Epigenetic Silencing of the \textit{RASSF1A} Tumor Suppressor Gene through HOXB3-Mediated Induction of DNMT3B Expression

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SUMMARY

The \textit{RASSF1A} tumor suppressor gene is epigenetically silenced in a variety of cancers. Here, we perform a genome-wide human shRNA screen and find that epigenetic silencing of \textit{RASSF1A} requires the homeobox protein HOXB3. We show that HOXB3 binds to the DNA methyltransferase DNMT3B gene and increases its expression. DNMT3B, in turn, is recruited to the \textit{RASSF1A} promoter, resulting in hypermethylation and silencing of \textit{RASSF1A} expression. DNMT3B recruitment is facilitated through interactions with Polycomb repressor complex 2 and MYC, which is bound to the \textit{RASSF1A} promoter. Mouse xenograft experiments indicate that the oncogenic activity of HOXB3 is due, at least in part, to epigenetic silencing of \textit{RASSF1A}. Expression analysis in human lung adenocarcinoma samples reveals that \textit{RASSF1A} silencing strongly correlates with overexpression of HOXB3 and DNMT3B. Analysis of human cancer cell lines indicates that the \textit{RASSF1A} epigenetic silencing mechanism described here may be common in diverse cancer types.

INTRODUCTION

RAS association domain family 1A (\textit{RASSF1A}, also known as \textit{RASSF1}) is a tumor suppressor gene whose inactivation has been implicated in the development of more than 40 types of sporadic human cancers (reviewed in Donninger et al., 2007; van der Weyden and Adams, 2007). \textit{RASSF1A} lacks apparent enzymatic activity but contains a RAS association domain and is thought to be an effector of the RAS oncoprotein. \textit{RASSF1A} regulates several biological processes, including cell-cycle progression, apoptosis, and microtubule stability. It is currently thought that \textit{RASSF1A} functions as a scaffold for the assembly of multiple tumor suppressor complexes and may relay proapoptotic signaling by K-RAS.

Inactivation of \textit{RASSF1A} can occur by several mechanisms, including gene deletion or mutation; however, the most common cause of loss of \textit{RASSF1A} function is transcriptional silencing through promoter hypermethylation. Loss of \textit{RASSF1A} expression due to epigenetic silencing appears to be one of the most common events in human cancers, with aberrant \textit{RASSF1A} promoter methylation detected in at least 37 tumor types (van der Weyden and Adams, 2007). However, the factors, regulatory pathways, and mechanisms underlying \textit{RASSF1A} epigenetic silencing remain to be identified.

To gain a better understanding of the molecular basis of \textit{RASSF1A} epigenetic silencing, we have performed a genome-wide RNA interference (RNAi) screen to identify factors that, when knocked down, result in derepression of an epigenetically silenced \textit{RASSF1A} reporter gene. Using this approach, we identified the homeobox protein HOXB3 as a factor required for \textit{RASSF1A} epigenetic silencing. Hyperactivity of HOX proteins, due to either overexpression or chromosomal translocation, has been implicated in a variety of malignancies (reviewed in Arigopulos and Humphries, 2007; Grier et al., 2005). Here, we delineate the mechanism by which HOXB3 epigenetically silences \textit{RASSF1A} and demonstrate that silencing of \textit{RASSF1A} is a critical aspect of HOXB3’s oncogenic activity.

RESULTS

A Genome-wide shRNA Screen Identifies HOXB3 as an Effector of \textit{RASSF1A} Epigenetic Silencing

To screen for factors involved in epigenetic silencing of \textit{RASSF1A}, we generated a reporter construct in which the \textit{RASSF1A} promoter was used to direct expression of a gene encoding red fluorescent protein (RFP) fused to the blasticidin resistance (BlastR) gene. This \textit{RASSF1A-RFP-BlastR} reporter construct was stably transduced into human MDA-MB-231 breast cancer cells in which the endogenous \textit{RASSF1A} gene is epigenetically silenced (Dammann et al., 2001). We then selected cells in which the reporter gene had been silenced as evidenced by loss of RFP expression and acquisition of blasticidin resistance (BlastR) gene. This \textit{RASSF1A-RFP-BlastR} reporter construct was stably transduced into human MDA-MB-231 breast cancer cells in which the endogenous \textit{RASSF1A} gene is epigenetically silenced (Dammann et al., 2001). We then selected cells in which the reporter gene had been silenced as evidenced by loss of RFP expression and acquisition of blasticidin sensitivity. Transcriptional repression of the reporter gene was due to DNA methylation of the \textit{RASSF1A} promoter as evidenced by the appearance of blasticidin-resistant colonies following treatment with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-AZA) (Figure 1A).
A human shRNA library (Silva et al., 2005) comprising ∼62,400 shRNAs directed against ∼28,000 genes was divided into 10 pools, which were packaged into retrovirus particles and used to stably transduce the MDA-MB-231/RASSF1A-RFP-BlastR reporter cell line. Blasticidin-resistant colonies, indicative of derepression of the epigenetically silenced reporter gene, were selected, and the shRNAs were identified by sequence analysis (Figure 1A).

Figure 1. A Genome-wide shRNA Screen Identifies HOXB3 as an Effector of RASSF1A Epigenetic Silencing
(A) Schematic summary of the screen. Growth of parental MDA-MB-231 cells and the stable MDA-MB-231/RASSF1A-RFP-BlastR clone are shown, cultured in the presence of blasticidin and 5-AZA, as indicated.
(B) Analysis of endogenous RASSF1A expression in MDA-MB-231 cells expressing a nonsilencing (NS) or HOXB3 shRNA (HOXB3-1) by qRT-PCR (left) or immunoblot (right) analysis. Error bars represent SEM. Table S1 provides a complete list of candidates isolated from the screen that, when knocked down, result in derepression of the endogenous RASSF1A gene.
(C) qRT-PCR analysis of RASSF1A expression in A549 cells following treatment with 5-AZA (left) or expressing a HOXB3-1 shRNA or a second, unrelated shRNA directed against HOXB3 (HOXB3-2) (middle). Error bars represent SEM. (Right) Immunoblot analysis monitoring RASSF1A levels following HOXB3 knockdown.
(D) qRT-PCR analysis of RASSF1A expression in NCI-H23 and NCI-H460 cells following treatment with 5-AZA (left) or with a luciferase or HOXB3 siRNA (right). Error bars represent SEM.
(E) Analysis of RASSF1A expression in NCI-H1437 cells stably expressing HOXB3 cDNA or empty vector by qRT-PCR (left) or immunoblot (right) analysis. Error bars represent SEM.
RASSF1A is silenced, the HOXB3 in of malignancies (reviewed in Argiropoulos and Humphries, 2007; Grier et al., 2005), we elected to investigate the role of HOXB3 in RASSF1A silencing in greater detail. The quantitative RT-PCR (qRT-PCR) experiment of Figure 1B (left) indicates that shRNA-mediated knockdown of HOXB3 in parental MDA-MB-231 cells also resulted in the derepression of the endogenous, epigenetically silenced RASSF1A gene. Immunoblot analysis confirmed that RASSF1A expression was derepressed following HOXB3 knockdown (Figure 1B, right).

Overexpression of HOX genes and epigenetic silencing of RASSF1A have both been reported to occur in nonsmall cell lung cancers (NSCLCs) (Abe et al., 2006; Chen et al., 2006; Maroulakou and Spyropoulos, 2003). Therefore, we investigated the relationship between HOXB3 expression, DNMT3B levels, and RASSF1A hypermethylation. Figure 2C shows that knockdown of HOXB3 in A549 cells, which increased RASSF1A expression (see Figure 1C), resulted in a substantial decrease in DNMT3B levels. By contrast, the levels of the two other known DNA methyltransferases, DNMT1 and DNMT3A, were unaffected by HOXB3 knockdown (Figure 2D).

Ectopic expression of HOXB3 in NCI-H1437 cells, which reduced RASSF1A expression (see Figure 1E), substantially increased the level of DNMT3B (Figure 2G). Collectively, these results show that HOXB3-mediated epigenetic silencing of RASSF1A occurs, at least in part, through upregulation of DNMT3B.

To determine whether HOXB3 and DNMT3B mediated RASSF1A promoter hypermethylation by direct binding to the RASSF1A promoter, we carried out a series of chromatin immunoprecipitation (ChIP) experiments. The ChIP results of Figure 2H (left) show that, in A549 cells, DNMT3B was directly associated with the RASSF1A promoter. Significantly, following knockdown of HOXB3, association of DNMT3B with the RASSF1A promoter substantially decreased. Conversely, in NCI-H1437 cells, there was only negligible association of DNMT3B with the RASSF1A promoter, but following ectopic expression of HOXB3, the amount of DNMT3B bound to the RASSF1A promoter was markedly elevated. Recruitment of DNMT3B inversely correlated with both binding of RNA polymerase II (Pol II) to the RASSF1A promoter (Figure 2H, right) and RASSF1A expression (see Figures 1C and 1E).

We considered the possibility that HOXB3 bound to the RASSF1A promoter directly and recruited DNMT3B. However, we were unable to detect association of HOXB3 with the RASSF1A promoter in a ChIP assay (data not shown). As an alternative possibility, we considered that HOXB3 could be a direct activator of DNMT3B transcription. Consistent with this idea, bioinformatic analysis identified multiple HOX consensus binding sites within the first intron of DNMT3B. The ChIP experiment of Figure 2I confirms that, in A549 cells, there was substantial binding of HOXB3 to the first intron of DNMT3B, which, as expected, decreased following HOXB3 knockdown. Conversely, in NCI-H1437 cells, there was a relatively low level of HOXB3 binding to DNMT3B, which was substantially increased following ectopic expression of HOXB3. In both A549 and NCI-H1437 cells, binding of HOXB3 to DNMT3B correlated with the level of promoter-bound Pol II.

**Polycomb Repressive Complex 2 Facilitates Binding of DNMT3B to the RASSF1A Promoter**

Previous studies have shown that EZH2, a subunit of Polycomb repressive complex 2 (PRC2), physically associates with...
DNMT3B and provides a platform that enables recruitment of DNMT3B to the promoter (Vire et al., 2006). Our finding that DNMT3B is associated with the epigenetically silenced RASSF1A promoter led us to examine the possibility that EZH2 was also involved in epigenetic silencing of RASSF1A. ChIP analysis indicates that, in A549 cells, EZH2 was associated with the RASSF1A promoter (Figure 3A), and siRNA-mediated knockdown of EZH2 (Figure S3) derepressed RASSF1A expression (Figure 3B).

EZH2 is a histone methyltransferase that methylates histone H3 on lysine 27 (H3K27) (Kuzmichev et al., 2002; Muller et al., 2002). The ChIP results of Figure 3C show that, in A549 cells,
The epigenetically repressed RASSF1A promoter contained substantial H3K27 trimethylation. Significantly, knockdown of EZH2 resulted in decreased H3K27 trimethylation and DNMT3B association with the RASSF1A promoter, as well as an increased level of bound Pol II. The bisulfite sequence analysis of Figure 3D shows, as expected, that EZH2 knockdown also resulted in decreased RASSF1A promoter methylation, consistent with the observed derepression of RASSF1A expression and decreased DNMT3B recruitment.

A previous study suggested that the histone methyltransferase SETDB1 binds to the RASSF1A promoter and is involved in epigenetic silencing (Li et al., 2006). Therefore, we investigated the role of SETDB1 in epigenetic silencing of RASSF1A in A549 cells. ChIP experiments failed to detect binding of SETDB1 to the RASSF1A promoter, although SETDB1 binding to TP53BP2, a known SETDB1 target gene (Sarraf and Stancheva, 2004), was readily detectable (Figure S4A). Moreover, shRNA-mediated knockdown of SETDB1 did not derepress RASSF1A expression in A549 cells (Figure S4B). Thus, SETDB1 is not required for RASSF1A epigenetic silencing in A549 cells, although our results do not exclude a role for SETDB1 in other cell types.

MYC Facilitates Recruitment of PRC2 and DNMT3B to the RASSF1A Promoter

We next investigated the basis by which PRC2 and DNMT3B are recruited to the RASSF1A promoter. Bioinformatic analysis of the RASSF1A promoter revealed putative binding sites for the oncoprotein MYC. Several previous studies have shown that MYC can function as a transcriptional repressor (Amin et al., 1993; Kurland and Tansey, 2008) and can directly interact with DNA methyltransferases (Brenner et al., 2005). Moreover, studies in Drosophila have shown that repression mediated by the MYC ortholog, dMyc, involves Polycomb complexes (Goodliffe et al., 2005). These observations raised the possibility that binding of MYC to the RASSF1A promoter facilitates recruitment of PRC2 and DNMT3B.

To test this idea, we first analyzed binding of MYC, EZH2, and DNMT3B by ChIP using a panel of PCR primer pairs that spanned the RASSF1A promoter. Significantly, for all three proteins, there was a peak of binding at the –235 to +18 region, which encompassed the transcription start site (Figure 4A). To test whether MYC had a functional role in RASSF1A epigenetic repression, we performed RNAi knockdown experiments. We found that siRNA-mediated knockdown of MYC (Figure 5A) derepressed RASSF1A expression (Figure 4B), which was accompanied by decreased recruitment of EZH2 and DNMT3B to the RASSF1A promoter (Figure 4C). Similar results were obtained when MYC was knocked down using an shRNA whose sequence was unrelated to that of the siRNA used in Figures 4B and 4C (Figures S5B and S5C).

An attractive explanation for our results, as well as those of previous studies showing a functional relationship between MYC and Polycomb complexes, is that MYC physically interacts with the PRC2/DNMT3B complex. To test this possibility, we performed coimmunoprecipitation experiments, which demonstrated a physical association between MYC, EZH2, and DNMT3B (Figure 4D). Taken together, the results of Figure 4 indicate that MYC interacts with the PRC2/DNMT3B complex and facilitates recruitment of the PRC2/DNMT3B complex to the RASSF1A promoter, resulting in epigenetic repression. Based on these findings, we propose a model in which MYC recruits Polycomb complexes to the RASSF1A promoter, leading to silencing of RASSF1A expression.
upon the collective results of Figures 1–4, we propose a model for RASSF1A epigenetic silencing that is summarized in Figure 4E.

**HOXB3 Increases Tumor Growth through Downregulation of RASSF1A Expression**

The fact that HOXB3 is overexpressed in cancers and downregulates expression of the RASSF1A tumor suppressor suggested that HOXB3 may be an oncogene that functions by epigenetically silencing RASSF1A. As a first test of this possibility, we asked whether knockdown of HOXB3 would decrease the oncogenic properties of A549 cells in vitro. Figure 5A shows that knockdown of HOXB3 in A549 cells resulted in decreased growth in soft agar, consistent with previous studies showing that overexpression of RASSF1A inhibits proliferation of A549 cells (Dammann et al., 2000). In a related manner, ectopic expression of RASSF1A decreased proliferation of NCI-H1437 cells that overexpress HOXB3 (Figure S6).

Previous studies have shown that RASSF1A exerts its tumor suppressor function primarily by promoting apoptosis (see, for example, Baksh et al., 2005). Therefore, it seemed likely that the decreased growth in soft agar following HOXB3 knockdown was due to increased apoptosis. To test this idea, we measured apoptosis in A549 cells following HOXB3 knockdown. Figure S7 shows, as expected, that HOXB3 knockdown resulted in increased apoptosis.

We then examined the ability of HOXB3 to act as an oncogene in a set of mouse xenograft experiments. In the experiments

**Figure 4. MYC Directs Recruitment of PRC2 and DNMT3B to the RASSF1A Promoter to Facilitate Epigenetic Repression**

(A) ChIP analysis monitoring binding of MYC (top), EZH2 (middle), and DNMT3B (bottom) to the RASSF1A promoter in A549 cells using a panel of PCR primer-pairs spanning the promoter. Error bars represent SEM.

(B) RASSF1A mRNA (left) and protein levels (right) in A549 cells treated with an NS or MYC siRNA. Error bars represent SEM.

(C) ChIP analysis monitoring binding of DNMT3B (left) and EZH2 (right) to the RASSF1A promoter in A549 cells treated with an NS or MYC siRNA. Error bars represent SEM.

(D) Coimmunoprecipitation experiments in A549 cells. Following immunoprecipitation with an α-DNMT3B, α-EZH2, or α-MYC antibody, the immunoprecipitate was analyzed for DNMT3B, EZH2, or MYC by immunoblotting.

(E) Model for HOXB3-mediated epigenetic repression of RASSF1A. For simplicity, a model in which promoter-bound MYC recruits the PRC2/DNMT3B complex to the RASSF1A promoter is shown. However, our results do not exclude the possibility that MYC is associated with PRC2 and DNMT3B, presumably as part of larger complex, prior to binding to the RASSF1A promoter.
described below, cells were injected subcutaneously into the flanks of nude mice, and tumor formation was measured over the course of several weeks. Figure 5B shows that, following knockdown of HOXB3 in A549 cells, tumor growth was substantially decreased. Conversely, ectopic expression of HOXB3 in NCI-H1437 cells markedly increased the rate of tumor growth.

Figure 5. HOXB3 Increases Tumor Growth through Downregulation of RASSF1A Expression

(A) A549 cells expressing an NS or HOXB3-1 shRNA were analyzed for their ability to grow in soft agar. Error bars represent SEM.

(B) Mouse xenograft experiments. A549 cells expressing an NS shRNA, a HOXB3 shRNA, both a HOXB3 and RASSF1A shRNA, or both a HOXB3 and RB1 shRNA were injected into the flanks of nude mice, and tumor volume was measured every 3 days. Error bars represent SEM.

(C) NCI-H1437 cells stably expressing either empty vector or HOXB3 cDNA were assayed for their ability to form tumors in a xenograft mouse model as described in (B). Error bars represent SEM.

(D) TUNEL assays on the tumor samples described in Figure 5B. Samples were stained with hematoxylin and eosin (H&E). Images are shown at 20x magnification.
(Figure 5C). These results indicate that HOXB3 promotes tumor growth, a characteristic property of an oncogene.

We next tested whether the ability of HOXB3 to promote tumor growth and its ability to epigenetically silence RASSF1A were related activities. To determine whether derepression of RASSF1A contributed to the decreased tumor growth rate, we knocked down both HOXB3 and RASSF1A in A549 cells. Remarkably, xenografts derived from this double knockdown cell line grew at a rate comparable to that of control A549 cells expressing a nonsilencing shRNA (Figure 5B). By contrast, knockdown of the retinoblastoma (RB1) protein, a known NSCLC tumor suppressor (reviewed in Kaye, 2002; Wikman and Kettunen, 2006) that is expressed in A549 cells (Figure S8), had no significant effect on tumor growth rate (Figure 5B). In conjunction with the experiments described above, the results of Figure 5 indicate that the oncogenic activity of HOXB3 is due, at least in part, to epigenetic silencing of RASSF1A.

To determine whether the decreased tumor growth rate upon HOXB3 knockdown was due to increased apoptosis, we performed terminal transferase uridyl nick end labeling (TUNEL) assays on the tumors derived from the experiment described in Figure 5B. The results of Figure 5D reveal that tumors formed from A549 HOXB3 knockdown cells had a higher level of apoptosis, as evidenced by increased TUNEL staining, than those formed from the control A549 cells expressing a nonsilencing shRNA. Moreover, the level of apoptosis in A549 HOXB3, RASSF1A double-knockdown tumors was comparable to that of the control A549 tumors. By contrast, the levels of apoptosis in tumors formed from A549 HOXB3, RB1 double-knockdown and A549 HOXB3 knockdown cells were comparable. Thus, these TUNEL results reveal, as expected, an inverse correlation between tumor growth rates and apoptotic levels.

Epigenetic Silencing of RASSF1A through HOXB3-Mediated Induction of DNMT3B Expression Is Common in Lung Adenocarcinomas and Diverse Human Cancer Cell Lines

To investigate whether the RASSF1A epigenetic silencing mechanism described above occurs in human tumors, we analyzed expression of RASSF1A, HOXB3, and DNMT3B in a series of human lung adenocarcinoma and squamous cell carcinoma samples. The qRT-PCR analysis of Figure 6A shows that, in six of ten lung adenocarcinomas, RASSF1A expression was significantly downregulated. Most importantly, in five of these six lung adenocarcinomas, the decreased RASSF1A expression was accompanied by elevated levels of HOXB3 and DNMT3B. By contrast, only one of ten lung squamous cell carcinoma samples had a similar expression profile. These results suggest that HOXB3-mediated silencing of RASSF1A occurs frequently in lung adenocarcinomas.

Finally, we investigated the generality of the HOXB3-mediated RASSF1A epigenetic silencing mechanism in other cancer types. Figure 6B shows that treatment of 12 diverse human cancer cell lines with 5-AZA increased RASSF1A expression, indicating that RASSF1A is silenced by promoter methylation in these cell lines. To assess the requirement of HOXB3, we chose four cell lines for further analysis: MDA-MB-231 and T47D (breast), SK-OV-3 (ovarian), and U251 (central nervous system).

In each of these four cell lines, knockdown of HOXB3 (Figure S9) substantially decreased DNMT3B levels and derepressed RASSF1A expression (Figure 6C). These results suggest that epigenetic silencing of RASSF1A through HOXB3-mediated induction of DNMT3B expression occurs in diverse cancer types.

DISCUSSION

In this report, we describe a genome-wide shRNA screening strategy, which is a general method that can be used to identify regulators of any epigenetically silenced gene. Using this approach, we have identified HOXB3 as a factor required for epigenetic silencing of the RASSF1A tumor suppressor. Additional functional experiments have enabled us to describe a mechanism for HOXB3-mediated epigenetic silencing of RASSF1A, which is summarized in Figure 4E and discussed below.

Several models have been proposed to explain how tumor suppressor genes become epigenetically silenced during cancer development. According to one model, silencing occurs by random acquisition of epigenetic marks that confer a selective growth advantage, whereas an alternative model posits that silencing occurs through a specific and targeted “instructive” pathway that is initiated by an oncogene (Keshet et al., 2006). The identification of a pathway that is initiated by the oncogene HOXB3, containing a defined set of components and culminating in the repression of a critical tumor suppressor, RASSF1A, provides strong support for an instructive model.

Model for Epigenetic Repression of RASSF1A

We have shown that increased expression of HOXB3 leads to upregulation of DNMT3B, which is recruited to the RASSF1A promoter and silences gene expression through promoter hypermethylation. We found that DNMT3B recruitment requires EZH2, a histone methyltransferase subunit of the PRC2 complex, which has been previously shown to physically associate with DNMT3B (Vire et al., 2006). Consistent with this conclusion, we found that there is a similar increase of RASSF1A expression following knockdown of either DNMT3B (~7- to 8-fold) or EZH2 (~9-fold). Significantly, EZH2 is overexpressed in several types of primary and metastatic cancers, including lung cancers, and has been shown to contribute to the malignant properties of cancer cells (Breuer et al., 2004; Tonini et al., 2008; Varambally et al., 2002).

Several lines of evidence indicate that the oncoprotein MYC facilitates recruitment of the PRC2/DNMT3B complex to the RASSF1A promoter. First, MYC binds near the RASSF1A transcription start site, which coincides with binding of EZH2 and DNMT3B. Second, RNAi-mediated knockdown of MYC results in decreased recruitment of EZH2 and DNMT3B to the RASSF1A promoter, as well as derepression of RASSF1A expression. Finally, coimmunoprecipitation experiments demonstrate physical associations between MYC, EZH2, and DNMT3B. The coimmunoprecipitation results raise the possibility that MYC may be associated with PRC2 and DNMT3B, presumably as part of a larger complex, prior to binding to the RASSF1A promoter; alternatively, MYC could bind to the RASSF1A promoter first, followed by interaction with and recruitment of the PRC2/DNMT3B complex.
Our results fit very well with previous reports of a functional relationship between MYC and Polycomb complexes in transcriptional repression. For example, in both flies and mammalian cell lines, MYC has been shown to autorepress its own transcription, which in both cases requires Polycomb complexes (Goodliffe et al., 2005). In addition, studies in Drosophila have demonstrated that the majority of dMyc repression targets require Polycomb for silencing (Goodliffe et al., 2005).

Although we find that MYC is required for Polycomb recruitment, several lines of evidence suggest that, in general, MYC is not sufficient to recruit Polycomb. For example, analysis of genome-wide occupancy results (Kidder et al., 2008) reveals that, of the 2189 promoters bound by Myc in mouse embryonic stem cells, only 108 (~5%) are also bound by Polycomb. In a related manner, at many promoters, MYC is an activator, not a repressor of transcription; Polycomb complexes antagonize transcription activation and therefore are not expected to be present on promoters at which MYC is an activator.

Continual Recruitment of DNMT3B Is Required to Maintain Epigenetic Silencing of RASSF1A

It has been thought that, once promoter hypermethylation is established, it will be maintained during DNA replication due to an intrinsic ability of DNA methyltransferases to recognize hemimethylated CpG residues and convert them to the fully methylated form. In support of this model are reports of interactions between DNA methyltransferases and DNA replication factors (see, for example, Chuang et al., 1997). However, we have found that knockdown of HOXB3, EZH2, or MYC results in loss of DNMT3B recruitment, decreased promoter methylation, and RASSF1A derepression. Likewise, we have previously shown that multiple factors are required to maintain hypermethylation of the epigenetically repressed Fas promoter (Gazin et al., 2007), and our study and others have demonstrated an essential role for EZH2 in maintaining hypermethylation (Vire et al., 2006). Thus, at least in these instances, maintenance of promoter hypermethylation is not simply due to intrinsic DNA methylation...
methyltransferase activity during DNA replication but instead requires continual recruitment of a DNA methyltransferase through a defined set of cofactors, which may differ depending upon both promoter and cell type (see, for example, McGarvey et al., 2007).

What is the basis for loss of RASSF1A promoter methylation following knockdown of HOXB3? Two mechanisms for DNA demethylation have been proposed. The first is the so-called passive mechanism in which methyl groups are lost simply as a result of DNA replication. The second is an active mechanism in which a DNA demethylase catalyzes the removal of the methyl groups. Support for the latter mechanism has been considerably strengthened by the recent definitive identification of DNA demethylases (Barreto et al., 2007; Rai et al., 2008). We found that, following blockade of passive DNA demethylation by treatment with the DNA replication inhibitor aphidicolin, knock-down of HOXB3 still results in derepression of RASSF1A expression, which is accompanied by decreased methylation of the RASSF1A promoter (Figure S10). Thus, an active demethylation mechanism is responsible, at least in part, for RASSF1A derepression following HOXB3 knockdown.

**Basis for HOXB3 Oncogenic Function**

HOX proteins have long been known as master regulators of gene transcription during development (Grier et al., 2005). However, it is becoming increasingly evident that HOX proteins can also play important roles in cancer initiation and progression. The oncogenic potential of a HOX protein has been most clearly established in leukemias that harbor a chromosomal translocation that deregulates HOXA9 activity (Argiropoulos and Humphries, 2008). In solid tumors, numerous studies have reported differences in HOX gene expression between normal and neoplastic tissue (for example, Cantile et al., 2003; Cillo et al., 1992, 1999; Lewis, 2000). How alterations in HOX protein activity in either leukemias or solid tumors lead to cancer has not been resolved. For example, the downstream target genes of the HOX protein and the basis by which the HOX protein/target gene interaction leads to transformation have not been determined.

We have provided several lines of evidence indicating that RASSF1A is the critical target of HOXB3. First, we have shown that HOXB3 is both necessary and sufficient to induce epigenetic silencing of RASSF1A (Figure 1). Second, the effect of HOXB3 on RASSF1A expression is highly specific. For example, we found that, in contrast to the effect on RASSF1A, HOXB3 knockdown either did not affect or, at most, modestly increased expression of a panel of 23 known NSCLC tumor suppressors and 9 housekeeping genes (Figure S11). Third, the decreased tumor growth rate following HOXB3 knockdown can be selectively rescued by concomitant knockdown of RASSF1A (Figure 5B), indicating that the oncogenic activity of HOXB3 is due, at least in part, to epigenetic silencing of RASSF1A. Thus, our results have elucidated a mechanism by which a HOX protein promotes tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**

MDA-MB-231, A549, NCI-H23, NCI-H460, and NCI-H1437 were obtained from ATCC. NCI-60 cell lines were obtained from NOI. All cell lines were main-

**Quantitative RT-PCR Analysis**

Total RNA was isolated and reverse transcription was performed as previously described (Wajapeyee et al., 2008), followed by qPCR using Platinum SYBR Green qPCR SuperMix-UDQ with Rox (Invitrogen). For all reactions, inputs were normalized, and the Ct values of samples were analyzed after subtracting the signal obtained with the nonsilencing shRNA (for RNAi) or no antibody (for ChIP) controls. Primer sequences are as listed in Table S3. For 5-AZA treatment, cells were treated with 10 μM 5-AZA (Calbiochem) for 72 hr prior to RNA isolation. Experiments were performed in triplicate.

**Bisulfite Sequencing Analysis**

Bisulfite conversion reactions were performed using an Epitext Bisulfite Kit (QIAGEN) according to the manufacturer’s instructions. Bisulfite-treated DNA was cloned into pGEM-T (Promega), and for each cell line, six clones were sequenced using primers (listed in Table S3) to analyze the RASSF1A promoter from ~220 to +162 relative to the transcription start site.

**Chromatin Immunoprecipitation Assays**

Bioinformatic analysis to identify transcription factor binding sites was performed using viSTa 2.0 (Loots and Ovcharenko, 2004). ChIP assays were performed as previously described (Raha et al., 2005) using the following antibodies: α-HOXB3 rabbit polyclonal (Santa Cruz, sc28606); α-DNMT3B rabbit polyclonal (Abcam, ab2851); α-Pol II mouse monoclonal (Covance, 8WG16); α-EZH2 mouse monoclonal (Cell Signaling Technology, AC22); α-H3K27-Me3 rabbit polyclonal (Upstate, 07-449); α-MYC rabbit polyclonal (Santa Cruz, sc764); and α-SETDB1 rabbit polyclonal (Millipore, 07-1568). ChIP products were analyzed by qPCR as described above. Primer sequences
for ChIP and qPCR are listed in Table S3. Unless otherwise stated, the RASSF1A promoter was analyzed using a primer pair that spanned the transcription start site (−235 to +18). Calculation of fold differences was done as previously described (Pfaffl, 2001). Experiments were performed in triplicate with at least two independent samples.

**Coimmunoprecipitation Assays**

To prepare cell extracts, cells were washed twice with cold PBS, resuspended in cytoplasmic extract buffer (50 mM HEPES pH 8, 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, and protease inhibitor) and incubated on ice for 5 min. Samples were centrifuged at 5000 rpm for 2 min at 4°C, and the nuclear pellet was resuspended in wash buffer (50 mM HEPES, 50 mM NaCl, 25% glycerol, 0.1 mM EDTA, and protease inhibitors). The pellet was then lysed in nuclear extract buffer (50 mM HEPES, 350 mM NaCl, 25% glycerol, 0.1 mM EDTA, 0.1% Triton X-100, and protease inhibitors) on ice for 30 min and centrifuged at the highest speed; the supernatant was taken as nuclear extract. For immunoprecipitations, 500 μg nuclear extract was incubated with 2 μg antibody (α-DNMT3B mouse monoclonal [Imgenex], α-EZH2 rabbit polyclonal [Millipore, 07-688], or α-MYC rabbit polyclonal [Santa Cruz]) overnight at 4°C on a rocking platform, followed by addition of 50 μl of either TrueBlot anti-mouse Ig IP beads or anti-rabbit Ig beads (eBioscience) and incubation for 1 hr on a rocking platform. The beads were then washed three times with nuclear extract buffer, and the protein complex was eluted by boiling in Laemmli buffer.

**Soft Agar Assays**

Soft agar assays were performed in triplicate using CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, Inc.) according to the manufacturer’s instructions.

**Mouse Xenograft Experiments**

Cells (5 × 10^5) were suspended in 100 μl of serum-free DMEM and injected subcutaneously into the right flank of three athymic Balb/c (nu/nu) mice (Taconic). Tumor dimensions were measured as described previously (Wajapeyee et al., 2008). Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

**TUNEL Assays**

TUNEL assays were performed as described previously (Wajapeyee et al., 2008).

**SUPPLEMENTAL DATA**

The Supplemental Data include three tables and twelve figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00744-8.

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