The Tale of Two Domains

PROTEOMICS AND GENOMICS ANALYSIS OF SMYD2, A NEW HISTONE METHYLTRANSFERASE*[S]

Mohamed Abu-Farha‡§, Jean-Philippe Lambert‡§, Ashraf S. Al-Madhoun§¶, Fred Elisma‡, Ilona S. Skerjanc§‖, and Daniel Figeys‡§**

Very little is known about SET- and MYND-containing protein 2 (SMYD2), a member of the SMYD protein family. However, the interest in better understanding the roles of SMYD2 has grown because of recent reports indicating that SMYD2 methylates p53 and histone H3. In this study, we present a combined proteomics and genomics study of SMYD2 designed to elucidate its molecular roles. We report the cytosolic and nuclear interactome of SMYD2 using a combination of immunoprecipitation coupled with high throughput MS, chromatin immunoprecipitation coupled with high throughput MS, and co-immunoprecipitation methods. In particular, we report that SMYD2 interacted with HSP90α independently of the SET and MYND domains, with EBP41L3 through the MYND domain, and with p53 through the SET domain. We demonstrated that the interaction of SMYD2 with HSP90α enhances SMYD2 histone methyltransferase activity and specificity for histone H3 at lysine 4 (H3K4) *in vitro*. Interestingly histone H3K36 methyltransferase activity was independent of its interaction with HSP90α similar to LSD1 dependence on the androgen receptor. We also showed that the SET domain is required for the methylation at H3K4. We demonstrated using a modified chromatin immunoprecipitation protocol that the SMYD2 gain of function leads to an increase in H3K4 methylation *in vivo*, whereas no observable levels of H3K36 were detected. We also report that the SMYD2 gain of function was correlated with the up-regulation of 37 and down-regulation of four genes, the majority of which are involved in the cell cycle, chromatin remodeling, and transcriptional regulation. *TACC2* is one of the genes up-regulated as a result of SMYD2 gain of function. Up-regulation of *TACC2* by SMYD2 occurred as a result of SMYD2 binding to the *TACC2* promoter where it methylates H3K4. Furthermore the combination of the SMYD2 interactome with the gene expression data suggests that some of the genes regulated by SMYD2 are closely associated with SMYD2-interacting proteins. *Molecular & Cellular Proteomics 7:560–572, 2008.*

The mapping of protein-protein interaction helps us understand the function of proteins. In recent years, our group (1) and others (2, 3) have performed protein interaction experiments for large sets of human proteins. Our approach for the mapping of protein-protein interactions is based on immunoprecipitation coupled with high throughput MS (IP-HTMS).[1]

The SMYD protein family consists of five proteins (SMYD1–5) that are not fully characterized and are grouped based on the presence of two conserved domains (MYND and SET domains). The MYND domain is a zinc finger motif that is involved in protein-protein interaction. It is named after Myeloid, Nervy, and DEAF-1, which are the three most characterized proteins that contain the MYND domain (4). The SET domain is an evolutionarily conserved sequence motif consisting of 130–140 amino acids. Its name is derived from the three proteins in which it was initially characterized: Su (var) 3-9, Enhancer-of-zeste, and Trithorax (5, 6). These enzymes add methyl groups to lysine residues of histone H3 using *S*-adenosylmethionine (AdoMet) as a donor substrate (7, 8). Interest in the SMYD family of proteins has grown significantly because of recent reports indicating that SMYD1, -2, and -3 control gene expression through histone methylation (7–9). In addition, *in vitro* studies have shown that both SMYD1 and SMYD3 specifically methylate histone at lysine 4 (H3K4) in the presence of HSP90α (7, 8). In contrary to reports on SMYD1 and -3, Brown *et al.* (9) have shown that SMYD2 dimethylates H3K36 *in vitro* in the absence of HSP90α (9). Beyond their function in histone methylation, proteins containing SET domain were also shown to methylate non-histone proteins. Examples of this activity are the methylation of p53 (10) and the TATA-binding protein-associated factor TAF10 by human SET7/9 (11). Human SET7/9 stabilizes p53 through Lys-372 methylation (10). Similarly SMYD2 regulates p53 activity through methylation at Lys-370, which represses p53-mediated transcriptional regulation (12). These data suggest that p53, like histones, can be activated or repressed through

From the ‡Ottawa Institute of Systems Biology and §Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Received, June 11, 2007, and in revised form, November 13, 2007
Published, MCP Papers in Press, December 7, 2007, DOI 10.1074/mcp.M700271-MCP200

[1] The abbreviations used are: IP-HTMS, immunoprecipitation coupled with high throughput MS; SMYD2, SET- and MYND-containing protein 2; SET, Su (var) 3-9, Enhancer-of-zeste, and Trithorax; MYND, Myeloid, Nervy, and DEAF-1; AdoMet, *S*-adenosylmethionine; ChIP-HTMS, chromatin immunoprecipitation coupled with high throughput MS; TACC2, transforming, acidic coiled-coil-containing protein 2; aa, amino acids; SAM, significance analysis of microarrays; nt, nucleotides; TPR, tetratricopeptide repeat; PPP, protein phosphatase; CHD9, chromodomain helicase DNA-binding protein 9; MT, microtubule; AKAP, A-kinase anchoring protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SWI/SNF, SWItch/Sucrose NonFermentable.

methylation. It also points to a potential role for SMYD2 in tumorigenesis through its direct methylation activity that regulates p53 and potentially other proteins (12).

This study presents a combined genomics and proteomics study of the molecular roles of SMYD2. Using interactome mapping methods, we identified novel SMYD2 interaction partners and demonstrated the role of the two domains (MYND and SET) of SMYD2 in mediating some of these interactions, in particular the interactions with EBP41L3, HSP90α, and p53. Furthermore we showed that SMYD2 acts as an H3K4 methyltransferase in the presence of HSP90α and acts as an H3K36 methyltransferase in the absence of HSP90α. Gene expression studies indicated that the SMYD2 gain of function in 293T cells leads predominantly to up-regulation of gene expression. ChIP data indicated that activation of downstream genes, such as transforming, acidic coiled-coil-containing protein 2 (*TACC2*), occurs as a result of the ability of SMYD2 to methylate H3K4 in promoter regions of its target genes. We also showed that SMYD2 binds to p53, which was recently found to be methylated by SMYD2 (12). Finally we showed using bioinformatics tools that SMYD2 protein interactors are close neighbors of the protein products of some of the genes targeted by SMYD2 in the human interactome map. This might indicate feedback mechanisms and combined regulation through gene expression and protein interactions.

MATERIALS AND METHODS

Cell Culture—293T cells were cultured in a high glucose Dulbecco's modified Eagle's medium at 37 °C in a 5% CO_2 humidified incubator. The Dulbecco's modified Eagle's medium was supplemented with 10% fetal bovine serum, 100 μg/ml of penicillin, 100 μg/ml streptomycin, and 50 μg/ml antimycotic.

FLAG-SMYD2 Cloning—Using the Promega Access RT-PCR, a full-length SMYD2 cDNA was amplified from a total RNA that was extracted from HEK293T cells using the RNeasy RNA extraction kit according to the manufacturer's protocol (Qiagen, Mississauga, Ontario, Canada). SMYD2 cDNA was then cloned into a directional Gateway Topo pENTR vector (Invitrogen). To transfer the SMYD2 cDNA into a mammalian expression vector, the GATEWAY™ cloning system was used. The pENTR vector contains attL1 and attL2 recombination sites on its 5′- and 3′-ends. These two sites were then utilized to transfer the SMYD2 clone into the mammalian expression vector containing FLAG tag at its C terminus (ATCC). Small batches of transient transfection of 293T cells were used to verify the FLAG-SMYD2 expression.

Site-directed Mutagenesis—The SET domain has two conserved sequences that are known to bind to AdoMet. These two conserved sequences in the SET domain, termed ΔNHSC (aa 206–209) and ΔGEE (aa 233–235), as well as the MYND domain (aa 51–90) were each deleted using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Mutagenesis primers were as follows: ΔNHSC F, 5′-cct gat gtt gca ttg atg tgc ccc aat gtc att gtg acc-3′; ΔNHSC R, 5′-ggt cac aat gac att ggg gca cat caa tgc aac atc agg-3′; ΔGEE F, 5′-gct gta cag gaa atc aag ccg gtt ttt acc agc tat att gat ctc c-3′; ΔGEE R, 5′-gga gat caa tat agc tgg taa aaa ccg gct tga ttt cct gta cag c-3′; MYND F, 5′-cgg tca acg agc ggg gca acc acc cca tgg ttg ttt ttg ggg-3′; and MYND R, 5′-ccc caa aaa caa cca tgg ggt ggt tgc ccc gct cgt tga ccg-3′. The resulting clones were screened for the presence of the mutation by sequencing.

IP-HTMS—IP-HTMS experiments for SMYD2 were performed as part of a large scale report published previously (1). Briefly transient transfections of SMYD2 wild type and mutant constructs into 293T, NIH3T3, and HeLa cells were done using Lipofectamine 2000 as instructed by the manufacturer (Invitrogen). Cells were plated into 75-cm^2 flasks the day before transfection in an antibiotic-free medium to produce an 80% confluent monolayer on the day of transfection. Following transfection, cells were split into two new flasks and left for 48 h, reaching about full confluence on the day of harvest. After 48 h of transfection, the cells were washed twice with ice-cold PBS and then harvested. The cells were then frozen in liquid nitrogen and stored at −80 °C until ready to be used. Immunoprecipitation experiments were performed using 2×10^7 cells. The cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing a mixture of protease inhibitors (Mini Complete, Roche Diagnostics) for 30 min at 4 °C. Cell debris were removed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatant was then precleared with agarose beads for 2 h with shaking at 4 °C. Agarose beads were removed and replaced with prewashed anti-FLAG M2 affinity gel and incubated in a shaker overnight at 4 °C. The beads were then washed five times with washing buffer (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl). Bound proteins were eluted by incubation with 100 μl of 3×FLAG peptide (150 ng/μl) for 30 min at 4 °C in 1× wash buffer. The proteins were analyzed by mass spectrometry as outlined below.

ChIP-HTMS—For the modified IP protocol, the 293T cells that were transfected with SMYD2-FLAG or the control vector (empty vector) were cross-linked in 1% formaldehyde for 10 min at 37 °C. The cross-linking reaction was then stopped by adding glycine to a final concentration of 125 mM. The cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing a mixture of protease inhibitors (Mini Complete) and centrifuged at 5000 × *g* for 5 min at 4 °C to remove cellular debris. The pellet was washed three times in 1× lysis buffer and then resuspended in RIPA buffer (150 mM NaCl,1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM NaF, and 1 mM sodium orthovanadate) with Mini Complete protease inhibitor mixture. The protein pellet was sonicated three times for 20 s with at least 1 min on ice between each pulse. The lysate was then centrifuged at 13,000 × *g* for 10 min. The supernatant was then immunoprecipitated using anti-FLAG antibodies and analyzed by mass spectrometry as outlined below.

Mass Spectrometry Analysis (HTMS)—Gel bands were excised with a scalpel and placed in fresh microtubes. The gel pieces were briefly washed with 50 mM ammonium bicarbonate solution and subsequently shrunk in a solution of 50% acetonitrile (v/v) and 25 mM ammonium bicarbonate at room temperature for 15 min. The supernatants were discarded, and the gel pieces were dried for ~10 min at low temperature in a SpeedVac. Protein disulfide bonds were reduced by swelling the gel pieces in 10 mM DTT and 50 mM ammonium bicarbonate at 56 °C for 15 min. The free sulfhydryl groups were then alkylated with 100 mM iodoacetamide in 50 mM ammonium bicarbonate for 15 min at room temperature. The gel pieces were washed with 50 mM ammonium bicarbonate and shrunk in a solution of 50% acetonitrile (v/v) and 25 mM ammonium bicarbonate at room temperature for 15 min. The pieces were dried for ~10 min. Trypsin in 50 mM ammonium bicarbonate was added to each tube, and the samples were placed at 37 °C overnight (12–16 h) for digestion. Peptides were extracted from the gel pieces with 5% formic acid (v/v) and 50% acetonitrile (v/v). The peptide solutions were then dried using a SpeedVac and stored at −20 °C until mass spectrometric analysis.

LC-MS/MS was performed by dissolving the peptide samples in 5% formic acid. The samples were injected on a 200-μm × 5-cm

precolumn packed in house with 5-μm YMC ODS-A C_{18} beads (Waters, Milford, MA) using an Agilent 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA). The peptides were desalted on line with 95% water, 5% acetonitrile, and 0.1% formic acid (v/v) for 10 min at 10 μl/min. The flow rate was then split before the precolumn to produce a flow rate of ~200 μl/min at the column. Following their elution from the precolumn, the peptides were directed to a 75-μm × 5-cm analytical column packed with 5-μm YMC ODS-A C_{18} beads. The peptides were eluted using a 1-h gradient (5–80% acetonitrile with 0.1% formic acid) into an LTQ linear ion trap mass spectrometer (Thermo Electron, Waltham, MA). MS/MS spectra were acquired in a data-dependent acquisition mode that automatically selected and fragmented the five most intense peaks from each MS spectrum generated.

Peak lists were generated from the MS/MS .raw file using Mascot Distiller 2.0.0.0 (Matrix Science, London, UK) to produce .mgf files. For each MS/MS individual peak lists were generated assuming a 2+ and a 3+ charge. The .mgf files were then analyzed and matched to the 248,060 *Homo sapiens* protein sequences in a concatenated database containing the National Center for Biotechnology Information (NCBI) forward and reverse database (released January 2007) using the Mascot database search engine version 2.1.0.4 (Matrix Science, Boston, MA) with trypsin as digestion enzyme, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. The searches were restricted to the *H. sapiens* protein sequences because the experiments were performed using a human cell line. Peptide and MS/MS mass tolerances were set at ±2 and 0.8 Da, respectively, with one miscleavage allowed. An ion score cutoff of 30 was used for acceptance of individual MS/MS spectra. This threshold was chosen based on the report by Elias *et al.* (45) to ensure a false positive rate of less than 1% in our MS data. A protein hit required at least one "bold red peptide," *i.e.* the most logical assignment of the peptide in the database selected, to be considered. Furthermore when peptides matched to more than one database entry, only the highest scoring protein was considered. All of the proteins observed in the negative control ChIP-HTMS were subtracted from the SMYD2 ChIP-HTMS. Hence only the proteins observed in the SMYD2 ChIP-HTMS experiments are reported in supplemental Table 1A. All of the proteins reported in supplemental Table 1A had a single protein match except for Hsp90α, which had multiple isoforms. The isoform of HSP90α reported in supplemental Table 1A (gi:83699649) is the one that had the highest number of unique peptide matches.

Cellular Protein Extraction and Western Blots—Total cellular proteins were prepared by lysing cells in RIPA buffer containing Mini Complete protease inhibitor mixture. Proteins were separated on a 4–12% SDS-PAGE gel (NuPAGE, Novex, San Diego, CA) and then transferred to nitrocellulose membranes. A Western blot analysis for SMYD2 and its mutants was performed using monoclonal horseradish peroxidase-conjugated mouse anti-FLAG M2 antibodies (Sigma). H3 methylated lysines were detected by rabbit polyclonal antibodies for mono-, di-, and trimethylated Lys-4 (Upstate Cell Signaling Solutions, Lake Placid, NY). HSP90α protein was detected using mouse monoclonal antibodies purchased from Upstate Cell Signaling Solutions. p53 and EBP41L3 were detected using mouse monoclonal antibodies purchased from Calbiochem and Abnova (Jhongli City, Taoyuan, Taiwan), respectively. Rabbit IgG was used as a negative control and was purchased from Sigma. Membranes were then probed with horseradish peroxidase-labeled goat anti-rabbit IgG or goat anti-mouse secondary antibody (Dako Cytomation Inc., Mississauga, Ontario, Canada).

In Vitro Methylation Assay—*In vitro* methylation assay was performed on extracts from 293T cells transfected with plasmids expressing the mock vector (pcDNA3.1), wild type SMYD2 (pcDNA3.1-FLAG-SMYD2), or one of the SMYD2 mutants (pcDNA3.1FLAG-SMYD2 ΔGEE, pCDNA3.1 FLAG-SMYD2 ΔNHSC, or pcDNA3.1-FLAG-SMYD2 ΔMYND). FLAG-tagged proteins were then immunoprecipitated according to the previously described protocol using anti-FLAG antibodies conjugated to agarose beads. The *in vitro* histone methyltransferase assay was performed using an *in vitro* methylation kit purchased from Upstate Cell Signaling Solutions. *In vitro* methylation assay was done on the beads before the FLAG protein elution. After incubating the cell lysate with the anti-FLAG M2 affinity gel, the beads were washed to remove nonspecific binding proteins. The beads containing FLAG-tagged proteins were mixed with 1.65 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (PerkinElmer Life Sciences), 4 μg of chicken core histone, 1.5 μg of HSP90α (Calbiochem) in 50 mM Tris, pH 9.0, and 0.5 mM DTT. The reaction mixture was incubated for 3 h at 30 °C with gentle mixing. The methyltransferase activity was measured by scintillation counting. To measure the H3K4-specific activity, SMYD2 was incubated with 15 μM unlabeled AdoMet and 4 μg of recombinant histone H3. H3K4 methylation was detected using an anti-mono-, di-, and trimethylated H3K4 antibody or dimethylated H3K36 (Upstate Cell Signaling Solutions).

Gene Expression Analysis by Microarray—Changes in gene expression resulting from overexpression of SMYD2 were measured using cDNA microarray. Human 293T cells of about 80% confluence were transfected with either SMYD2 or a mock vector. After 24 h, cells were harvested and frozen in liquid nitrogen. Total RNA was extracted from 293T cells using an RNeasy kit according to the manufacturer's protocol (Qiagen). Total RNA samples were treated with DNase to remove potential genomic DNA contamination. Transfection efficiency was determined to be higher than 95% using immunocytochemistry with anti-FLAG antibodies conjugated to FITC. Samples were sent for analysis at the University of Toronto Microarray Center. The two different mRNA samples (sample *versus* mock control) were labeled with Cy3 or Cy5 dye and subjected to co-hybridization onto a single spotted human 19K Array (H19K) containing 19,008 characterized and unknown human expressed sequence tags. The gene expression analysis was done in triplicate. The data generated were analyzed by the SAM software to generate statistically significant hits. Reported genes were shown by the SAM software to be statistically significant and were filtered to eliminate all genes with an absolute value of $\log_2$ ratio >0.6 in less than two observations.

Chromatin Immunoprecipitation Assays—ChIP assay was performed using a chromatin IP assay kit from Upstate Cell Signaling Solutions. 293T cells transfected with SMYD2-FLAG or the control vector were cross-linked in 1% formaldehyde for 10 min. The cross-linking reaction was then stopped by adding 125 mM glycine. The samples were then immunoprecipitated using the anti-FLAG M2 affinity gel. The following primers were used to examine whether the promoter sequence of the DNA of interest was pulled down. The primers used for the PCR amplification were: Tacc2 chip1 F, 5′-aaccaatcagcggcactatc; Tacc2 chip1 R, 5′-ttcttcatagcacttacaatgacc; Tacc2 chip2 F, 5′-ggctaaagggataggtgga; Tacc2 chip2 R, 5′-gcacagtgactcatgcctgt; Tacc2 chip3 F, 5′-cccgacctgaaagttgctat; Tacc2 chip3 R, 5′-cttatcaggaagcggctgac; Tacc2 chip4 F, 5′-gatctcaggagttgggcaag; and Tacc2 chip4 R, 5′-agcgggtgacttgagaaatc.

Semiquantitative RT-PCR—Human 293T cells at about 80% confluency were transfected with a mock control, wild type SMYD2, ΔNHSC, or ΔGEE for 24 h. The cells were harvested and frozen in liquid nitrogen. Total RNA was extracted using an RNeasy kit according to the manufacturer's protocol (Qiagen). Total RNA samples were treated with DNase to remove potential genomic DNA contamination. The gene expression level was measured using semiquantitative RT-PCR using the iScript One Step RT-PCR kit (Bio-Rad). The primers used were: *TACC2* F, 5′-cacccactgaggagttggat; *TACC2* R, 5′-acttgacagggctctcctga; SMYD2 F, 5′-tgcaagcaggcattttactg; SMYD2 R, 5′-tttctgtttggccagaatcc; GAPDH F, 5′-tgatgacatcaagaaggtggtgaag; and GAPDH R, 5′-tccttggaggccatgtgggccat.

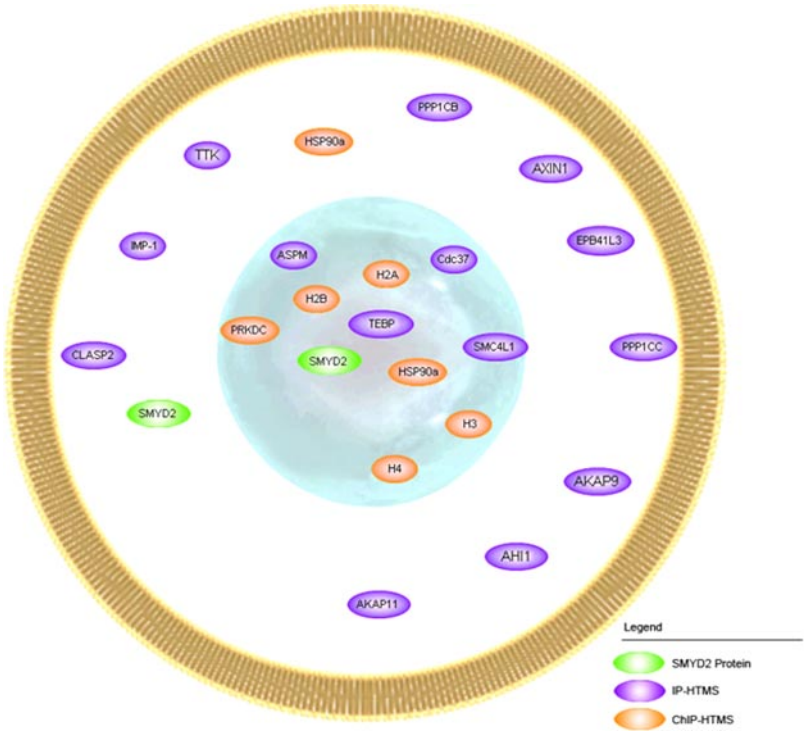

FIG. 1. **A diagram showing the SMYD2 protein-protein interaction partners with their cellular localization as identified by Ewing *et al.* (1) using IP-HTMS and with our modified ChIP-HTMS method as described under "Materials and Methods."** The SMYD2 interaction partners identified by IP-HTMS were located outside the nucleus, whereas the ChIP-HTMS interactors were located in the nucleus.

Microarray data for five genes was validated using the previous method with the following primer pairs: Smarca2 F, 5′-tccgaggcaaaatcagtcaag; Smarca2 R, 5′-ttcctcgatttggccttttct; Wdr9 F, 5′-ttccagagctcgtgaatcct; Wdr9 R, 5′-cgggaccagtttttctttga; Bat1 F, 5′-acagctctggctttcgtgac; Bat1 R, 5′-gcactcatgctggacttctg; Chd9 F, 5′-catctggaaaccggacagat; Chd9 R, 5′-ctgccactgatggcctctat; Akap13 F, 5′-agttctcttccgctccaaca; and Akap13 R, 5′-actcaccaaaccctgaagga.

Combining SMYD2 Interactions and Protein Products of Genes Regulated by SMYD2—The UniGene list generated by the microarray data and the names of proteins generated by the interaction mapping were imported into Pathway Studio 5.0. This text-mining software uses a database of molecular networks that was assembled from scientific abstracts and a manually curated dictionary of synonyms to recognize biological terms (13). The combined interaction network was created by searching the database for direct interactions between the imported gene/protein and for other proteins that are one edge neighbor from the imported list of gene/protein. The protein-protein interactions are represented as a network graph where the vertices are the proteins and the edges are the references (see Fig. 7).

RESULTS

SMYD2, a 49.7-kDa protein, is composed of 433 amino acids and is a member of the SMYD family. Sequence alignment of SMYD2 with other SMYD family members reveals regions that are known to possess methyltransferase activity (14). SMYD2 shares 31% sequence identity and 52% sequence similarity with SMYD3, which is another member of the SMYD family. The sequence homology of SMYD2 and SMYD3 is primarily due to the MYND and SET domains.

Identification of SMYD2 Protein Interactions—To understand the role of SMYD2 in the cell, we mapped its interactome using two approaches. The first approach, termed IP-HTMS, looked at the global interactors of SMYD2 (1). In this approach, FLAG-tagged SMYD2 was transiently transfected in 293T cells, 48 h after the transfection the FLAG-tagged SMYD2 was immunoprecipitated, and its protein interactors were identified by mass spectrometry. The results from the global IP-HTMS method are represented in Fig. 1 and Ref. 1. One limitation of the IP-HTMS method is a bias against nuclear proteins. Therefore, a second approach, termed ChIP-HTMS, was used. The approach consists of a modified chromatin IP protocol (see "Materials and Methods") coupled to HTMS to enrich SMYD2 interaction partners from the nucleus and from SMYD2 bound to DNA. The ChIP-HTMS method enlarged the list of SMYD2 nuclear proteins interactors (Fig. 1). In total, 21 proteins were found to interact with SMYD2.

Proteins identified as SMYD2 interaction partners were divided into two groups according to their functions: histone regulation and microtubule dynamics. The former group includes histone H3, histone H4, and Epb4113 (regulator of arginine-*N*-methyltransferase specifically PRMT3 and -5) (15, 16). Histone H3 is a known substrate for SMYD2. Another protein that is known to interact with SMYD2 is p53, which was recently shown to be methylated by SMYD2. The second group includes CLASP2, a protein that binds to the end of growing microtubules in association with cytoplasmic linker proteins (clips) (17). Other microtubule-related proteins were observed including Axin and TTK, which are known to play a role in microtubule dynamics. TTK is localized to the centrosome and is known to phosphorylate *TACC2* (18). We showed that *TACC2* is up-regulated as a result of SMYD2 overexpression (see below). Co-immunoprecipitation was performed to

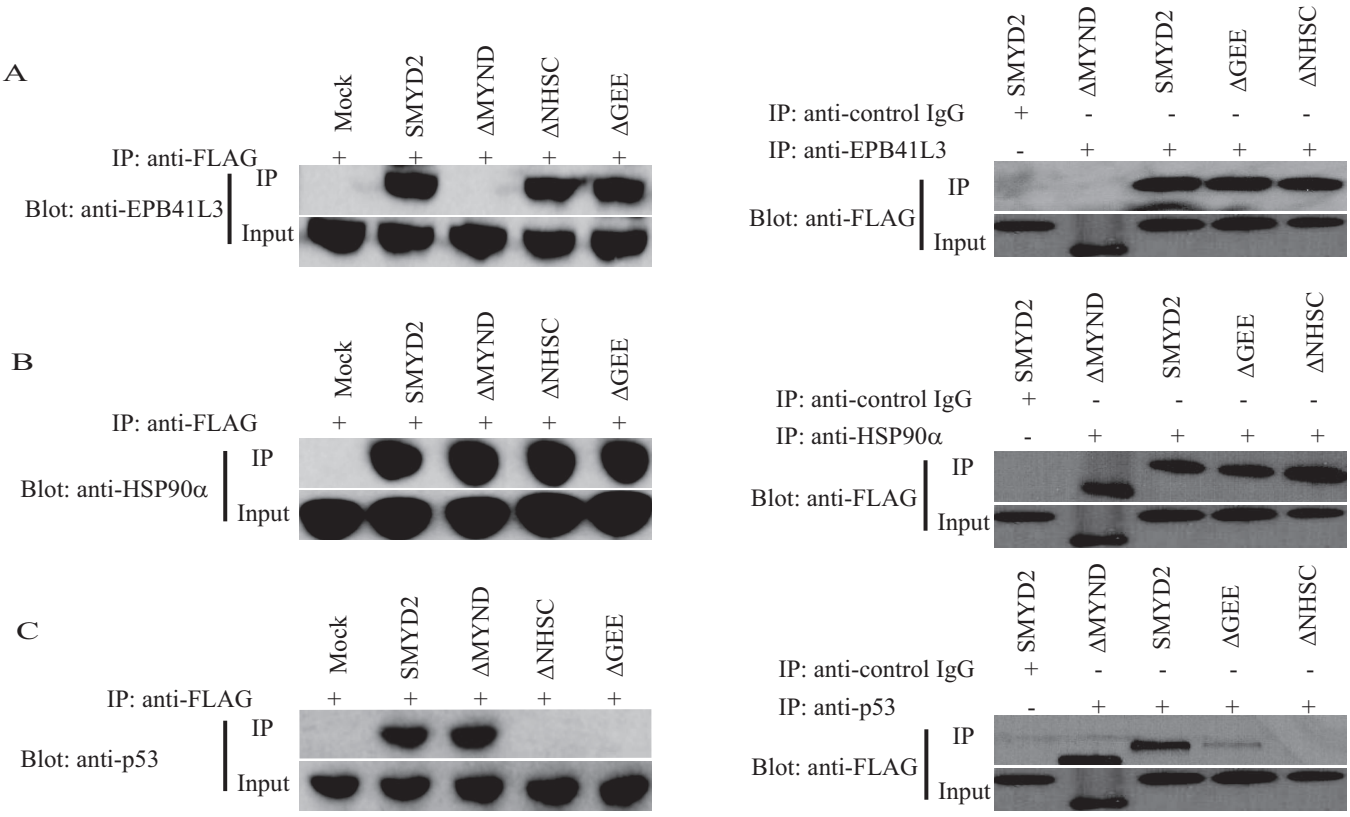

FIG. 2. **Validation of some physical interactions of SMYD2 by co-IP.** *A*, Western blots showing co-IP assays. Transiently expressed wild type FLAG-SMYD2 and its mutants, endogenous EPB41L3, and control IgG were immunoprecipitated from 293T cell extracts, and precipitates were probed for the immunoprecipitants (*IP*; *top panel*). The *bottom panel* shows the Western blot analysis of input whole cell lysate. SMYD2 interaction with EPB41L3 requires a functional MYND domain as shown by the inability of the MYND domain deletion to immunoprecipitate EPB41L3 and vice versa. *B*, similar experiments performed for FLAG-SMYD2, endogenous HSP90α, and control IgG. Interaction between HSP90α and SMYD2 is not mediated through the MYND domain as shown by the ability of the MYND-deleted form of SMYD2 to immunoprecipitate HSP90α and vice versa. *C*, similar experiments performed for FLAG-SMYD2, endogenous p53, and control IgG. Interaction between p53 and SMYD2 is not mediated through the MYND domain as shown by the ability of the MYND-deleted form of SMYD2 to immunoprecipitate p53 protein and vice versa.

validate the interactions of SMYD2 with HSP90α, EPB41L3, and p53. The interactions with these proteins are explored in more details below.

The MYND Domain of SMYD2 Interacts with PXLXP Motif-containing Proteins and SMYD2 Interacts with its Substrates through the SET Domain—The MYND domain mediates protein-protein interactions by binding to the conserved P*X*L*X*P motif (19). Interestingly five of the 21 proteins found to interact with SMYD2 possess a P*X*L*X*P motif, including EPB41L3. EPB41L3 was chosen for further validation because of its role in regulating arginine methyltransferases 3 and 5 (15, 16). To test whether the MYND domain of SMYD2 was required for its interaction with EPB41L3, co-IP assays were performed between EPB41L3 and the wild type or the MYND-deleted form of SMYD2 and the SET deletions. As shown in Fig. 2*A*, EPB41L3 co-immunoprecipitated with the wild type SMYD2 but not with the MYND mutant. Other proteins that did not contain the P*X*L*X*P motif such as HSP90α and p53 interacted with SMYD2 independently of the MYND domain (Fig. 2, *B* and *C*). EPB41L3 interaction with SMYD2 was not affected by deletions of the SET domain consensus sequences (NHSC and GEE). This clearly indicates that the interaction between SMYD2 and EPB41L3 is dependent on the MYND domain and that the P*X*L*X*P motif in interactors is a good predictor that interactions occur with the MYND domain.

Other interactions depend on the activity of the SET domain. For example, the interaction between p53 and SMYD2 was abolished by the deletion of the two SET domain consensus sequences (Fig. 2*C*). This correlates with the nature of interaction between p53 and SMYD2 in which p53 acts as a substrate for SMYD2.

The interaction between SMYD2 and HSP90α was not affected by any of the deletions, raising the possibility that HSP90α interacts with another part of SMYD2 or indirectly through another protein that is also not affected by the deletion of the MYND and SET domains (Fig. 2*B*). See supplemental Fig. 3 for a silver-stained gel showing the effect of SMYD2 mutants on its interactions with other proteins.

The Interaction between HSP90α and SMYD2 Changes SMYD2 Methyltransferase Activity and Specificity—Our pro-

tein-protein interaction data showed that HSP90α interacts with SMYD2; this was further confirmed by co-IP (Figs. 1 and 2*B*). Previous studies have shown that SMYD1 and -3 can methylate H3K4 in the presence of HSP90α (7, 8). However, a recent report published by Brown *et al.* (9) suggested that SMYD2 dimethylates Lys-36 of H3 and not Lys-4, although their *in vitro* experiments did not include HSP90α.

The interaction of SMYD2 with HSP90α suggests that HSP90α plays an important role in the functions of SMYD2. Hence we performed *in* vitro methylation assays to verify whether HSP90α affects the activity and specificity of SMYD2 as a methyl transferase. Briefly SMYD2 was immunoprecipitated using anti-FLAG antibodies, and the immunoprecipitate was incubated with recombinant histone H3 and AdoMet with or without HSP90α. Proteins were fractionated on SDS-PAGE and immunoblotted with anti-histone H3 antibodies (Fig. 3*A*). Clearly the *in vitro* methyltransferase activity of SMYD2 toward histone H3 was dependent on the presence of HSP90α. A second set of experiments was performed to determine the specificity of the methylation site using antibodies directed against mono-, di-, or trimethylated H3K4. In short exposure experiments, no methylation activity toward Lys-4 or Lys-36 was observed without HSP90α (supplemental Fig. 1). The addition of recombinant HSP90α caused SMYD2 to methylate histone H3 at Lys-4. H3K4 methylation was readily detectable after a short exposure of the autoradiography film (Fig. 3*B* and supplemental Fig. 1). Moreover methylation at Lys-36 was only weakly observable in the absence of recombinant HSP90α and following much longer exposure of the autoradiography film (supplemental Fig. 1).

Role of SMYD2 SET Domain in Its Methyltransferase Activity—The SET domain is a known histone lysine methyltransferase domain. The presence of this domain in SMYD2 indicates a potential role for this protein as a histone methyltransferase. Two homologues of SMYD2 (SMYD3 and SMYD1) have methyltransferase activity for H3K4 (7, 8). SMYD3 and SUV39H1 have two conserved regions in its SET domain, ΔNHSC*X*PN and ΔGEE*XXXX*Y to which AdoMet binds (7). These two regions are also present in the SET domain of SMYD2. To investigate the role of the SET domain and the AdoMet binding motif in the SET domain, these two sites (ΔNHSC and ΔGEE) were mutated (Fig. 4*A*). The wild type SMYD2 and its mutants were transfected into 293T cells, immunoprecipitated using anti-FLAG antibodies, and used for methyltransferase assays in the presence of tritium ^{3}H-labeled AdoMet and HSP90α. The methylation activity was measured using a scintillation counter as described previously (7). Wild type and the MYND deletion mutant showed, respectively, a 10- and 7-fold increase in methylation activities compared with the mock control. Purified human SET7/9 protein was used as a positive control (Fig. 4*B*). Furthermore the deletion of either the AdoMet binding consensus sequence ΔGEE or ΔNHSC resulted in a sharp reduction of SMYD2 methylation activity that indicated that both AdoMet

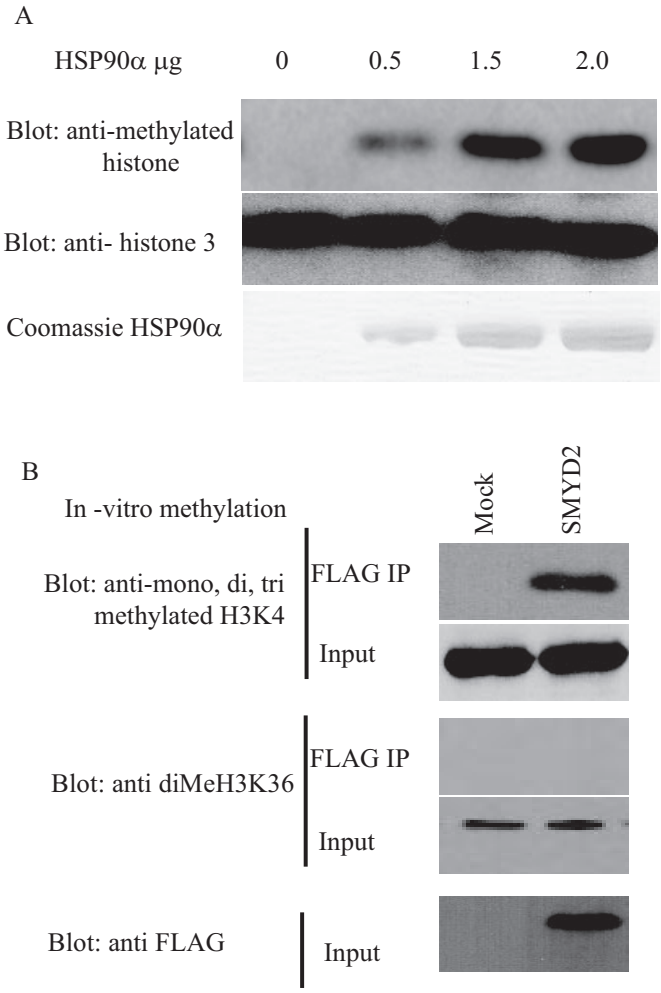

FIG. 3. **HSP90α enhances SMYD2 methyltransferase activity.** *A*, *in vitro* histone methylation assay using an increasing amount of HSP90α recombinant protein. This shows that SMYD2 requires HSP90α for its activity. *B*, *in vitro* methylation assay showing the ability of SMYD2 to methylate histone H3 at Lys-4 and not Lys-36. In the *top panel*, mock control and immunoprecipitated SMYD2 from 293T cells were added to the *in vitro* assay, and methylation was identified on Western blot using specific antibodies. *FLAG IP* refers to immunoprecipitated proteins using anti-FLAG antibodies from 293T cells. The *bottom* Western blot shows analysis of input whole cell lysate (*Input*). *diME*, dimethylated.

binding domain sequences were critical for SMYD2 methyltransferase activity (Fig. 4*B*). These results were further confirmed following an *in vitro* methylation assay using mono-, di-, tri-methylated H3K4 antibody. Wild type and a MYND deletion mutant of SMYD2 were found to methylate H3K4, whereas deleting the GEE or NHSC sequence rendered SMYD2 inactive.

Modified Chromatin IP Reveals Increased H3K4 Methylation in Vivo in the Vicinity of SMYD2 Binding to DNA—To further validate our *in vitro* methylation assay, we performed a chromatin IP experiment to look at histone modifications in the vicinity of DNA bound by SMYD2. However, instead of follow-

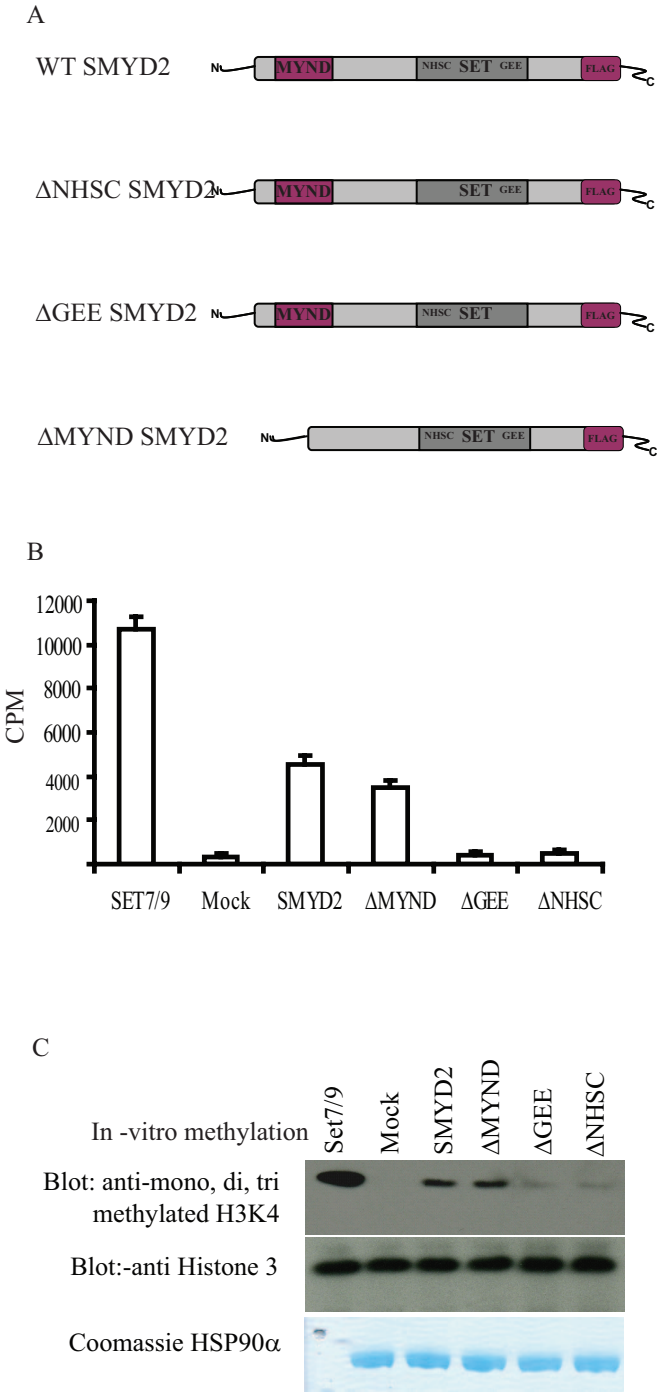

FIG. 4. **Role of SMYD2 SET domain in its methyltransferase activity.** *A*, diagram showing the structure of the wild type (*WT*) form of SMYD2 as well as the mutants introduced to its MYND domain and to the two conserved sequences in the SET domain, ΔNHSC and ΔGEE. *B*, *in vitro* methylation activity of SMYD2 and its mutants showing the decrease in its activity after point mutations in the two conserved sequences of the SET domain. Deletion of the MYND domain does not seem to affect the histone methylation activity of SMYD2. *C*, methylation of H3K4 by wild type SMYD2 as detected by mono-, di-, and tri-methylated H3K4-specific antibody. SET domain mutations obliterate the methylation activity of SMYD2. The MYND domain deletion does not have any effect on the methylation activity of SMYD2. Histone H3 is used as a loading control. The Coomassie-stained SDS-PAGE gel shows an equal amount of HSP90α used for the different methylation reactions except for the positive control (SET7/9).

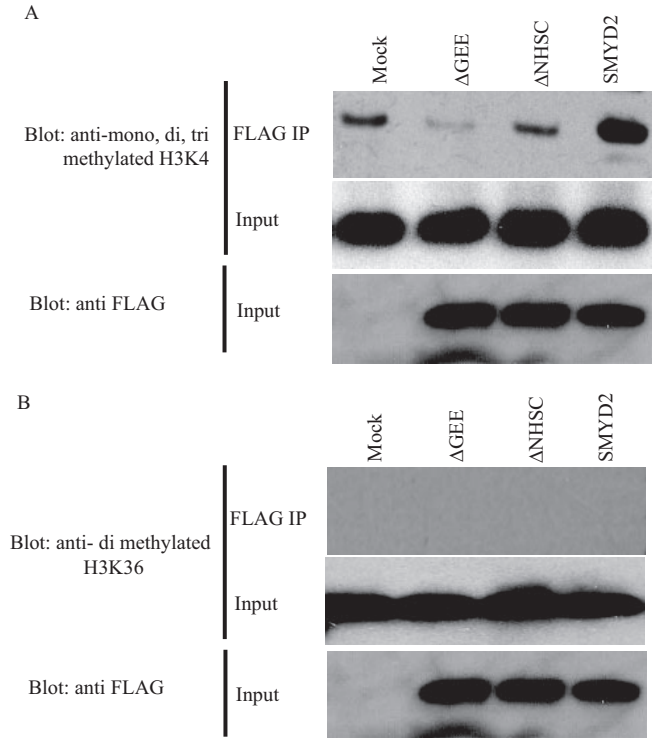

FIG. 5. **Enrichment of H3K4 methylation as a result of SMYD2 overexpression by chromatin IP.** *In vivo* methylation activity was assessed by performing chromatin IP of overexpressed SMYD2, SMYD2 mutants, and a mock control with a subsequent analysis by Western blot to measure the level of enrichment of H3K4 methylation (*A*) and H3K36 methylation (*B*) (*top panel*). The *middle panels* in *A* and *B* are the Western blot of the input whole cell lysate probed for H3K4 methylation (*A*) and H3K36 methylation (*B*). *FLAG IP* refers to immunoprecipitated proteins using anti-FLAG antibodies from 293 cells. The *bottom panels* are Western blot analyses of input whole cell lysate (*Input*) for the presence of FLAG-tagged proteins.

ing the chromatin IP by gene chips, we performed gel electrophoresis and Western blot analysis to look for the enrichment of lysine methylation on histone H3. Briefly wild type SMYD2 and ΔNHSC and ΔGEE deletion mutants were individually overexpressed in 293T cells, a cross-linker was used to fix the interactions, the cell lysate was sonicated to reduce the size of the DNA, and anti-FLAG antibodies were used to purify SMYD2 and its associated proteins. Immunoblot analyses using antibodies directed against either mono-, di-, and trimethylated H3K4 or dimethylated H3K36 revealed that chromatin IP against wild type SMYD2 specifically enriches methylated H3K4 but not dimethylated H3K36 (Fig. 5, *A* and *B*). In contrast, methylated H3K36 was not detected in SMYD2 methylation even after long exposure (data not

shown). Furthermore chromatin IP performed with the two SET domain deletion mutants showed no enrichment for H3K4 (Fig. 5*A*).

SMYD2 Role in Gene Expression Regulation—Histone methylation has been associated with the regulation of gene expression. In particular, methylation at H3K4 is generally associated with the activation of gene expression (20, 21). We studied the impact of the overexpression of SMYD2 on gene expression levels. Briefly wild type SMYD2 was overexpressed in 293T cells, and the total RNA extracted was compared with RNA extracted from cells transfected with a mock vector using microarrays (see "Materials and Methods"). A total of 41 genes showed statistically significant changes in expression according to the SAM software (22); 37 genes were up-regulated, whereas only four genes were down-regulated following the overexpression of SMYD2 (supplemental Table 3). Validation of microarray data was done by semiquantitative RT-PCR for six of the differentially expressed genes, *TACC2*, *WDR9*, *AKAP13*, *Bat1*, chromodomain helicase DNA-binding protein 9 (*CHD9*), and *SMARCA2* (supplemental Fig. 2). As shown in supplemental Table 3, some of these genes function in chromatin remodeling as well as in the cell cycle and transcription regulation. Our data show that SMYD2 overexpression will lead to the overall activation of gene expression in agreement with the observed H3K4 methylation activity of SMYD2.

We proposed that SMYD2, similar to other SMYD family members, regulates gene expression by directly binding to specific DNA consensus sites and/or indirectly through protein-protein interactions with cofactors. To investigate the binding of SMYD2 to regulatory regions of its target genes, the promoter region of *TACC2* was used for chromatin IP. Sequence analyses revealed that the promoter region of *TACC2* contains the CCCTCC motif, which is a binding site for SMYD3 and is localized at the chip 3 region (Fig. 6*A*). The promoter of the *TACC2* gene spanning the region −1279 to +130, with respect to the transcription start site, was divided into four regions: chip 1 (nt −1279 to −949), chip 2 (nt −949 to −629), chip 3 (nt −629 to −200), and chip 4 (nt −200 to +130). Specific primers for each region were used to identify the region to which SMYD2 specifically binds (Fig. 6*A*). Chromatin IP followed by PCR amplification demonstrated that SMYD2 binds to the *TACC2* promoter sequence in the region spanned by chip 4 (Fig. 6*C*). IP with a mock antibody (mouse IgG) was used as a negative control to show that the PCR product obtained in this experiment was specific to the presence of SMYD2. Our data indicate that SMYD2 binds to the promoter region of *TACC2* at a site that is different from the site recognized by SMYD3.

We then tested whether SMYD2 regulated *TACC2* activity by its histone methyltransferase activity or indirectly through a different mechanism. Briefly 293T cells were transfected with wild type SMYD2 or ΔGEE or ΔNHSC deletion mutant as well as with the mock control. Gene expression of *TACC2* was measured by semiquantitative RT-PCR on RNA extracted from the transfected cells. As shown in Fig. 6*D*, only the wild type form had the ability to increase *TACC2* gene expression. The two histone methyltransferase-inactive mutants and the mock control did not increase the gene expression of *TACC2*. These data indicate that the methylation activity of SMYD2 directly affected gene expression of *TACC2*.

DISCUSSION

Very little is known about SMYD2 other than that it contains two evolutionary conserved domains (MYND and SET domains), it methylates p53, and it methylates histone H3 at Lys-36. Hence the proteomics and genomics study of SMYD2 is likely to enhance our understanding of its molecular roles.

Our mapping of the interactome of SMYD2 clearly points to different molecular roles for SMYD2 and indicates that the MYND and SET domains are critical to many of these interactions. In particular, we found that five of the interaction partners of SMYD2 contain the P*X*L*X*P motif (supplemental Table 2). It was demonstrated previously that the MYND domain of the human protein BS69 binds to proteins that contain the motif P*X*L*X*P (19). This is also the case for SMYD1 where the P*X*L*X*P motif in nascent polypeptide-associated complex α-subunit, muscle-specific form, is required for their interaction (23). Hence we hypothesized that SMYD2 interactors that contain the P*X*L*X*P motif, such as EBP41L3, are likely to interact with the MYND domain of SMYD2. EBP41L3, also known as DAL-1, has been implicated in the molecular pathogenesis of breast, lung, and brain cancers, and it is thought to be a tumor suppressor (15). Our co-IP data support the view that the interaction between SMYD2 and EBP41L3 is mediated by the MYND domain through the P*X*L*X*P motif. It is likely that the seven other proteins that have the P*X*L*X*P motif also interact with the MYND domain of SMYD2. In addition to the MYND domain, the SET domain also appeared to be important in mediating some of the protein-protein interactions. We showed that the two conserved regions in the SET domain were important for its interaction with p53, one of its substrates.

HSP90α interaction with SMYD2 was mediated through a region other than the MYND and SET domains as shown by its ability to interact with SMYD2 deletion mutants. We found that interaction between SMYD2 and HSP90α is important for the histone methyltransferase activity of SMYD2 in agreement with previous results for SMYD1 and -3 (7, 8). The manner in which HSP90α contributes as a cofactor of SMYDs is still unclear. Interestingly interactions between HSP90 and its binding partners are mediated through a tetratricopeptide repeat (TPR) domain; for example, its interaction with protein phosphatase 5 (PPP5) (24–26). HSP90 acts as a mediator for the association between PPP5 and glucocorticoid receptor heterocomplexes. An expressed PPP5 TPR domain acts as a dominant negative mutant that strongly inhibits glucocorticoid receptor-mediated transcriptional activation (25). The crystal structure of autoinhibited PPP5 reveals an extensive interface

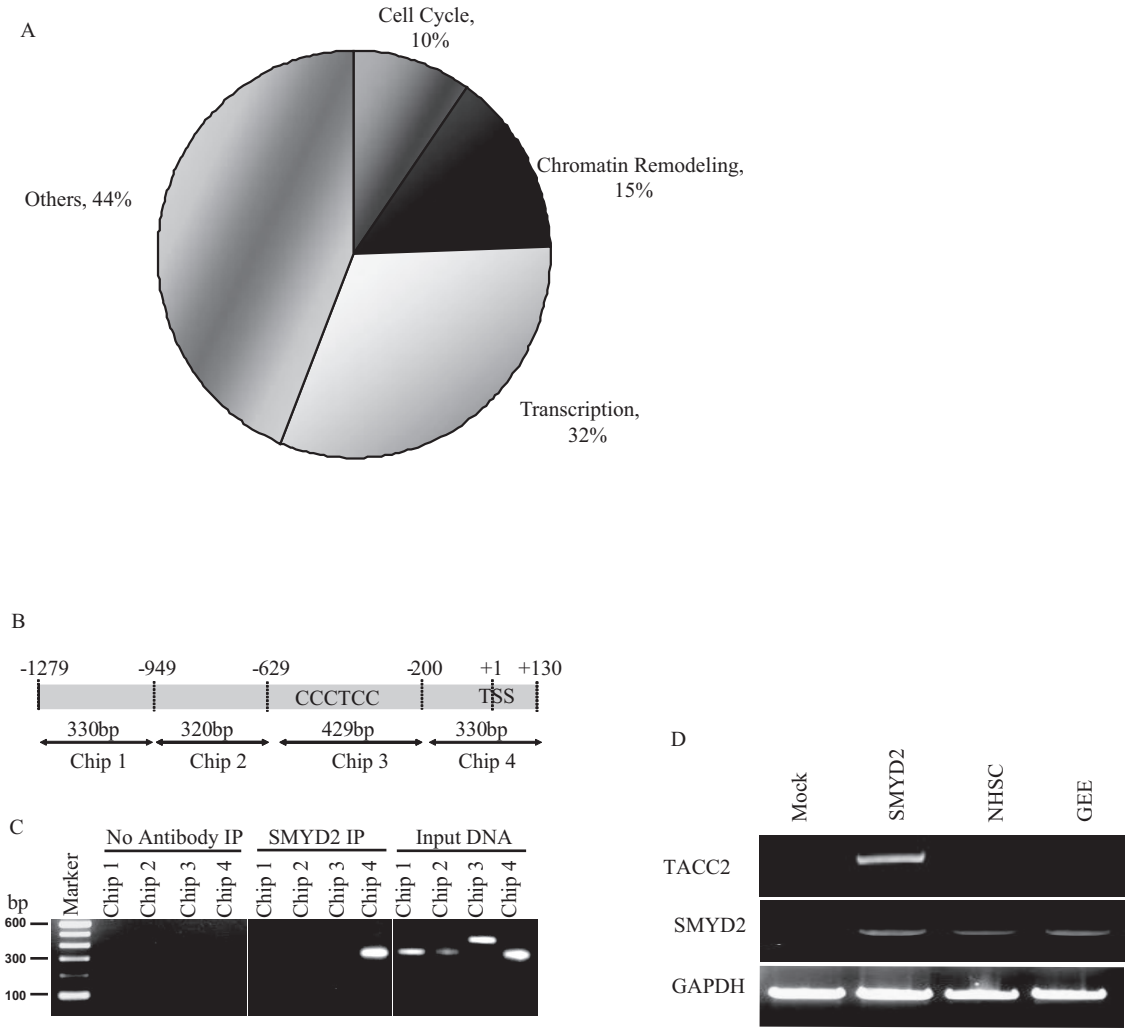

FIG. 6. **SMYD2 affects gene expression and binds the promoter of *TACC2*.** *A*, pie chart showing the classification of the genes with altered expression following overexpression of SMYD2 in 293T cells after 24 h of transient transfection compared with cells transfected with a mock vector. Genes were classified into four categories according to their function: transcription, chromatin remodeling, cell cycle, and other. *B*, promoter sequence of *TACC2* gene showing the location of primers used in the ChIP assay and the transcription start site (*TSS*). *C*, PCR results of the chromatin IP showing a PCR product for Chip 4 primers only when anti-FLAG-SMYD2 antibodies were used, indicating that SMYD2 can bind to the *TACC2* promoter. The *last panel* shows PCR amplification products for the input DNA as a PCR amplification control for the primers. *D*, semiquantitative RT-PCR analysis of the level of expression of *TACC2* transcripts following the overexpression of the wild type SMYD2, its SET domain deletion mutants, and a mock control in 293T cells. The *middle panel* shows the level of expression of wild type SMYD2 and its SET domain deletion mutants by semiquantitative RT-PCR. GAPDH was used as an internal control.

between the TPR domain and the phosphatase catalytic subunit (26). This interface includes the region of the TPR domain that interacts with HSP90, leading the authors to conclude that the binding between the TPR domain and HSP90 activates PPP5 by dissociating the TPR domain from the phosphatase domain (26, 27). SMYD2 has a TPR-like domain predicted by the InterPro database. We hypothesize that SMYD2 activation by HSP90α may be mediated through its TPR domain. This TPR-like domain could act as an inhibitor of SMYD2 activity by binding to its SET domain. Similar to PPP5, SMYD2 interaction with HSP90α might be occurring through the TPR domain leading to the enhancement of SMYD2 methylation activity. At this stage, further work is required to validate this hypothesis.

Histone methylation of different lysine residues correlates with either the activation or the repression of gene expression (20, 21). For example, methylation of H3K4 is associated with gene activation, whereas methylation of Lys-9 on the same histone is associated with gene repression (20). Our data clearly show that SMYD2 specifically methylates H3K4 in the presence of HSP90α, similarly to SMYD1 and SMYD3. Furthermore our *in vitro* data show that, in the absence of HSP90α, SMYD2 has some weak activity for dimethylating Lys-36. The Lys-36 methylation is in agreement with the data

reported by Brown *et al.* (9). However, our *in vivo* experiments strongly suggest that H3K4 is the predominant site of methylation. We did not observe *in vivo* any methylation at Lys-36 above the basal level. Our *in vitro* experiments also suggest that Lys-36 methylation does not occur in the presence of HSP90α. This dual specificity has been observed for other proteins such as LSD1. LSD1 is a histone demethylase that interacts with the androgen receptor leading to demethylation of the repressive histone marks mono- and dimethyl-H3K9 (28). When not bound by the androgen receptor, LSD1 demethylates the activating histone marks mono- and dimethyl-H3K4 (29). Hence we suggest that SMYD2 when bound to HSP90α specifically methylates H3K4 and predominantly leads to the activation of gene expression. We also suggest that, in the absence of HSP90α, SMYD2 has some level of activity to methylate H3K36 that could lead to gene repression.

The overexpression of SMYD2 causes changes in expression of genes associated with chromatin remodeling, cell cycle, and transcription regulation. The majority of genes regulated by SMYD2 were up-regulated in agreement with H3K4 methylation. Examples of proteins involved in chromatin remodeling that were up-regulated by the overexpression of SMYD2 are the three proteins from the SWI/SNF complex (30, 31). The SWI/SNF complex is recruited to chromatin to remodel nucleosomes using ATP hydrolysis as a source of energy (32). The SWI/SNF complex has been implicated in cancer through different mechanisms (30, 33). Furthermore we have observed a down-regulation of the protein WRD9 (bromodomain and WD repeat domain-containing 1), which is known to interact with SMARCA4 (SWI2-related gene 1) (34). Bromodomains are known to bind to acetylated histone H3 (35). As well we have observed the up-regulation of *CHD9*, also named the chromatin-related mesenchymal modulator, which is known to bind to methylated histones H3. Another gene that was up-regulated by SMYD2 overexpression is *MEF2C*, which has transcription factor activity and interestingly regulates SMYD1 expression (36). Finally many zinc finger proteins that are known to bind DNA were up-regulated by SMYD2. The up-regulation of members of the SWI/SNF complex points to a chromatin remodeling effect on the cell. As well the down-regulation of a bromodomain-containing protein and the up-regulation of a chromodomain-containing protein point to potential feedback mechanisms for histone acetylation/methylation.

To gain a global understanding of how SMYD2 protein-protein interactions affect gene expression, we combined the protein interaction data with the protein products of the genes up-regulated by SMYD2 with a bioinformatics tool called Pathway Studio. Combining the data in Pathway Studio allowed us to search for the interacting partners of the combined data in the network and to find the direct links and the shortest paths between entities (13). As shown in Fig. 7, linkages were identified between the list of SMYD2 protein interactors and the protein products of the genes up-regulated by SMYD2. These proteins were divided for simplicity into two groups (Fig. 7). The color code shows the source of entries in the list. The first group (A) includes a set of proteins that are associated with the centrosome and MTs. An interesting part of group A is the interaction of SMYD2 with members of the protein phosphatase 1 (PPP1) complex that is essential for cell division (37). PPP1CC that interacts with SMYD2 forms an essential complex with another protein called Repo-Man that is required for the recruitment of PPP1 to chromatin (37). This complex plays important roles at multiple stages of the cell cycle, including both interphase and mitosis (37). Inhibition of PPP1CC results in an aberrant chromatin condensation (38). AKAP9, another SMYD2 interaction protein, is a scaffolding protein that assembles several protein kinases and phosphatases, including PPP1CC, at the centrosome throughout the cell cycle (39, 40). AKAP9 interacts with members of the TACC protein family (41), one of which (*TACC2*) was up-regulated by SMYD2 overexpression. *TACC2* is particularly interesting because it is phosphorylated by TTK (18), which we found to interact with SMYD2. Other members of the AKAP and PPP1 families were also found to associate with SMYD2 as shown in Fig. 7. These include PPP1CA and -B as well as AKAP11 and -13. The link to the centrosome is further supported by the up-regulation of PPP1CA by SMYD2. PPP1CA has been shown to form a complex with Nek2, a serine/threonine kinase that is localized to the centrosome (42). These protein-protein interaction data and the gene expression data show that SMYD2 might have an important role in the centrosomal MT dynamics. Other proteins that were also observed to associate with SMYD2 also suggest a role for SMYD2 in microtubule dynamics. In particular, CLASP2 (cytoplasmic linker-associated protein 2) is a mammalian MT plus end-binding protein, and it binds to EB1 and to MTs. It mediates interactions between MT plus ends and the cell cortex (43).

The second group (B) includes genes such as p53 and PRKDC. The link between these proteins is probably centered on the role of SMYD2 in its regulation of p53. SMYD2 methylates p53, which leads to its dissociation from promoter regions of its target genes (12). In response to DNA damage, equilibrium is shifted toward SET7/9-mediated methylation of p53, which blocks SMYD2 methylation (12). Finally the other protein that interacts with SMYD2 is PRKDC, which is a serine/threonine-protein kinase that acts as a molecular sensor for DNA damage. PRKDC also acts as a scaffold protein to aid the localization of DNA repair proteins to the site of damage (44). Although it requires further experimental validations, combining our proteomics and genomics data using new bioinformatics tools demonstrates clear links to different cellular processes that support existing data on SMYD2 and provides new venues for understanding the function of SMYD2.

In conclusion, we have established, through a combination of proteomics and genomics methods, some molecular roles

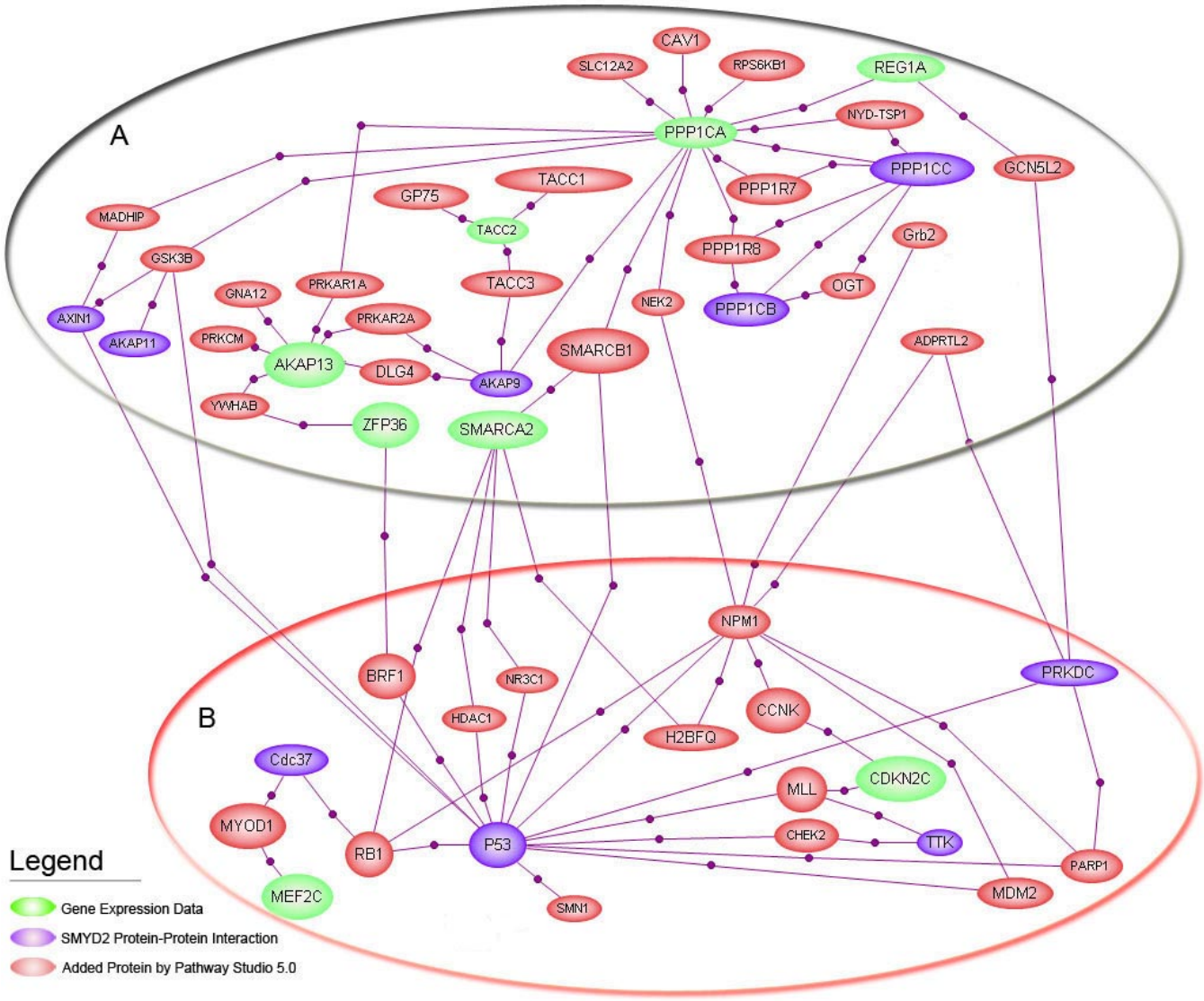

FIG. 7. **Combination of SMYD2 protein-protein interaction data with microarray data obtained from SMYD2 overexpression in 293T cells regrouped in two major networks.** *A*, enrichment of SMYD2 protein interaction partners to the centrosome; proteins anchored to the centrosome might have a role in centrosomal microtubule dynamics and the regulation of cell cycle. *B*, potential role of SMYD2 as a DNA damage response gene through its interaction with genes like p53 and PRKDC.

of SMYD2. Our interactome mapping identified 21 protein interactors of SMYD2. We demonstrated that the MYND domain present in SMYD2 interacts with proteins that have the P*X*L*X*P motif, such as EPB41L3. Five of the SMYD2 interactors contain the P*X*L*X*P motif. We also established that SMYD2 interacts with HSP90α. We demonstrated that SMYD2, through its SET domain, acts as a methyltransferase that specifically methylates H3K4 in the presence of HSP90α and methylates H3K36 in the absence of HSP90α *in vitro*. We also showed that *in vivo* SMYD2 predominantly methylates H3K4. We established that SMYD2 interaction with its substrates like p53 is dependent on the SET domain. Our genomics experiments indicated that SMYD2 overexpression leads to the up-regulation of 37 genes involved in chromatin remodeling, cell cycle, and transcription regulation such as *TACC2*. We established that the regulation of downstream target genes like *TACC2* is mediated through the ability of SMYD2 to associate with the promoter region of target genes and methylate histones in the promoter region. Furthermore we demonstrated that SMYD2 and SMYD3 do not bind to the same promoter regions of *TACC2*. The combination of proteomics and genomics data indicates possible feedback mechanisms and combined regulation at the interaction and expression levels. Finally data provided here will greatly contribute to our understanding of SMYD2 and the SMYD family of proteins.

* This work was supported in part by the Canada Research Chair, the Canadian Institutes of Health Research (CIHR), MDS Inc., the Ontario Genomics Institute, and the University of Ottawa. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The microarray data were submitted to ArrayExpress under accession number E-MEXP-963.

S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

¶ Supported by an Ontario Women's Health Council/CIHR Institute of Gender and Health fellowship.

‖ Supported by a Canadian Institute of Aging Investigator Award.

** A Canada Research Chair in Proteomics and Systems Biology. To whom correspondence should be addressed. Tel.: 613-562-5800 (ext. 8674); Fax: 613-562-5655; E-mail: dfigeys@uottawa.ca.

REFERENCES

1. Ewing, R. M., Chu, P., Elisma, F., Li, H., Taylor, P., Climie, S., McBroom-Cerajewski, L., Robinson, M. D., O'Connor, L., Li, M., Taylor, R., Dharsee, M., Ho, Y., Heilbut, A., Moore, L., Zhang, S., Ornatsky, O., Bukhman, Y. V., Ethier, M., Sheng, Y., Vasilescu, J., Abu-Farha, M., Lambert, J. P., Duewel, H. S., Stewart, II, Kuehl, B., Hogue, K., Colwill, K., Gladwish, K., Muskat, B., Kinach, R., Adams, S. L., Moran, M. F., Morin, G. B., Topaloglou, T., and Figeys, D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol. Syst. Biol.* **3,** 89–91
2. Rual, J. F., Venkatesan, K., Hao, T., Hirozane-Kishikawa, T., Dricot, A., Li, N., Berriz, G. F., Gibbons, F. D., Dreze, M., Ayivi-Guedehoussou, N., Klitgord, N., Simon, C., Boxem, M., Milstein, S., Rosenberg, J., Goldberg, D. S., Zhang, L. V., Wong, S. L., Franklin, G., Li, S., Albala, J. S., Lim, J., Fraughton, C., Llamosas, E., Cevik, S., Bex, C., Lamesch, P., Sikorski, R. S., Vandenhaute, J., Zoghbi, H. Y., Smolyar, A., Bosak, S., Sequerra, R., Doucette-Stamm, L., Cusick, M. E., Hill, D. E., Roth, F. P., and Vidal, M. (2005) Towards a proteome-scale map of the human protein-protein interaction network. *Nature* **437,** 1173–1178
3. Stelzl, U., Worm, U., Lalowski, M., Haenig, C., Brembeck, F. H., Goehler, H., Stroedicke, M., Zenkner, M., Schoenherr, A., Koeppen, S., Timm, J., Mintzlaff, S., Abraham, C., Bock, N., Kietzmann, S., Goedde, A., Toksoz, E., Droege, A., Krobitsch, S., Korn, B., Birchmeier, W., Lehrach, H., and Wanker, E. E. (2005) A human protein-protein interaction network: a resource for annotating the proteome. *Cell* **122,** 957–968
4. Gross, C. T., and McGinnis, W. (1996) DEAF-1, a novel protein that binds an essential region in a Deformed response element. *EMBO J.* **15,** 1961–1970
5. Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998) SET domain proteins modulate chromatin domains in eu- and heterochromatin. *CMLS Cell. Mol. Life Sci.* **54,** 80–93
6. Sims, R. J., III, Nishioka, K., and Reinberg, D. (2003) Histone lysine methylation: a signature for chromatin function. *Trends Genet.* **19,** 629–639
7. Hamamoto, R., Furukawa, Y., Morita, M., Iimura, Y., Silva, F. P., Li, M., Yagyu, R., and Nakamura, Y. (2004) SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. *Nat. Cell Biol.* **6,** 731–740
8. Tan, X., Rotllant, J., Li, H., De Deyne, P., and Du, S. J. (2006) SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 2713–2718
9. Brown, M. A., Sims, R. J., III, Gottlieb, P. D., and Tucker, P. W. (2006) Identification and characterization of Smyd2: a split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase that interacts with the Sin3 histone deacetylase complex. *Mol. Cancer* **5,** 26
10. Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barlev, N. A., and Reinberg, D. (2004) Regulation of p53 activity through lysine methylation. *Nature* **432,** 353–360
11. Kouskouti, A., Scheer, E., Staub, A., Tora, L., and Talianidis, I. (2004) Gene-specific modulation of TAF10 function by SET9-mediated methylation. *Mol. Cell* **14,** 175–182
12. Huang, J., Perez-Burgos, L., Placek, B. J., Sengupta, R., Richter, M., Dorsey, J. A., Kubicek, S., Opravil, S., Jenuwein, T., and Berger, S. L. (2006) Repression of p53 activity by Smyd2-mediated methylation. *Nature* **444,** 629–632
13. Nikitin, A., Egorov, S., Daraselia, N., and Mazo, I. (2003) Pathway studio—the analysis and navigation of molecular networks. *Bioinformatics* **19,** 2155–2157
14. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406,** 593–599
15. Singh, V., Miranda, T. B., Jiang, W., Frankel, A., Roemer, M. E., Robb, V. A., Gutmann, D. H., Herschman, H. R., Clarke, S., and Newsham, I. F. (2004) 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. *Oncogene* **23,** 7761–7771
16. Jiang, W., Roemer, M. E., and Newsham, I. F. (2005) The tumor suppressor DAL-1/4.1B modulates protein arginine N-methyltransferase 5 activity in a substrate-specific manner. *Biochem. Biophys. Res. Commun.* **329,** 522–530
17. Lansbergen, G., Grigoriev, I., Mimori-Kiyosue, Y., Ohtsuka, T., Higa, S., Kitajima, I., Demmers, J., Galjart, N., Houtsmuller, A. B., Grosveld, F., and Akhmanova, A. (2006) CLASPs attach microtubule plus ends to the cell cortex through a complex with LL5β. *Dev. Cell* **11,** 21–32
18. Dou, Z., Ding, X., Zereshki, A., Zhang, Y., Zhang, J., Wang, F., Sun, J., Huang, H., and Yao, X. (2004) TTK kinase is essential for the centrosomal localization of TACC2. *FEBS Lett.* **572,** 51–56
19. Ansieau, S., and Leutz, A. (2002) The conserved Mynd domain of BS69 binds cellular and oncoviral proteins through a common PXLXP motif. *J. Biol. Chem.* **277,** 4906–4910
20. Lachner, M., and Jenuwein, T. (2002) The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.* **14,** 4906–4910
21. Holbert, M. A., and Marmorstein, R. (2005) Structure and activity of enzymes that remove histone modifications. *Curr. Opin. Struct. Biol.* **15,** 673–680
22. Tusher, V. G., Tibshirani, R., and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5116–5121
23. Sims, R. J., III, Weihe, E. K., Zhu, L., O'Malley, S., Harriss, J. V., and Gottlieb, P. D. (2002) m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart- and muscle-specific transcription factor. *J. Biol. Chem.* **277,** 26524–26529
24. Silverstein, A. M., Galigniana, M. D., Chen, M. S., Owens-Grillo, J. K., Chinkers, M., and Pratt, W. B. (1997) Protein phosphatase 5 is a major component of glucocorticoid receptor·hsp90 complexes with properties of an FK506-binding immunophilin. *J. Biol. Chem.* **272,** 16224–16230
25. Chen, M. S., Silverstein, A. M., Pratt, W. B., and Chinkers, M. (1996) The tetratricopeptide repeat domain of protein phosphatase 5 mediates binding to glucocorticoid receptor heterocomplexes and acts as a dominant negative mutant. *J. Biol. Chem.* **271,** 32315–32320
26. Yang, J., Roe, S. M., Cliff, M. J., Williams, M. A., Ladbury, J. E., Cohen, P. T., and Barford, D. (2005) Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. *EMBO J.* **24,** 1–10
27. Chen, M. X., and Cohen, P. T. (1997) Activation of protein phosphatase 5 by limited proteolysis or the binding of polyunsaturated fatty acids to the TPR domain. *FEBS Lett.* **400,** 136–140
28. Metzger, E., Wissmann, M., Yin, N., Muller, J. M., Schneider, R., Peters, A. H., Gunther, T., Buettner, R., and Schule, R. (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* **437,** 436–439
29. Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119,** 941–953
30. Roberts, C. W., and Orkin, S. H. (2004) The SWI/SNF complex—chromatin and cancer. *Nat. Rev. Cancer* **4,** 133–142
31. Lee, D. Y., Teyssier, C., Strahl, B. D., and Stallcup, M. R. (2005) Role of protein methylation in regulation of transcription. *Endocr. Rev.* **26,** 147–170

32. Johnson, C. N., Adkins, N. L., and Georgel, P. (2005) Chromatin remodeling complexes: ATP-dependent machines in action. *Biochem. Cell Biol.* **83,** 405–417
33. Gibbons, R. J. (2005) Histone modifying and chromatin remodelling enzymes in cancer and dysplastic syndromes. *Hum. Mol. Genet.* **14,** R85–R92
34. Huang, H., Rambaldi, I., Daniels, E., and Featherstone, M. (2003) Expression of the Wdr9 gene and protein products during mouse development. *Dev. Dyn.* **227,** 608–614
35. Yang, X. J. (2004) Lysine acetylation and the bromodomain: a new partnership for signaling. *BioEssays* **26,** 1076–1087
36. Phan, D., Rasmussen, T. L., Nakagawa, O., McAnally, J., Gottlieb, P. D., Tucker, P. W., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2005) BOP, a regulator of right ventricular heart development, is a direct transcriptional target of MEF2C in the developing heart. *Development* **132,** 2669–2678
37. Trinkle-Mulcahy, L., Andersen, J., Lam, Y. W., Moorhead, G., Mann, M., and Lamond, A. I. (2006) Repo-Man recruits PP1γ to chromatin and is essential for cell viability. *J. Cell Biol.* **172,** 679–692
38. Swain, J. E., Ding, J., Brautigan, D. L., Villa-Moruzzi, E., and Smith, G. D. (2007) Proper chromatin condensation and maintenance of histone H3 phosphorylation during mouse oocyte meiosis requires protein phosphatase activity. *Biol. Reprod.* **76,** 628–638
39. Takahashi, M., Yamagiwa, A., Nishimura, T., Mukai, H., and Ono, Y. (2002) Centrosomal proteins CG-NAP and kendrin provide microtubule nucleation sites by anchoring gamma-tubulin ring complex. *Mol. Biol. Cell* **13,** 3235–3245
40. Witczak, O., Skalhegg, B. S., Keryer, G., Bornens, M., Tasken, K., Jahnsen, T., and Orstavik, S. (1999) Cloning and characterization of a cDNA encoding an A-kinase anchoring protein located in the centrosome, AKAP450. *EMBO J.* **18,** 1858–1868
41. Steadman, B. T., Schmidt, P. H., Shanks, R. A., Lapierre, L. A., and Goldenring, J. R. (2002) Transforming acidic coiled-coil-containing protein 4 interacts with centrosomal AKAP350 and the mitotic spindle apparatus. *J. Biol. Chem.* **277,** 30165–30176
42. Helps, N. R., Luo, X., Barker, H. M., and Cohen, P. T. (2000) NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. *Biochem. J.* **349,** 509–518
43. Mimori-Kiyosue, Y., Grigoriev, I., Lansbergen, G., Sasaki, H., Matsui, C., Severin, F., Galjart, N., Grosveld, F., Vorobjev, I., Tsukita, S., and Akhmanova, A. (2005) CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. *J. Cell Biol.* **168,** 141–153
44. Cahill, D., Connor, B., and Carney, J. P. (2006) Mechanisms of eukaryotic DNA double strand break repair. *Front. Biosci.* **11,** 1958–1976
45. Elias, J. E., Haas, W., Faherty, B. K., and Gygi, S. P. (2005) Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat. Methods* **2,** 667–675