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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 used genomic analysis of copy number and gene expression to perform a detailed analysis of the 8p11-12 amplicon for identifying candidate oncogenes in breast cancer. We identified Wolf-Hirschhorn syndrome candidate 1-like 1 (WHSC1L1) as a candidate oncogene 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 nontumorigenic 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 recently been shown to bind methylated histones, specifically H3K36 methylation marks. Thus WHSC1L1 may contribute to transformation through the alteration of epigenetic histone marks in a subset of aggressive breast cancers. We published 2 research papers and 1 abstract based on this award in the past year.

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

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Introduction

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 (1-5). Earlier, we used genomic analysis of copy number and gene expression to perform a detailed analysis of the 8p11-12 amplicon for identifying candidate oncogenes in breast cancer (4). We identified Wolf-Hirschhorn syndrome candidate 1-like 1 (WHSC1L1) as a candidate oncogene based on statistical analysis of copy number increase and overexpression (4). The WHSC1L1 gene encodes a PWWP domain protein that regulates gene transcription and differentiated function of cells through regulation of histone methylation (6, 7). In this proposal, we hypothesize that WHSC1L1 is the major driving oncogene in the 8p11 amplicon that is found in aggressive forms of ER positive, luminal breast cancers. Furthermore, we hypothesize 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 are involved in 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 are involved in transforming function through the alteration of the epigenetic histone code in human breast cancer cells**. *Month 1-16*

In our previous report, we demonstrated that we identified 21 candidate oncogenes within the 8p11-12 amplicon in breast cancer based on statistical analysis of copy number increase and gene overexpression. Using gain- and loss-of- function approaches, we found that WHSC1L1 is the most potently transforming oncogene we tested from the 8p11-12 region. 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 methytransferase domain. The short isoform encodes a 645 amino acid protein containing only a PWWP domain. Importantly, we found that amplification and overexpression of the WHSC1L1 short isoform was predominant in a subset of aggressive breast cancers, suggesting an important role for the short isoform of the protein in breast cancer development.

PWWP proteins, including WHSC1L1, constitute a new family of methyl lysine histone binders. Recently, the PWWP domain has been identified to bind trimethylated Lys36 on histone H3 (8, 9). Thus, WHSC1L1 likely involves the regulation of epigenetic methylation on histone tails. In our previous report, we detailed that we performedexpression profiling and identified a large set of genes with altered expression upon WHSC1L1 knockdown in breast cancer cells. To determine WHSC1L1 binding sites across the human genome, and to identify the

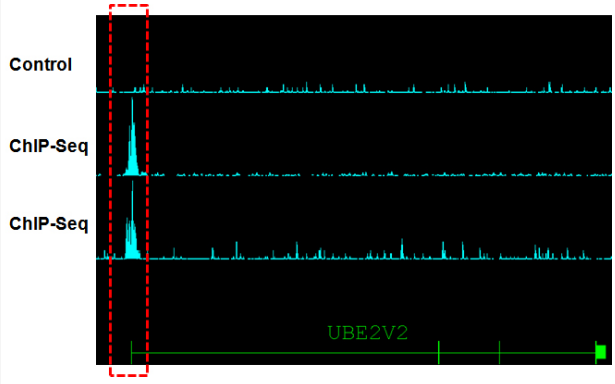

Figure 1. Representative view of ChIP-seq peak of a histone modifying factor at the UBR2V2 genomic locus in the Affymetrix Integrated Genome Browser.

direct target genes of WHSC1L1 short isoform, we expected to profile the genome-wide occupancy of WHSC1L1 in an unbiased manner by using chromatin immunoprecipitation (ChIP)-on-chip or ChIP-sequencing (ChIP-Seq) assays. To successfully establish ChIP assays and ensure ChIP efficacy, especially given the absence of good commercially available ChIP-grade antibodies to most histone-modifying factors, including WHSC1L1, we chose to perform the ChIP with an anti-V5 ChIP-grade antibody (Abcam, #ab9116) in MCF10A model cells stably expressing the V5-tagged histone-modifying factors [60, 61]. Briefly, cells were fixed with formaldehyde, harvested, and rinsed with ice-cold PBS. The resultant cell pellet was sonicated, and DNA fragments were enriched by immunoprecipitation with ChIP-grade anti-V5 antibody. The DNA samples obtained from ChIP were subjected to chip and sequence analysis. As shown in Fig. 1, we demonstrated that ChIP assays with the anti-V5 ChIP-grade antibody can identify specific ChIP peaks (bound regions) of the histone-modifying factor (unpublished data). We further confirmed the ChIP-on-chip and ChIP-seq data with the conventional ChIP-PCR assays. Thus, we demonstrated that ChIP assays with anti-tag antibodies in MCF10A models are feasible. We initiated the ChIP assay in MCF10A cells stably expressing the V5-tagged WHSC1L1 short isoform. Unfortunately, our first ChIP-on-chip assay did not show the specific ChIP peaks in MCF10A-WHSC1L1 model cells. During the no-cost extension period, we will continue to test and modify the experimental condition for the ChIP assays using MCF10A-WHSC1L1 models cells. Alternatively, we will test commercially available WHSC1L1 antibodies for the ChIP assay in our WHSC1L1-amplified breast cancer cells. Since WHSC1L1 family proteins have been identified to modify H3K36 methylation, we will modify our original plan in which we proposed to test the association between WHSC1L1 expression and H3K4- and H3K27-methylation levels to H3K36 methylation in breast cancer cells in the no-cost extension period.

Task2. **To determine whether the histone modulation function of WHSC1L1 is linked to cancer stem cell phenotypes**. *Months 12-30*

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 (10-12). 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 (13). In the sphere formation assay, cells are plated at a clonal density so that individual cells will form spatially distinct spheres (10, 14). To determine whether overexpression of WHSC1L1 enhances the colony-formation *in vitro*, we seeded MCF10A cells stably expressing the WHSC1L1short isoform, and control cells in soft agar plates. As shown in Fig. 2, MCF10A cells overexpressing WHSC1L1 short isoform grew into robust colonies in soft agar, a property not observed

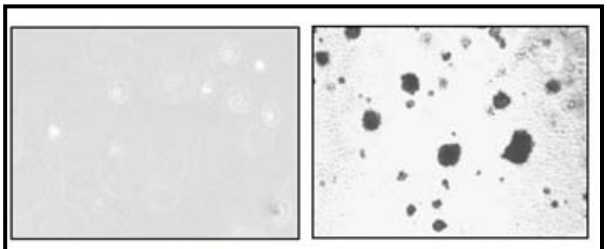

Figure 2. Representative pictures of MCF10A cells that stably over express WHSC1L1 short isoform and control cell soft agar colonies. Cells were grown for 3 weeks in soft agar and stained with the vital dye p-iodonitrotetrazolium violet.

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 (15, 16). ALDH can be assessed by the Aldefluor assay to detect cells displaying aldehyde dehydrogenase activity (Stem Cell Technologies, Inc). As demonstrated in previous reports, we have successfully detected ALDH expression by using an immunohistochemistry staining assay in breast cancer cells with high cancer stem-like cell population. 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 short isoform likely, or 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.

***Task 3*. To examine the potential of WHSC1L1 as a therapeutic target in aggressive, ER-positive breast cancers that harbor the 8p11 amplicon.** *Months 18-36*

In this task, the loss-of-function approach with the lentirviral vector-based RNAi specifically targeting WHSC1L1 was performed to investigate the contribution of endogenous WHSC1L1 overexpression on the expression of transformed phenotypes in the luminal breast cancer cells with 8p11-12 amplification. To perform RNAi knock-down experiments, we obtained eight pGIPZ-WHSC1L1 shRNA expression constructs from OpenBiosystems. (http://www.openbiosystems.com/). We identified the two most efficient shRNAs with respect to knockdown of WHSC1L1 expression levels in WHSC1L1 amplified breast cancer cells. Quantitative RT-PCR and Western blot data revealed that the

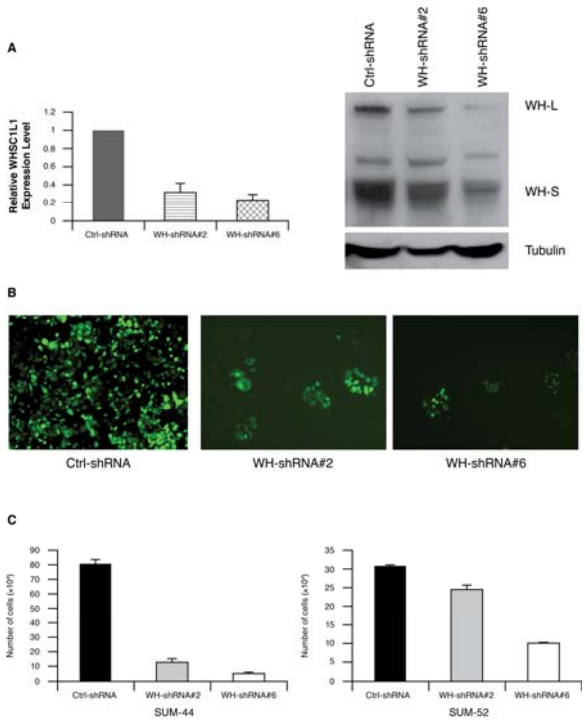

Figure 3. (A) WHSC1L1 expression in SUM-44 cells was analyzed by semiquantitative RT-PCR and western blot after infection with non-silencing control shRNA or WHSC1L1 specific shRNA (shRNA#2 and #6). (B). The images show the TurboGFP fluorescence of pGIPZ-WHSC1L1 shRNAs in SUM-44 cells after 3 weeks.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

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. 3). WHSC1L1 knock-down with these shRNA#2 and #6 suppressed proliferation of WHSC1L1 amplified breast cancer cell lines, including aggressive, ER-positive SUM-44 and SUM-52 lines, while WHSC1L1 shRNAs had an undetectable effect on the cell growth of WHSC1L1 non-amplifed breast cancer cells, as well as MCF10A control cells. In addition, recently published data from the other lab also indicated that knockdown of WHSC1L1 inhibits cell growth of the 8p12 amplified, ER-positive breast cancer cells (17).

Remaining work for no-cost extension: Very recently, WHSC1L1 family proteins have been shown to bind methylated histones, specifically H3K36 methylation marks. Thus, chromatin immunoprecipitation-sequence (ChIP-seq) and ChIP-PCR experiments are required to determine the WHSC1L1 binding sites across the human genome and identify the direct targets of WHSC1L1. We need to examine the correlation between genome-wide WHSC1L1 binding and histone H3K36 methylation marks in normal and cancer cells. Furthermore, we will determine if overexpression of WHSC1L1 in MCF10A cells results in expansion of cell pools with other stem cell-surface markers.

Key Research Accomplishments

In the present study, 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 nontumorigenic 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 successfully established ChIP-seq and ChIP-PCR assays with anti-tags antibodies in MCF10A models. During the course of these studies, we found a high frequency of genetic alterations in histone-modifying genes, including GASC1, UHRF2 and KDM5A, in breast cancer. We further demonstrated that KDM5A plays an important role in mediating both transforming and drug resistance phenotypes in a subset of aggressive breast cancer (Appendices).

Reportable Outcomes

Abstracts:
"Oncogenic role of PWWP-domain protein WHSC1L1 in breast cancer" DOD BCRP Era of Hope Meeting, August 2-5, 2011, Orlando, Florida.

Manuscript:
"Identification and functional analysis of 9p24 amplified genes in human breast cancer" Oncogene. 2012. 31:333-41. (see Appendices)
"Genomic amplification and a role in drug-resistance for the KDM5A histone demethylase in breast cancer" American Journal of Translational Research, Accepted (see Appendices)

Conclusion

In the past year, we continued to investigate whether WHSC1L1 is the major driving oncogene in the 8p11-12 amplicon in a subset of breast cancer, and how over-expression of WHSC1L1 is linked to transforming and cancer stem cell phenotypes. 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. The WHSC1L1 protein is involved in histone code modification and epigenetic regulation of gene expression. We have established ChIP-sequencing assays that will be used to determine how WHSC1L1 contributes to its transformation through the alteration of epigenetic histone marks in breast cancer cells.

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Appendices

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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.

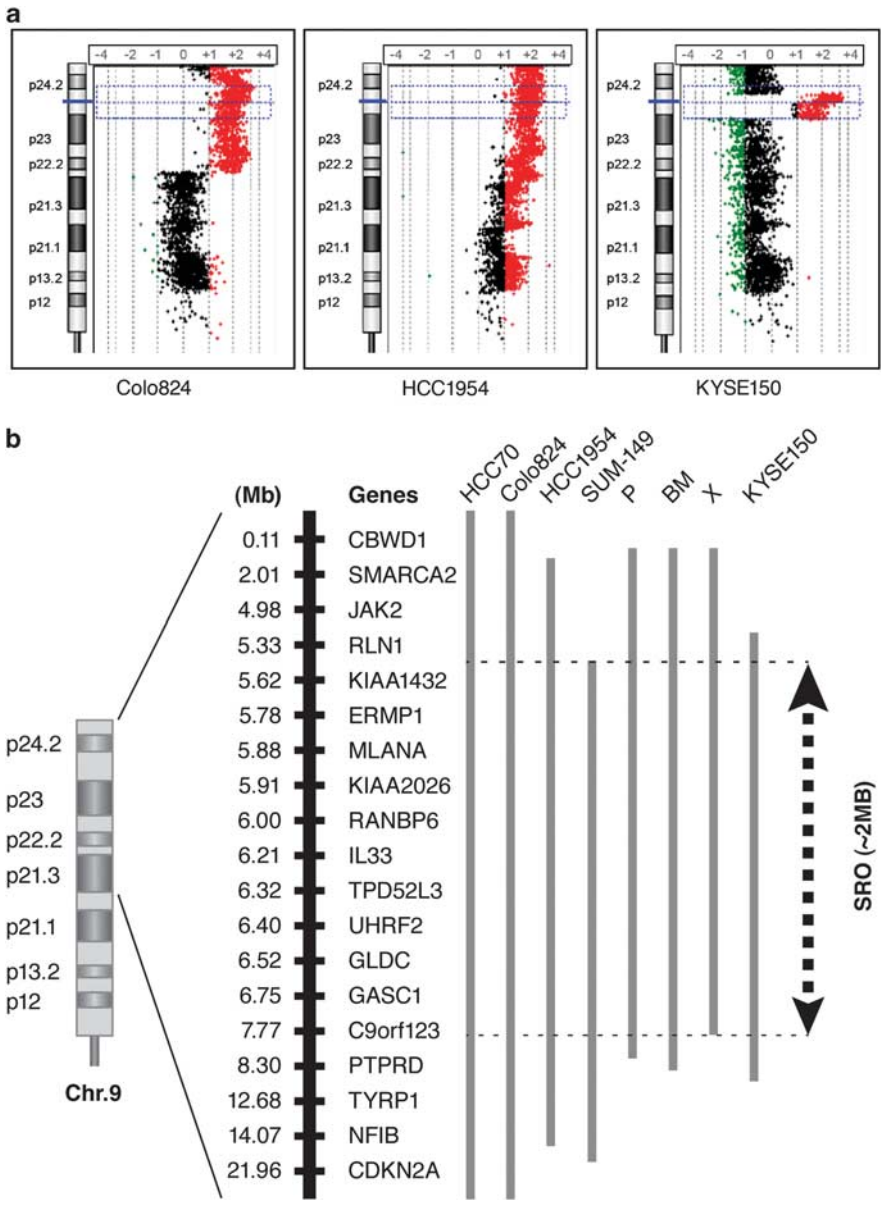

Figure 1 Genomic analysis of the 9p24 region in human cancer cell lines. (**a**) Genome view of chromosome 9p analyzed on the Agilent oligonucleotide array (Agilent Technology) in Colo824, HCC1954 and KYSE150 cells. (**b**) Schematic representation of the 9p24-amplified region in four breast cancer cell 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-

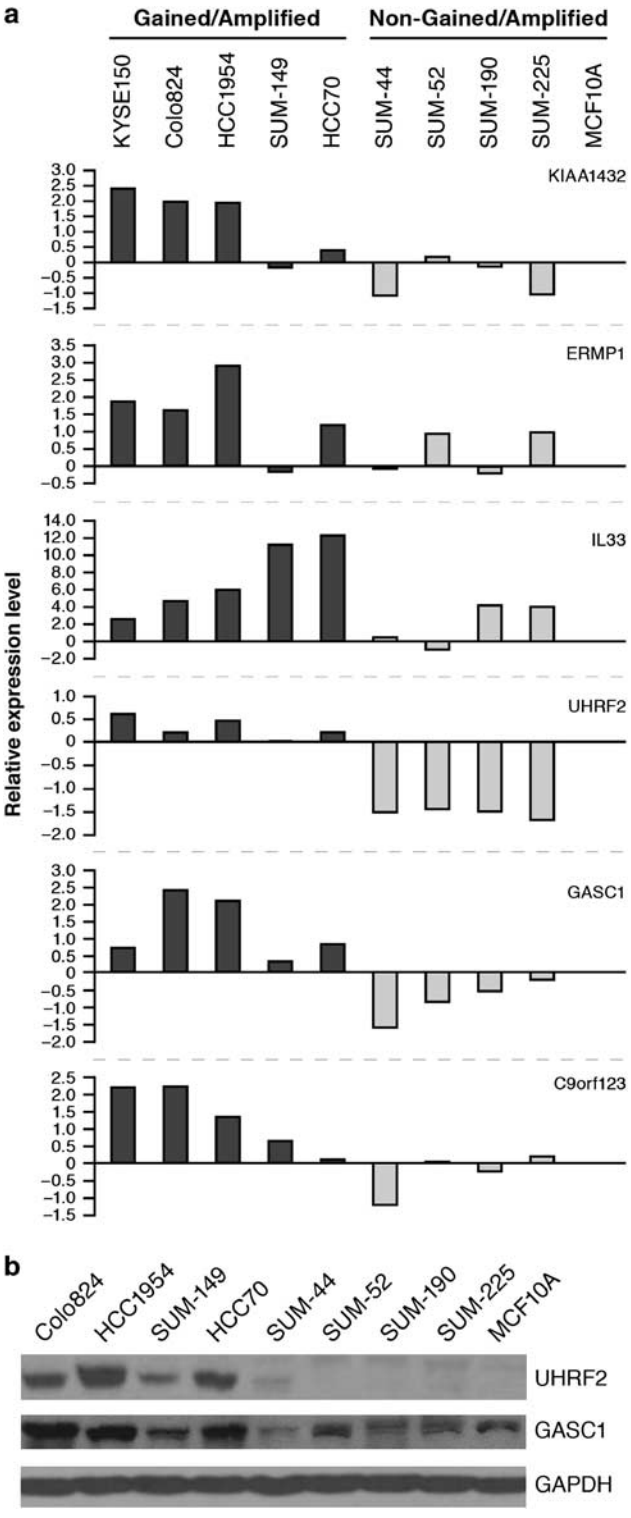

Figure 2 (**a**) Expression level of six genes in the 9p24 amplicon. Gene expression was examined in cancer cells with 9p24 gain/amplification (KYSE150, Colo824, HCC1954, SUM-149 and HCC70) or without the gain/amplification (SUM-44, SUM-52, SUM-190 and SUM-225). mRNA expression levels in the MCF10A cells, 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.

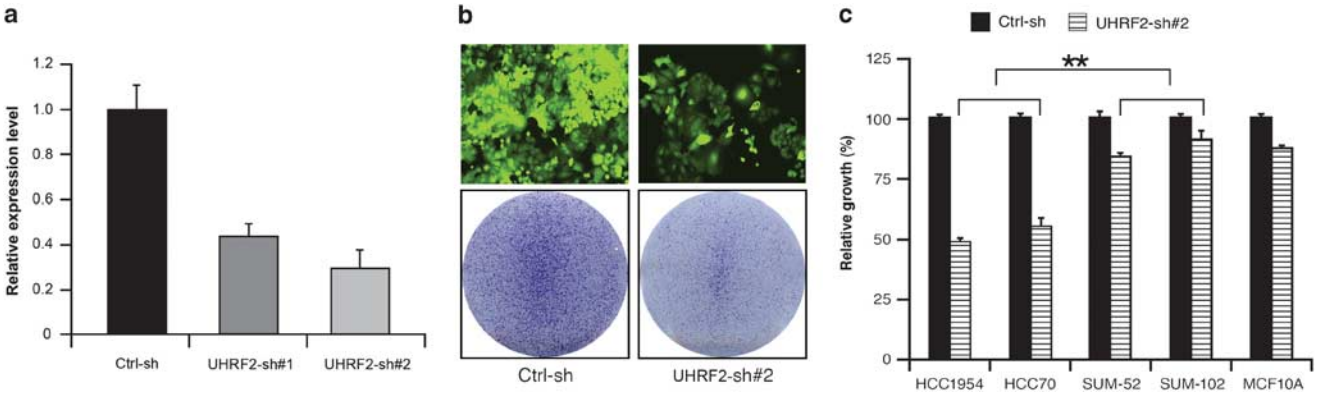

Figure 3 Effect of UHRF2 knockdown on cancer cell growth. (**a**) Knockdown of UHRF2 mRNA in HCC1954 cells with two different shRNAs was confirmed by real-time RT–PCR. The real-time RT–PCR data were normalized with a GAPDH control and is shown as the mean ± s.d. of triplicate determinations from two 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 (**$P<0.01$).

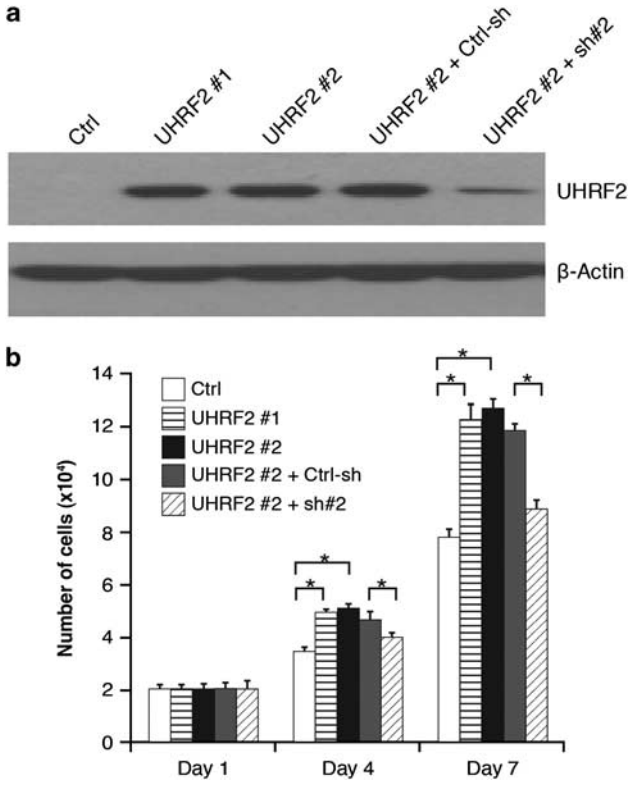

Figure 4 (**a**) Stable overexpression of UHRF2 in MCF10A cells with the pLenti6/V5-UHRF2 construct (MCF10A-UHRF2). Overexpression of UHRF2 protein in two cell clones (UHRF2#1 and UHRF2#2), and knockdown of UHRF2 in clone #2 cells were confirmed by western blot. (**b**) Ectopic overexpression of UHRF2 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

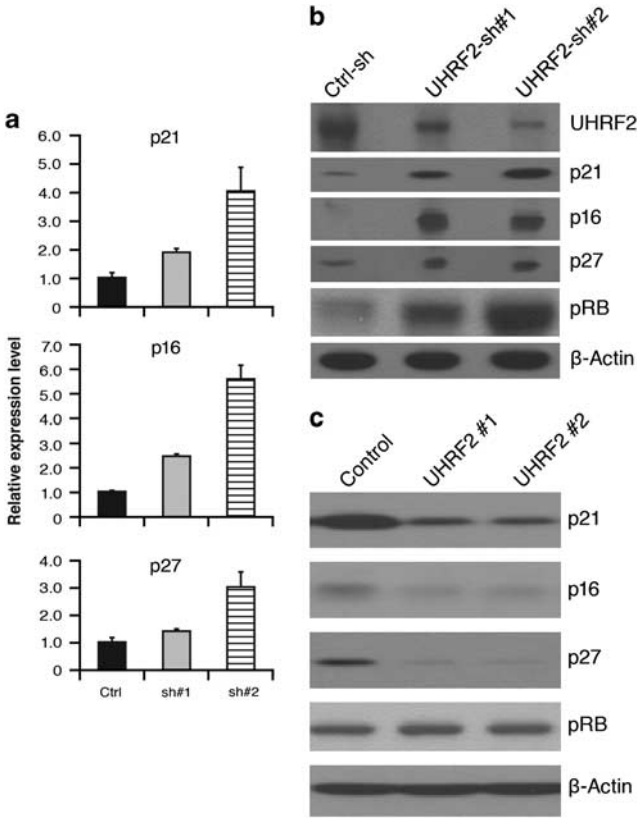

Figure 5 UHRF2 influences expression of $p16^{INK4a}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and pRB. (**a**) mRNA levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$ and $p27^{Kip1}$ were examined by real-time RT–PCR after knocking down UHRF2 in HCC1954 cells. The baseline for the cells infected with control shRNA 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.

Acknowledgements

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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.

Key words: 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 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 a further 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).

Immnuoblotting 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 Singnaling #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: Colo824, ZR-75-1, HCC1937, HCC1428, SUM-149, HCC3153, HCC2185, HCC1187 and HBL100. To obtain further support for the involvement of the 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 tumors, 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 short hairpin RNA (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 over-expression. We found that HCC1937 and SUM149 cells (with KDM5A amplification) exhibited significantly higher EGFR inhibitor IC_{50} values as compared with SUM102 and MCF10A cells (without KDM5A amplification), although all cell lines expressed high-levels of EGFR protein (Figure 3a and data no 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 with 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 [28]. 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 upregulation of CDK inhibitors and genes mediating apoptotic cell death.

Because KDM5A is the key histone demethylase 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 no 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 no 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 reistance phenotypes associated with KDM5A amplification and over expression [19]. To validate these array-based observations, we examined 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 development 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 knock-down 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 the 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 no 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, indicates 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 up-regulation 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 reistance, our findings suggest that BAK1 migh 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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Competing interests

The authors declare that they have no competing interest.

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Figure Legend

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.

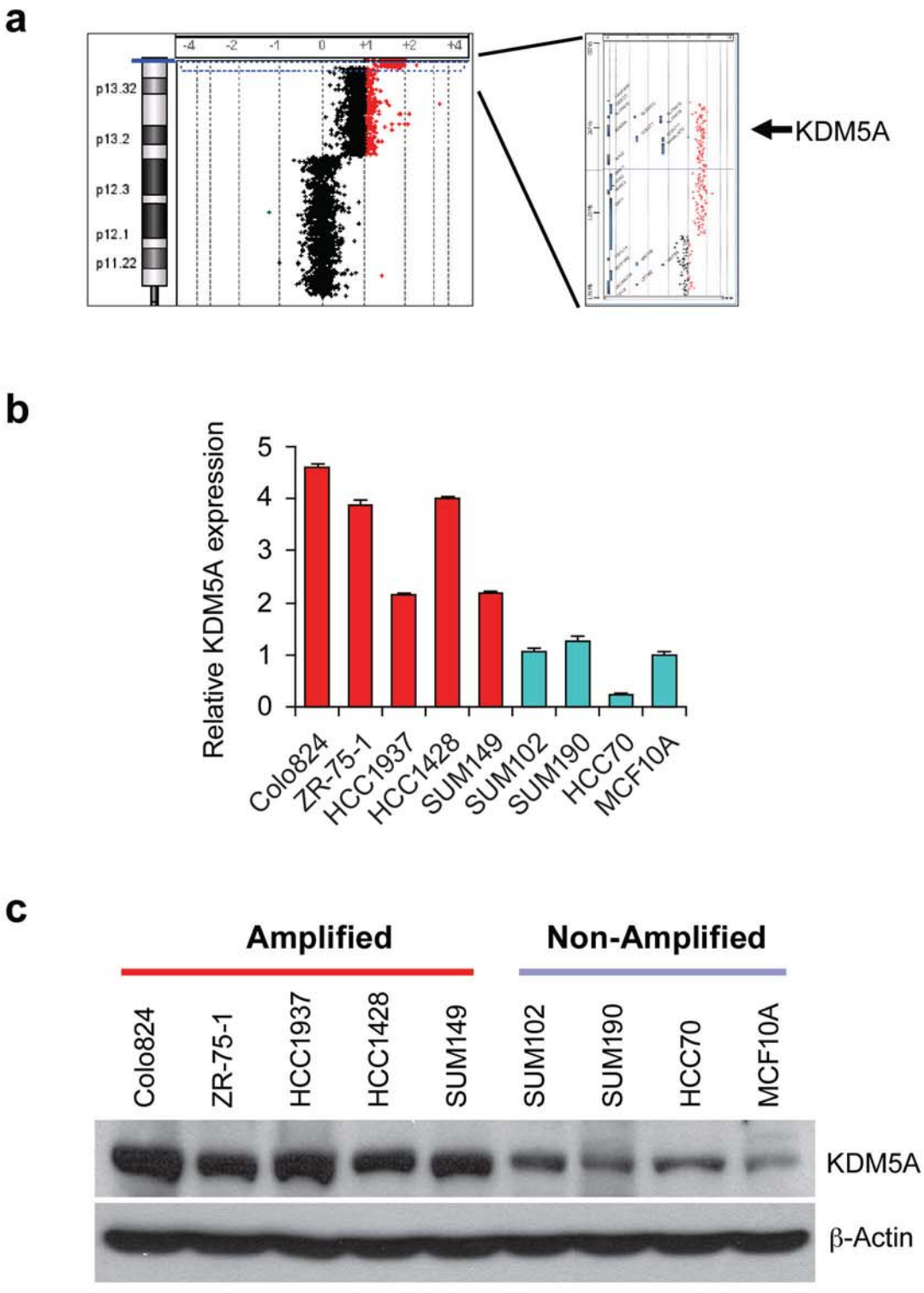
Figure 2. Reducing KDM5A expression by shRNA knockdown resulted in decreased cell proliferation and colony formation in soft agar. (a) Knockdown of KDM5A in four breast cancer cell lines with two different shRNAs was confirmed by Western blot assays. (b) shRNA-mediated knockdown of KDM5A inhibits cell 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 $\pm$ 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 $\pm$ 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 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 indicated concentration erlotinib for 30 days. Cell counting was shown as the mean $\pm$ 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 upregulation 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 was 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.

Figure 1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-2
-1
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+1
+2
+4
p13.32
p13.2
p12.3
p12.1
p11.22
KDM5A
b
Relative KDM5A expression
0
1
2
3
4
5
Colo824
ZR-75-1
HCC1937
HCC1428
SUM149
SUM102
SUM190
HCC70
MCF10A
c
Amplified
Non-Amplified
Colo824
ZR-75-1
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SUM102
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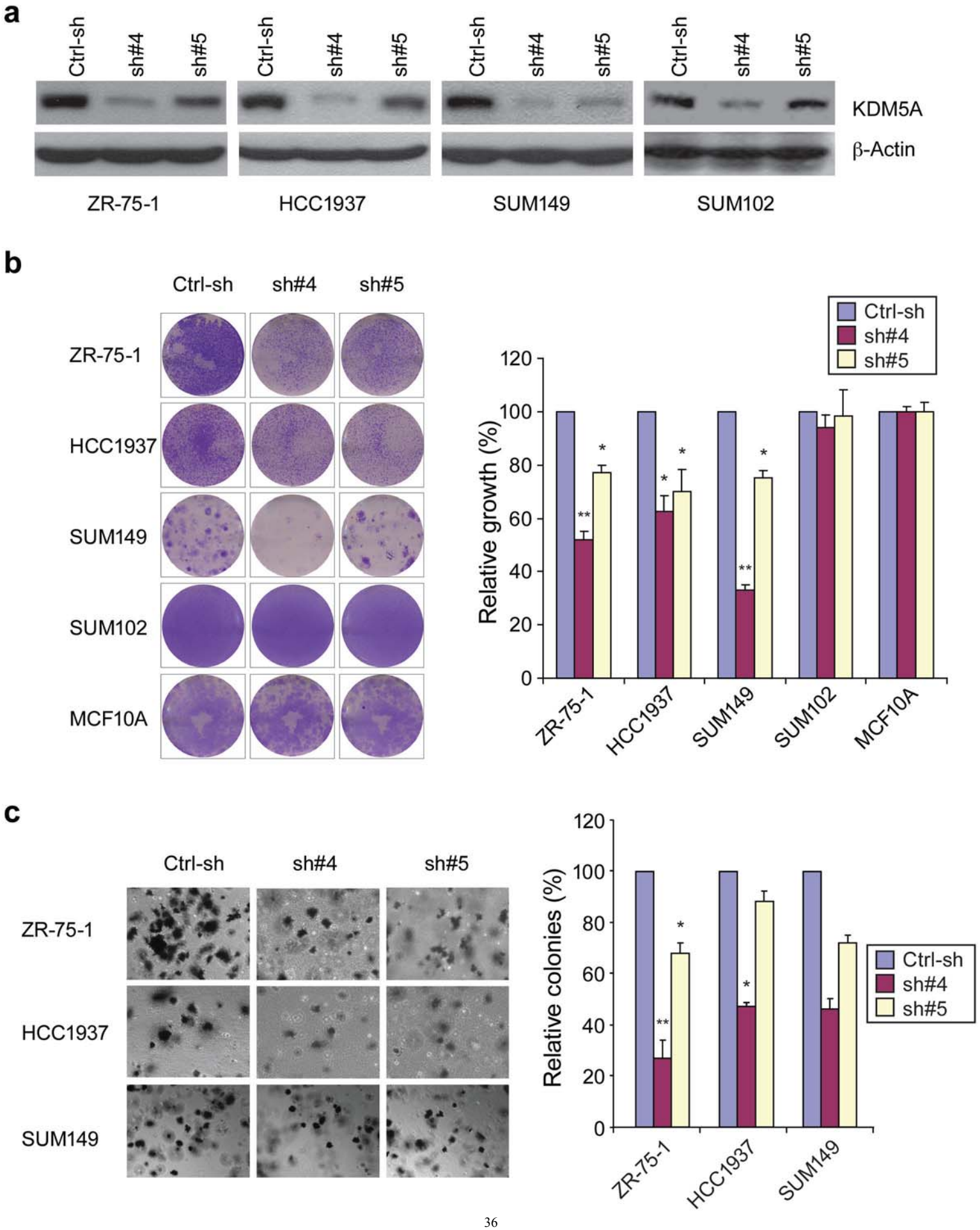
HCC70
MCF10A
KDM5A
β-Actin

Figure 2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KDM5A
β-Actin
ZR-75-1
HCC1937
SUM149
SUM102
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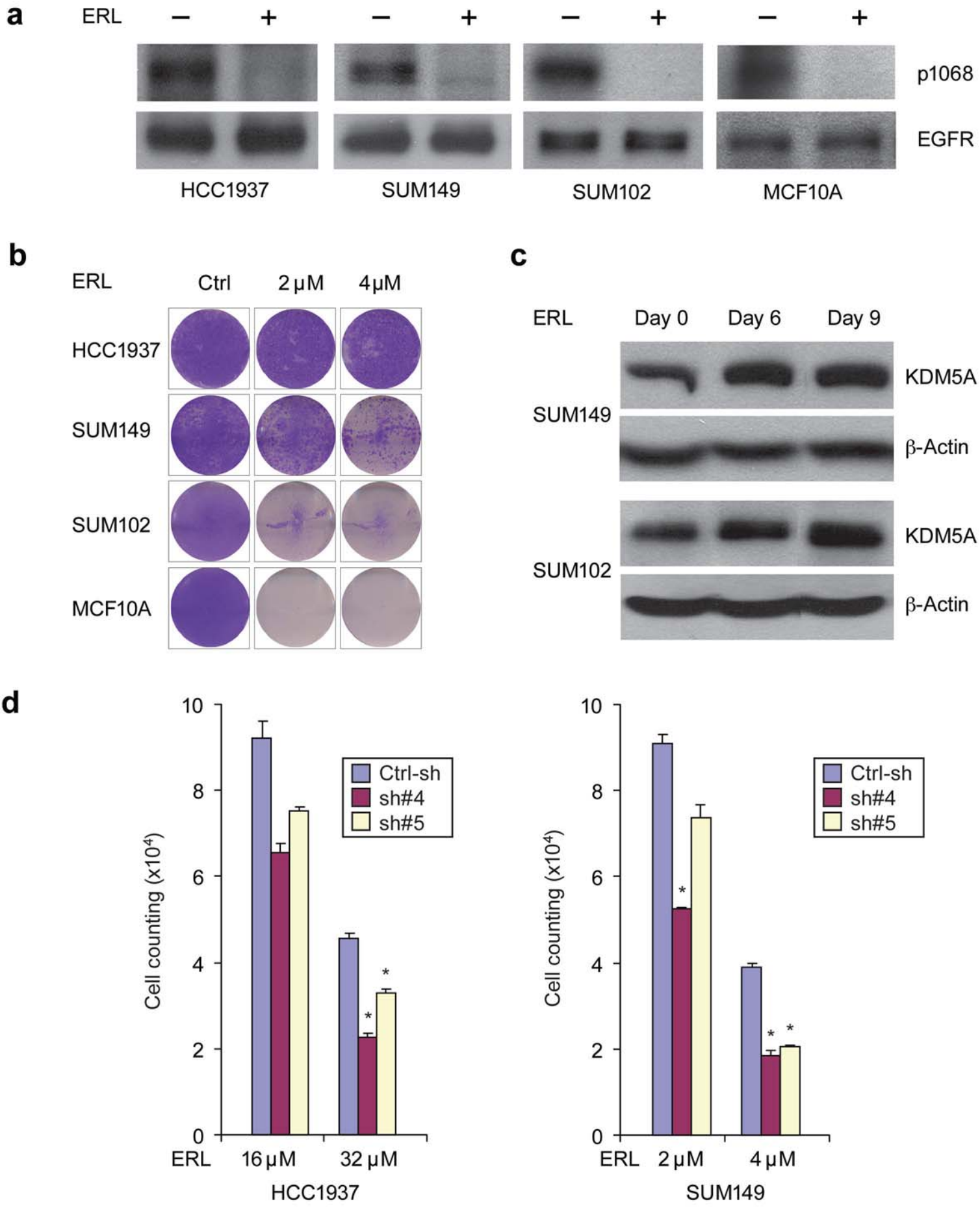
Ctrl-sh
sh#4
sh#5
ZR-75-1
HCC1937
SUM149
SUM102
MCF10A
Relative growth (%)
120
100
80
60
40
20
0
**
*
c
Relative colonies (%)

Figure 3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ERL
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+
–
+
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+
–
+
p1068
EGFR
HCC1937
SUM149
SUM102
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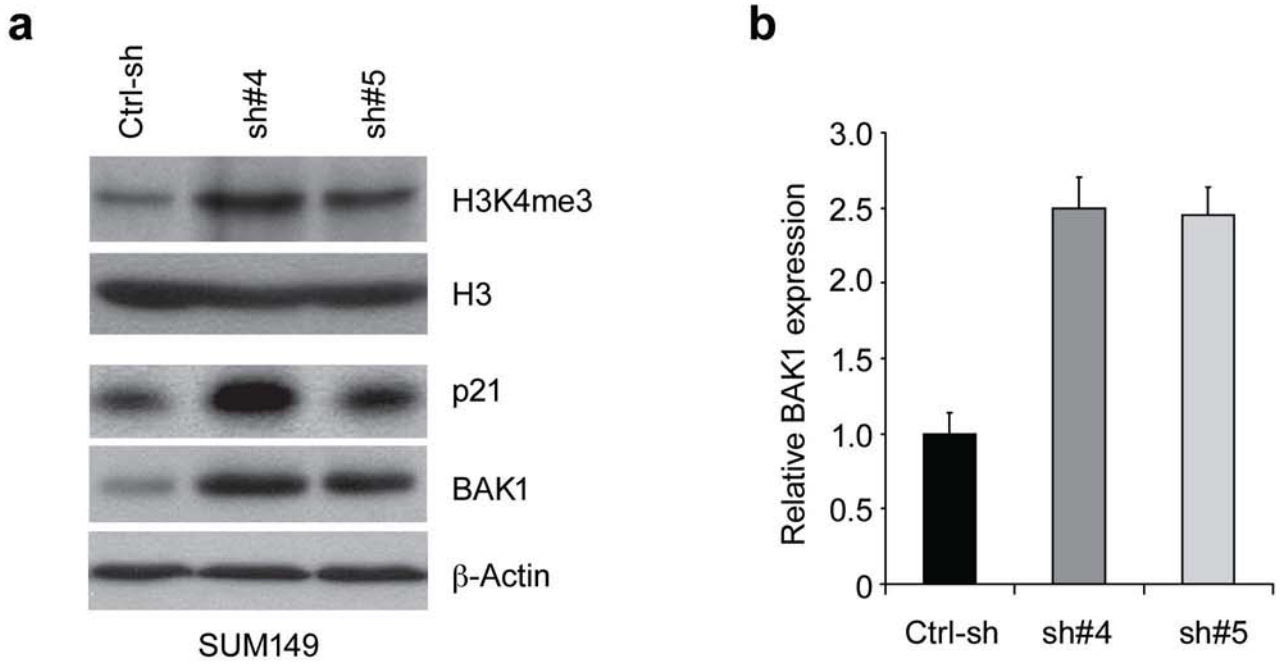
b
ERL
Ctrl
2 μM
4 μM
HCC1937
SUM149
SUM102
MCF10A
c
ERL
Day 0
Day 6
Day 9
KDM5A
SUM149
β-Actin
KDM5A
SUM102
β-Actin
d
Cell counting (x10⁴)
Ctrl-sh
sh#4
sh#5
ERL
16 μM
32 μM
HCC1937
Cell counting (x10⁴)
Ctrl-sh
sh#4
sh#5
ERL
2 μM
4 μM
SUM149

Figure 4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Ctrl-sh
sh#4
sh#5
H3K4me3
H3
p21
BAK1
β-Actin
SUM149
b
Relative BAK1 expression
3.0
2.5
2.0
1.5
1.0
0.5
0
Ctrl-sh
sh#4
sh#5