: AT-rich interacting domain (orange); C5HC2 zinc-finger domain (grey); TPR: tetratricopeptide domain (light blue).

dominantly expressed in the mouse testes [1, 28-30]. To further investigate the expression of human KDM4 demethylases, we conducted meta-analyses of next-generation sequencing profiles for normal tissues using the RNA-Seq Atlas, and for normal and diseased tissues using GENT databases [31, 32]. Generally, *KDM4A*, *B*, and *C* are broadly expressed in normal human tissues, with high expression in the spleen, ovary and colon (**Figure 1**). Based on RPKM (Reads Per Kilobase per Million) values, expression levels of *KDM4A* and *C* are approximately 3-6 fold higher than those of *KDM4B*. For instance, in the spleen, *KDM4A* and *KDM4C* have RPKM values of about 6 and 9 respectively, compared to 1.3 for *KDM4B*. Both *KDM4D* and *E* are predominantly expressed in the human testes. However, the RPKM values of *KDM4E* in human tissues are very low (<0.25) as compared to other *KDM4* genes. The variation in expression levels of the KDM4 subfamily members in human tissues suggest these proteins may be regulated by distinct pathways and have non-overlapping biological functions in different cell types.

To study the physiological function of KDM4, knockout and/or transgenic models have been

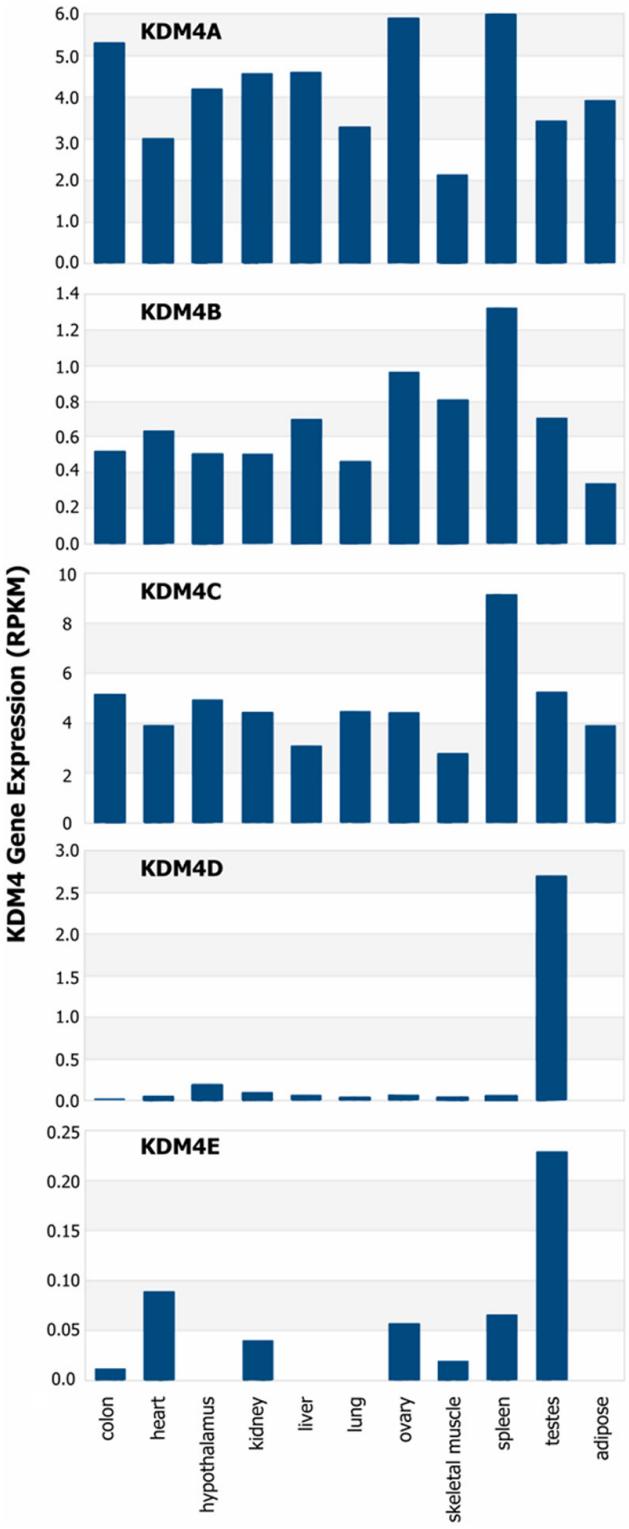

Figure 1. Analysis of gene expression levels for KDM4 member genes in normal human tissues using the RNA-Seq Atlas [28]. Next-generation sequencing profiles for each of *KDM4A* (NM_014663), *KDM4B* (NM_015015), *KDM4C* (NM_015061), *KDM4D* (NM_018039) and *KDM4E* (NM_001161630) are presented as Reads Per Kilobase per Million mapped reads (RPKM).

established in model organisms including *Drosophila melanogaster, C. elegans* and mice. Double homozygous mutants of both *Drosophila KDM4* orthologs, *dKDM4A* and *B,* are not viable and die in the second instar larval stage [33]. Depletion of the single *C. elegans KDM4* gene results in germ line apoptosis and slows DNA replication [34]. Studies in mice using conditional heart-specific *KDM4A* knockout as well as transgenic mice have demonstrated that KDM4A promotes cardiac hypertrophy in response to hypertrophic stimuli [35]. Knockout mouse models for *KDM4B* and *D* are viable without gross abnormalities [36]. Conditional knockout of *KDM4B* in mammary epithelial cells exhibit delayed mammary gland development with reduced branching [37]. Though absent in other tissues, KDM4D is highly expressed in spermatocytes and spermatids [30]. Mutant *KDM4D* male mice have globally higher levels of H3K9me3 than control mice [30]. However, adult *KDM4D* mutant mice are as fertile as control mice, possibly through *KDM4B* compensation.

During development, several KDM4 members are known to play important roles in maintaining the open chromatin state required in embryonic stem (ES) cells to ensure efficient proliferation and readiness for differentiation [38]. At an epigenetic level, this euchromatic state relies on the absence of H3K9 methylation, which is insured by KDM4 demethylase activity. In mouse development, *KDM4A, B* and *C* are expressed early in the fertilized egg and in undifferentiated ES cells [19, 39]. The functions of KDM4 proteins during development are diverse, as they promote pluripotency in some instances and direct differentiation in others. KDM4A for instance, which is essential for mouse embryonic development, also drives neural crest specification in the chick embryo [40, 41]. In humans, embryonic skeletal, bone and fat cell differentiation depends on KDM4A, KDM4B, and KDM4C, respectively [42-44].

Paradoxically, while KDM4 proteins appear to direct differentiation during embryogenesis, they also participate in maintaining the gene expression signa-

ture typical of undifferentiated stem cells. KDM4 proteins interact with, or prompt the expression of many pluripotency factors including Oct4, Sox2 and c-Myc, which together with Klf4, are sufficient to induce the reprogramming of differentiated cells to a pluripotent state [45]. KDM4A can induce expression of Oct4, which is required for the de-differentiation of adult neural stem cells to induced pluripotent stem (iPS) cells [46]. In undifferentiated human ES cells, KDM4C is conversely induced by Oct4 [19, 28, 39, 47]. Evidence also supports the participation of KDM4C in the Oct-4/Sox2/Nanog expression feedback loop... described by Wagner and Cooney [48]. When KDM4C expression is ablated, Oct-4, Sox2 and Nanog signalling is eliminated [47]. In this context, H3K9me3 demethylation by KDM4C directs the expression of pluripotency factors with critical implications in cellular reprogramming [39, 47]. Together, the interactions between KDM4 proteins with several other molecular regulators likely play important roles for directing stem cell functions during organismal development.

Regulatory factors of the KDM4 subfamily

Considering the significant biological functions of KDM4 proteins, it is not surprising that cells have developed various mechanisms for controlling their expression, activity and localization. Recent studies have revealed that the abundance of KDM4A in the cell can be regulated by the ubiquitination pathway. For example, KDM4A is mediated by two SCF complexes, SKP1-CUl1-F-Box and FBXO22, which control its turnover and ubiquitination during cell cycle progression [49, 50]. Furthermore, KDM4A and B, but not C or D, are also regulated by ubiquitination in response to DNA damage by the RNF8 and RNF168 complexes [51]. The Hsp90 molecular chaperone also interacts with, and stabilizes the KDM4B protein [52]. Pharmacological inhibition of Hsp90 with geldanamycin consequently leads to ubiquitin-dependent proteasomal degradation of KDM4B, but not KDM4C. A recent study also revealed that the JmjN domain of KDM4D is poly(ADP-ribosyl) ated by PARP-1, affecting its H3K9 demethylation function [53]. It is likely that KDM4A, B and C are regulated by PARP-1 in a similar manner, as the two glutamic acid residues predisposed to poly(ADP-ribosyl)ation are conserved in all KDM4 family members. Very recently, Burton *et al.,* revealed that inositol hexakisphosphate kinase 1 (IP6K1) also interacts with KDM4C and regulates its demethylation function [54]. Over-expression of IP6K1 induces KDM4C dissociation from chromatin and increases H3K9me3 levels [54].

Expression of KDM4B and C are also regulated by several transcription factors in physiological and/or pathological conditions. HIF1, a master regulator of cellular and systemic homeostatic response to hypoxia, can induce KDM4B and C expression in both normoxic and hypoxic conditions [55]. Interestingly, KDM4C selectively interacts with HIF1α, which mediates its recruitment to the HIF1α target gene response elements in breast cancer [56]. KDM4B is also an androgen-regulated demethylase, which can influence AR transcriptional activity not only via demethylation but also by modulation of AR ubiquitination [57]. KDM4B is further a direct transcriptional target of p53 [57].

To fine-tune epigenetic regulation, KDM4 proteins interact with each other as well as with protein complexes, such as those associated with transcriptional activity or DNA mismatch repair. All KDM4 proteins appear to have the capacity to form homodimers, though only KDM4A, B, and C form heterodimers [32]. KDM4 proteins also associate and demethylate non-histone protein substrates such as polycomb 2, the G9a methyltransferase and the chromodomain Y-like protein (CDYL1) [58, 59]. KDM4A, B, and C are known to participate in multiprotein complexes with members of the SWI/SNF chromatin-remodeling complex [36] and can interact with inhibitory complexes including histone deacetylases (HDAC1-3), N-CoR, or the pRb tumor suppressor [17, 40, 60]. Through these interactions, KDM4 demethylases are significant players in directing gene expression in development, homeostasis and disease.

Alterations and roles of the KDM4 subfamily in cancer

It is now well established that alterations in the expression of both methyltransferases and demethylases trigger the progression of cancer. Though only recently apparent, mounting evidence points to the role of histone demethylases in disrupting the proliferative balance, survival and metastatic potential of cells from multiple tissues. Many histone demethylases

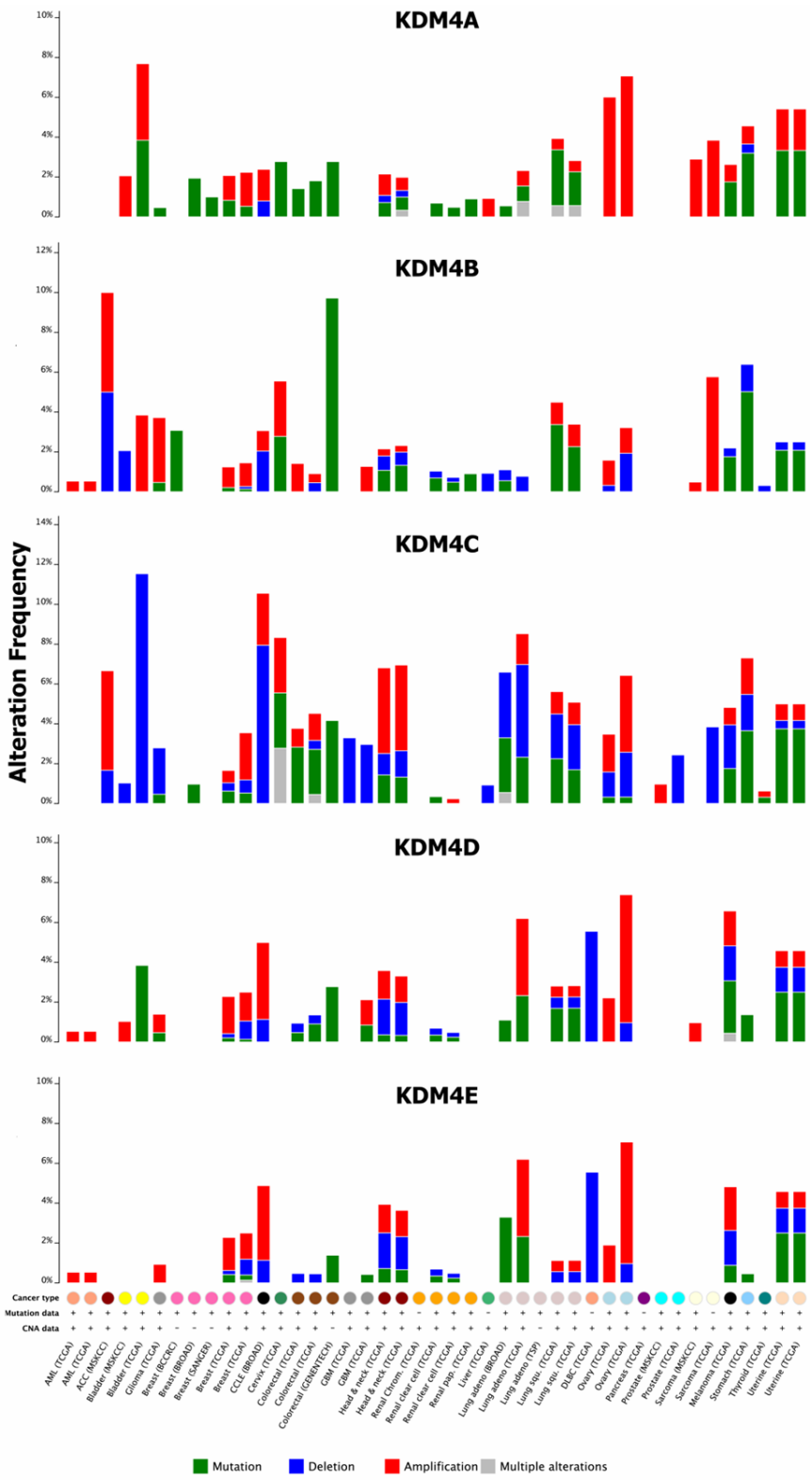

Figure 2. Alteration frequencies of *KDM4* subfamily genes identified in human tumors of multiple origins reported across 52 databases held in the Cancer Genomics cBioPortal [67, 68]. Alteration frequencies are displayed for each of four categories, including: genetic amplifications (red), deletions (blue), mutations (green) or multiple alterations (grey).

are dysregulated in cancer, where the effect is either to activate expression of oncogenes, repress expression of tumor suppressors, alter DNA mismatch repair, disrupt chromosomal stability, or interact with key hormonal receptors which control cellular proliferation [61-63]. Previous studies have demonstrated that *KDM4* genes are amplified and overexpressed in various tumor types, including lung, breast, esophageal, prostate cancers and lymphoma [28, 57, 64-66]. To establish a comprehensive profile of genomic alterations for *KDM4A-E* in human cancer, we conducted a large-scale meta-analysis of the genetic amplifications, deletions and mutations reported across 52 databases in the Cancer Genomics cBioPortal [67, 68]. An overview of this data reveals that *KDM4A-E* are altered across many tumor types (**Figure 2**). This data is complemented by a recent analysis of 4,934 cancer copy number profiles from The Cancer Genome Atlas (TCGA) Pan-Cancer data set, which has revealed significant amplifications of the *KDM4C* genomic region in human cancer cells [69]. The involveme-

nt of KDM4 proteins in cancer is further supported by findings of several independent research groups.

KDM4A

KDM4A amplification and overexpression is highly prevalent in ovarian cancer and in squamous cell carcinoma [6, 62]. More importantly, the overexpression of KDM4A in tumors specifically triggers highly localized chromosomal instability, consisting of site specific copy gains at 1q12, 1q21 and Xq13.1 [62]. *KDM4A* knockdown has been shown to not only impact cell growth but also metastasis *in vitro* and in mouse models [6]. KDM4A interacts with the activating protein 1 (AP1) transcription factors which control cell proliferation, apoptosis and differentiation [6]. KDM4A histone demethylation can induce the expression of AP1 genes including *JUN* and *FOSL1*, which promote cell growth and metastasis [6]. It also directly facilitates AP1 complex binding to *JUN* and *FOSL* promoters, creating a positive feedback loop which maintains AP1 activation. Furthermore, it is reported that KDM4A promotes cellular transformation by blocking senescence through transcriptional repression of the CHD5 tumor suppressor [70].

KDM4B

Of the demethylases that mediate nuclear receptor responsiveness in breast and prostate cancer, much is known about the role played by KDM4B. KDM4B is highly expressed in estrogen receptor (ER)-positive, aggressive subtypes and can be induced by the ER in an estrogen-dependent manner in breast cancer [36, 71]. KDM4B can bind to the ER, which together demethylate repressive H3K9me3 marks and recruit members of the SWI/SNF-B and MLL2 chromatin remodeling complexes to induce gene expression in an estrogen dependent manner [36]. Targets of the KDM4B-ER complex include not only oncogenic MYB, MYC and CCND1, which induce proliferation, but also the ER and KDM4B themselves, resulting in an activating feedback loop [71, 72]. Conversely, knockdown of KDM4B greatly inhibits estrogen dependent gene expression, and stabilizes p53 which halts breast tumor cell proliferation [73]. In prostate cancer cells, KDM4B expression, which positively correlates with the severity of cancer, can cooperate with the AR to induce the AR transcriptional response [57]. KDM4B also stabilizes the AR through inhibiting its ubiquitination and degradation. Knockdown of *KDM4B* results in a near complete depletion of AR protein levels [57]. Together, the interaction between KDM4B and nuclear receptors in prostate and breast cancers consist of major drivers that can dictate the aggressiveness of disease.

KDM4B also appears to contribute to metastasis and hypoxia. Overexpressed in colorectal cancer, KDM4B can induce expression of the plasma membrane signaling protein, PRL-3, which triggers lymph node metastasis [74]. KDM4B also promotes a pro-survival gene expression response in renal cancer cells through the accumulation of HIF1α [75]. Consequently, KDM4B mediates hypoxic conditions, frequently associated with highly proliferative and therapeutically refractory cancer cells.

KDM4C

KDM4C, also referred to as GASC1 (Gene Amplified in Squamous Cell Carcinoma), is overexpressed in numerous cancers including esophageal squamous cell carcinoma, breast and prostate cancers, medulloblastoma, metastatic lung sarcomatoid carcinoma, in primary mediastinal B-cell lymphoma and Hodgkin's lymphoma, and in acute myeloid leukemia [22, 28, 64, 65, 76-79]. In a high-resolution SNP analysis of 212 medulloblastoma genomes, KDM4C was among several histone modifying enzymes aberrantly expressed, specifically enriched in a significant 15% fraction of genomes [78]. Accordingly, high level chromosome 9 gains observed correspond to hypomethylation of H3K9 residues in medulloblastoma tumors, supporting the substantial role played by the methylome in aberrant gene transcription [76, 78]. Recurring evidence supports that *KDM4C* overexpression results from aberrant amplification of chromosome 9 at the 9p23-24 foci [65]. It is also aberrantly expressed as a fusion partner to the immunoglobulin heavy chain gene (IGH) in mucosa-associated lymphoma, following 9p translocation [66].

On a functional basis, KDM4C can act to promote tumorigenesis through several mechanisms. It activates expression of oncogenes

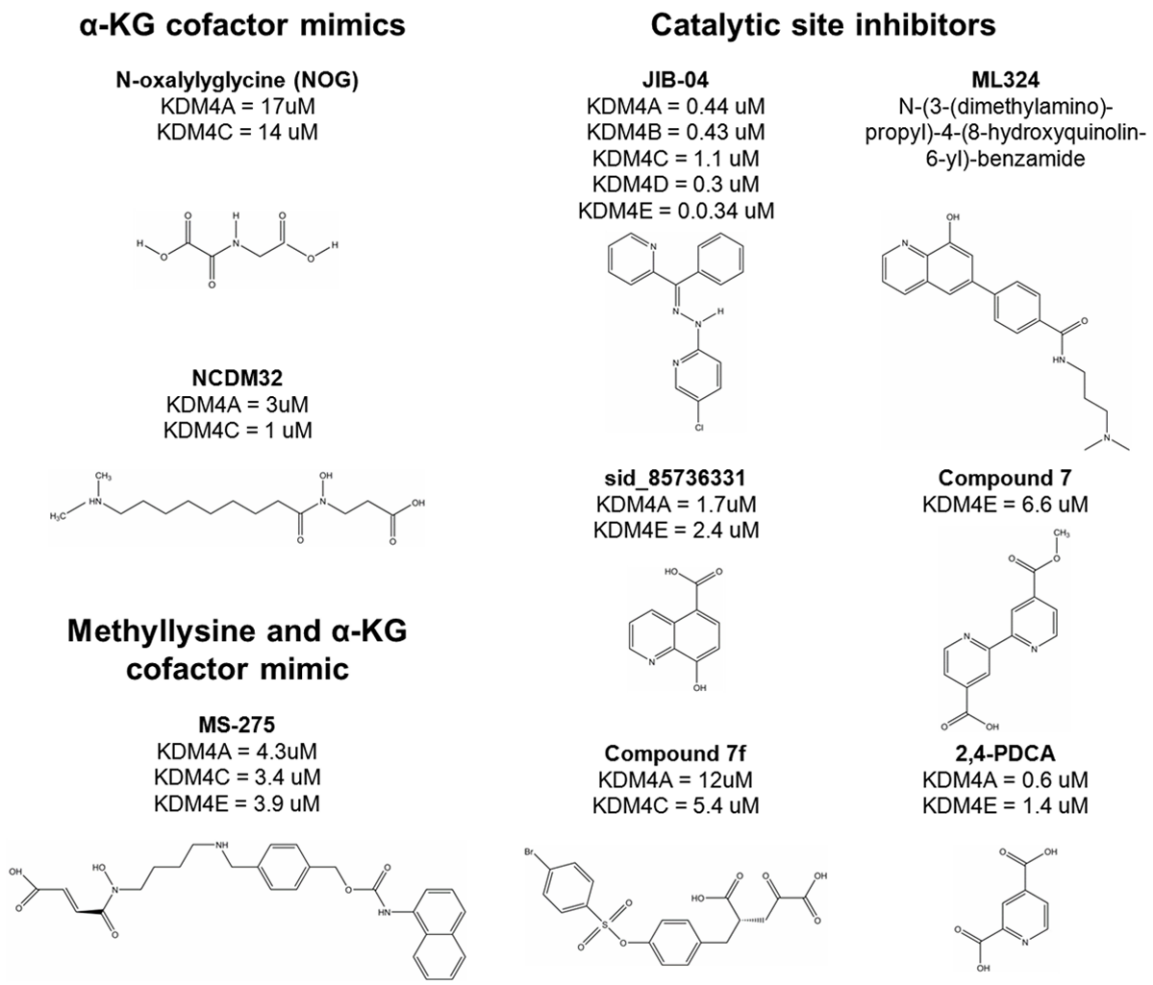

Figure 3. Chemical structure and half maximal inhibitory concentration (IC50) for representative KDM4 inhibitors.

such as MDM2, a regulator of p53, and binds to the AR to stimulate androgen dependent gene expression and tumour cell proliferation [80, 81]. In breast cancer, KDM4C amplification and overexpression are prevalent in aggressive basal-subtypes. Recent studies indicate that KDM4C is a transforming breast oncogene: stable KDM4C overexpression in non-tumorigenic cells induces transformed phenotypes, whereas KDM4C knock down inhibits tumor proliferation and metastasis [56, 82]. KDM4C overexpression also confers stem cell-like characteristics such as the ability to form mammospheres in culture and induces expression of NOTCH1, a pro-survival factor in breast cancer stem cells [65, 83]. Such KDM4C mediated genetic programs in cancer cells reiterate its functions in ES cells, supporting the hypothesis that it functions in establishing stem cell-like transcriptional programs in cancer cells [65].

KDM4D and KDM4E

In contrast to other KDM4 members, KDM4D and E are structurally divergent proteins, lacking both C-terminal PHD and Tudor domains, which may reason why no conclusive evidence exists of their contribution to cancer establishment or progression. However, as with KDM4A, KDM4D can interact with nuclear receptors such as the AR, suggesting it may function to regulate gene expression in tissues such as the prostate [84]. The mechanism of KDM4D binding to the AR is distinct from KDM4A, which binds at its C-terminus. Yet, the roles of KDM4D and E in cancer remain unclear and require further investigation.

KDM4 subfamily in other diseases

Beyond the role of KDM4 proteins in cancer, their dysregulation can severely disrupt normal

cellular functions in other diseases [73, 85]. Aberrant KDM4A expression has been linked to cardiac failure, cardiac hypertrophy, to the progression of viral infections, as well as disorders such as alopecia areata [29, 86-88]. SNPs in KDM4C genes are also associated with autism and alcohol withdrawal symptoms [89, 90]. Together, these instances demonstrate the breadth of KDM4 protein functions in establishing disrupted gene expression programs.

KDM4 inhibitors

Considering the significant implication of KDM4 demethylases in the development of various diseases, a thorough understanding of their molecular mechanism and effective therapeutic inhibition is of considerable interest. On the basis of the three-dimensional structures available and studies of their catalytic mechanisms, a number of KDM4 inhibitors have been identified and reported. These inhibitors can be categorized into three major groups: α-KG cofactor mimics and disruptors, metal cofactor mimics, as well as histone substrate analogs (**Figure 3**). Here, we describe the historical development of KDM4 inhibitors and describe novel molecules recently proven to have good efficacy and specificity in both biochemical and cellular assays.

α-KG cofactor mimics and disruptors

The vast majority of KDM4 inhibitors currently consist of α-ketoglutarate (α-KG) or 2-oxoglutarate (2-OG) cofactor competitive inhibitors which bind the iron Fe(II) molecule in the catalytic site (reviewed in [91]). All JmjC-domain containing demethylases require α-KG as a cofactor in the demethylation reaction. Thus, α-KG cofactor mimics appear to inhibit multiple members of the histone lysine demethylases. Hamada *et al.* first explored the inhibitory potential of α-KG analogues including N-oxalylglycine (NOG) and subsequently presented hydroxamate analogues such as NCDM32, which has a 500 fold better KDM4C inhibitory activity compared to NOG (**Figure 3**) [92, 93]. Other KDM4 subfamily cofactor disruptors include the α-KG analog 2,4-pyridindicarboxylic acid (PDCA), the PDCA derivative, compound 15c and a 4-carboxylate containing 2,2-bipyridyl derivative compound 7 [94, 95]. Following report on these inhibitors, Rose *et al.* used crystallographic analyses to discover a sub pocket within the KDM4 active site which was significantly larger and more open than in other oxygenases [96]. This sub pocket also extends into the substrate binding groove. Accordingly, a series of N-oxalyl-D-phenylalanine derivatives, thought to occupy this sub-pocket, were developed with the intention of selectively inhibiting KDM4 proteins among all cellular oxygenases. This effort led to the identification of molecules such as compound 7f (**Figure 3**).

In addition to these inhibitors, compounds structurally unrelated to α-KG were also found to bind and inhibit the KDM4 catalytic site. Among 236,000 compounds assayed in a high throughput screen by King *et al.,* were 8-hydroxyquinoline derivatives such as sid_85736331 [97]. Further cellular assays confirmed that these compounds potently inhibited H3K9 demethylation in HeLa cells. Within this novel class of inhibitors, Liang *et al.* showed that the related compound, ML324 effectively inhibited intermediate early viral gene replication mediated by KDM4A in herpes virus infected cells [88, 97]. These experiments stand as proof of principle for the development of therapeutically active compounds against KDM4 proteins *in vivo*.

Metal cofactor disruptors

Disruption of iron and zinc cofactors also inhibits KDM4 protein catalytic activity, and can be accomplished by both non-iron metals and organic molecules. Non-iron metals such as nickel have the potential to disable the catalytic activity of KDM4A and C through occupancy of the iron binding pocket [98]. Structural and bioinformatics analyses have also revealed a Zn(II) Cys3-His binding site in the KDM4A catalytic domain, which is absent in other α-KG dependent oxygenases [94]. In KDM4A, the $Zn2^{+}$ ion, required for its catalytic activity, is specifically ejected through the binding of disulfiram, and ebselen [94]. These metal cofactor disruptors offer an alternative inhibitory mechanism which may be used to selectively target KDM4 demethylases.

Histone substrate competitive inhibitors

Thus far, few methyllysine histone substrate mimics have been designed or tested, with the exception of WAG-003 and a derivative of the well characterized histone deacetylase (HDAC)

inhibitor, MS-275 [99, 100]. WAG-003 is a Tudor domain inhibitor analogous to the antiarrhythmic drug amiodarone, which modestly inhibits KDM4A *in vitro*. The MS-275 derivative, in contrast, was designed as a methyllysine cofactor mimic linked to an α-KG mimic, inhibiting both key sites of KDM4 proteins (**Figure 3**). *In vitro* assays have demonstrated that while this molecule and its prodrug, methylstat, potently inhibit KDM4A, C and E, its inhibition of non-target oxygenases is much weaker [100]. Thus, the development of comparable dual targeting molecules has the advantages of disrupting multiple KDM4 domain functions while offering good selective inhibition.

To date, only one other structurally distinct KDM4 subfamily inhibitor, JIB-04, identified in an unbiased cellular screen, effectively and specifically inhibits KDM4 activity *in vivo* as well as *in vitro*. In biochemical assays, JIB-04 potently inhibited the catalytic activity of KDM4 member proteins including KDM4A, B, C and E [101]. Furthermore, JIB-04 has an unprecedented capacity to specifically inhibit KDM4 protein function in cancer cells, as well as in tumors *in vivo* [101]. JIB-04 is not a competitive inhibitor of α-KG, and the exact molecular mechanism is unclear. Yet, JIB-04 does not appear to affect the function of other α-KG-dependent enzymes, nor alter transcriptional growth programs in normal cells. As such, this inhibitor stands as an important breakthrough in the field of epigenetic drugs research, which will likely serve as a model in the development of analogs with excellent *in vivo* potency and specificity.

Conclusions

KDM4 demethylases function extensively in multiple cellular events throughout organismal development and homeostasis. Despite the recent discovery that the KDM4 subfamily plays an essential role in regulating gene expression and chromatin architecture via H3K9 and H3K36 demethylation, there is still much to learn about how KDM4 proteins are recruited to genomic loci, how they modulate histone demethylation and subsequently activate specific downstream targets in different cell types. Moreover, it is clear that KDM4 proteins cooperate in similar macromolecular complexes and processes, yet the redundancies and interactions between them are still not well understood. Considering the enormous potential of these epigenetic master regulators in modulating gene transcriptional programs, it is not surprising that their alterations are implicated in human diseases, particularly in cancer. However, the molecular mechanisms by which KDM4-dependent chromatin regulation translates into oncogenicity and cancer progression remain poorly understood. Thus, deeply understanding the biology and mechanism of KDM4 demethylases will be a significant component of future research.

Considering that epigenetic changes are reversible and histone demethylases are druggable, KDM4 proteins are promising therapeutic targets. However, one caveat remains that most KDM4 inhibitor scaffolds are borrowed from studies of structurally or mechanistically related enzymes and are often also active against related non-target proteins. In addition, most inhibitors are cofactors and/or substrate mimics and so far have only very limited or undetermined specificity for the KDM4. It is thus anticipated that the next decade of KDM4 demethylase research will intensely focus on developing specific and effective small molecule inhibitors for experimental and therapeutic applications.

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Disclosure of conflict of interest

None.

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