The Telomerase Reverse Transcriptase (hTERT) Gene Is a Direct Target of the Histone Methyltransferase SMYD3

Cheng Liu, Xiaolei Fang, Zheng Ge, Marit Jalink, Satoru Kyo, Magnus Björkholm, Astrid Gruber, Jan Sjöberg, and Dawei Xu

Abstract
Recent evidence has accumulated that the dynamic histone methylation mediated by histone methyltransferases and demethylases plays key roles in regulation of chromatin structure and transcription. In the present study, we show that SET and MYND domain-containing protein 3 (SMYD3), a histone methyltransferase implicated in oncogenesis, directly trans-activates the telomerase reverse transcriptase (hTERT) gene that is essential for cellular immortalization and transformation. SMYD3 occupies its binding motifs on the hTERT promoter and is required for maintenance of histone H3-K4 trimethylation, thereby contributing to inducible and constitutive hTERT expression in normal and malignant human cells. Knocking down SMYD3 in tumor cells abrogated trimethylation of H3-K4, attenuated the occupancy by the trans-activators c-MYC and Sp1, and led to diminished histone H3 acetylation in the hTERT promoter region, which was coupled with down-regulation of hTERT mRNA and telomerase activity. These results suggest that SMYD3-mediated trimethylation of H3-K4 functions as a licensing element for subsequent transcription factor binding to the hTERT promoter. The present findings provide significant insights into regulatory mechanisms of hTERT/telomerase expression; moreover, identification of the hTERT gene as a direct target of SMYD3 contributes to a better understanding of SMYD3-mediated cellular transformation.

Introduction
SET and MYND domain-containing protein 3 (SMYD3) is a histone H3-K4-specific dimethyltransferase and trimethyltransferase and plays an important role in oncogenesis (1, 2). Expression of SMYD3 is undetectable or very weak in many types of normal human tissues whereas significantly up-regulated in the great majority of colorectal carcinoma, hepatocellular carcinoma, and breast cancer (1–3). It has been shown that non-transformed cells overexpressing SMYD3 exhibit increased capabilities of proliferation and colony formation, whereas suppressing SMYD3 gene expression by small interference RNA (siRNA) leads to growth inhibition or apoptosis of colorectal carcinoma and hepatocellular carcinoma cells (1, 2, 4).

Evidence has accumulated that SMYD3 elicits its oncogenic effect via activating transcription of its downstream target genes, and two mechanisms have been proposed for its trans-activating function: First, SMYD3 recruits RNA polymerase II through RNA helicase, forming a transcriptional complex to elongate transcription. Second, SMYD3 interacts with its binding motif CCCTCC in the promoter region of its target genes and dimethylates or trimethylates H3-K4 (1). The correlation between histone methylation pattern in promoter regions and gene transcriptional activity has been well established: trimethyl-H3-K4 modification is in general enriched at 5’ ends of actively transcribed genes and associated with a high transcriptional activity (5–7). The methylated H3-K4 alters the chromatin folding, leading to increased accessibility of DNA to proteins that mediate transcription. Moreover, trimethyl-H3-K4 provides specific binding sites for certain proteins or complexes that induce transcription activation (8, 9).

However, the mechanism underlying SMYD3-mediated oncogenesis is incompletely understood and its target genes essential for transformation remain to be characterized further. Several lines of evidence have recently shown that activation of telomerase, a cellular ribonucleoprotein responsible for elongation of telomeres, is prerequisite for cell immortalization and transformation (10, 11). Telomerase activity is indeed detectable in up to 90% of human malignancies, whereas it is repressed in most normal human cells (10, 11). It has been shown that the transcriptional regulation of the catalytic component of the telomerase complex, telomerase reverse transcriptase (hTERT), is a predominant determinant for controlling telomerase activity (10, 12–14). More recently, roles for histone modification-mediated chromatin remodeling in regulating hTERT transcription have been revealed (15): Increased histone acetylation and/or phosphorylation at the hTERT promoter leads to trans-activation of the hTERT gene (16–24). Interestingly, Atkinson et al. observed that highly trimethylated H3-K4 was associated with the actively transcribed hTERT gene in telomerase-proficient tumor cells (25). However, it is unclear what is responsible for H3-K4 methylation at the hTERT promoter. Given the above findings, together with SMYD3 as a histone H3-K4–specific methyltransferase, we sought to determine whether the hTERT gene is a transcriptional target of SMYD3. In the present study, we provide evidence that SMYD3-mediated H3-K4 methylation is required for inducible and constitutive hTERT expression in both normal human fibroblasts and cancer cell lines.

Materials and Methods
Cell lines and culture conditions. Human normal foreskin (BJ) fibroblasts and lung fetal fibroblasts (LFI; ref. 20), Saos-2, HCT116, Hep3B, and L1236 tumor cell lines were cultured at 37°C in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% FCS, 100 units/mL penicillin, and 2 mmol/L L-glutamine.

Plasmids and transfection. The flag-tagged pcDNA-SMYD3 expression plasmid and the corresponding empty vector pcDNA-flag were provided by

Requests for reprints: Dawei Xu, Hematology Lab, Karolinska University Hospital, CMM, L 803, SE-171 76, Stockholm, Sweden. Phone: 46-8-5177-6552; Fax: 46-8-5177-3054; E-mail: Dawei.Xu@ki.se.
Drs. Nakamura and Furukawa (1). BJ and LF1 fibroblasts were transfected with either the SMYD3 plasmid or empty control vector using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol, and the cells were then harvested for hTERT mRNA analysis 48 h after transfection.

**siRNA treatment.** Chemically modified Stealth siRNA targeting SMYD3 (sequence: AAUCCUGUAAGGCUCCAGGUCC) and control siRNA were purchased from Invitrogen. siRNA transfaction was done using LipofectAMINE 2000.

**RNA extraction and reverse transcription-PCR.** Total cellular RNA was extracted using an RNeasy total RNA isolation kit (Qiagen GmbH, Hilden, Germany). cDNA synthesis, reverse transcription-PCR primers, and conditions for hTERT mRNA were described previously (13, 26). β-2 Microglobulin mRNA expression was used as a control for RNA loading and reverse transcription efficiency and was amplified with 24 cycles (20). PCR for both hTERT and β-2-microglobulin mRNA was optimized to keep amplification in a linear phase, which allowed a semiquantitative evaluation for the level of hTERT transcripts as described (26). The PCR primers for SMYD3 mRNA are 5'-GACCTTCTGGCAGATTCG-3' (forward) and 5'-GTGAAAAGAGTTGCG-3' (reverse) as described (1).

**Western blot.** Total cellular proteins were extracted with SDS lysis buffer, and 20 μg of the protein were resolved by SDS-PAGE and transferred to an intracellular membrane. The membranes were probed with the antibody against SMYD3 (Abcam, Cambridge, United Kingdom) followed by anti-mouse horseradish peroxidase–conjugated IgG and developed with the enhanced chemiluminescent method (Amersham, Uppsala, Sweden).

**Telomerase activity assay.** Telomerase activity was determined by using a dual luciferase reporter assay system (Promega, Madison, WI) 48 h after transfection. The hTERT promoter–driven firefly luciferase activity was normalized to the thymidine kinase promoter–containing plasmid, which is always included in transfection to control transfection efficiency. Luciferase containing plasmid, which is driven by thymidine kinase promoter, was used to determine telomerase activity in all samples in duplicate according to manufacturer’s protocol. One microgram of cellular proteins was added into PCR reaction, and 4 μL of products was used for ELISA assay (26).

** Luciferase activity assay.** The hTERT luciferase reporter (hTERT-Luc p181) harbors the core promoter sequence of the hTERT 5'-flanking region (27, 28). The targeting mutation of the hTERT-Luc p181 was made using a mutagenesis kit (Stratagene, La Jolla, CA). Cells cultured in 24-well plates were transfected with hTERT-Luc p181 or mutant variants and pcDNA-SMYD3 or pcDNA using LipofectAMINE 2000. A Renilla luciferase–containing plasmid, which is driven by thymidine kinase promoter, was always included in transfection to control transfection efficiency. Luciferase activity was determined by using a dual luciferase reporter assay system (Promega, Madison, WI) 48 h after transfection. The hTERT promoter-driven firefly luciferase activity was normalized to the thymidine kinase Renilla luciferase activity.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation assay was carried out as described (20). Cells were cross-linked by incubating them in 1% (v/v) formaldehyde-containing medium for 10 min at 37°C and then sonicated to make soluble chromatin with DNA fragments between 200 and 1,000 bp. The antibodies against flag-tag (M2, Sigma, St. Louis, MO), trimethylated H3-K4, acetylated histone H3 (Upstate, Lake Placid, NY), c-MYC, and Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used to precipitate DNA fragments bound by their corresponding elements. The protein-DNA complex was collected with protein A or G-Sepharose beads (Upstate, Lake Placid, NY), eluted, and reverse cross-linked. Following a treatment with Protease K (Sigma), the samples were extracted with phenol-chloroform and precipitated with ethanol. The recovered DNA was resuspended in TE buffer and used for PCR amplification as described (20).

**Results**

**SMYD3 induces hTERT mRNA expression in human primary and cancer cells lacking telomerase activity.** Normal human fibroblasts lack detectable telomerase activity due to the stringent hTERT gene repression and do not express endogenous SMYD3 (1, 3, 10, 14). In an effort to investigate the effect of SMYD3 on hTERT gene expression, we ectopically expressed SMYD3 in BJ and LF1 fibroblasts. As shown by the reverse transcription-PCR analysis (Fig. 1A), mock control cells expressed no hTERT mRNA, whereas transfection of the SMYD3 expression vector (pcDNA-SMYD3) induced hTERT mRNA expression in both BJ and LF1 fibroblasts. We next determined the effects of SMYD3 overexpression on hTERT expression in the osteosarcoma cell line Saos2 in which hTERT is transcriptionally silent. hTERT mRNA was readily detectable in Saos2 cells expressing ectopic SMYD3 (Fig. 1A). These data show that SMYD3 positively regulates hTERT mRNA expression in human primary fibroblasts and cancer cells with a repressed hTERT gene.

**SMYD3 expression is required for constitutive expression of hTERT mRNA and telomerase activity in telomerase-proficient cancer cells.** SMYD3 is overexpressed in the majority of colorectal cancer cells. SMYD3 is overexpressed in the majority of colorectal cancer cells.

![Figure 1. SMYD3 regulates hTERT expression in human primary fibroblasts and cancer cells.](image-url)
carcinoma and hepatocellular carcinoma (1), whereas constitutive expression of hTERT and telomerase is also observed in most of these tumors (29). Thus, we further asked whether SMYD3 was required for constitutive hTERT mRNA expression. For this purpose, we knocked down SMYD3 expression with specific SMYD3 siRNAs in human colorectal carcinoma HCT116 and hepatocellular carcinoma Hep3B cell lines. The efficient silencing of SMYD3 expression was verified by both reverse transcription-PCR and Western blot analyses, and a significant reduction in hTERT mRNA was seen following the inhibition of SMYD3 expression in these cells (Fig. 1B). Consistent with down-regulation of hTERT expression, telomerase activity decreased substantially in HCT116 and Hep3B cells treated with SMYD3 siRNA, as determined using a semiquantitative, telomeric repeat amplification protocol–based telomerase ELISA kit (Fig. 1C).

In addition, because the transcription factor E2F-1 is involved in the transcriptional activation of SMYD3, and because Hodgkin’s lymphoma cell lines have high E2F-1 expression (3, 30), we sought to define the relationship between expression of SMYD3 and hTERT in a Hodgkin’s cell line L1236. As expected, SMYD3 mRNA and protein was detected in L1236 cells (Fig. 1B). Knocking down SMYD3 expression similarly resulted in diminished hTERT expression accompanied by a decline in telomerase activity (Fig. 1B and C). Collectively, SMYD3 is required for constitutive hTERT expression and telomerase activity in colorectal carcinoma, hepatocellular carcinoma, and Hodgkin’s lymphoma cell lines examined.

**SMYD3 activates the hTERT promoter.** To determine whether SMYD3 up-regulates hTERT expression at the transcriptional level, we examined the effect of SMYD3 on the hTERT promoter activity. SMYD3 is known to bind to a putative motif CCCTCC in its target promoters (1). Interestingly, we identified five potential SMYD3 binding sites CCCTCC within the hTERT core promoter region (Fig. 2A). Cotransfection of the SMYD3 expression vector with a luciferase reporter driven by the hTERT core promoter sequence (hTERT-Luc p181) into the telomerase-negative cell line Saos2 led to a 100% increase in reporter gene activity in a dose-dependent manner (Fig. 2B). Through substitution mutation of each SMYD3 binding site (MT1–MT5), we found that two of them (MT3 and MT5) were important for the transcriptional activity of the hTERT gene, and disruption of them did not only abolish SMYD3-mediated increases in hTERT-Luc p181 activity but also resulted in a significant reduction in baseline of the hTERT promoter activity (Fig. 2C). In contrast, the mutation of remaining three CCCTCC motifs (MT1, MT2, and MT4) affected neither the response of hTERT-Luc p181 to SMYD3 expression (Fig. 2B).
The wild-type hTERT-Luc p181 and its mutant variants were then transfected into HCT116, Hep3B, and L1236 cells for further evaluation. The MT3 mutant reporters exhibited significantly lower luciferase activity compared with wild-type hTERT-Luc p181 in all the three cell lines (Fig. 2D). The activity of the reporter with MT5 also declined although varying from cell line to cell line (Fig. 2D). These results were highly consistent with inhibitory effects of SMYD3 knocking down on constitutive hTERT mRNA expression in HCT116, Hep3B, and L1236 cells. However, cotransfection with pcDNA-SMYD3 only slightly increased the wild-type hTERT-Luc p181 activity in HCT116 and Hep3B cells, whereas it had no effects in L1236 cells (Fig. 2D). This was not surprising because all the three cell lines expressed endogenous SMYD3 that may already be saturated.

SMYD3 is associated with the hTERT promoter. Having shown the presence of two functional SMYD3 binding sites on the hTERT core promoter, we next examined the ability of this region to in vivo interact directly with SMYD3 using chromatin immunoprecipitation assay. Because the commercially available SMYD3 antibody was not qualified for chromatin immunoprecipitation analysis (data not shown), we transiently transfected Saos2 cells with the Flag-tagged SMYD3 expression vector and did chromatin immunoprecipitation assay using an anti-Flag antibody (M2). The primers encompassing the two functional SMYD3 binding elements in the hTERT proximal promoter were used for PCR amplification as described (20). As shown in Fig. 3, Flag-tagged SMYD3 was bound to the hTERT promoter containing the SMYD3 binding motifs. This specific interaction was verified by the absence of specific sequence amplifications when omission of binding motifs. This specific interaction was verified by the absence of specific sequence amplifications when omission of antibodies was applied for chromatin immunoprecipitation, or when primers for the unrelated GAPDH gene were used in the PCR reaction.

SMYD3 inhibition leads to diminished trimethylation of histone H3-K4 in the hTERT promoter. Because SMYD3 induces transcriptional activation of its target genes by dimethylating or trimethylating H3-K4 in their promoter regions (1), we sought to determine whether the level of SMYD3 expression affected methylation patterns of H3-K4 at the hTERT promoter. We knocked down SMYD3 expression in HCT116 and L1236 cells using siRNA and then examined alterations in H3-K4 trimethylation in the hTERT promoter region by chromatin immunoprecipitation assay. As shown in Fig. 4, H3-K4 trimethylation was abolished in the hTERT core promoter region when SMYD3 expression was inhibited. Similar reduction of trimethylated H3-K4 at the hTERT promoter was observed in Hep3B cells treated with SMYD3 siRNA (data not shown). These data suggest that SMYD3 is responsible for trimethylation of H3-K4 in the hTERT promoter region.

Hypo-trimethylated H3-K4 at the hTERT promoter leads to defects in binding the transcription factors c-MYC and Sp1 and in maintaining histone H3 acetylation. Because H3-K4 methylation alters chromatin folding that in turn contributes to increased accessibility of DNA to transcription factors and provides specific binding sites for certain proteins including histone acetyltransferases, we wanted to determine whether the abolished H3-K4 trimethylation affected the occupancy of the transcription factors c-MYC and Sp1. Because c-MYC and Sp1 are essential transactivators for the hTERT gene, on the hTERT promoter in the SMYD3-silent cells. The chromatin immunoprecipitation assay showed that both c-MYC and Sp1 were present at the hTERT promoter in control HCT116 cells, whereas they absent in the same cells with SMYD3 knocking down (Fig. 4). Moreover, histone H3 acetylation at the hTERT promoter was detected in the control cells but not in the cells treated with SMYD3 siRNA. The diminished or abolished Sp1 occupancy and histone H3 acetylation at the hTERT promoter were similarly observed in L1236 cells treated with the SMYD3 siRNA. However, we failed to detect the presence of c-MYC on the hTERT promoter in L1236 cells, likely because these cells predominantly express I-MYC that competes for E-boxes with c-MYC (31).
Discussion

Recent identification of histone methylases and demethylases has led to the conclusion that histone methylation is a dynamic process and, like other histone modifications, governs chromatin structure and transcription of the cell (5, 6, 9). There are a number of lysine residues in the histone NH2 termini that are prominently methylated, and the methylation of different residues leads to either repression or activation of genes (5, 6, 9). For instance, H3-K4 methylation is a prevalent mark associated with transcription activation. Several mammalian SET-domain containing proteins are capable of methylating H3-K4. However, compared with other histone methyltransferases, SMYD3 is unique because it not only has methyltransferase activity but also specifically binds to a DNA sequence CCCTCC in its target promoters as do transcription factors (1). In the study presented here, we have observed that SMYD3 induces hTERT transcription by directly binding to the hTERT promoter and affecting abundance of trimethylated H3-K4 associated with the hTERT chromatin. This finding thus reveals the hTERT gene as a direct target of SMYD3. Given the fact that hTERT/telomerase plays key parts in cellular immortalization and transformation, the identification of hTERT as a target of SMYD3 might gain new insights into SMYD3-mediated oncogenic activity.

Interestingly, the disruption of the functional SMYD3 binding motifs in the hTERT core promoter led to a significant reduction in promoter activity. This, together with down-regulation of hTERT mRNA caused by silencing SMYD3 expression in tumor cells, strongly suggests that the basic or constitutive hTERT expression depends heavily on the presence of SMYD3 in the examined cells. Maintenance of H3-K4 trimethylation by SMYD3 should be crucial for the constitutive transcription of the hTERT gene. It has been proposed that methylated H3-K4 affects chromatin folding through a non-electrostatic mechanism (5, 32). More importantly, methylated H3-K4 could serve as a specific binding site of proteins or protein complexes mediating transcription activation. Furthermore, a recent study showed that high H3-K4 methylation was one of the strict prerequisites for recognition of any target site by the c-Myc oncoprotein (33). Consistent with these observations, we found impaired occupancy of the transcription factors c-Myc and Sp1 on the hTERT promoter and diminished histone H3 acetylation following SMYD3 silencing and subsequent inhibition of H3-K4 trimethylation in the examined cancer cells. It is well established that the transcription factors c-Myc and Sp1 play a key role in activation of the hTERT gene transcription (10, 14). Thus, by governing the access of positive transcription factors to and histone acetylation at the hTERT promoter, SMYD3-mediated H3-K4 methylation may function as a critical licensing element through which the trans-activation of the hTERT gene is initiated (Fig. 5).

Deregulation of the E2F family members occurs in most human cancers through different mechanisms (34), and it has been shown that E2F-1 represses the hTERT transcription in tumor cells (35, 36). Not withstanding this, up to 90% of human malignancies express high levels of telomerase activity (10, 14, 29). It is unclear how the hTERT gene escapes from E2F-1-mediated repression in cancer cells with high E2F1 activity. A recent study showed that E2F-1 was involved in the transcriptional activation of SMYD3, and a variable number of tandem repeat polymorphism in an E2F-1 binding element in the 5′ flanking region of SMYD3 was a risk factor for human cancers (3). It is thus likely that E2F-1 drives a higher expression of SMYD3 in that in turn compensates for an inhibitory effect of E2F-1 on the hTERT transcription. Indeed, we found abundant amounts of SMYD3 in the E2F1-overexpressing Hodgkin’s lymphoma cell line L1236, and when SMYD3 was knocked down, hTERT expression and telomerase activity were inhibited in these cells.

It is evident from the present study that SMYD3 increases trimethylation of H3-K4 in the hTERT promoter, thereby trans-activating the hTERT gene. However, SMYD3 is unlikely the sole histone methyltransferase responsible for the trimethylation of H3-K4 at the hTERT chromatin because certain cells such as HEK293 express undetectable SMYD3 but contain high levels of hTERT mRNA and telomerase activity (1). It thus remains to be determined whether different histone methyltransferases are associated with the hTERT chromatin in different types of cells. In addition, H3-K4 methylation and demethylation are associated with transcriptional activation and repression of the hTERT gene, respectively (25). It will be highly interesting to elucidate which demethylases occupy the hTERT promoter and maintain H3-K4 demethylation in normal somatic cells lacking hTERT expression. Our preliminary results showed that inhibition of LSD1, a newly identified histone demethylase, indeed induced hTERT expression and activated the hTERT promoter in human fibroblasts and cancer cells. Collectively, delineation of the relationship between H3-K4 methylation/demethylation and hTERT expression might contribute to profound insights into mechanisms underlying telomerase activation during the oncogenic process.

Acknowledgments


Grant support: Swedish Cancer Society, Cancer Society in Stockholm, Swedish Research Council, Swedish Medical Society, Karolinska Institute Funds (D. Xu), National Natural Science Foundation of China grant 30540099 (X. Fang), and Dutch Cancer Foundation (M. Jalink).

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 1754 solely to indicate this fact.

We thank Drs. Nakamura and Furukawa (University of Tokyo) for providing the SMYD3 expression plasmid.

\(^{4}\) C. Liu and D. Xu, unpublished data.
References


The Telomerase Reverse Transcriptase (hTERT) Gene Is a Direct Target of the Histone Methyltransferase SMYD3

Cheng Liu, Xiaolei Fang, Zheng Ge, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/6/2626

Cited articles
This article cites 36 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/6/2626.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/6/2626.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.