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Epigenetic Silencing of the *RASSF1A* Tumor Suppressor Gene through HOXB3-Mediated Induction of *DNMT3B* Expression

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Abstract

SUMMARY—The *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 *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 *RASSF1A* promoter, resulting in hypermethylation and silencing of *RASSF1A* expression. DNMT3B recruitment is facilitated through interactions with Polycomb repressor complex 2 and MYC, which is bound to the *RASSF1A* promoter. Mouse xenograft experiments indicate that the oncogenic activity of HOXB3 is due, at least in part, to epigenetic silencing of *RASSF1A*. Expression analysis in human lung adenocarcinoma samples reveals that *RASSF1A* silencing strongly correlates with over-expression of *HOXB3* and *DNMT3B*. Analysis of human cancer cell lines indicates that the *RASSF1A* epigenetic silencing mechanism described here may be common in diverse cancer types.

INTRODUCTION

RASSF1A (RAS association domain family 1A, also known as *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). RASSF1A lacks apparent enzymatic activity but contains a RAS-association domain and is thought to be an effector of the RAS oncoprotein. RASSF1A regulates several biological processes including cell cycle progression, apoptosis and microtubule stability. It is currently thought that RASSF1A functions as a scaffold for the assembly of multiple tumor suppressor complexes and may relay pro-apoptotic signaling by K-RAS.

Inactivation of *RASSF1A* can occur by several mechanisms including gene deletion or mutation, however the most common cause of loss of *RASSF1A* function is transcriptional silencing through promoter hypermethylation. Loss of *RASSF1A* expression due to

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SUPPLEMENTAL DATA
The Supplemental Data include 12 figures and three tables can be found with this article at http://www.cell.com/molecular-cell/supplemental/

epigenetic silencing appears to be one of the most common events in human cancers, with aberrant *RASSF1A* promoter methylation detected in at least 37 tumor types (van der Weyden and Adams, 2007). However, the factors, regulatory pathways and mechanisms underlying *RASSF1A* epigenetic silencing remain to be identified.

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

RESULTS

A Genome-Wide shRNA Screen Identifies HOXB3 as an Effector of *RASSF1A* Epigenetic Silencing

To screen for factors involved in epigenetic silencing of *RASSF1A*, we generated a reporter construct in which the *RASSF1A* promoter was used to direct expression of a gene encoding red fluorescent protein (RFP) fused to the blasticidin-resistance ($Blast^R$) gene. This *RASSF1A*-RFP-$Blast^R$ reporter construct was stably transduced into human MDA-MB-231 breast cancer cells, in which the endogenous *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 *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-$Blast^R$ reporter cell line. Blasticidin-resistant colonies, indicative of de-repression of the epigenetically silenced reporter gene, were selected and the shRNAs identified by sequence analysis (Figure 1A).

One of the shRNAs isolated multiple times from the screen was directed against the homeobox protein HOXB3. Because over-expression of HOX proteins, and chromosomal translocations involving HOX proteins, have been implicated in a variety 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 panel) indicates that shRNA-mediated knockdown of HOXB3 in parental MDA-MB-231 cells also resulted in the de-repression of the endogenous, epigenetically silenced *RASSF1A* gene. Immunoblot analysis confirmed that RASSF1A expression was de-repressed following HOXB3 knockdown (Figure 1B, right panel).

Over-expression of *HOX* genes and epigenetic silencing of *RASSF1A* have both been reported to occur in non-small cell lung cancers (NSCLCs) (Abe et al., 2006; Chen et al., 2006; Maroulakou and Spyropoulos, 2003). We therefore investigated the role of HOXB3 in *RASSF1A* epigenetic silencing in human A549 NSCLC cells, in which *RASSF1A* is silenced by promoter methylation (Figure 1C, left panel and Dammann et al., 2001). The

qRT-PCR experiment of Figure 1C (middle panel) shows that knockdown of HOXB3 in A549 cells resulted in de-repression of the epigenetically silenced *RASSF1A* gene. *RASSF1A* expression was also reactivated in A549 cells by a second, unrelated shRNA directed against HOXB3. De-repression of RASSF1A expression following HOXB3 knockdown was confirmed by immunoblot analysis (Figure 1C, right panel). In addition, qRT-PCR and immunoblot analyses verified that both HOXB3 shRNAs knocked down expression of the target gene (Figure S1A). *RASSF1A* was also de-repressed following HOXB3 knockdown in two independent human NSCLC cell lines, NCIH23 and NCI-H460, in which *RASSF1A* is also epigenetically silenced (Figure 1D and Ross et al., 2000).

To test whether over-expression of HOXB3 was sufficient to silence *RASSF1A*, we ectopically expressed HOXB3 in NCI-H1437 cells (Figure S1B), a human NSCLC cell line in which *RASSF1A* is active (Dallol et al., 2004). Both qRT-PCR and immunoblot analyses show that ectopic expression of HOXB3 in NCI-H1437 cells decreased *RASSF1A* expression (Figure 1E). Collectively, the results of Figure 1 show that HOXB3 is both necessary and sufficient to induce epigenetic silencing of *RASSF1A*.

HOXB3 Silences *RASSF1A* through a Pathway Involving Direct Binding of DNMT3B to the *RASSF1A* Promoter

To ask whether HOXB3 regulated *RASSF1A* expression through promoter DNA hypermethylation, we performed bisulfite sequence analysis. Figure 2A shows that in A549 cells, in which *RASSF1A* is silenced, the *RASSF1A* promoter contained a region of dense DNA hypermethylation. Significantly, following knockdown of HOXB3 in A549 cells, hypermethylation of the *RASSF1A* promoter was substantially decreased, consistent with the increased *RASSF1A* expression. Conversely, the *RASSF1A* promoter was not hypermethylated in NCI-H1437 cells, but became hypermethylated following ectopic expression of HOXB3 (Figure 2B).

The DNA methyltransferase DNMT3B is over-expressed in a variety of cancers including NSCLCs (Eads et al., 1999; Robertson et al., 1999; Saito et al., 2002), and previous studies have shown that depletion of DNMT3B using an antisense oligonucleotide can lead to reactivation of *RASSF1A* (Beaulieu et al., 2002). Based upon these considerations, we hypothesized that HOXB3-induced *RASSF1A* promoter hypermethylation may be a consequence of increased levels of DNMT3B, and therefore investigated the relationship between HOXB3 expression, DNMT3B levels, and *RASSF1A* promoter 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). Likewise, knockdown of DNMT3B (Figure S2A) increased expression of *RASSF1A* (Figure 2E), whereas knockdown of either DNMT1 or DNMT3A (Figure S2B) had no effect (Figure 2F).

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 up-regulation 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 panel) 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 (POL2) to the *RASSF1A* promoter (Figure 2H, right panel) 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 *DNMT3*B, 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 POL2.

Polycomb Repressive Complex 2 (PRC2) 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 enabling 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) de-repressed *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 tri-methylation and DNMT3B association with the *RASSF1A* promoter, and an increased level of bound POL2. The bisulfite sequence analysis of Figure 3D shows, as expected, that EZH2 knockdown also resulted in decreased *RASSF1A* promoter methylation, consistent with the observed de-repression 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). We therefore 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 de-repress *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 S5A) de-repressed *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 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 Down-regulation of *RASSF1A* Expression

The fact that HOXB3 is over-expressed in cancers and down-regulates 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 over-expression of *RASSF1A* inhibits proliferation of A549 cells (Dammann et al., 2000). Relatedly, ectopic expression of *RASSF1A* decreased proliferation of NCI-H1437 cells over-expressing 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). It therefore 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 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 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 to epigenetically silence *RASSF1A* were related activities. To determine whether de-repression 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 non-silencing 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 non-silencing 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 level 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 de-repressed *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 novel 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 initiated by an oncogene (Keshet et al., 2006). The identification of a pathway 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 up-regulation 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 over-expressed 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, and de-repression of *RASSF1A* expression. Finally, co-immunoprecipitation 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. Relatedly, 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 methylransferases 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* de-repression. Likewise, we have previously shown that multiple factors are required to maintain hypermethyation 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 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 blockage of passive DNA demethylation by treatment with the DNA replication inhibitor aphidicolin, knockdown of HOXB3 still results in de-repression 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* de-repression 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 harboring a chromosomal translocation that deregulates HOXA9 activity (Argiropoulos and Humphries, 2007; Eklund, 2007). 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; Cillo et al., 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 NCI. All cell lines were maintained according to the supplier's instructions. NCI-H1437 cells were stably transfected with a retroviral vector expressing HOXB3 (MSCV-HOXB3; kindly provided by Keith Humphries) or empty vector (MSCV-Neo) and G418 selected (800 μg/ml) for 2 weeks. Total RNA from normal lung, adenocarcinoma and squamous cell carcinoma samples were obtained from the UMass Cancer Center Tissue Bank.

Screening Strategy

To construct the *RASSF1A*-RFP-Blast^R reporter, 2.5 kb of the *RASSF1A* promoter was amplified from a BAC and cloned into a derivative of DsRed2N1 (Clontech) in which the CMV promoter had been excised and the Blast^R gene (PCR amplified from PEF6V5-HisB; Invitrogen) had been inserted to generate an in-frame fusion with DsRed2. Stable clones were isolated by neomycin selection and clonally purified. A single clone that showed epigenetic silencing of the *RASSF1A* promoter (as observed by blasticidin sensitivity) was selected for the genome-wide RNAi screen. Blasticidin sensitivity was monitored by culturing cells in the presence of 1, 2, 5, 7.5 or 10 μg/ml blasticidin. Retroviral shRNA pools were generated and used to transduce the reporter cell line, and shRNAs from blasticidin resistant colonies were identified by sequence analysis as described previously (Wajapeyee et al., 2008).

RNA interference

Individual knockdown cell lines were generated by stable transduction of cells with a single shRNA and selection with puromycin (1.5 μg/ml) for 1 week. For double-knockdown cell lines, A549 cells stably transduced with a HOXB3 shRNA were infected with a lentivirus expressing a RASSF1A or RB1 shRNA and 48 hrs later GFP-positive cells were sorted and cultured with 1.5 μg/ml puromycin. For siRNAs, 100 nM of siRNA was transfected with Lipofectamine 2000 (Invitrogen). Fold knockdown was calculated by normalizing the expression of the target gene following knockdown to that obtained using a non-silencing shRNA or luciferase siRNA. Source identification numbers for shRNAs and sequences for siRNAs are listed in Table S2.

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-UDG with Rox (Invitrogen). For all reactions, inputs were normalized and the Ct values of samples were analyzed after subtracting the signal obtained with the non-silencing 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 hrs prior to RNA isolation.

Immunoblot Analysis

Protein extracts were prepared as previously described (Santra et al., 2009). Blots were probed with α-RASSF1A mouse monoclonal (eBiosciences, 14-6888), α-Tubulin mouse

monoclonal (Sigma, T6074), α-DNMT3B mouse monoclonal (Imgenex, IMG-184A), or α-RB1 mouse monoclonal (Calbiochem, OP66) antibody.

Bisulfite Sequencing Analysis

Bisulfite conversion reactions were performed using an EpiTect 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 rVISTA 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-POL2 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.

Co-immunoprecipitation 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, 07-689) 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 3 times with nuclear extract buffer, and the protein complex was eluted by boiling in Laemmli buffer.

Soft Agar Assays

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

Mouse Xenograft Experiments

~5×10^6 cells were suspended in 100 μl of serum-free DMEM and injected subcutaneously into the right flank of 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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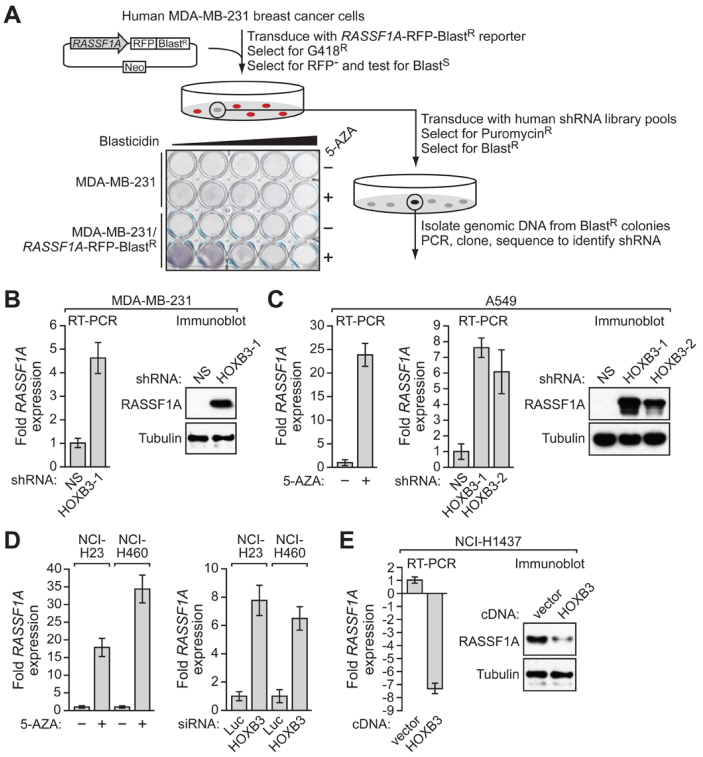

Figure 1. A Genome-Wide shRNA Screen Identifies HOXB3 as an Effector of *RASSF1A* Epigenetic Silencing

(A) Schematic summary of the screen. Growth of parental MDA-MB-231 cells and the stable MDA-MB-231/*RASSF1A*-RFP-Blast^R 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 non-silencing (NS) or HOXB3 shRNA (HOXB3-1) by qRT-PCR (left) or immunoblot (right) analysis. Error bars represent SEM. A complete list of candidates isolated from the screen that, when knocked down, result in de-repression of the endogenous *RASSF1A* gene, is provided in Table S1.

(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.

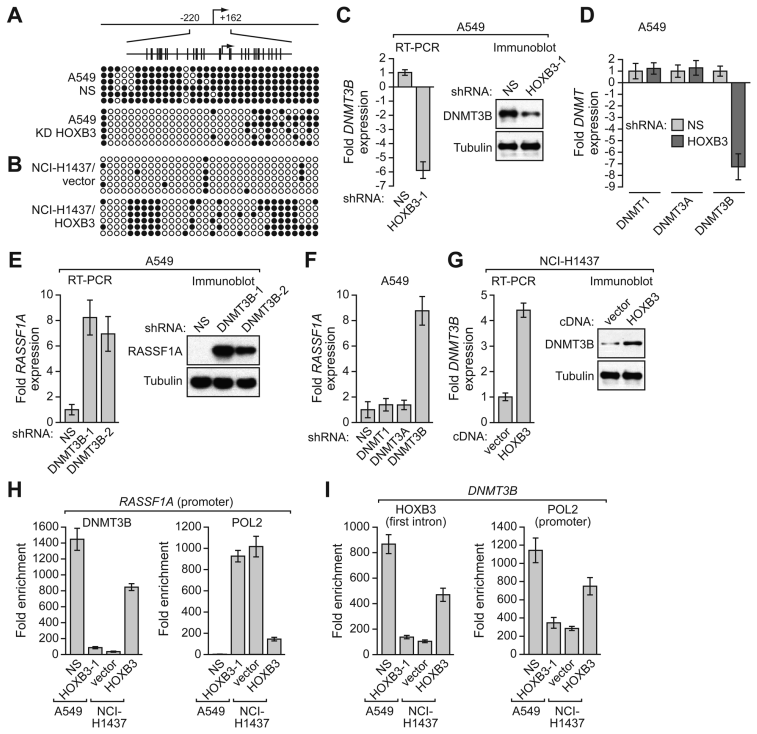

Figure 2. Direct Binding of DNMT3B to the *RASSF1A* Promoter and HOXB3 to *DNMT3B*

(A) Bisulfite sequencing analysis of the *RASSF1A* promoter in A549 cells expressing an NS or HOXB3-1 shRNA. (Top) Schematic of the *RASSF1A* promoter; positions of CpGs are shown to scale by vertical lines. (Bottom) Each circle represents a methylated (black) or unmethylated (white) CpG dinucleotide. Each row represents a single clone.

(B) Bisulfite sequencing analysis of the *RASSF1A* promoter in NCI-H1437 cells stably expressing HOXB3 cDNA or empty vector.

(C) *DNMT3B* mRNA (left) or protein (right) levels in A549 cells expressing an NS or HOXB3-1 shRNA. Error bars represent SEM.

(D) qRT-PCR analysis of *DNMT1*, *DNMT3A* or *DNMT3B* expression in A549 cells expressing an NS or HOXB3-1 shRNA. Error bars represent SEM.(E) *RASSF1A* mRNA (left) or protein (right) levels in A549 cells expressing an NS or one of two unrelated DNMT3B shRNAs. Error bars represent SEM.

(F) qRT-PCR analysis of *RASSF1A* expression in A549 cells expressing an NS shRNA or an shRNA directed against DNMT1, DNMT3A or DNMT3B. Error bars represent SEM.

(G) DNMT3B mRNA (left) or protein (right) levels in NCI-H1437 cells expressing HOXB3 cDNA or empty vector. Error bars represent SEM.

(H) ChIP analysis monitoring binding of DNMT3B (left) or POL2 (right) to the *RASSF1A* promoter in A549 cells expressing an NS or HOXB3 shRNA, or in NCI-H1437 cells expressing HOXB3 cDNA or empty vector. Error bars represent SEM.

(I) Binding of HOXB3 (left) and POL2 (right) to the *DNMT3B* first intron and promoter, respectively, as described in (H). Error bars represent SEM.

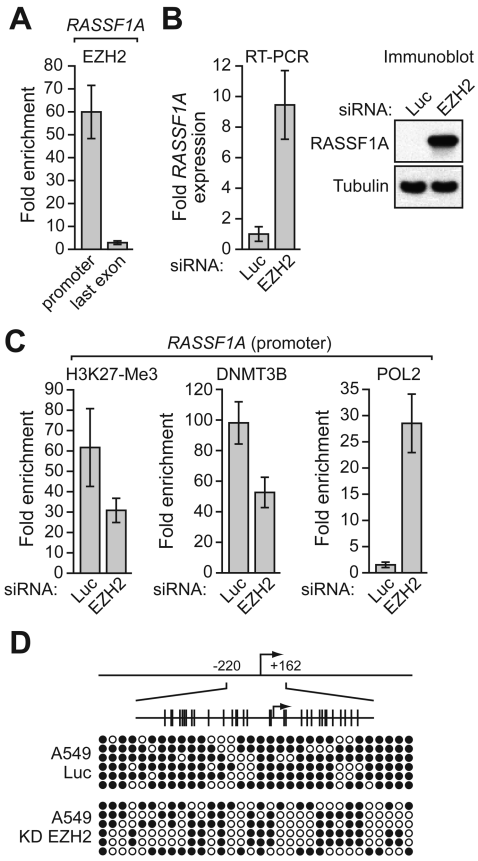

Figure 3. PRC2 Facilitates Recruitment of DNMT3B to the*RASSF1A* Promoter

(A) ChIP analysis monitoring binding of EZH2 to the *RASSF1A* promoter and last exon in A549 cells. Error bars represent SEM.

(B) *RASSF1A* mRNA (left) and protein (right) levels in A549 cells treated with a luciferase (Luc) or EZH2 siRNA. Error bars represent SEM.

(C) Association of H3K27-Me3, DNMT3B and POL2 with the *RASSF1A* promoter in A549 cells treated with a luciferase or EZH2 siRNA. Specific association of DNMT3B, H3K27-Me3 and POL2 with the *RASSF1A* promoter, and HOXB3 and POL2 with the *DNMT3B* promoter, was evidenced by the negligible association with a downstream region in the last exon of each gene (Figure S12).

(D) Bisulfite sequencing analysis of the *RASSF1A* promoter in A549 cells treated with a luciferase or EZH2 siRNA.

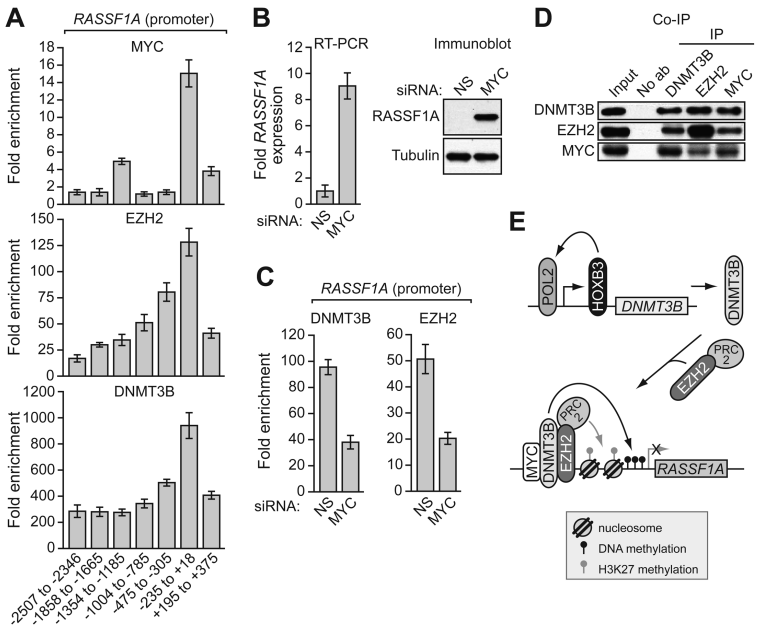

Figure 4. MYC Directs Recruitment of PRC2 and DNMT3B to the *RASSF1A* Promoter to Facilitate Epigenetic Repression

(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) Co-immunoprecipitation 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.

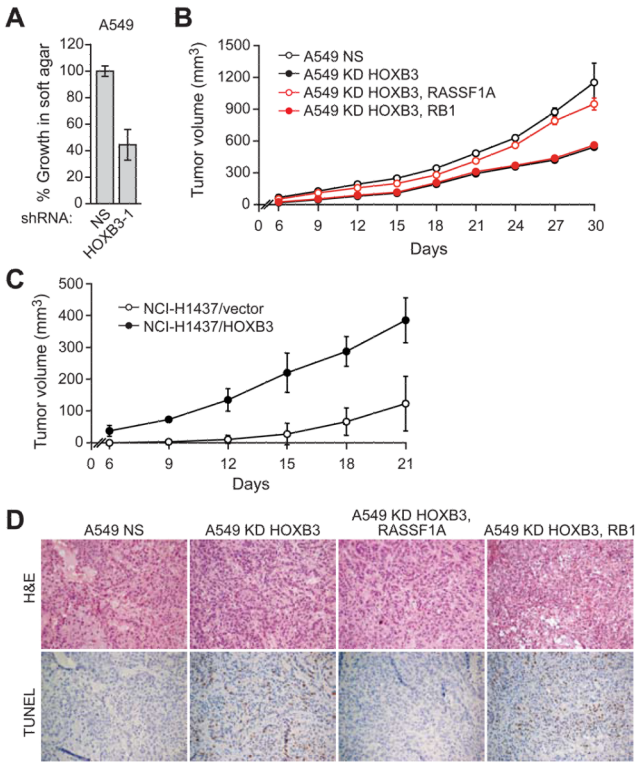

Figure 5. HOXB3 Increases Tumor Growth through Down-regulation of *RASSF1A* Expression

(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 three 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.

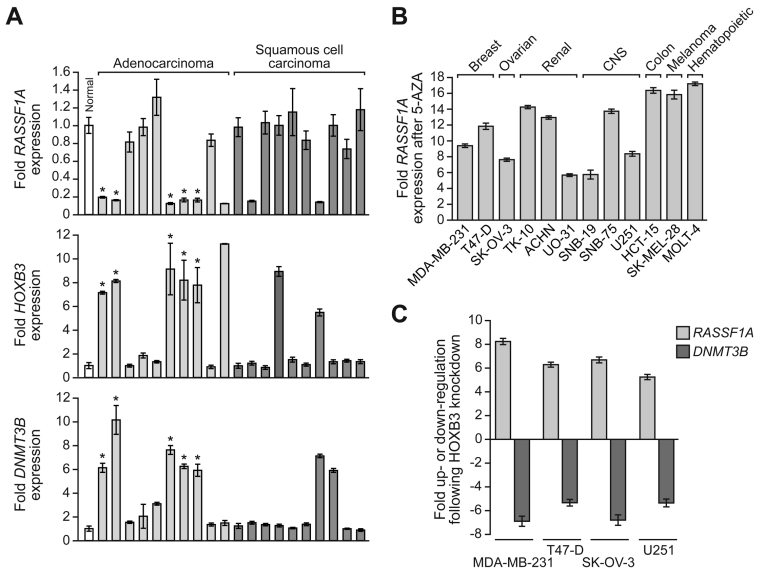

Figure 6. Epigenetic Silencing of *RASSF1A* through HOXB3-Mediated Induction of *DNMT3B* Expression is Common in Lung Adenocarcinomas and Diverse Human Cancer Cell Lines

(A) qRT-PCR analysis of *RASSF1A*, *HOXB3* and *DNMT3B* expression in 10 individual human adenocarcinoma and squamous cell carcinoma samples. As a control, expression of each gene was also analyzed in normal cells. Adenocarcinomas in which decreased *RASSF1A* expression was accompanied by elevated levels of *HOXB3* and *DNMT3B* are indicated by an asterisk. Error bars represent SEM.

(B) qRT-PCR analysis monitoring *RASSF1A* expression in a variety of human cancer cell lines treated with 5-AZA. The results have been normalized to *RASSF1A* expression observed in the absence of 5-AZA. Error bars represent SEM.

(C) MDA-MB-231, T-47D, SK-OV-3 and U251 cell lines were analyzed for expression of *RASSF1A* and *DNMT3B* following treatment with an NS or HOXB3 shRNA. Error bars represent SEM.