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DAL-1/4.1B tumor suppressor interacts with protein arginine *N*-methyltransferase 3 (PRMT3) and inhibits its ability to methylate substrates *in vitro* and *in vivo*

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DAL-1 (differentially expressed in adenocarcinoma of the lung)/4.1B is a tumor suppressor gene on human chromosome 18p11.3 whose expression is lost in >50% of primary non-small-cell lung carcinomas. Based on sequence similarity, DAL-1/4.1B has been assigned to the Protein 4.1 superfamily whose members interact with plasma membrane proteins through their N-terminal FERM (4.1/Ezrin/Radixin/Moesin) domain, and cytoskeletal components via their C-terminal SAB (spectrin–actin binding) region. Using the DAL-1/4.1B FERM domain as bait for yeast two-hybrid interaction cloning, we identified protein arginine *N*-methyltransferase 3 (PRMT3) as a specific DAL-1/4.1B-interacting protein. PRMT3 catalyses the post-translational transfer of methyl groups from *S*-adenosyl-L-methionine to arginine residues of proteins. Coimmunoprecipitation experiments using lung and breast cancer cell lines confirmed this interaction in mammalian cells *in vivo*. *In vitro* binding assays demonstrated that this was an interaction occurring via the C-terminal catalytic core domain of PRMT3. DAL-1/4.1B was determined not to be a substrate for PRMT3-mediated methylation but its presence inhibits the *in vitro* methylation of a glycine-rich and arginine-rich methyl-accepting protein, GST (glutathione-*S*-transferase-GAR (glycine- and arginine-rich), which contains 14 'RGG' consensus methylation sites. In addition, induced expression of DAL-1/4.1B in MCF-7 breast cancer cells showed that the DAL-1/4.1B protein significantly inhibits PRMT3 methylation of cellular substrates. These findings suggest that modulation of post-translational methylation may be an important mechanism through which DAL-1/4.1B affects tumor cell growth.

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Introduction

The human tumor suppressor gene DAL-1/4.1B (differentially expressed in adenocarcinoma of the lung) was identified using Differential Display PCR (DDPCR) as a gene whose expression was lacking in primary non-small-cell lung cancer (NSCLC) when compared with matched normal tissue (Tran *et al*., 1999). This gene product was determined to be a new member of the Protein 4.1 superfamily by virtue of the presence of a 336 amino-acid N-terminal region sharing significant identity to the FERM (4.1/Ezrin/Radixin/Moesin) domain present in all 4.1 family proteins (Chishti *et al*., 1998). Members of this family, which include the neurofibromatosis 2 tumor suppressor protein merlin or schwannomin (Rouleau *et al*., 1993; Trofatter *et al*., 1993), are proteins that localize to the cytoplasmic side of the plasma membrane and link membrane proteins with the spectrin/actin cytoskeleton (Tsukita *et al*., 1994). Like merlin and now 4.1R, which have been shown to suppress growth in schwannoma and meningioma cell lines (Gutmann *et al*., 2000; Robb *et al*., 2004), DAL-1/4.1B negatively regulates cell growth in several different cell types. For example, when introduced into DAL-1/4.1B-null lung and breast cancer cells, this new Protein 4.1 family member significantly suppresses growth (Tran *et al*., 1999; Charboneau *et al*., 2002). Similar results are obtained when DAL-1/4.1B is overexpressed in meningioma cell lines (Gutmann *et al*., 2000, 2001) and the glioma cell line U87 (Newsham, data not shown).

DAL-1/4.1B maps to a region on human chromosome 18p11.3 that undergoes frequent loss of heterozygosity (LOH) in a significant proportion of lung, breast and brain tumors (Tran *et al*., 1998). Extensive analysis on

patients presenting with multiple stages of breast cancer revealed that 18p11.3 allelic deletions occur in greater than 55% of ductal carcinoma *in situ* (DCIS) tumors (Kittiniyom *et al*., 2001), suggesting that events disrupting DAL-1/4.1B expression occur early in the progression of this disease although intragenic mutations are uncommon (Kittiniyom *et al*., 2004). Furthermore, significant LOH and concomitant loss of DAL-1/4.1B protein has also been reported in greater than 70% of sporadic meningiomas (Gutmann *et al*., 2000). Interestingly, combined loss of DAL-1/4.1B and merlin is common (Perry *et al*., 2000), suggesting that both proteins contribute to a tumorigenic phenotype, at least in these central nervous system tumors, despite being structurally related members.

The mechanism(s) by which DAL-1/4.1B suppresses tumor cell growth is not clearly understood. Preliminary studies in MCF-7 breast cancer cells show that DAL-1/4.1B increases cell attachment while inducing apoptosis (Charboneau *et al*., 2002). Other studies performed on rat schwannoma cell lines suggest that DAL-1/4.1B inhibits cell motility and adhesion (Gutmann *et al*., 2001). Defining the interactions of DAL-1/4.1B with other proteins in different tumor types might elucidate the pathway(s) in which this tumor suppressor protein participates. An important domain for protein interactions in 4.1 family members is the shared N-terminal localization domain or FERM domain (Chishti *et al*., 1998). Previous studies of merlin and ERM (Ezrin/Radixin/Moesin) proteins have revealed binding of this N-terminal domain to a charged amino-acid motif in the cytoplasmic tail of transmembrane proteins CD44, CD43 and intracellular adhesion molecule 1 (ICAM1) (Legg and Isacke 1998; Yonemura *et al*., 1998). Contact inhibition of cell growth in rat schwannoma cells is mediated by merlin's regulated interaction with CD44 (Morrison *et al*., 2001). Characterizing such FERM-mediated protein–protein interactions will be important for understanding the function of DAL-1/4.1B.

To determine how DAL-1/4.1B functions to suppress the growth of human tumor cells, yeast two-hybrid interaction cloning was performed on a human lung cDNA library using the 336 amino-acid FERM domain of DAL-1/4.1B as bait. Screening of over 2 million colonies identified several interacting proteins, one of which represented 45% of the isolated cDNA clones. This protein, protein arginine *N*-methyltransferase 3 (PRMT3) is a member of an expanding family of enzymes that catalyse the sequential transfer of methyl groups from *S*-adenosyl-L-methionine to the guanidino nitrogens of arginine residues in proteins (Aletta *et al*., 1998; Gary and Clarke, 1998; Tang *et al*., 1998). Sequence comparisons indicate that all mammalian PRMT family members contain a common catalytic domain, but variable N- and C-terminal regions (Frankel and Clarke, 2000). Protein methylation by PRMT enzymes has been shown to regulate transduction of signals to the nucleus, transcription regulation through nuclear receptors, and RNA transport between the nucleus and cytoplasm (McBride and Silver, 2001). In this study, an association of PRMT3 with the tumor suppressor protein DAL-1/4.1B was confirmed by coimmunoprecipitation and *in vitro* binding assays. DAL-1/4.1B interaction is not exclusive within the arginine methyltransferase family although only one other Protein 4.1 family member tested, 4.1G, was found to complex with PRMT3. DAL-1/4.1B protein is not itself an *in vitro* substrate for PRMT3, but its interaction with this methyltransferase does inhibit the methylation of 'RGG' consensus sequences *in vitro* and cellular substrates *in vivo*. These results suggest that the interaction between a tumor suppressor and a post-translational methylation enzyme might be biologically important in controlling tumorigenesis.

Results

Tumor suppressor DAL-1/4.1B interacts with PRMT3

Yeast two-hybrid interaction cloning was performed to isolate potential binding partners of protein DAL-1/4.1B, originally identified as a tumor suppressor in lung tumor cells. A lung cDNA library was screened using a bait construct encoding the FERM domain (residues 1–337) of DAL-1/4.1B (Figure 1a). Analysis of more than 2 million cotransfectants led to the isolation of 20 positive colonies after two rounds of screening. Sequence analysis revealed that nine of these colonies contained cDNAs encoding human protein PRMT3. cDNA-insert sizes for PRMT3 ranged between 1.5 and 2.0 kb. In 1998, Tang *et al*. (1998) reported the amino-acid sequence for human PRMT3, lacking approximately 10 amino acids at the N-terminus. Sequence analyses indicated that only cDNAs encoding parts of the C-terminal region, which encodes the catalytic-core domain of the protein, were present in these positive clones, suggesting that this region is sufficient for interaction with DAL-1/4.1B (Figure 1a). To investigate protein expression profiles for human PRMT3, a polyclonal anti-PRMT3 antibody, raised against rat PRMT3 amino acids 166–528 (Tang *et al*., 1998), was used to screen several human tumor cell lines (Figure 1b). The lung cancer cell line NCI-H460, breast cancer lines MCF-7 and T47D and kidney cell line 293GP show expression of the 68 kDa band corresponding to the expected size of human PRMT3 when compared with the protein band found in rat heart homogenate. Based on equivalent loading of protein (confirmed by Coomassie staining), 293GP cells express high levels of the approximately 68 kDa PRMT3 protein, whereas the brain tumor cell line U87 expresses minimally detectable amounts of PRMT3 protein. Additional bands at 95 and 60 kDa may represent antibody crossreactivity with other related protein species sharing homology to PRMT3 and/or PRMT3 variants produced by alternate splicing. Lysates were also analysed for the presence of endogenous DAL-1/4.1B (Figure 1b). NCI-H460, T47D and 293GP cells expressed DAL-1/4.1B, while MCF-7 and U87 cell lines did not. Growth of both MCF-7 (Charboneau *et al*., 2002) and U87 cells (Newsham, unpublished data) have

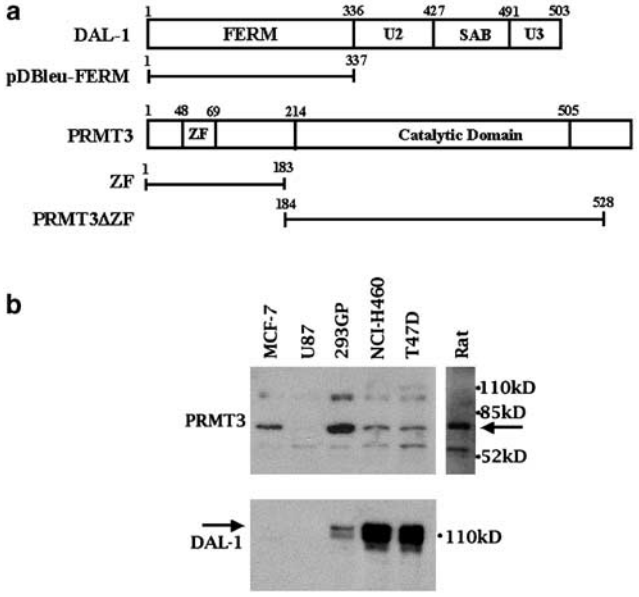

Figure 1 PRMT3 and DAL-1/4.1B expression profile. (**a**) Schematic representation of the specific domains of DAL-1/4.1B (GenBank no. AF069072) and PRMT3 (GenBank no. AF059530) as well as the constructs used for analysis in this study. Approximate amino-acid positions flanking these domains are shown above in each gene. FERM: 4.1/Ezrin/Radixin/Moesin domain; U2, U3: unique domains; SAB: spectrin/actin-binding domain; Zn: zinc-finger domain; Catalytic: methyltransferase catalytic domain; pDBleu-FERM: DAL-1/4.1B construct, containing the FERM domain, used for yeast two-hybrid analysis; ZF: region of PRMT3 protein containing the ZF domain used in binding studies; PRMT3ΔZF: PRMT3 protein fragment lacking the ZF domain used in binding studies. (**b**) Western analysis of human breast (MCF-7, T47D), brain (U87), kidney (293GP) and lung (NCI-H460) tumor cell lines was performed using 18 μg of total protein. Proteins were subjected to electrophoresis on 10% denaturing SDS–polyacrylamide gels, transferred to PVDF$^+$ membrane and probed with anti-PRMT3 antibody (upper panel) or anti-DAL-1/4.1B antibody 3A-1 (lower panel). Arrows indicate expected migration positions of endogenous DAL-1/4.1B and PRMT3 proteins. PRMT3 expression was detectable in all cell lines except the brain tumor cell line U87. In addition to the human PRMT3 protein, several other proteins crossreacted with the rat PRMT3 antibody. For DAL-1/4.1B, a doublet around 110 kDa was detected in 293GP, NCI-H460 and T47D cells, while no DAL-1/4.1B protein was expressed in MCF-7 or U87 cell lines. Coomassie blue staining confirmed equivalently loaded lanes (not shown)

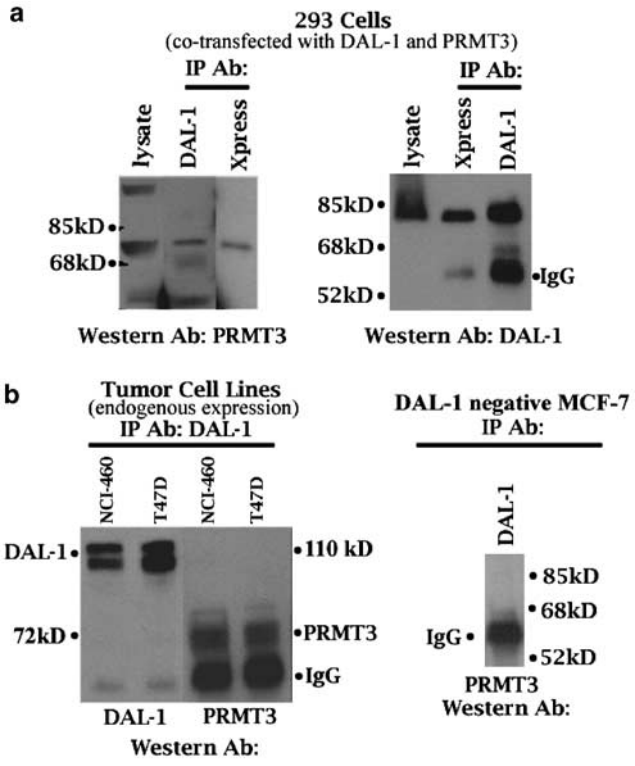

Figure 2 DAL-1/4.1B coimmunoprecipitates with PRMT3. DAL-1/4.1B and PRMT3 proteins from 293GP, NCI-H460 and T47D cell lines were tested for their ability to coimmunoprecipitate. (**a**) 293GP cells, transfected with pcDNA3-DAL-1/4.1B and pcDNA4-PRMT3, were immunoprecipitated with the polyclonal anti-DAL-1/4.1B antibody 3A-1 or monoclonal anti-Xpress antibody (Invitrogen) detecting an N-terminal epitope on PRMT3. Immunoprecipitated products were analysed by Western analysis with either the DAL-1/4.1B antibody or a polyclonal anti-PRMT3 antibody. Coimmunoprecipitated products were compared to proteins in the total cell lysate. Analysis showed that cotransfected DAL-1/4.1B and PRMT3 proteins are found complexed to each other in 293GP cells. (**b**) Endogenously expressed DAL-1/4.1B and PRMT3 were tested for interaction in NCI-H460 and T47D. Western analysis with DAL-1/4.1B following immunoprecipitation with DAL-1/4.1B antibody 3A-1 showed the expected doublet for DAL-1/4.1B. Western analysis of the same immunoprecipitate with the PRMT3 antibody revealed a strong band for PRMT3, indicating that endogenous PRMT3 and DAL-1/4.1B interact in cells expressing both proteins. The DAL-1/4.1B-negative cell line MCF-7 reveals no nonspecific crossreactivity to other cellular proteins exists for the 3A-1 antibody. IP: immunoprecipitation; IgG: immunoglobulin heavy chain; kDa: kilodalton; Ab: antibody

been shown to be suppressed upon re-expression of the DAL-1/4.1B protein.

To confirm the interaction of DAL-1/4.1B and PRMT3 in mammalian cells, expression vectors containing the original 2A3 cDNA clone for DAL-1/4.1B (pcDNA3-DAL-1/4.1B) and Xpress epitope-tagged PRMT3 (pcDNA4-PRMT3) were transiently transfected into 293GP cells. These cells were chosen for their high transfection efficiency. An epitope-tagged PRMT3 construct was used as preliminary studies indicated that the rat PRMT3 polyclonal antibody was inadequate at immunoprecipitating human PRMT3. The 2A3 DAL-1/4.1B cDNA produces an 80 kDa variant of the 110 kDa endogenous protein, which possesses all sequences required for growth suppression in tumor cell lines (Gutmann *et al*., 2001). Western analysis of immunoprecipitates generated with both DAL-1/4.1B-specific and PRMT3 epitope-tag Xpress antibodies revealed that human PRMT3 coimmunoprecipitates with DAL-1/4.1B (Figure 2a). The interaction of PRMT3 and DAL-1/4.1B was also assessed in human cell lines expressing both endogenous DAL-1/4.1B and PRMT3. Immunoprecipitation of DAL-1/4.1B in the NCI-H460 and T47D cell lines, followed by Western analysis with the polyclonal anti-PRMT3 or anti-DAL-1/4.1B antibodies show that endogenous PRMT3 associates with endogenous DAL-1/4.1B (Figure 2b), further supporting the results obtained in the 293GP overexpression experiments.

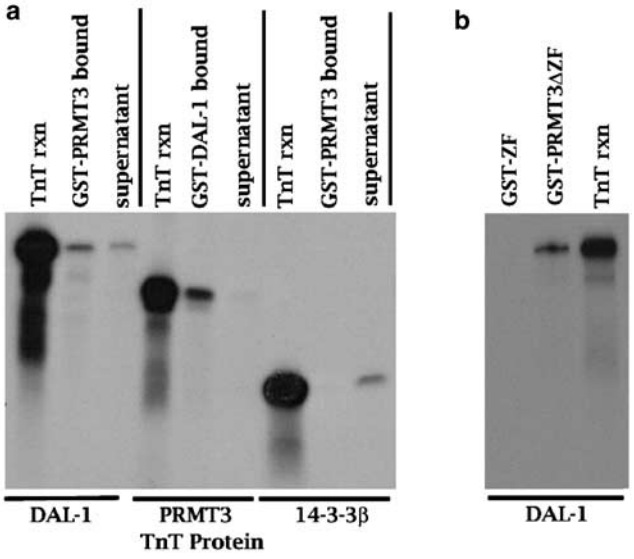

Figure 3 DAL-1/4.1B interacts directly with PRMT3. GST-fusion products (2 μg each) of DAL-1/4.1B, PRMT3, PRMT3ΔZF and ZF were generated and confronted *in vitro* with 5 μl of TnT-radiolabeled proteins DAL-1/4.1B, PRMT3 and 14-3-3β in various combinations. Aliquots of each radiolabeled protein were run as a reference. Equivalent amounts of protein collected on GST-beads and remaining in the supernatant were analysed by electrophoresis and autoradiography. (**a**) Both GST-PRMT3 and GST-DAL-1/4.1B directly interact with their respective radiolabeled protein partners. This interaction is specific for DAL-1/4.1B and PRMT3, as PRMT3 was not able to bind the ubiquitously expressed cytoplasmic-binding protein 14-3-3β. (**b**) Radiolabeled DAL-1/4.1B was confronted with either the N-terminal zinc-finger (GST-ZF) or catalytic domain of PRMT3 (GST-PRMT3ΔZF). DAL-1/4.1B interacted directly with the catalytic domain but not with the zinc-finger domain. TnT rxn: transcription and translation reaction protein

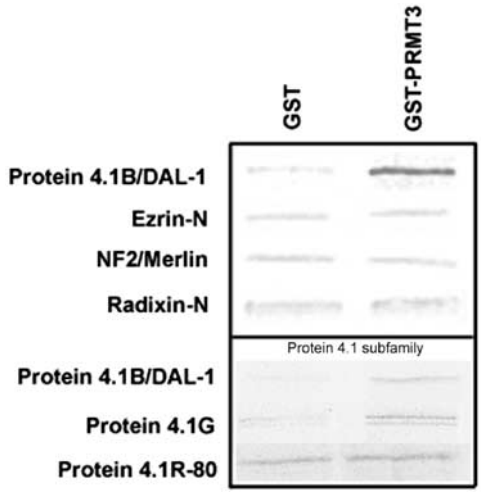

Figure 4 Protein 4.1 family binding to PRMT3. Protein 4.1 family members DAL-1/4.1B, 4.1G, 4.1R80 as well as the FERM domains of NF2/merlin, ezrin and radixin were examined for their ability to bind to PRMT3 *in vitro*. Each TnT-radiolabled 4.1 family protein was incubated with equimolar amounts of either GST-PRMT3 or GST control proteins. GST-protein complexes were purified on glutathione Sepharose beads and separated by SDS–PAGE electrophoresis as described in the Materials and methods. In addition to DAL-1/4.1B, protein 4.1G was the only other 4.1 family member tested that showed an affinity for binding to PRMT3

In vitro binding assays were also performed to further confirm this interaction. Radiolabeled DAL-1/4.1B or PRMT3 proteins were incubated with either glutathione-*S*-transferase (GST)-PRMT3 or GST-DAL-1/4.1B, respectively, and protein complexes were retrieved on GST affinity beads. Following centrifugation, the presence of bound radiolabeled protein was analysed by SDS–PAGE separation and autoradiography. As shown in Figure 3, fractions of bound [^{3}H]DAL-1/4.1B and [^{3}H]PRMT3 proteins were significantly higher than in the supernatant when confronted with the opposing GST-fusion protein. As a negative control, an *in vitro* binding assay was performed using [^{3}H]14-3-3β and GST-PRMT3. Protein 14-3-3 molecules are abundantly expressed proteins that have been shown to have affinity for a wide variety of cellular proteins (Fu *et al*., 2000). Protein 14-3-3β did not interact with PRMT3. Binding assays were also performed with a zinc-finger domain fragment (GST-ZF) and a catalytic domain fragment (GST-PRMT3ΔZF) of PRMT3 (see Figure 1a). The C-terminal end of PRMT3 (encompassed in GST-PRMTΔZF; amino acids 184–528) interacted with DAL-1/4.1B (Figure 3b), supporting the original yeast two-hybrid results that identified cDNAs containing the C-terminal end of the PRMT3 coding region. The N-terminal zinc-finger domain of PRMT3 (GST-ZF; amino acids 1–183) showed no affinity for DAL-1/4.1B. Thus, DAL-1/4.1B interacts with PRMT3 both *in vivo* and *in vitro* via the catalytic C-terminal core region of the protein.

PRMT and Protein 4.1. family interactions

Previous work by our group has shown that NF2/merlin and DAL-1/4.1B are structurally similar and function to suppress growth in meningiomas (Gutmann *et al*., 2000). In addition, NF2/merlin and DAL-1/4.1B have been shown to share a portion of the spectrum of binding partners (Gutmann *et al*., 2001). Therefore, the ability of PRMT3 to interact with other members of the Protein 4.1 family was examined. Radiolabeled N-terminal FERM domain-containing regions of NF2/merlin, ezrin and radixin as well as full-length 4.1B-like subfamily members 4.1G and 4.1R80 were confronted with GST or GST-PRMT3 proteins and assayed for their ability to interact *in vitro*. Results of these binding assays are shown in Figure 4. Protein 4.1 family member 4.1G was the only other Protein 4.1 family member other than DAL-1/4.1B to show an affinity for interacting with PRMT3.

Given that the catalytic core domain of PRMT3 is the region of interaction with DAL-1/4.1B and that the core domains of the PRMT family members are highly conserved, we also determined whether the DAL-1/4.1B interaction was mutually exclusive to PRMT3. PRMT proteins 1–6 were examined for their ability to interact by both *in vitro* binding assays and coimmunoprecipitation reactions in 293GP cells (Figure 5). PRMT 3, 5 and

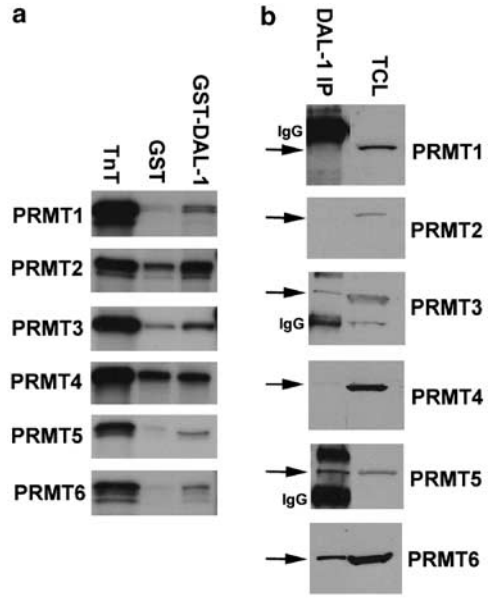

Figure 5 Binding of DAL-1/4.1B to members of the PRMT family. The interaction of DAL-1/4.1B and other members of the PRMT family was investigated using both *in vitro* confrontation assays and coimmunoprecipation experiments in 293GP cells. (**a**) Equimolar amounts of GST and GST-DAL-1/4.1B were incubated with 5 μl of TnT radiolabeled PRMT 1, 2, 3, 4, 5 and 6 proteins as described in the Materials and methods. GST complexes were purified by affinity chromatography and the proteins fractionated by SDS gels. The region of the gel where PRMT proteins migrated is displayed. A 2–5 μl aliquot of each TnT-radiolabeled protein was run as a control. These assays show that PRMT4 is the only family member which does not show affinity for binding to DAL-1/4.1B *in vitro*. (**b**) Individual PRMT proteins and DAL-1/4.1B were coexpressed in 293GP cells and the complexes were immunoprecipitated using the rabbit polyclonal anti-DAL-1 antibody 3A-1. Complexes were centrifuged, fractionated by SDS gel electrophoresis and analysed by Western blot analysis. PRMT proteins were visualized using PRMT-specific antibodies for PRMT 1, 3 and 5, while PRMT 2, 4 and 6 were identified via their 5′ Xpress epitope tag. A fraction of the total cell lysate (TCL) was used to confirm expression levels and PRMT protein sizes. PRMT 3, 5 and 6 were found to coimmunoprecipitate in these experiments, while PRMT 1, 2 and 4 did not form complexes in these cells. The apparent binding of PRMT 1 and 2 with DAL-1/4.1B *in vitro* (Figure 4) but lack of interaction in 293GP cells suggests there may be additional cellular proteins which may cooperate to regulate these interactions *in vivo*. IgG, immunoglobulin heavy chain

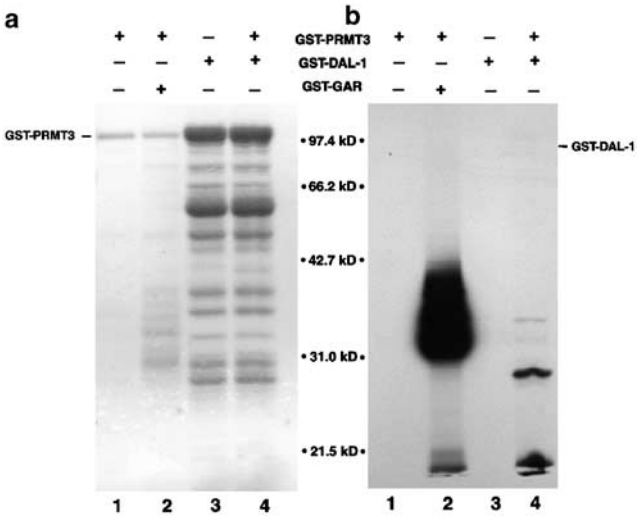

Figure 6 Full-length GST-DAL-1/4.1B is not a substrate for PRMT3. Reaction mixtures containing GST-GAR (5.0 μg), GST-DAL-1/4.1B (10.5 μg) or no methyl-accepting protein were incubated with 2 μg of GST-PRMT3 (or with no enzyme) in the presence of 0.8 μm [^{3}H]AdoMet at 37°C for 60 min in a final volume of 30 μl phosphate-buffered saline. The polypeptides were then fractionated by 10% SDS–PAGE as described in the Materials and methods. (**a**) Coomassie-stained SDS–PAGE gel of GST-tagged proteins used for the PRMT3/DAL-1/4.1B *in vitro* methylation assays. Both GST-DAL-1/4.1B (lane 3, 10.5 μg) and GST-PRMT3 (lane 1, 2 μg) migrate at 97 kDa. The GAR protein substrate can be seen in lane 2 at 31–35 kDa. (**b**) Fluorograph, representing a 2-week exposure at −80°C, showing methylation of GST-GAR but no methylation of GST-DAL-1/4.1B

6 were found to complex with DAL-1/4.1B in both assays, whereas PRMT 1 and 2 bound *in vitro* but not when coexpressed in 293GP cells. It is possible that a cellular substrate exists which regulates the interaction of DAL-1/4.1B with these particular PRMTs. PRMT4 did not appear to interact with DAL-1/4.1B in either assay suggesting that it is not a binding partner for this tumor suppressor protein, although the apparent high background binding to GST may be masking such an interaction.

DAL-1/4.1B inhibits PRMT3 activity in vitro

PRMT3 is a type I arginine methyltransferase responsible for methylating the terminal guanidino nitrogens of arginine residues to produce mono- and asymmetric dimethylarginine in proteins (Aletta *et al*., 1998; Tang *et al*., 1998). *In vitro* assays measuring the potential methylation of DAL-1/4.1B by PRMT3 were performed using GST fusion proteins and the *in vitro* methyl-accepting substrate glycine- and arginine-rich (GAR). GAR contains 14 arginine residues within a glycine-rich region, the majority of which reside within 'RGG' consensus methylation sites, and has been shown previously to be a PRMT3 methylation substrate (Lischwe *et al*., 1985; Tang *et al*., 1998). Incubation of GST-PRMT3 in the presence of GAR and [^{3}H]AdoMet results in the generation of a diffusely radiolabeled band at 30–35 kDa, which represents partially proteolized, methylated fragments of GST-GAR (Figure 6b). When GST-PRMT3 and GST-DAL-1/4.1B proteins were combined, no methylation of the DAL-1/4.1B protein was observed, indicating that DAL-1/4.1B is not itself a substrate for PRMT3. Such methylation products would be expected to migrate around 97 kDa (Figure 6a). The ^{3}H-labeled product migrating at 28 kDa was shown to represent a bacterial methyl-acceptor, which copurified with DAL-1/4.1B during the preparation of the GST-DAL-1/4.1B fusion protein was cleared when more stringent washing conditions were applied during protein purification (data not shown). This 28 kDa species is not observed in the lane with the GST-PRMT3 protein alone.

Although the DAL-1/4.1B protein is not methylated by PRMT3, the PRMT3/DAL-1/4.1B-complex might

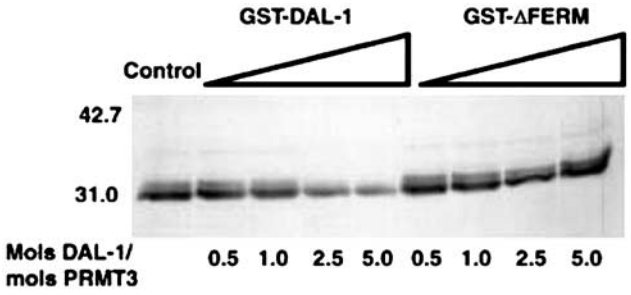

Figure 7 Effect of DAL-1/4.1B on the activity of PRMT3. GST-PRMT3 was incubated with the GST-GAR methylation substrate in the presence of increasing amounts of GST-DAL-1/4.1B fusion protein. GST-PRMT3 (1.0 μg) was incubated with GST-GAR (2.0 μg) and 0.4 μM [^{3}H]AdoMet at 37°C for 30 min in 100 mM sodium phosphate (pH 7.5) in a final volume of 70 μM. As indicated, increasing amounts of either GST-DAL-1/4.1B (0.5–5.0 μg) or GST-ΔFERM (0.5–5.0 μg) were added to the reaction mixture. GST-DAL-1/4.1B clearly decreases the ability of PRMT3 to methylate the GST-GAR substrate (3.3-fold decrease at 5.0 μg) when compared to GST-ΔFERM domain alone or no additional protein (control). These results are representative of at least three individual experiments

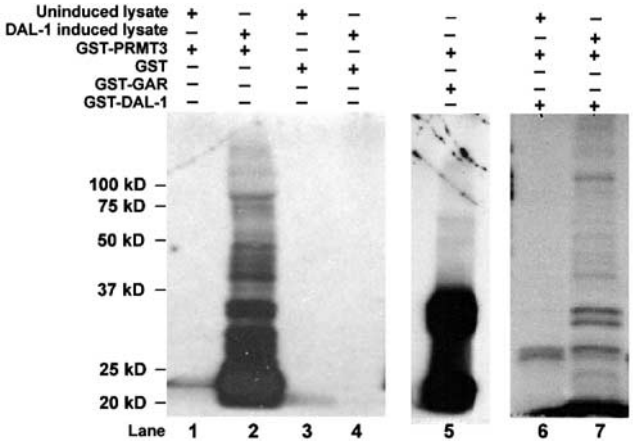

Figure 8 Induced DAL-1/4.1B expression inhibits PRMT3-associated methylation in MCF-7 breast cancer cells. The ability of DAL-1/4.1B to inhibit PRMT3 methylation of cellular substrates *in vivo* was examined using a DAL-1/4.1B-inducibile MCF-7 breast cancer cell line, cl27. Cell lysates were harvested following 48 h with and without induction of DAL-1/4.1B protein expression. Purified GST-PRMT3 (10 μg) or GST (7.5 μg) control protein were added in the presence of the methyl-donating [^{3}H]AdoMet. Left panel, induced endogenous DAL-1/4.1B protein expression inhibited PRMT3-associated methylation (compare lanes 1 and 2) making a variety of substrates available for *in vitro* methylation upon addition of GST-PRMT3. No methylation is observed with either the induced or uninduced extracts with the addition of GST protein only (lanes 3 and 4). Middle panel, the active nature of the purified GST-PRMT3 protein was confirmed by the addition of 2 μg of the *in vitro* methylation substrate GST-GAR (lane 5). Right panel, the addition of purified GST-DAL-1/4.1B protein (7.5 μg) to the *in vitro* methylation assay following endogenous expression of DAL-1/4.1B protein significantly inhibits the ability of GST-PRMT3 protein to methylate hypomethylated cellular substrates (compare lanes 6 and 7), further supporting a role for DAL-1/4.1B in the inhibition of PRMT3. Additionally, the presence of GST-DAL-1/4.1B does not stimulate *in vitro* methylation directly in the uninduced or DAL-1/4.1B-induced cell extracts. kD, kilodaltons; GST, glutathione-*S*-transferase

redirect or alter PRMT3 catalytic activity. To test this hypothesis, GST-PRMT3 was incubated in the presence of GST-GAR substrate, along with increasing amounts of either GST-DAL-1/4.1B or a GST-DAL-1/4.1B sequence deleted for the interacting FERM domain (GST-ΔFERM). Addition of GST-DAL-1/4.1B showed dose-dependent inhibition of PRMT3-mediated GAR methylation (Figure 7). Densitometric analysis measured a 3.3-fold decrease in GST-GAR methylation in the presence of 5 times as much DAL-1/4.1B as PRMT3 protein. In the presence of GST-ΔFERM protein, no inhibition of GAR methylation was observed, indicating a requirement for the PRMT3-binding FERM domain in the inhibition of *in vitro* methylation of GST-GAR by GST-PRMT3.

DAL-1/4.1B expression inhibits PRMT3 methylation in vivo *in MCF-7 cells*

If the overexpression of DAL-1/4.1B results in the hypomethylation of PRMT3 cellular substrates *in vivo*, then protein extracts from DAL-1/4.1B-expressing cells should be good methyl acceptors for *in vitro* methylation reactions using the GST-PRMT3 enzyme. To assess this, we used a breast cancer cell line, MCF-7 cl27, which has been engineered to be inducible for expression of the DAL-1/4.1B protein (Charboneau *et al*., 2002). MCF-7 cl27 cells were induced to express DAL-1/4.1B protein for 48 h. Cell lysates were collected and *in vitro* methylation reactions were performed in the presence of GST or GST-PRMT3 proteins (Figure 8). In uninduced cl27 cell lysates, GST-PRMT3 did not catalyse the labeling of proteins in the presence of [^{3}H]AdoMet indicating that the endogenous PRMT3 activity in MCF-7 cells had successfully methylated its cellular substrates *in vivo*. However, in cells grown in the presence of induced DAL-1/4.1B protein, addition of GST-PRMT3 to the cell lysate catalysed significant *in vitro* methylation of a variety of cellular proteins as shown by the ladder of labeled bands in lane 2 of Figure 8. Such *in vitro* methylation indicates that the induced DAL-1/4.1B protein inhibited the ability of endogenous PRMT3 to methylate cellular substrates, consistent with our *in vitro* methylation assay results shown in Figure 7. No methylation was found in either cell lysate when only GST control protein was added.

To confirm that the GST-PRMT3 protein used in these assays was active, the *in vitro* substrate GST-GAR was added to the methylation reaction. This produced the expected radiolabeled band at 30–35 kDa (Figure 8, middle panel) representing the methylated fragments of GST-GAR previously shown in Figures 6 and 7. Furthermore, we hypothesized that if additional GST-DAL-1/4.1B protein was added to reactions using lysates from DAL-1/4.1B-expressing cells, this additional protein should serve to quench the GST-PRMT3-mediated *in vitro* methylation of cellular substrates. This was indeed the case where the ladder of labeled proteins produced is significantly diminished (Figure 8, right

panel) from that shown without additional GST-DAL-1/4.1B (Figure 8, left panel). Additionally, the lack of methylation seen in lane 6 shows that GST-DAL-1/4.1B does not stimulate methylation *in vitro*, further supporting the ability of endogenous PRMT3 to methylate substrates *in vivo* (lane 1) in the absence of induced DAL-1/4.1B protein expression.

Discussion

The tumor suppressor DAL-1/4.1B shares significant sequence similarity in its N- terminal FERM domain with members of the Protein 4.1 superfamily that includes the NF2 tumor suppressor merlin. Like other 4.1 family member proteins, DAL-1/4.1B has been localized to regions of the plasma membrane at points of cell-to-cell contact by immunocytochemistry (Tran *et al*., 1999). While merlin has been shown to interact with several proteins including NHERF, SCHIP and CD44 (Murthy *et al*., 1998; Yonemura *et al*., 1998; Goutebroze *et al*., 2000), comparatively less is known about DAL-1/4.1B-binding partners. To determine the protein interactions that might transduce the DAL-1/4.1B growth suppressor signal, yeast two-hybrid analysis of a lung cDNA library was performed using the FERM domain of DAL-1/4.1B as bait. We identified the human protein PRMT3 gene (Tang *et al*., 1998) as a binding partner of DAL-1/4.1B. This interaction was also seen in mammalian cells by coimmunoprecipitation, and supported by *in vitro* confrontation experiments. Despite significant homology in the N-terminal FERM domain within the Protein 4.1 superfamily, the domain responsible for interaction of DAL-1/4.1B and PRMT3, Protein 4.1G was the only other family member tested that was also found to complex with PRMT3 (Figure 4).

PRMT3 protein has been immunolocalized to the cytoplasm in rat cells (Tang *et al*., 1998) and as a cytoplasmic GFP fusion protein in human HeLa cells (Frankel *et al*., 2002). Expression analysis in a variety of tissues demonstrates almost universal expression for this methyltransferase (Tang *et al*., 1998). The fact that DAL-1/4.1B and PRMT3 are expressed in the same tissues and associate *in vitro* and *in vivo* suggests biological relevance for a DAL-1/4.1B and PRMT3 complex. Despite their interaction, DAL-1/4.1B was determined not to be a methyl-accepting substrate for PRMT3 as assayed by *in vitro* methylation (Figure 6) but rather appears to inhibit PRMT3-mediated methylation activity *in vitro* and *in vivo* (Figures 7 and 8).

Currently, the mammalian protein methyltransferase family consists of seven gene products (Frankel and Clarke, 2000; Frankel *et al*., 2002; Miranda *et al*., 2004). These enzymes mediate methylation, typically although not exclusively within RGG consensus sequences, in the context of GAR regions through the transfer of methyl groups from the donor *S*-adenosyl-L-methionine (AdoMet) (Lischwe *et al*., 1985; Tang *et al*., 1998). All PRMT proteins share a relatively conserved catalytic core domain – the domain through which PRMT3 was shown to interact with DAL-1.4B (Figure 3). A complex binding pattern for the PRMT family and DAL-1/4.1B was determined where PRMT 3, PRMT 5 and PRMT 6 were found to interact both *in vitro* and *in vivo*, while PRMT 1 and PRMT 2 bound *in vitro* but did not co-immunoprecipitate from 293GP cells. PRMT4 did not interact with DAL-1/4.1B in either assay.

The biological interaction of DAL-1/4.1B and PRMT proteins may be additionally regulated by the widely varying length and sequence of the N- and C-terminal ends of these family members or by the presence of other binding partners. In the case of PRMT3, a 194 amino acid N-terminal portion contains two motifs – a zinc-finger domain (Cys^{48}–His^{69}) and a tyrosine phosphorylation consensus sequence (Tyr^{85}–Phe^{93}) (Tang *et al*., 1998). This phosphorylation sequence (GLEFYGYIK) is similar to the JAK kinase substrate site GPKGYIK present in STAT1 (Shuai *et al*., 1992), an identified substrate of PRMT1 (Mowen *et al*., 2001). PRMT2, whose activity has not yet been demonstrated, contains an SH3-binding domain in its N-terminal portion (Aletta *et al*., 1998). The presence or absence of N-terminal-interacting or regulatory domains may be important for defining the specificity of these protein-methylating enzymes.

If arginine methylation is an irreversible modification, as is generally believed, then regulation of protein methylation would presumably have to occur at the level of modulating either the activity or the level of expression of the methyltransferase enzyme itself. The possibility that interacting proteins critically modulate PRMT3 kinetics and specificity is worth considering. Support for this hypothesis comes from previous studies on PRMT1. Yeast two-hybrid analysis with the immediate early gene TIS21/BTG1 revealed a strong interaction with the catalytic domain of PRMT1. TIS21 was found not to be a substrate for PRMT1 methylation, but its binding enhanced the ability of PRMT1 to methylate substrates in hypomethylated RAT1 extracts (Lin *et al*., 1996). Another family member, BTG2, is a candidate tumor suppressor regulating the G1/S transition of the cell cycle. It too has been shown to interact with PRMT1 and enhance methylation of histone H2A (Berthet *et al*., 2002).

This current study shows an alternate situation in which DAL-1/4.1B appears to inhibit the level of post-translational modification of arginine residues by PRMT3 as measured by decreased methylation of a GST-GAR substrate *in vitro* (Figure 7) and more importantly, as detected in cells grown in the presence of endogenously induced DAL-1/4.1B protein (Figure 8). Interestingly, little PRMT3 methylation was detected in RAT1 cell extract assays unless the protein lysate had been pretreated with RNase (Frankel and Clarke, 1999), suggesting methylation substrates might exist in complexes with RNA species that may also inhibit methyltransferase action. Binding of DAL-1/4.1B may inhibit the release of such inhibitors from methylation substrates (indirect inhibition) and this may involve the zinc-finger domain of PRMT3 or it may structurally prevent the interaction of these protein substrates with

the catalytic domain of PRMT3 (direct inhibition). DAL-1/4.1B has also been shown to complex with several isoforms of the adapter protein 14-3-3 (Yu *et al*., 2002). These proteins stimulate protein–protein interactions as well as regulate the subcellular location of proteins and have been implicated in cell cycle control, signal transduction and apoptosis (reviewed in Muslin and Xing, 2000). Recent data show that 14-3-3 η binds both DAL-1/4.1B and 4.1G, the same Protein 4.1 family interaction profile exhibited by PRMT3 (Robb *et al*., 2004). In addition, the DAL-1/4.1B FERM sequences required for 14-3-3 (Robb *et al*., 2004) and PRMT3 (Newsham, unpublished data) protein binding are different, suggesting that DAL-1/4.1B and its ability to regulate PRMT3 could be affected by the simultaneous or alternate presence of a 14-3-3 molecule. In the context of the mammalian cell, therefore, the mechanisms responsible for regulating the binding of such protein and RNA species would be crucial to the functionality of PRMT3.

Many different types of proteins have been identified as targets of arginine methylation by PRMT proteins. The most common targets are RNA-binding proteins, some of which play roles in pre-mRNA processing and nucleocytoplasmic RNA transport. In fact, PRMT3 has recently been shown in the fission yeast *Schizosaccharomyces pombe* to be a ribosomal protein methyltransferase that affects the cellular level of ribosomal subunits (Bachand and Silver, 2004). Other general PRMT-methylated proteins include heterogeneous nuclear ribonucleoproteins (hnRNPs) (Kumar *et al*., 1986; Kim *et al*., 1997), poly(A)-binding protein II (Smith *et al*., 1999) and spliceosomal snRNP proteins SmD1 and SmD3 (Brahms *et al*., 2000). Additionally identified targets include fibrillarin (Tang *et al*., 2000a), the mitotic Src kinase substrate Sam^{68} (Bedford *et al*., 2000), Signal Transducer and Activator of Transcription STAT1 (Mowen *et al*., 2001) and interleukin enhancer-binding factor (Tang *et al*., 2000b). Recent studies have implicated arginine methylation in modulation of specific intra- and intermolecular interactions of target proteins (Frankel and Clarke, 2000). For example, methylation in the proline-rich region of Sam^{68}, an adaptor protein in signaling pathways, decreases its binding to SH3-containing proteins without affecting binding to WW domain-containing proteins (Bedford *et al*., 2000). Although methylation does not affect the overall charge of an arginine residue, it does increase steric hindrance and potentially disrupts hydrogen bonding by removing guanidine hydrogen atoms. Arginine methylation has also been implicated in the regulation of signaling and transcription activation. Methylation of STAT-1 decreases its affinity for the inhibitor PIAS1 and impairs STAT-1's ability to bind DNA (Mowen *et al*., 2001). Likewise, methyltransferase interactions with the p160 family of transcriptional coactivators of nuclear hormone receptors also result in an enhancement of gene expression (Chen *et al*., 1999). Such expression may also be indirectly affected by alteration of chromatin structure through PRMT methylation of histones (Wang *et al*., 2001).

Protein methylation, therefore, represents an important post-translational modification system that can impact the ability of a variety of proteins to perform their biological functions. The diversity of proteins found to be substrates for PRMT molecules suggests that arginine methylation or protein methylation in general may begin to parallel protein phosphorylation in its complexity. In this report, we show that the tumor suppressor DAL-1/4.1B, which suppresses growth in multiple cell types, interacts with PRMT3 and inhibits its ability to methylate at the archetypal 'RGG' consensus methylation sequences as well as endogenous PRMT3 cellular substrates. How the PRMT3/DAL-1/4.1B interaction directly or indirectly relates to DAL-1/4.1B growth suppression may partially be explained through the identification of those proteins that are substrates for PRMT3 and whose methylation is altered by the presence of DAL-1/4.1B protein. The identification of these methylation substrates may support the hypothesis that DAL-1/4.1B impacts cell growth through mediating post-translational protein modifications, thereby regulating their ability to function in pathways important in controlling cell growth. This would be a unique mechanism for such tumor suppressor gene function in mammalian cells.

Materials and methods

Yeast two-hybrid analysis to identify proteins that interact with the FERM domain of DAL-1/4.1B

Yeast two-hybrid analysis to identify proteins interacting with the FERM domain of DAL-1/4.1B was performed using the ProQuest™ Two-Hybrid system (InVitrogen). The FERM domain of DAL-1/4.1B, representing the first 336 amino acids of the DAL-1/4.1B cDNA clone 2A3 (Figure 1a) (Tran *et al*., 1999) was amplified using primers 5′- ACG-TGT-CGA-CCA TGC-AGT-GCA-AAG-TGA-TAC-3′ and 5′-ACG-TGC-GG C-CGC-CTC-TCC-ATC-CAA-GCT-GCG-3′ containing *Sal*I and *Not*I linkers, respectively. The resulting 1015 bp fragment was cloned into the pDBLeu 'bait' vector creating a fusion protein with the GAL-4 transcription factor DNA-binding domain. An adult human lung cDNA library (InVitrogen) encoding expressed cDNAs fused with the GAL-4 transcription factor activation domain was used for screening. More than 2 million cDNA clones were screened with 45 positively interacting colonies being identified based upon growth on leucine-, tryptophan-, uracil- and histidine-lacking plates, as well as the ability to process lacZ substrate. Following secondary screening, 20 colonies were selected. pPC86-cDNA constructs were isolated from the interacting yeast colonies using the Yeast DNA Isolation System (Stratagene) and the inserts were sequenced and analysed with either Thermosequenase Cy 5 or Cy 5.5 terminator sequencing kits (AP Biotech, Inc.) on the Long – Read™ System sequencer (Visible Genetics Inc). Homology searches were performed using the Blast algorithm through the National Center for Biotechnology Information (Altschul *et al*., 1990).

Cell culture and Western analysis

Lung tumor cell line NCI-H460, and breast carcinoma cell lines T47D and MCF-7 were obtained from the American Type Culture Collection (Manassas,VA, USA). T47D cells

were maintained in RPMI 1640 with 10% fetal calf serum (FCS) and insulin. NCI-H460 cells were maintained in the same medium without insulin. The MCF-7 cl27 cell line is a DAL-1/4.1B-inducible cell line generated from the parental MCF-7 cell line using the Ecdysone muristerone-inducible Expression Kit (InVitrogen) (Charboneau *et al*., 2002). This cell line is grown in MEM with 10% FCS, sodium pyruvate, nonessential amino acids and insulin. DAL-1/4.1B expression is induced by the addition of 1–2 μM muristerone to the culture medium for 48 h. Cell lysates were prepared in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40) with protease inhibitors (Boehringer Mannheim). Electrophoresis was performed on either 10 or 12% SDS–PAGE gels (BioRad) and transferred onto PVDF Plus membrane (MSI Inc.) using the BioRad Mini Protean II transfer system as previously described (Tran *et al*., 1999).

Coimmunoprecipitation assays

The full-length rat PRMT3 cDNA was cloned in frame into the pcDNA4-Xpress/His vector (InVitrogen), which provides both 5′ Xpress and His epitope tags. Constructs pcDNA4-PRMT3 and pcDNA3-DAL-1/4.1B (Tran *et al*., 1999) were cotransfected into 293GP cells using calcium phosphate precipitation. At 2 days after transfection, protein lysates were prepared as described above and immunoprecipitates generated from 1–2 mg of lysate using either the monoclonal antibody against the Xpress epitope tag (InVitrogen) or the polyclonal anti-DAL-1/4.1B antibody 3A-1 (Tran *et al*., 1999). Immunoprecipitation was also performed on DAL-1/4.1B and PRMT3 endogenously expressing cell lines NCI-H460 (lung cancer) and T47-D (breast cancer) using the anti-DAL-1/4.1B antibody. Immunoprecipitates were collected on Protein A Agarose beads (Roche Molecular Biochemicals) and washed 3–5 times in RIPA buffer. Protein A agarose bound proteins were analysed by Western blotting using either the rat polyclonal anti-PRMT3 antibody (Tang *et al*., 1998) or the anti-DAL-1/4.1B antibody (Tran *et al*., 1999) detected with either mouse IgG or rabbit IgG ImmunStar Chemiluminescence kits (BioRad).

Coimmunoprecipitation analysis of other PRMT family members PRMT1, 2, 4, 5 and 6 with DAL-1/4.1B were performed as described above except that Lipofectamine 2000 was used for some transfections. Immunoprecipitates collected on Protein A agarose beads were washed with either 1 × PBS (9 g/L NaCl, 0.144 g/L KH_2PO_4, 0.795 g/L NH_2HPO_4, pH 7.5) or 1 × TTBS (0.1% Tween, 20 mM Tris, pH 7.5. 0.5 M NaCl). Immunoprecipitated PRMT1 was detected using a rat polyclonal anti-PRMT1 antibody (Lin *et al*., 1996) (1 : 5000), while PRMT 2, 4 and 6 were detected using the mouse anti-Xpress antibody (InVitrogen) (1 : 2500). PRMT5 was detected using a mouse anti-PRMT5 antibody (Upstate Biotech) (1 : 1000).

In vitro *binding assays*

For binding domain localization, pGEX plasmids expressing rat recombinant GST fusion proteins GST-PRMT3, GST-PRMT3ΔZF (PRMT3 C-terminal amino acids 184–528) and GST-ZF (PRMT3 N-terminal amino acids 1–183) described previously (Frankel and Clarke, 2000) were used and are schematically illustrated in Figure 1. Fusion proteins were expressed in BL-21 cells (Novagen) after induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside(IPTG). Purified GST-fusion proteins were isolated using the Bug-Buster™ GST-Bind™ purification kit (Novagen). Full-length [^{3}H]leucine-labeled DAL-1/4.1B and PRMT3 proteins were generated by coupled *in vitro* transcription and translation (TnT; Promega). Individually labeled proteins were confronted with an excess of GST-DAL-1/4.1B, GST-PRMT3, GST-14-3-3β, GST-PRMT3ΔZF or GST-ZF in Bug Buster binding/wash buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM $NaCl_2$, 2.7 mM KCl, pH 7.3). Complexes were collected on GST-Bind resin (Novagen), washed several times with Bug Buster binding/wash buffer and separated on 10% SDS–PAGE gels. Gels were fixed in an isopropanol : water : acetic acid (25 : 65 : 10) solution and fluorographic signals were enhanced using Amplify™ (Amersham). Vacuum-dried gels were exposed to autoradiographic film for 1–5 days.

In vitro binding assays assessing PRMT and Protein 4.1 family interactions were also performed. Protein 4.1 family member binding to PRMT3 (Figure 4) was performed with NF2/merlin, ezrin, radixin, 4.1G and 4.1R80 as previously described (Yu *et al*., 2002). *In vitro* binding of DAL-1/4.1B to PRMT family members 1–6 (Figure 5) was performed as described above except Glutathione Sepharose 4B (Pharmacia Biotech) was used to collect complexes followed by several washes in 1 × TTBS.

In vitro *methylation reactions*

The preparation of GST-PRMT3 and GST-GAR and the procedure for the methylation reactions have been described previously (Tang *et al*., 1998; Frankel and Clarke, 2000). GST-DAL-1/4.1B and GST-ΔFERM (a DAL-1/4.1B cDNA deleted for the FERM domain (Tran *et al*., 1999)) were prepared as described above but purified as described in Frankel and Clarke, (2000) with more extensive washing of the glutathione-beads prior to elution. Here, 10 ml of 50 mM Na_2HPO_4, 137 mM $NaCl_2$ and 2.7 mM KCl, pH 7.5 are added to the beads and the mixture rotated for 10 min at 4°C. This wash step is repeated twice more. A second wash step is then performed by rotating the beads for 10 min at 4°C with 10 ml of 137 mM $NaCl_2$, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4) and this was repeated twice more. Finally, the fusion protein was eluted from the beads with 30 mM glutathione, 50 mM Tris-HCl and 120 mM $NaCl_2$.

For the methylation reactions, GST-PRMT3 and GST-GAR were incubated with either GST-DAL-1/4.1B or GST-ΔFERM in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; 77 Ci/mmol from a 10.4 μM stock solution in dilute HCl/ethanol 9 : 1, pH 2.0–2.5; Amersham Biosciences) for 30–60 min at 37°C. GST-GAR is a protein arginine methyltransferase substrate containing the first 148 amino acids of the human fibrillarin protein that includes 14 arginine residues within a glycine-rich region, the majority of which reside in 'RGG' consensus methylation sites (Lischwe *et al*., 1985). Methylation reactions were quenched by the addition of an equal volume of 2 × SDS–PAGE sample buffer (120 mM Tris-HCl, pH 6.8, 1.43 M 2-mercaptoethanol, 4% SDS, 24% glycerol, 0.002% bromphenol blue), heated at 100°C for 5 min, and separated on SDS–PAGE gels for 4 h. Following electrophoresis, gels were soaked in EN^{3}HANCE (Perkin Elmer Life Sciences) according to the manufacturer's instructions, then dried under vacuum. Radioactivity was visualized by autoradiography at −80°C with exposure times varying from 3 h to 1 week.

Methylation assays with DAL-1/4.1B-inducible. MCF-7 cell line Cl27

The MCF-7 cl27 DAL-1/4.1B-inducible cell line has been described previously (Charboneau *et al*., 2002). Cells were grown to approximately 80–90% confluence with and without 2 μM DAL-1/4.1B expression-inducing muristerone

(InVitrogen), washed twice with cold 1 × PBS, and harvested in 50 mM sodium phosphate, pH 7.5, with a commercial mixture of protease inhibitors (cat. #1836170: Roche Molecular Biochemicals). Washed cells were lysed by four 10-s sonicator pulses (50% duty; setting '2') on ice, and subjected to a 15 min centrifugation at 14 000 r.p.m. at 4°C. The resulting supernatant was collected and stored at −20°C. MCF-7 cl27 cell extracts were pretreated with bovine pancreatic RNase A (10 mg/ml; Sigma) at a 0.2 mg/ml final concentration for 30 min at room temperature and heated for 10 min at 70°C to abolish endogenous methyltransferase activity prior to methylation reactions.

Purified GST-PRMT3 (10 μg) or GST (7.5 μg) were incubated with 10 μg of DAL-1/4.1B-induced or uninduced MCF7 CL27 cell extract in the presence of 1.23 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; 81 Ci/mmol, AP Biotech) for 1 h at 37°C. In some experiments, GST-GAR (2 μg) or GST-DAL-1/4.1B (7.5 μg) was also added. Each reaction mixture was buffered with 50 mM sodium phosphate at pH 7.5. Methylation reactions were quenched by the addition of 1/5 volume of 6 × SDS–PAGE sample buffer (360 mM Tris-HCl, pH 6.8, 4.26 M 2-mercaptoethanol, 12% SDS, 30% glycerol, 0.006% bromphenol blue), heated at 70°C for 10 min, and separated on 10% Tris-HCl ready gels (Bio-Rad). Following electrophoresis, gels were stained in Coomassie Brilliant Blue R-250 for 20–30 min, destained in a 25% methanol (v/v), 7% acetic acid (v/v) destain solution to visualize protein bands, and then soaked in NAMP100V Amplify (Amersham Bioscience) for 30 min. Gels were vacuum dried and radioactivity visualized by fluorography at −80°C for 5 days.

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References

Aletta JM, Cimato TR and Ettinger MJ. (1998). *Trends Biochem. Sci.*, **23,** 89–91.
Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ. (1990). *J. Mol. Biol.*, **215,** 403–410.
Bachand F and Silver PA. (2004). *EMBO J.*, **23,** 2641–2650.
Bedford MT, Frankel A, Yaffe MB, Clarke S, Leder P and Richard S. (2000). *J. Biol. Chem.*, **275,** 16030–16036.
Berthet C, Guehenneux F, Revol V, Samarut C, Lukaszewicz A, Dehay C, Dumontet C, Magaud JP and Rouault JP. (2002). *Genes Cells*, **7,** 29–39.
Brahms H, Raymackers J, Union A, de Keyser F, Meheus L and Luhrmann R. (2000). *J. Biol. Chem.*, **275,** 17122–17129.
Charboneau AL, Singh V, Yu T and Newsham IF. (2002). *Int. J. Cancer*, **100,** 181–188.
Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, Aswad DW and Stallcup MR. (1999). *Science*, **284,** 2174–2177.
Chishti AH, Kim AC, Marfatia SM, Lutchman M, Hanspal M, Jindal H, Liu SC, Low PS, Rouleau GA, Mohandas N, Chasis JA, Conboy JG, Gascard P, Takakuwa Y, Huang SC, Benz Jr EJ, Bretscher A, Fehon RG, Gusella JF, Ramesh V, Solomon F, Marchesi VT, Tsukita S, Tsukita S, Arpin M, Louvard D, Tonks NK, Anderson JM, Fanning AS, Bryant PJ, Woods DF and Hoover KB. (1998). *Trends Biochem. Sci.*, **23,** 281–282.
Frankel A and Clarke S. (1999). *Biochem. Biophys. Res. Commun.*, **259,** 391–400.
Frankel A and Clarke S. (2000). *J. Biol. Chem.*, **275,** 32974–32982.
Frankel A, Yadav N, Lee J, Branscombe TL, Clarke S and Bedford MT. (2002). *J. Biol. Chem.*, **277,** 3537–3543.
Fu H, Subramanian RR and Masters SC. (2000). *Annu. Rev. Pharmacol. Toxicol.*, **40,** 617–647.
Gary JD and Clarke S. (1998). *Prog. Nucleic Acid Res. Mol. Biol.*, **61,** 65–131.
Goutebroze L, Brault E, Muchardt C, Camonis J and Thomas G. (2000). *Mol. Cell. Biol.*, **20,** 1699–1712.
Gutmann DH, Donahoe J, Perry A, Lemke N, Gorse K, Kittiniyom K, Rempel SA, Gutierrez JA and Newsham IF. (2000). *Hum. Mol. Genet.*, **9,** 1495–1500.
Gutmann DH, Hirbe AC, Huang ZY and Haipek CA. (2001). *Neurobiol. Dis.*, **8,** 266–278.
Kim S, Merrill BM, Rajpurohit R, Kumar A, Stone KL, Papov VV, Schneiders JM, Szer W, Wilson SH, Paik WK and Williams KR. (1997). *Biochemistry*, **36,** 5185–5192.
Kittiniyom K, Gorse KM, Dalbegue F, Lichy JH, Taubenberger JK and Newsham IF. (2001). *Breast Cancer Res.*, **3,** 192–198.
Kittiniyom K, Mastronardi M, Roemer M, Wells WA, Greenberg ER, Titus-Ernstoff L and Newsham IF. (2004). *Genes, Chrom. & Cancer*, **40,** 190–203.
Kumar A, Williams KR and Szer W. (1986). *J. Biol. Chem.*, **261,** 11266–11273.
Legg JW and Isacke CM. (1998). *Curr. Biol.*, **8,** 705–708.
Lin WJ, Gary JD, Yang MC, Clarke S and Herschman HR. (1996). *J. Biol. Chem.*, **271,** 15034–15044.
Lischwe MA, Cook RG, Ahn YS, Yeoman LC and Busch H. (1985). *Biochemistry*, **24,** 6025–6028.
McBride AE and Silver PA. (2001). *Cell*, **106,** 5–8.
Miranda TB, Miranda M, Frankel A and Clarke S. (2004). *J. Biol. Chem.*, **279,** 22902–22907.
Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H and Herrlich P. (2001). *Genes Dev.*, **15,** 968–980.
Mowen KA, Tang J, Zhu W, Schurter BT, Shuai K, Herschman HR and David M. (2001). *Cell*, **104,** 731–741.
Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, Solomon F, Gusella J and Ramesh V. (1998). *J. Biol. Chem.*, **273,** 1273–1276.
Muslin AJ and Xing. (2000). *Cell Signal.*, **12,** 703–709.
Perry A, Cai DX, Scheithauer BW, Swanson PE, Lohse CM, Newsham IF, Weaver A and Gutmann DH. (2000). *J. Neuropathol. Exp. Neurol.*, **59,** 872–879.
Robb VA, Li W and Gutmann DH. (2004). *Oncogene*, **23,** 3589–3596.
Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C and Plougastel B. (1993). *Nature*, **363,** 515–521.
Shuai K, Schindler C, Prezioso VR and Darnell Jr JE. (1992). *Science*, **258,** 1808–1812.
Smith JJ, Rucknagel KP, Schierhorn A, Tang J, Nemeth A, Linder M, Herschman HR and Wahle E. (1999). *J. Biol. Chem.*, **274,** 13229–13234.

Tang J, Frankel A, Cook RJ, Kim S, Paik WK, Williams KR, Clarke S and Herschman HR. (2000a). *J. Biol. Chem.*, **275,** 7723–7730.

Tang J, Gary JD, Clarke S and Herschman HR. (1998). *J. Biol. Chem.*, **273,** 16935–16945.

Tang J, Kao PN and Herschman HR. (2000b). *J. Biol. Chem.*, **275,** 19866–19876.

Tran Y, Benbatoul K, Gorse K, Rempel S, Futreal A, Green M and Newsham I. (1998). *Oncogene*, **17,** 3499–3505.

Tran YK, Bogler O, Gorse KM, Wieland I, Green MR and Newsham IF. (1999). *Cancer Res.*, **59,** 35–43.

Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, Kley N, Menon AG, Pulaski K, Haase VH, Ambrose CM, Numroe D, Bove C, Haines JL, Martuza RL, MacDonald ME, Seizinger BR, Short MP, Buckler AJ and Gusella JF. (1993). *Cell*, **75,** 826.

Tsukita S, Oishi K, Sato N, Sagara J, Kawai A and Tsukita S. (1994). *J. Cell Biol.*, **126,** 391–401.

Wang H, Huang ZQ, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, Briggs SD, Allis CD, Wong J, Tempst P and Zhang Y. (2001). *Science*, **293,** 853–857.

Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S and Tsukita S. (1998). *J. Cell Biol.*, **140,** 885–895.

Yu T, Robb VA, Singh V, Gutmann DH and Newsham IF. (2002). *Biochem. J.*, **365,** 783–789.