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SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells

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Colorectal and hepatocellular carcinomas are some of the leading causes of cancer deaths worldwide, but the mechanisms that underly these malignancies are not fully understood. Here we report the identification of *SMYD3*, a gene that is over-expressed in the majority of colorectal carcinomas and hepatocellular carcinomas. Introduction of *SMYD3* into NIH3T3 cells enhanced cell growth, whereas genetic knockdown with small-interfering RNAs (siRNAs) in cancer cells resulted in significant growth suppression. SMYD3 formed a complex with RNA polymerase II through an interaction with the RNA helicase HELZ and transactivated a set of genes that included oncogenes, homeobox genes and genes associated with cell-cycle regulation. SMYD3 bound to a motif, 5′-CCCTCC-3′, present in the promoter region of downstream genes such as *Nkx2.8*. The SET domain of SMYD3 showed histone H3-lysine 4 (H3-K4)-specific methyltransferase activity, which was enhanced in the presence of the heat-shock protein HSP90A. Our findings suggest that SMYD3 has histone methyltransferase activity and plays an important role in transcriptional regulation as a member of an RNA polymerase complex. Furthermore, activation of SMYD3 may be a key factor in human carcinogenesis.

Colorectal carcinoma (CRC) is the leading cause of cancer deaths in developed countries. In 2000, nearly 940,000 individuals were diagnosed with CRC and approximately 579,000 died from it[1,2]. Hepatocellular carcinoma (HCC) is also one of the most common malignancies among human populations worldwide, especially in Asia and Africa, and its incidence is increasing significantly in Japan and the United States[3]. In the past few years, much progress has been made towards a better understanding of the molecular mechanisms of CRC and HCC, including the role of histone modification[4,5].

Histone modification functions in the regulation of chromatin structure, as well as transcriptional activation and repression. Histone modifications occur at selected residues and multiple modifications that function in a combinational or sequential fashion, in single or multiple tails, dictate 'histone codes' that are closely linked to the biological consequences[6]. Among the modifications, histone lysine methylation on lysine residues 4, 9, 27 and 36 in H3, and on residue 20 in H4 (ref. 7), is considered to be critical for transcriptional regulation[8,9]. In addition, as it seems to be relatively stable compared with other histone modifications, such as acetylation or phosphorylation, histone methylation may provide an epigenetic hallmark for long-term transcriptional memory[7]. The increasing knowledge of histone methylation suggests that methylation of lysine 9 in histone H3 (H3-K9) is involved in transcriptionally-repressive heterochromatin formation with heterochromatin-associated proteins[10]; and, furthermore, that lysine 4 methylation of histone H3 (H3-K4) is important for transcriptional activation[9,11,12]. However, the precise mechanisms of histone methylation and transcriptional regulation still remain unclear.

Proteins containing a SET domain constitute a family and are classified into at least four groups on the basis of structural or sequence similarities. Among them, the SUV39 protein — a mammalian homologue of *Drosophila* position-effect variegation modifier Su(var)3-9 — has H3-K9 methyltransferase activity. Set1 and Set2 show H3-K4 or H3-K36 methyltransferase activity, respectively[13,14], whereas G9a functions as a 'dual' methyltransferase, catalysing histone H3-K9 and H3-K27 (ref. 15). In addition, SET7/9 has specific histone methyltransferase (HMTase) activity on H3-K4, and mono- and di-methylates this site[16]. Although a number of SET domain proteins function in histone modification, how they modulate transcription remains unclear.

Here, we report the identification and characterization of *SMYD3*, a human gene that contains a SET domain and is frequently up-regulated in CRCs and HCCs. SMYD3 methylates histone H3-K4 in the presence of HSP90A (heat shock 90kDa protein 1, α) and activates the transcription of downstream genes including *Nkx2.8*. Our data suggest that SMYD3 could function as a novel molecular target in the treatment of these tumours.

RESULTS

Isolation and expression of *SMYD3*

Among the genes up-regulated in CRCs and HCCs, as determined by cDNA microarray analysis[17,18] and semi-quantitative RT–PCR (Fig. 1a), we focused on the gene from an EST (archived as Hs.8109 in

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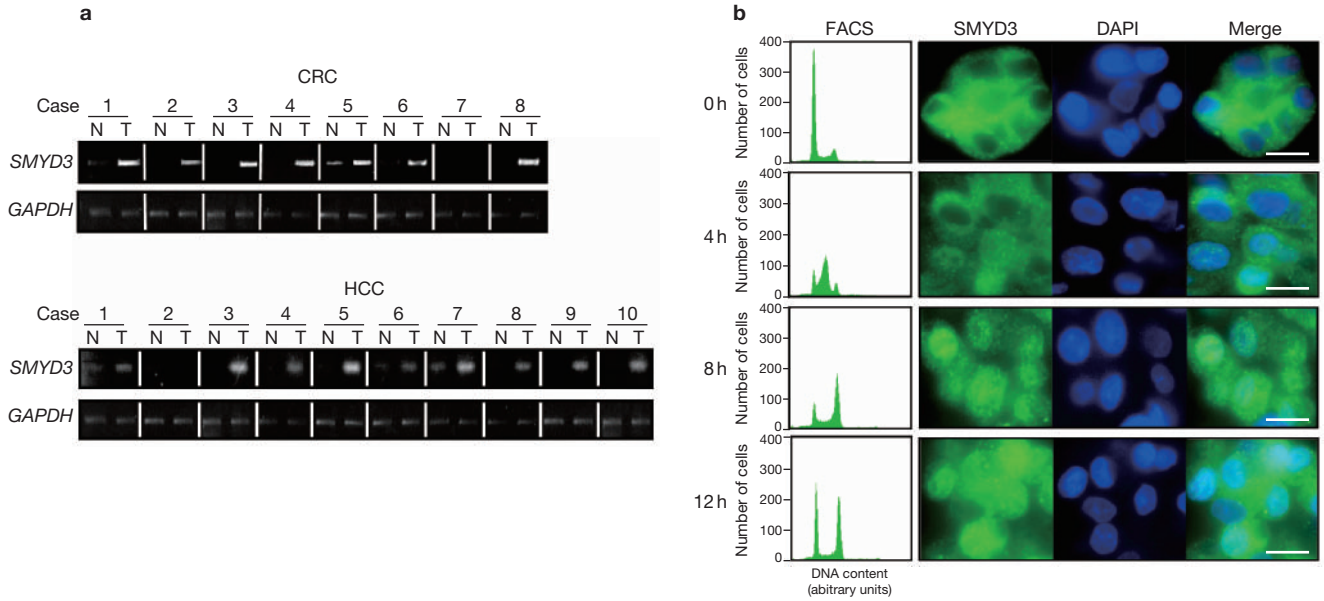

Figure 1 Expression of *SMYD3* and its subcellular localization. (**a**) Semi-quantitative RT–PCR analysis of *SMYD3* in eight CRC tissues and ten HCC tissues (T), and corresponding non-cancerous tissues (N). Case denotes the code number of the patients. Expression of *GAPDH* was used as an internal control. (**b**) Cell-cycle dependent localization of SMYD3. Antibodies to SMYD3 were stained with an anti-rabbit secondary antibody conjugated to FITC (green); nuclei were stained blue with DAPI; Sclae bars represent 15 μm. Huh7 cells were growth-arrested in G1 phase by incubation with 5 μg/ml aphidicolin for 36 h and released from G1 by the removal of aphidicolin. FACS analysis and immunocytochemical staining were performed at times 0, 4, 8 and 12 h after the withdrawal of aphidicolin.

the Unigene database) that has a 1.7-kb transcript expressed specifically in testis and skeletal muscle (see Supplementary Information, Fig. S1a). As the assembled cDNA sequence (GenBank accession number AB057595) of 1,622 nucleotides encoded a putative 428-amino-acid protein containing a SET domain (codons 148–239) and a zf-MYND domain (codons 49–87), we termed this gene *SMYD3* (SET- and MYND-domain containing 3).

Immunohistochemical staining with an anti-SMYD3 antibody indicated that the subcellular localization of SMYD3 was altered by the density of cultured Huh7 cells (see Supplementary Information, Fig. S1b). Therefore, we synchronized the cells using aphidicolin and examined SMYD3 localization during cell-cycle progression. Notably, the protein was located mainly in the cytoplasm when the cells were arrested at G0/G1, but accumulated in the nuclei at S phase and G2/M (Fig. 1b).

Oncogenic activity of SMYD3

To investigate the effect of SMYD3 on cell growth, a colony formation assay was performed by transfecting NIH3T3 cells with a plasmid that expresses SMYD3 (pcDNA–SMYD3). Compared with mock- and antisense-SMYD3-expressing plasmids (pcDNA–antisense-SMYD3), pcDNA–SMYD3 induced markedly more colonies (a result that was confirmed in three independent experiments; Fig. 2a). To confirm the growth-promoting effect, we established NIH3T3 cells that stably express exogenous *SMYD3* (NIH3T3–SMYD3 cells). As expected, the growth rate of NIH3T3–SMYD3 cells was much higher than that of cells transfected with antisense-SMYD3 (NIH3T3–antisense-SMYD3), or of cells transfected with mock plasmid (Fig. 2b).

To test whether suppression of *SMYD3* may result in growth suppression of CRC or HCC cells, the activity of different *SMYD3* siRNAs (corresponding to the different regions of SMYD3) was examined in SNU475 cells that express SMYD3 at high levels. Western blot analysis with the extracts of transfected cells demonstrated a significant reduction of endogenous SMYD3 expression by psiU6BX–SMYD3-12, moderate suppression by psiU6BX–SMYD3-4 or -14, and no suppression by psiU6BX–SMYD3-1 compared with cells transfected with psiU6BX–Mock or psiU6BX–EGFP as controls (Fig. 2c). In addition, two forms of mutant plasmid containing a 2-bp mismatch or a scrambled sequence of SMYD3-12 (psiU6BX–SMYD3-12mm or psiU6BX–SMYD3-12scr, respectively) were used. Neither plasmids suppressed SMYD3 expression, indicating sequence-specific inhibition of SMYD3 by psiU6BX–SMYD3-12. SNU475 cells were transfected with each plasmid (also containing the neomycin-resistance gene) and were cultured with an appropriate concentration of G418 (a neomycin analogue). MTT assays performed nine days after transfection revealed that psiU6BX–SMYD3-12 had the highest growth inhibitory effect and that psiU6BX–SMYD3-1 had no effect on the number of surviving cells compared with cells transfected with control plasmids (Fig. 2d). The growth inhibitory effect of the plasmids correlated well with their gene silencing effect. A similar growth inhibitory effect was observed in two CRC cell lines, SW948 and HCT116, as well as in other hepatoma cell lines, including SNU398, SNU423, SNU449, Huh7, Alexander and HepG2 (Fig. 2e), all of which expressed SMYD3 endogenously (see Supplementary Information, Fig. S1c). Furthermore, FACS analysis demonstrated that transfection of psiU6BX–SMYD3-12 increased the number of cells that were at sub-G1, suggesting that apoptosis was induced in response to suppression of SMYD3 (Fig. 2f).

Interaction of SMYD3 with an RNA helicase and RNA polymerase II

To examine how SMYD3 functions in oncogenesis, we searched for interacting proteins by yeast two-hybrid analysis and identified an RNA helicase (HELZ) and a heat shock protein with a relative molecular mass (M_r) of 90,000 (HSP90A). Association of Flag-tagged SMYD3

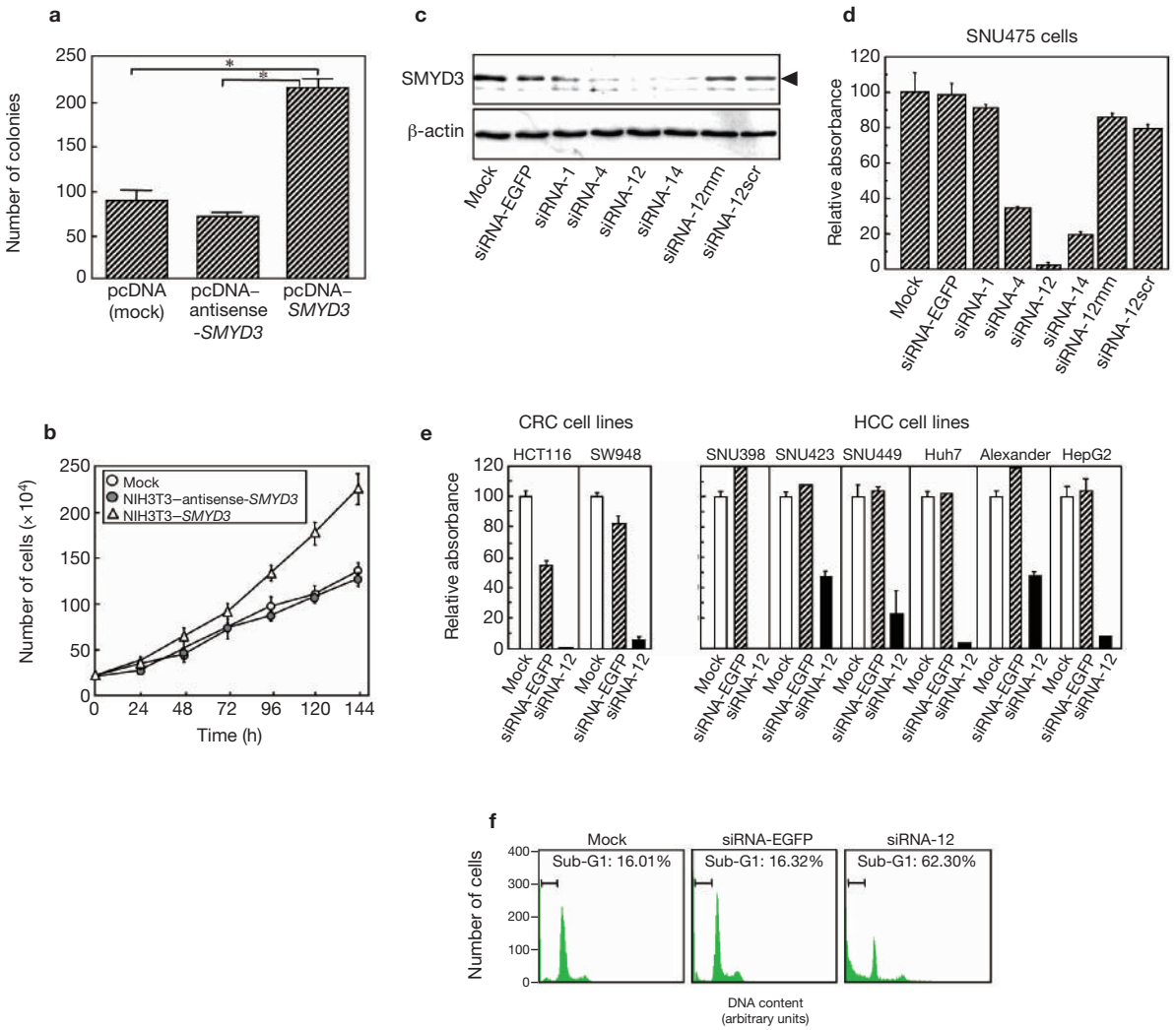

Figure 2 Effect of SMYD3 on proliferation. (**a**) Colony-formation assay in NIH3T3 cells. The number of colonies was assessed by electronic densitometry. Bars, ± SD. Asterisk denotes a significant difference ($p < 0.05$) determined by a Fisher's protected least-significant test. (**b**) Growth of NIH3T3–SMYD3 cells, NIH3T3 cells transfected with antisense SMYD3, and mock cells, as measured by trypan blue staining. (**c**) Effect of plasmids expressing *SMYD3* siRNAs in SNU475 cells. Western blot analysis of the cells at 48 h after transfection by Nucleofector. (**d**) Effect of *SMYD3* siRNAs on the growth of SNU475 cells. MTT assays 9 days after transfection and subsequent incubation with media containing G418. (**e**) Effect of *SMYD3* siRNAs on the growth of CRC and HCC cells. MTT assays 9 days after transfection and subsequent incubation with media containing G418. (**f**) FACS analysis of the SNU475 cells. The percentages of cells in sub-G1 population were determined from at least 20,000 ungated cells.

with haemagglutinin (HA)-tagged HELZ was confirmed in HeLa cells (Fig. 3a). In addition, simultaneous-transformation assays using different mutants of SMYD3 identified codons 100–250, which encompass the SET domain, as being responsible for the association with HELZ (Fig. 3b). As RNA helicases have important functions in transcription, through binding to transcription factors and RNA polymerase II, we reasoned that SMYD3 might form a transcriptional complex with HELZ and RNA polymerase II. To verify this, immunoprecipitation assays were performed using extracts from HeLa cells that had been co-transfected with pFlag–CMV-SMYD3 and pCMV–HA-HELZ, as HeLa cells abundantly express endogenous RNA polymerase II. HA-tagged HELZ and RNA polymerase II co-immunoprecipitated (Fig. 3c). In addition, endogenous RNA polymerase II co-immunoprecipitated with HA-tagged HELZ and Flag-tagged SMYD3 (Fig. 3c). However, the interaction between Flag-tagged SMYD3 and RNA polymerase II was weak when cells were transfected with pFlag–CMV–SMYD3 alone (Fig. 3c). These results suggest that HELZ functions as a bridge between SMYD3 and RNA polymerase II to produce a ternary complex. The interaction between endogenous SMYD3 protein and RNA polymerase II or HSP90A in HCT116 and HepG2 cells was also confirmed (Fig. 3d, e, for HCT116 cells; see Supplementary Information, Fig. S1d, for HepG2 cells).

Histone H3-methyltransferase activity

SMYD3 contains a SET domain that mediates lysine-directed histone methylation[7,8,19]. Therefore, we investigated a possible role for SMYD3 as an HMTase. A number of HMTases recruit S-adenosyl-L-methionine (SAM) as a cofactor. Two conserved amino-acid sequences (NHSCXPN and GEELXXXY) in the SET domain of SUV39H1 are thought to be responsible for the interaction with SAM and the HMTase activity[10]. These two sequence motifs were conserved in the SET domain of SMYD3, and two forms of mutant protein that lacked

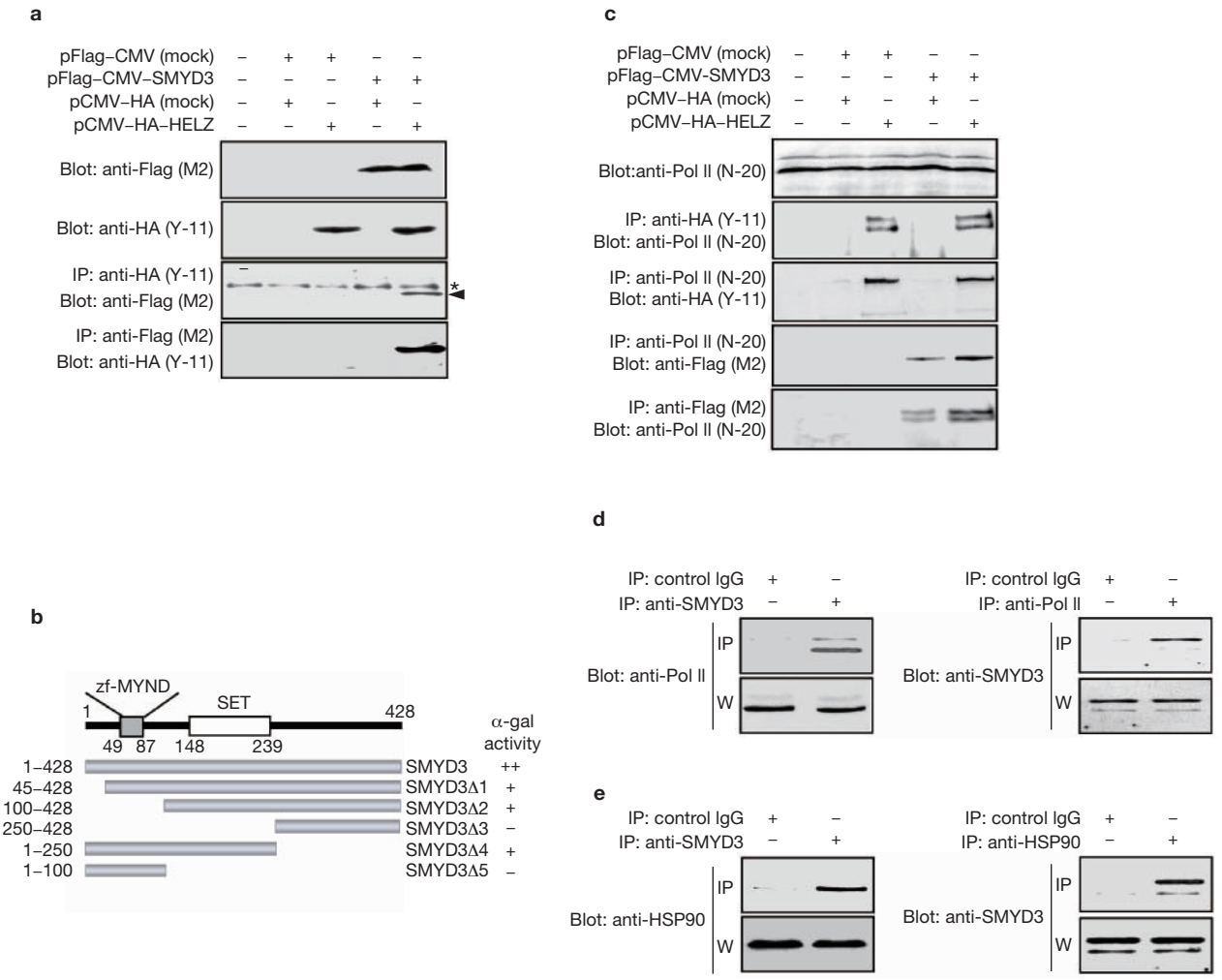

Figure 3 Association of SMYD3 with HELZ, RNA polymerase II and HSP90 *in vivo*. (**a**) Co-immunoprecipitation assay of SMYD3 and HELZ. Lysates from HeLa cells transfected with the indicated plasmids were immunoprecipitated with anti-HA or anti-Flag antibodies. Asterisk denotes antibody heavy chain; arrow indicates Flag–SMYD3. (**b**) Interaction between deletion mutants of SMYD3 and HELZ in yeast cells. (**c**) Association of endogenous RNA polymerase II with SMYD3 and HELZ. Expression of endogenous RNA polymerase II is shown in the top panel. (**d**) Interaction between endogenous RNA polymerase II and endogenous SMYD3 in HCT116 cells. Top, western blot analysis of immunoprecipitants (IP); bottom, whole-cell lysates (W). (**e**) Interaction between endogenous HSP90A and endogenous SMYD3 in HCT116 cells. Top, western blot analysis of immunoprecipitants (IP); bottom, whole-cell lysates (W).

either of the two motifs did not interact with ^{3}H-labelled SAM; however, the wild-type SET domain did interact (Fig. 4a, b). Next, methylation of histone H3 was measured after incubation of wild-type SMYD3 (or SET7/9 as a control) with ^{3}H-labelled SAM and recombinant histone H3 as a substrate. A 17K band corresponding to ^{3}H-labelled histone H3 was observed with the control SET7/9 in the fluorogram (Fig. 4c, lane 2). However, when the substrate was incubated with wild-type SMYD3, only a faint band corresponding to labelled histone H3 was observed (Fig. 4c, lane 3), although a methylated band was not observed in the control (Fig. 4c, lane 1). As HSP90A interacts with SMYD3, we hypothesized that HSP90A might assist with SMYD3 protein folding and be required for its biological activity. Therefore, SAM and histone H3 were incubated in combination with wild-type SMYD3 and recombinant HSP90A. HMTase activity on H3 increased in response to HSP90A in a dose-dependent manner (Fig. 4c, lanes 4–6). However, no HMTase activity was observed with mutant SMYD3, highlighting the importance of the two conserved motifs of the SET domain for the HMTase activity (Fig. 4c, lanes 7, 8). Notably, these mutants preserved their HSP90A-binding capacity (see Supplementary Information, Fig. S1e).

As proteins containing SET domains have an important function in the methylation of histone H3 Lysine 4 (H3-K4) or Lysine 9 (H3-K9), we asked whether SMYD3 might methylate H3-K4 or H3-K9. Recombinant histone H3 was incubated with SAM and HSP90A, as well as wild-type or the HMTase-inactive mutant SMYD3 or SET7/9 *in vitro*. In agreement with previous reports[11,16], incubation with SET7/9 enhanced mono- and di-methylation of H3-K4, but did not induce its tri-methylation (Fig. 4d, lane 2). However, incubation with wild-type SMYD3 resulted in di- and tri-methylation of H3-K4, but did not induce its mono-methylation. (Fig. 4d, lane 3). This methylation activity was completely inhibited by the addition of di-methylated H3-K4 peptides, but was unaffected by the addition of di-methylated H3-K9 peptides (Fig. 4e). Furthermore, H3-K9 was

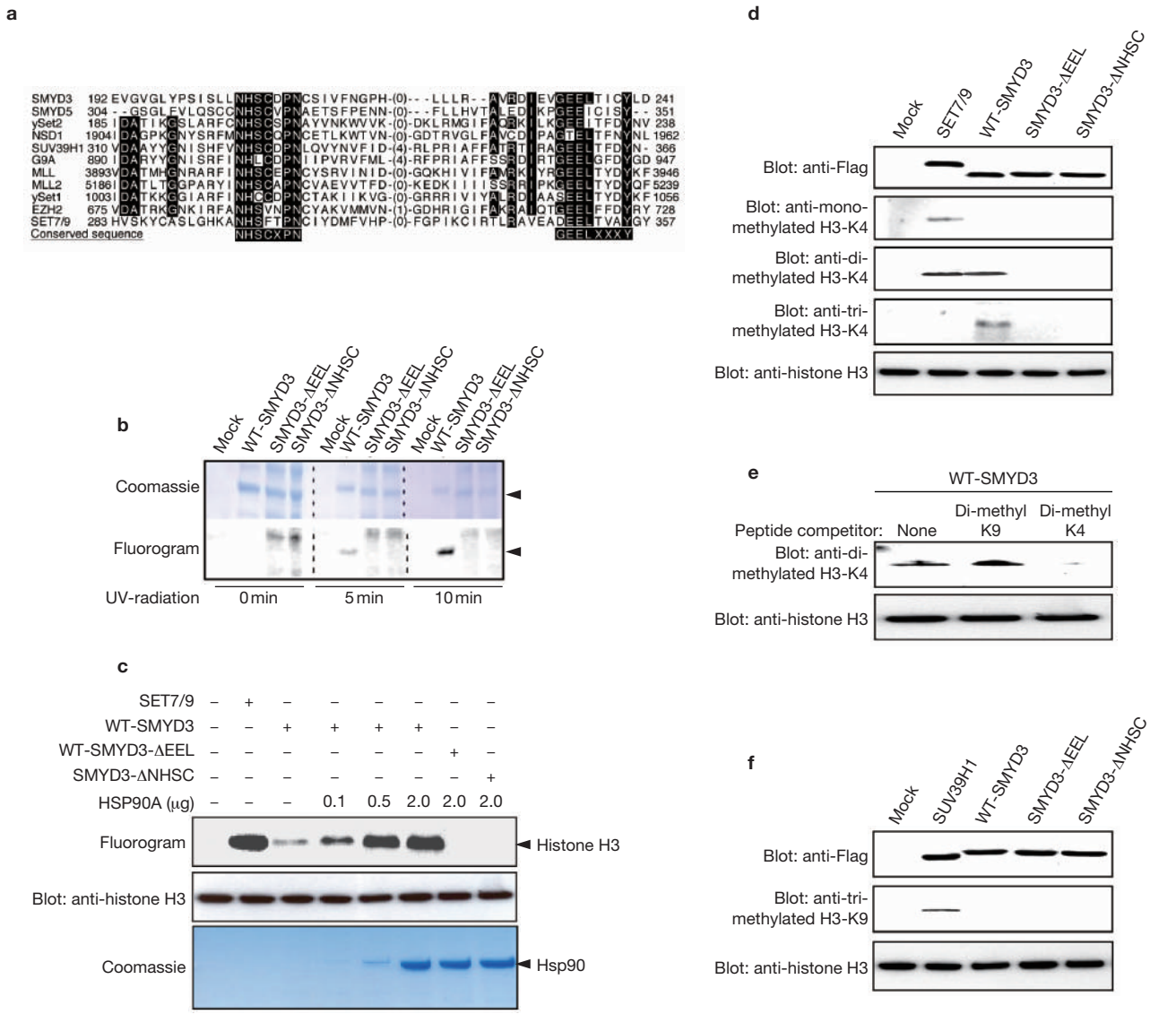

Figure 4 Histone methyltransferase activity of SMYD3. (**a**) Conserved sequences in the SET domains of methyltransferases. (**b**) Interaction between wild-type SMYD3 and SAM. An equal amount of wild-type or mutant recombinant SMYD3 was incubated with ^{3}H-labelled SAM and irradiated with ultraviolet light (at 254 nm) for the indicated time. Protein levels were assessed by staining with Coomassie on an SDS–PAGE gel (top) and the association of SAM with SMYD3 detected by fluorography (bottom). (**c**) *In vitro* HMTase assay of immunoprecipitated Flag-tagged SMYD3 protein with or without recombinant HSP90A. Recombinant *Xenopus laevis* histone H3 was used as a substrate. Immunoprecipitated Flag-tagged SET7/9 protein was used as a control. (**d**) Methylation of histone H3-K4 by SMYD3 *in vitro*. Immunoprecipitated wild-type or mutant Flag-tagged SMYD3, or SET7/9, was incubated with histone H3 in the presence of SAM and HSP90A. (**e**) Inhibition of H3-K4 di-methylation by the addition of specific peptides to di-methylated H3-K4. (**f**) Methylation of histone H3-K9 *in vitro*. SUV39H1 was used as a positive control.

not methylated by either wild-type or mutant SMYD3 (Fig. 4f), suggesting that it has H3-K4-specific HMTase activity.

The importance of the methyltransferase activity of SMYD3 for its oncogenic activity was investigated by colony formation assays using plasmids expressing wild-type or HMTase-inactive mutant-SMYD3 in HEK293 cells. Mutant SMYD3 had no effect on the number of colonies, whereas wild-type SMYD3 enhanced transformation activity, highlighting the importance of the HMTase activity for the oncogenic function of SMYD3 (see Supplementary Information, Fig. S2a).

Putative DNA-binding motifs of SMYD3

The presence of a MYND-type zinc-finger domain in SMYD3 suggested that SMYD3 might recognize and bind particular sequences of genomic DNA. Therefore, we searched for putative DNA-binding sequences of SMYD3 *in vitro* by selecting double-stranded random oligonucleotides using a glutathione *S*-transferase fusion protein (GST–SMYD3) immobilized with glutathione Sepharose 4B. The sequences of 92 selected nucleotides revealed that specific elements (5′-CCCTCC-3′ or 5′-GGAGGG-3′) were present in 32/92 sequences (34.8%). This corresponds to a 102-fold greater incidence than the calculated probability.

Identification of SMYD3 target genes

To identify downstream genes regulated by SMYD3, pcDNA–SMYD3 was transfected into HEK293 cells in which SMYD3 expression was barely detectable, even by RT–PCR, and alterations in gene expression were monitored using a cDNA microarray that contained 13,824 genes.

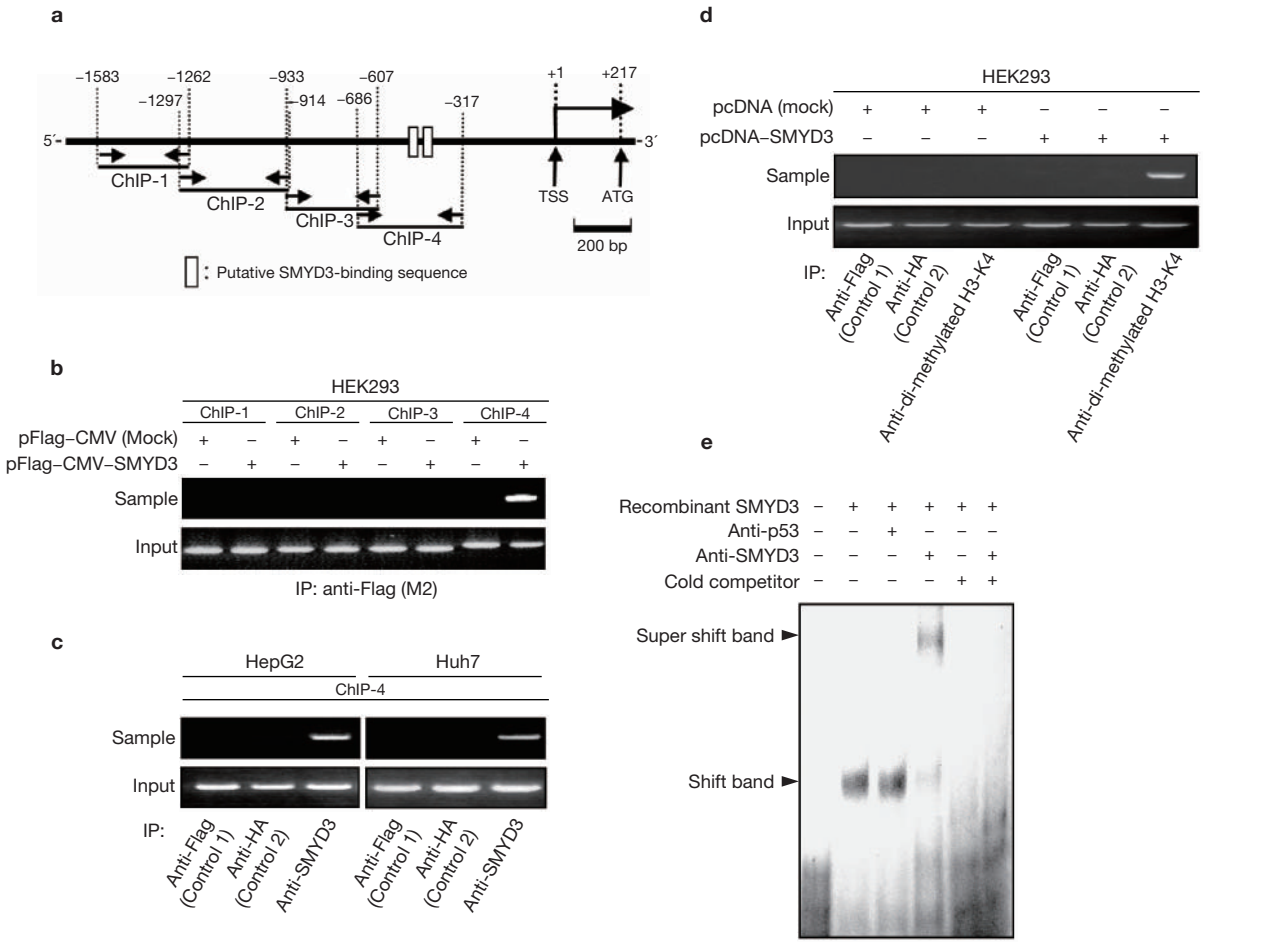

Figure 5 Identification of a SMYD3-binding element in the 5′ flanking region of *Nkx2.8*. (**a**) Primer positions for ChIP assay in the 5′-flanking region of *Nkx2.8*. (**b**) Analysis of association of SMYD3 with DNA in the 5′-flanking region by ChIP assay. DNA from HEK293 cells transfected with mock or pFlag–CMV–SMYD3 was immunoprecipitated with anti-Flag antibody (M2). (**c**) Interaction between endogenous SMYD3 and the 5′-flanking region in hepatoma cells. (**d**) Association of the 5′-flanking region with di-methylated histone H3 in cells expressing SMYD3. (**e**) Sequence-specific binding of SMYD3 with a SMYD3-binding element (SBE). Recombinant SMYD3 was incubated with a DNA probe containing the two putative binding sequences with or without anti-SMYD3 antibody and analysed by EMSA.

Expression profile analysis identified 80 genes with altered expression, including 61 genes that were up-regulated greater than threefold in pcDNA–SMYD3-transfected, compared with mock transfected, cells and 19 genes that were down-regulated less than threefold (see Supplementary Information, Table S1).

We selected 11 up-regulated genes, including *Nkx2.8*, *C/EBPδ*, *Nkx2.5*, *Wnt10B*, *PIK3CB*, *NEURL*, *PSMD9*, *ECEL1*, *CRKL*, *APS*, and *Seb4D*, and performed semi-quantitative RT–PCR using RNA from cells transfected with pcDNA-SMYD3 or pcDNA (mock). The result was consistent with enhanced expression of these genes by SMYD3 (see Supplementary Information, Fig. S2c). Although *Nkx2.8* mRNA levels have been reported to be elevated in HCC compared with normal adult liver[20] the function of Nkx2.8 remains unclear; therefore, we chose *Nkx2.8* for further analysis. After searching for the putative SMYD3-binding sequences within a 1.5-kb region upstream of the transcription start sites of *Nkx2.8*, two candidate sequences were found (Fig. 5a). Chromatin immunoprecipitation (ChIP) assays using cells transfected with pFlag–CMV–SMYD3, and anti-Flag antibody (M2), showed that one genomic segment (ChIP-4) containing the two candidate sequences (CCCTCC and GAGGGG) associated with SMYD3, but that other segments (ChIP-1, -2, and -3) without SMYD3-binding sequences were not able to associate (Fig. 5b). An additional ChIP assay was performed using an anti-SMYD3 antibody and extracts from HepG2 or Huh7 hepatoma cells that abundantly express SMYD3. Endogenous SMYD3 interacted with the ChIP-4 region (Fig. 5c). Further ChIP assays with an anti-di-methylated H3-K4 antibody revealed an association between di-methylated H3-K4 and the ChIP-4 region in HEK293 cells transfected with wild-type SMYD3 (Fig. 5d), supporting the notion that SMYD3 binds to the promoter region of *Nkx2.8* and induces di-methylation at K4 of the associated histone H3 *in vivo*. An electrophoretic mobility shift assay (EMSA) was performed using a double-stranded oligonucleotide probe that corresponds to the possible binding sequences. A band shift was detected in the presence of recombinant SMYD3 protein (Fig. 5e), which became further super-shifted after the addition of anti-SMYD3 antibody, but not after the addition of anti-p53 antibody. No band shifts were observed after cold competitive oligonucleotides were added, highlighting the specific interaction between the oligonucleotide probe and SMYD3. An *in vitro* binding assay, using recombinant GST and GST–SMYD3, and GST–WT-Tcf4

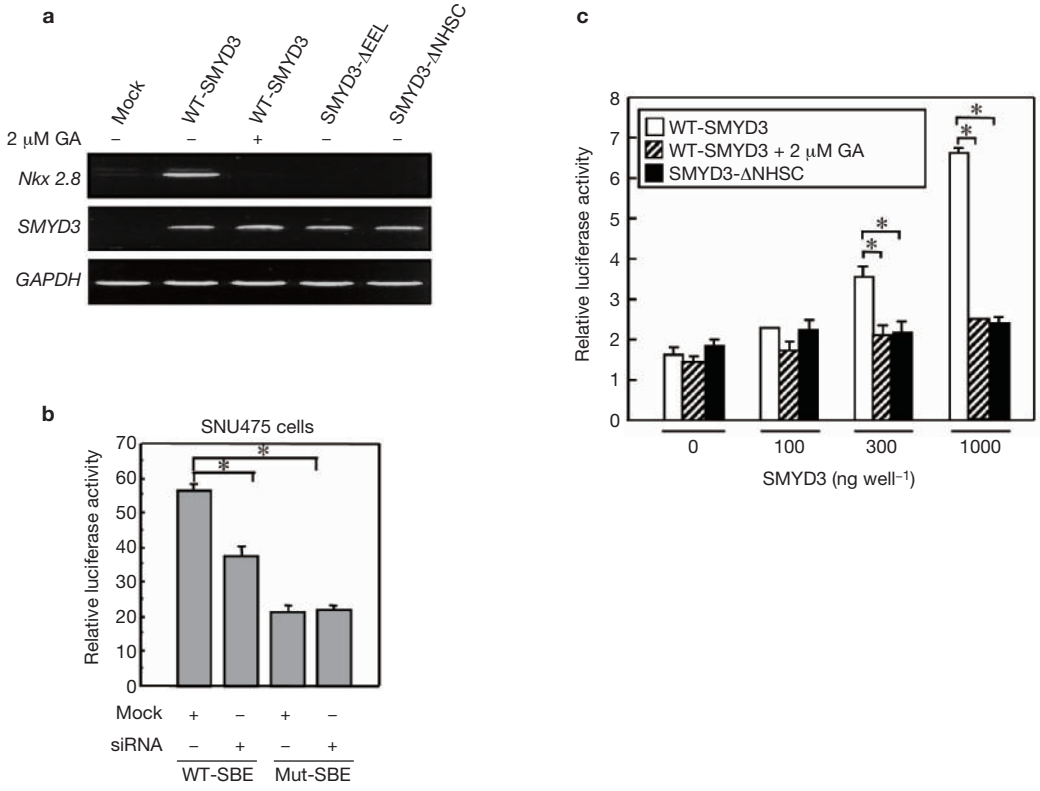

Figure 6 Transcriptional activation of *Nkx2.8* by SMYD3. (**a**) Expression of *Nkx2.8* in response to exogenous expression of wild-type (lane 2 and 3) or mutant (lane 4 and 5) SMYD3 in HEK293 cells. Addition of HSP90-specific inhibitor, geldanamycin (GA), diminished the enhanced expression of *Nkx2.8* caused by wild-type SMYD3 (lane 3). Expression of *GAPDH* was used as an internal control. (**b**) Transcriptional assay of *Nkx2.8* containing wild-type or mutant SMYD3-binding element (SBE) in the presence or absence of SMYD3-siRNA in HCC cells. Bars, ± SD. Asterisk denotes a significant difference ($p < 0.05$) determined by a Fisher's protected least-significant test. (**c**) Effect of wild-type or mutant SMYD3 on the luciferase activity in HEK293-Nkx2.8Luc cells that contain integrated Nkx2.8 promoter-luciferase gene in the genome. Bars, ± SD. Asterisk denotes a significant difference ($p < 0.05$) determined by a Fisher's protected least-significant test.

(T cell factor 4) as a control, confirmed the sequence-specific association of GST–SMYD3 protein with the SMYD3-binding element (SBE; see Supplementary Information, Fig. S2d).

Transcriptional regulation of downstream genes by SMYD3

To determine whether the HMTase activity of SMYD3 is associated with the expression of *Nkx2.8*, and whether HSP90A is involved in the regulation of *Nkx2.8*, HEK293 cells were transfected with plasmids expressing wild-type or HMTase-inactive mutant-SMYD3 (SMYD3-ΔEEL and SMYD3-ΔNHSC), and semi-quantitative RT–PCR was performed with RNAs isolated from the transfected cells (Fig. 6a). Although the wild-type plasmid enhanced the expression of *Nkx2.8*, both types of mutant plasmid failed to induce expression. Furthermore, the addition of geldanamycin (a specific inhibitor of HSP90) to the culture media, suppressed the enhancement of *Nkx2.8* expression caused by wild-type SMYD3 (Fig. 6a, lane 3). This is consistent with the finding that HSP90A enhances the HMTase activity of SMYD3 *in vitro* (Fig. 4c). To examine the significance of SBE in the transactivation of *Nkx2.8*, a reporter plasmid containing the −791 to +109 region of *Nkx2.8* with wild-type SBE (pGL3–Nkx2.8–WT-SBE) and a plasmid with mutant SBE (pGL3–Nkx2.8–Mut-SBE) at an upstream region of the luciferase gene were transfected into HepG2 or SNU475 cells (Fig. 6b). The mutant reporter plasmid displayed significantly lower luciferase activity than the wild-type plasmid, indicating that SBE is responsible for the transactivation of *Nkx2.8* in these cells. Notably, co-transfection with plasmids expressing *SMYD3* siRNAs (psiU6BX–SMYD3-12) reduced the luciferase activity of wild-type reporter plasmids compared with a mock plasmid (psiU6BX–Mock), but did not affect the activity of mutant plasmids.

Furthermore, HEK293–Nkx2.8–Luc cells were established that integrated the promoter region of *Nkx2.8* and the luciferase gene (pGL3–Nkx2.8–WT-SBE) into the genome. Transfection with plasmids expressing wild-type SMYD3 increased the luciferase activity in a dose-dependent manner, whereas transfections that included the addition of 2 μM geldanamycin or transfections with the HMTase-inactive mutant did not enhance luciferase activity (Fig. 6c). Taken together, these data suggest that SMYD3 directly regulates the transcriptional activity of *Nkx2.8* through an interaction with SBE in an HSP90A-dependent manner.

DISCUSSION

Many proteins that contain a SET domain possess histone methyltransferase activity for specific lysine residues on the histone tail, and these residues are able to accept one, two or three methyl groups to generate mono-, di- or tri-methylated products. These methylation events are crucial for transcriptional regulation, but there are still very important

points to be addressed: first, how each of the SET proteins recognizes their specific substrate; second, how the number of methyl groups (mono-, di-, or tri-methylation) is determined; third, how gene-specific modification is carried out; and finally, when and how the HMTase activity of SET proteins is switched on. SET-domain-containing proteins are classified into at least four families, including the SUV39, SET1, SET2 and RIZ families, according to their similarities within the SET domain[9]. βSUV39H1, G9A and ESET belong to the SUV39 family and contain H3-K9 HMTase activity, whereas SET1 and MLL/ALL-1 of the SET1 family possess H3-K4 HMTase activity. In addition, Set2 has H3-K36 HMTase activity. These data suggest that structural differences within the SET domain define the target substrate on the histone tail. A similarity analysis (using the clustal W program) among the SET domains of SMYD3, SET7/9, EZH, EZH2, MLL, MLL2, yeast Set1, SUV39H1, SUV39H2, G9A, ESET, RIZ and CLLL8, classified SMYD3 and SET7/9 into a unique branch distinct from those consisting of other SET proteins (data not shown), indicating that SMYD3 and SET7/9 may constitute a new class of the SET family.

Our data clearly demonstrate the HMTase activity of SMYD3 on H3-K4, but interestingly SMYD3 induced di- and tri-methylation whereas SET7/9 induced mono- and di-methylation. Structural analysis of the SET-domain methyltransferases suggests that the ternary complex of the SET-domain protein provides an accessible channel for the substrate and cofactors[21]. The different level of methylation may result from the ability to accommodate the increasing bulk of the lysine ε-amino group in the channel. However, it is quite intriguing whether H3-K4 tri-methylation induced by SMYD3 occurs as the next event after modification by SET7/9 *in vivo*. Further structural and biological analyses of H3-K4 methyltransferases should uncover the mechanisms underlying different levels of methylation on H3-K4 and their biological consequences.

As tri-methylation at H3-K4 is present exclusively at transcriptionally active genes[12,22], overexpression of SMYD3 should activate the transcription of target genes. Here we have revealed the interaction between SMYD3 and RNA polymerase II. In *Saccharomyces cerevisiae*, the Set1 domain of histone H3-K4 methyltransferase recruits RNA polymerase II for transcription elongation[13,23]. Taken together, our data suggests that H3-K4 methyltransferases modulate transcription by at least two distinct mechanisms: chromatin opening through the histone modification that enables the transcriptional machinery to become accessible to the promoter region, and transcription elongation by the recruitment of RNA polymerase II. These modes of action highlight new features of the 'histone code', which are dictated and recognized by the transcriptional machinery. Similarly to the indirect association of COMPASS (complex of proteins associated with Set1) with RNA polymerase II (ref. 23), we identified an RNA helicase as a bridging protein between SMYD3 and RNA polymerase II. As the interaction between COMPASS and RNA polymerase II requires components of the Paf1 complex[23], the set of proteins including SMYD3, HELZ and RNA polymerase II should constitute a transcriptional complex.

We have also demonstrated that the histone methyltransferase activity of SMYD3 was enhanced in the presence of HSP90A; this is the first demonstration of HSP90A functioning as an essential co-factor for a histone methyltransferase activity. Our data suggest that the methyltransferase activity of a SET-domain-containing protein may be enhanced in the presence of an appropriate co-factor, such as HSP90A for SMYD3.

There is accumulating evidence that highlights the involvement of deregulated SET-domain proteins in human neoplasms. In human leukaemia, for example, frequent translocations are observed in *MLL*[24,25], the human homologue of the *Drosophila trithorax* gene. MLL activates transcription of the *Hox* gene through H3-K4-specific methylation, which is mediated by the methylase activity of the SET domain[26] through direct binding to the *Hox* promoter sequences. Furthermore, *MLL2* is amplified in pancreatic cancer and glioma cell lines[27]. One of the polycomb group genes *EZH2,* is up-regulated in hormone-refractory, metastatic prostate cancers[28,29]. Double knockout mice of Suv39h1/h2 develop late-onset B-cell lymphomas[30]. These data indicate that deregulated HMTase activity is involved in the development and/or progression of cancer.

We have shown that expression of *SMYD3* is enhanced in CRC and HCC, and is absent or very weak in non-cancerous cells of other tissues. In addition, suppression of SMYD3 expression significantly inhibited the growth of CRC and HCC cells. Therefore, SMYD3 could be an ideal therapeutic target for treating primary CRCs and HCCs. Also, SMYD3 associates directly with HELZ to form a transcriptional complex with RNA polymerase II and regulates downstream genes by interacting with their promoter region as a transcription factor containing histone methyltransferase activity. Thus, small compounds that antagonize the association of SMYD3 with HELZ, or its target DNA sequences, and specific inhibitors of the SMYD3 methyltransferase activity, may be effective anticancer drugs for these tumours. The data provided here should contribute not only to a better understanding of the transcriptional control mediated by H3-K4 methylation, but also to the development of novel therapeutic approaches to CRC and HCC. □

METHODS

RT–PCR. Extraction of total RNA and subsequent cDNA synthesis were performed as described[31]. RT–PCR experiments were performed in 20 μl volumes of PCR buffer (Takara, Tokyo, Japan) with initial denaturation at 94 °C for 3 min before 18 cycles (for *GAPDH*) or 30 cycles (for *SMYD3*) at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. The sets of primers used for RT–PCR are listed in Supplementary Information Table S2.

Colony formation assay. Plasmids expressing the sense strand (pcDNA–SMYD3), antisense strand (pcDNA3–antisense-SMYD3), HMTase-inactive mutant form of SMYD3 (pcDNA–SMYD3ΔEEL), or control plasmid (Mock), were transfected into NIH3T3 or HEK293 cells using FuGENE6 reagent according to the manufacturer's instructions (Roche, Basel, Switzerland). Transfected cells were maintained for 9–14 days after transfection in culture media supplemented with 0.9 μg μl^{-1} geneticin (NIH3T3 and HEK293). The number of cells was measured with cell counting kit-8 (Dojindo, Kumamoto, Japan).

Gene silencing effect of SMYD3 siRNAs. A psiU6BX vector was created that contained the snRNA U6 promoter region as described[31]. Plasmids expressing siRNAs were prepared by cloning double-stranded oligonucleotides into the psiU6BX vector. The oligonucleotides used for SMYD3 and EGFP siRNAs are listed in Supplementary Information Table S2.

Yeast two-hybrid assay. A yeast two-hybrid assay was performed using the MATCHMAKER GAL4 Two-Hybrid System 3 according to the manufacturer's protocols (Clontech, Palo Alto, CA). The entire coding sequence of *SMYD3* was cloned into the *Eco*RI-*Sal*I site of the pAS2-1 vector and used as bait to screen a human-testis cDNA library (Clontech).

Immunoprecipitation assay. The coding regions of SMYD3 and HELZ were amplified by RT–PCR, and the products were cloned into pFlag–CMV5 (Sigma, St Louis, MO) and pCMV–HA (Clontech) vectors, respectively. HeLa cells transfected with combinations of pFlag–CMV, pFlag–CMV–SMYD3, pCMV–HA, pCMV–HA–HELZ, or untransfected HCT116 and HepG2 cells, were washed with PBS and lysed in NETN150 buffer containing 150 mM NaCl, 0.5% NP-40, 20 mM Tris-HCl at pH 8.0, 1 mM EDTA and a complete protease inhibitor cocktail (Roche). In a typical immunoprecipitation reaction, 300 μg of whole-cell extract was incubated with an optimum concentration of the following antibodies: anti-Flag (M2; Sigma), anti-HA (Y-11; Santa Cruz, Santa Cruz, CA), anti-RNA Polymerase II (N-20; Santa Cruz), anti-SMYD3 (prepared in our laboratory), anti-HSP90 (F-8; Santa Cruz), or anti-RNA Polymerase II (A-10; Santa Cruz), and 20 μl of protein A or protein G Sepharose beads at 4 °C for

1–2 h. After the beads were washed five times in 1 ml of NETN150 buffer, proteins that bound to the beads were eluted by boiling in Laemmli sample buffer.

***In vitro* histone methyltransferase assay.** 293T cells were transfected with plasmids expressing Flag-tagged wild-type SMYD3 (p3XFlag–CMV–SMYD3), mutant SMYD3 (p3XFlag–CMV–SMYD3ΔEEL or p3XFlag–CMV–SMYD3ΔNHSC), SET7/9, SUV39H1 protein or p3xFlag (mock), and tagged proteins were purified by immunoprecipitation with an anti-Flag antibody. The *in vitro* HMTase assay was performed as described[32], except for slight modifications. Briefly, immunoprecipitated protein was incubated with 1 μg of recombinant histone H3 protein (Upstate) as substrates and 2 μCi S-adenosyl-L- [*methyl*-^{3}H] methionine (SAM; Amersham Biosciences) as the methyl donor in a mixture of 20 μl of methylase activity buffer (50 mM Tris-HCl at pH 8.5, 100 mM NaCl, 10 mM dithiothreitol), for 3 h at 30 °C. Proteins were resolved on an 18% SDS–PAGE gel and visualized by Coomassie blue staining and fluorography.

To examine H3-K4 specific methyltransferase activity, recombinant histone H3 protein was incubated with the immunoprecipitants in the presence of 20 μM unlabelled SAM (Sigma) and 2 μg of HSP90A at 30 °C for 60 min, and western blot analysis performed using antibodies against mono-methylated H3-K4 (Abcam, Cambridge, MA), di-methylated H3-K4 (Abcam), tri-methylated (Abcam) H3-K4, tri-methylated H3-K9 (Upstate), or an anti-histone H3 (Santa Cruz) antibody. We investigated specificities by competition assay using peptides to di-methylated H3-K4.

Identification of putative binding motifs of SMYD3. Selection of a putative DNA binding motif of SMYD3 was performed as described[33], with a slight modification. A GST–SMYD3 fusion protein was prepared in *Escherichia coli* and purified with glutathione Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ). The immobilized protein was incubated with double-stranded oligonucleotides (5′-GGGAGAATTCCGACACGCGT(N_{20})CTCGAGCGTCTACATGGATCCTCA-3′) and washed three times with binding buffer (120 mM KCl, 10 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 25mM Hepes at pH 7.5, 0.1% NP-40, 5% glycerol and 1 mM DTT) . Bound DNA was eluted with elution buffer (120 mM NaCl, 50 mM Tris-HCl at pH 8.0 and 20 mM glutathione) and amplified by PCR with specific primers, 5′-GGGAGAATTCCGACACGCG-3′ and 5′-TGAGGATCCATGTAGACGCTC-3′. After ten rounds of selection by binding with GST–SMYD3 and subsequent amplification, the final PCR products were cloned using the TA cloning kit (Invitrogen, Carlsbad, CA) and the sequences of the products determined.

Identification of downstream genes by cDNA microarray. HEK293 cells were transfected with either pcDNA–SMYD3 or pcDNA (mock). RNA was extracted 18 h after transfection, labelled with Cy3 or Cy5 dye and subjected to co-hybridization onto in-house cDNA microarray slides containing 13,824 genes as described[17,34]. After normalization of the data, genes with signals higher than the cut-off value were analysed further.

Chromatin immunoprecipitation (ChIP) assays. Cells were cross-linked in 1% formaldehyde for 10 min. The fixed chromatin samples were subjected to immunoprecipitation using a ChIP assay kit according to the manufacturer's instructions (Promega, Madison, WI). The sets of primers used for ChIP assay are listed in Supplementary Information Table S2.

Luciferase assay. The fragment of the *Nkx2.8* promoter was amplified by PCR using the following primers: 5′-AGCGGGCCTGGTACCAAATTTGTG-3′ and 5′-CCGGGATGCTAGCGCATTTACAGC-3′, and cloned into the pGL3 basic vector (pGL3–Nkx2.8–WT-SBE). Mutant reporter plasmids (pGL3–Nkx2.8–Mut-SBE) were prepared by replacing the SMYD3-binding sequences (CCCTCCT to CCGACCT and GAGGGG to GTCGGG) in pGL3–Nkx2.8–WT-SBE using the QuickChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Luciferase assays were performed using a Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

Establishment of HEK293–Nkx2.8-Luc cells. Stable transformants of HEK293–Nkx2.8-Luc cells were established by transfection with pGL3–Nkx2.8–WT-SBE and pcDNA(+)3.1 plasmids (10:1) into HEK293 cells using FuGENE6 reagent, according to the manufacturer's instructions (Roche). Transfected cells were maintained in culture media supplemented with 0.9 μg μl^{-1} of geneticin, and single colonies were selected two weeks after transfection.

Electrophoretic mobility shift assay (EMSA). EMSA was performed by incubating GST–SMYD3 with ^{32}P-labelled oligonucleotide as described[35]. The sequences of oligonucleotides for the double-stranded DNA probe (SBE) were: 5′-TTACGCCCTCCTGAAACTTGTCATCCTGAATCTTAGAGGGGCCC-3′ and 5′-GGGCCCCTCTAAGATTCAGGATGACAAGTTTCAGGAGGGCGTAA-3′.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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