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Original Article

Proteome identification of proteins interacting with histone methyltransferase SET8

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SET8 (also known as PR-Set7/9, SETD8, KMT5A), a member of the SET domain containing methyltransferase family, which specifically catalyzes mono-methylation of K20 on histone H4 (H4K20me1), has been implicated in multiple biological processes, such as gene transcriptional regulation, cell cycle control, genomic integrity maintenance and development. In this study, we used GST-SET8 fusion protein as bait to search for SET8 interaction partners to elucidate physiological functions of SET8. In combination with mass spectrometry, we identified 40 proteins that potentially interact with SET8. DDX21, a nucleolar protein, was further confirmed to associate with SET8. Furthermore, we discovered a novel function of SET8 in the regulation of rRNA transcription.

Keywords SET8; H4K20me1; mass spectrometry; DDX21; rRNA

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Introduction

SET8 (also known as PR-Set7/9, SETD8, KMT5A), a member of the SET domain containing methyltransferase family [1], which specifically catalyzes mono-methylation of K20 on histone H4 (H4K20me1), has been shown to have important functions in diverse biological processes, such as gene transcriptional control, replication origin modulation, genome integrity maintenance, cell cycle progression and development [2–8]. Interestingly, recent reports indicated that SET8 participates in both gene transcriptional activation and repression. For example, SET8-mediated mono-methylation of H4K20 is associated with L3MBTL1 to generate a repressive transcription marker [9–11]; however, SET8 and H4K20me1 have also been observed to occupy the promoter and coding regions of actively transcribed genes [2,12] and mediate the activation of Wnt target genes [2]. Moreover, SET8 has been shown to mono-methylate non-histone protein substrates. SET8 can methylate p53 at lysine 382 and repress proapoptotic and cell cycle arrest functions of p53 [13]. This phenomenon suggests that SET8 may participate in tumor oncogenesis process. It has also been reported that SET8 plays an important role in tumor metastasis by triggering epithelial–mesenchymal transition program in association with TWIST [14].

On the other hand, the mechanisms for the diverse functions of SET8 remain elusive and identification of SET8-interacting partners may give helpful hints to address this issue and point to novel functions of SET8. In this study, we utilized GST-SET8 fusion protein as bait in pull-down assays to search for SET8-interacting proteins from HeLa cell extracts. In combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), we identified 40 potential binding proteins of SET8. Of the proteins identified by MS, an interaction between SET8 and DDX21, a nucleolar protein, was further confirmed, which suggested a function of SET8 in the nucleolus. Subsequent results indicated that SET8 could be involved in the regulation of rRNA transcription.

Materials and Methods

Antibodies and plasmids

Monoclonal antibodies (MAbs) against FLAG, hemagglutinin tag and anti-FLAG M2 affinity gel were from Sigma (St Louis, USA). Antibodies against SET8, H4K20me1 and HP1γ were from Upstate (New York, USA). Coding sequence of SET8 was amplified from HeLa cell cDNA and cloned into pGEX-4T-1 (GE Healthcare, Wisconsin, USA) and pcDNA3-FLAG vector constructed by our lab based on pcDNA3 vector (Invitrogen, Carlsbad, USA), respectively. cDNA of proliferating cell nuclear antigen (PCNA) or DDX21 was amplified from HeLa cDNA and constructed into pcDNA-N-HA vector (Hemagglutinin tag at the N-terminus, kindly provided by Prof. Pei of the Institute of Biochemistry and Cell Biology, Shanghai, China). DDX21 was inserted into

pEGFP-N1 vector (Clontech, Mountain View, USA) to generate EGFP-fusion protein of DDX21.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells and HeLa cells (ATCC, Manassas, USA) were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, USA) supplemented with 10% newborn calf serum (Gibco). Calcium-phosphate transfection method was employed for exogenous expression of recombinant proteins in cells [15].

Reverse transcription-polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacture's instructions. Polymerase chain reaction (PCR) was performed using the primer sets listed below (forward/reverse): pre-rRNA: 5′-GAACGGTGGT GTGTCGTTC-3′/5′-GCGTCTCGTCTCGTCTCACT-3′; and *β-actin*: 5′-ATCGTCCACCGCAAATGCTTCTA-3′/5′-AGC CATGCCAATCTCATCTTGTT-3′.

Expression and purification of GST-SET8

Escherichia coli BL21 (DE3) strain was transformed with pGEX-4T-1-SET8 and cultured in Luria-Bertani medium. Expression of GST-SET8 was induced using 0.1 mM isopropyl-β-D-thiogalactoside at 24°C for 12 h. Purification was performed using glutathione beads according to the manufacturer's instruction (GE Healthcare). The protein purity was higher than 95% according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

GST-pull-down assay

GST-pull-down was performed as described [16]. In brief, GST-SET8 was incubated with HeLa cell lysate pre-cleared by glutathione beads immobilized with GST. After being washed several times with RIPA buffer, the glutathione beads were boiled in protein loading buffer. The samples were separated using SDS-PAGE and stained with silver nitrate [17].

For confirmation of the interaction between SET8 and DDX21, ^{35}S-methionine-labeled DDX21 was produced using TNT quick-coupled transcription/translation system (Promega, Madison, USA) according to the manufacturer's instructions. Each pull-down assay was carried out with 1 μg of GST-SET8 and 10 μl of labeled DDX21.

Protein identification by MALDI-TOF MS

The bands visible in the GST-SET8 lane on the SDS-PAGE gel were grouped into nine sections and each section was cut off. Proteins were digested in gel and extracted. The extracted peptides were mixed with matrix and then spotted on sample plate. The mass of peptides were identified by TOF-MS (ABI 4700 protein analyzer; ABI, Foster City, USA). Data of MS and MS/MS were searched against Swiss-Prot database (*Homo sapiens*). Candidates of proteins were ranked by protein scores CI%.

Co-immunoprecipitation

HEK293T cells were co-transfected with the expression constructs expressing FLAG-SET8 and HA-DDX21 using the calcium-phosphate transfection method (7.5 μg DNA/100 mm dish). Co-immunoprecipitation was performed as described [15]. Immunoblotting of FLAG-SET8 and HA-DDX21 (1/20 input) was performed using anti-FLAG MAb (1 : 5000 dilution; Sigma) and anti-HA MAb (1 : 5000 dilution; Sigma) as primary antibody, respectively, and HRP-labeled rabbit anti-mouse IgG (1 : 1000; Dako, Copenhagen, Denmark) as secondary antibody.

Immunofluorescent microscopy

Cells grown on glass cover slips were washed twice with ice-cold phosphate-buffered saline and fixed with 3.7% formaldehyde for 5 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked in TBST buffer containing 1% bovine saline albumin (TBST/BSA) for 1 h at room temperature. Primary antibody incubation was performed at room temperature for 2 h in TBST/BSA, followed by secondary antibody incubation for 1 h in TBST. Cover slips were mounted using VECTASHIELD mounting medium with DAPI (Vector Labs, Burlingame, USA). Cells were analyzed using laser scanning Confocal microscopy.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed following a published protocol [18]. In brief, chromatins were sheared by sonication (three times, 10 s on, 60 s off). Pre-cleared extracts were immunoprecipitated by rabbit anti-SET8, anti-H4K20me1, anti-HP1γ (Upstate) at 4°C overnight, respectively. DNA was isolated from precipitated complexes and analyzed by PCR using primers for human rRNA promoter: 5′-GGTATATCTTTCGCTCC GAG-3′/5′-GACGACAGGTCGCCAGAGGA-3′. An aliquot of total input nuclear extract was used as loading control.

Lentivirus-mediated RNA silencing

Two siRNA targets in *SET8* sequence were selected (5′-GTTTCCTGAAACTGGGTTAAT-3′ and 5′-CGCAA CAGAATCGCAAACTTA-3′). Short hairpin oligos (shRNA) were synthesized and inserted into pLKO.1-TRC vector kindly provided by Dr David Root [19] (Addgene plasmid 10878). The control pLKO.1-GFPshRNA was a gift from Dr David Sabatini [20] (Addgene plasmid 30323). Procedures to make constructs and produce lentivirus followed the online protocol http://www.addgene.org/tools/protocols/plko/.

Results

Identification of proteins potentially interacting with SET8

To better understand the physiological functions of SET8, we employed GST-pull-down assay coupled with MS to identify proteins associated with SET8. In total, 40 proteins potentially associated with SET8 were identified (**Fig. 1**). After bioinformatic analysis, these proteins could be grouped into several categories based on their reported or presumed cellular functions, including DNA replication, cell cycle progression, chromatin modification, nucleosome remodeling, RNA processing, transcriptional regulation, and signal transduction (**Table 1**).

Validation of PCNA and DDX21 as binding partners of SET8

To verify the effectiveness of the protein identification process, PCNA and DDX21 were selected for further study.

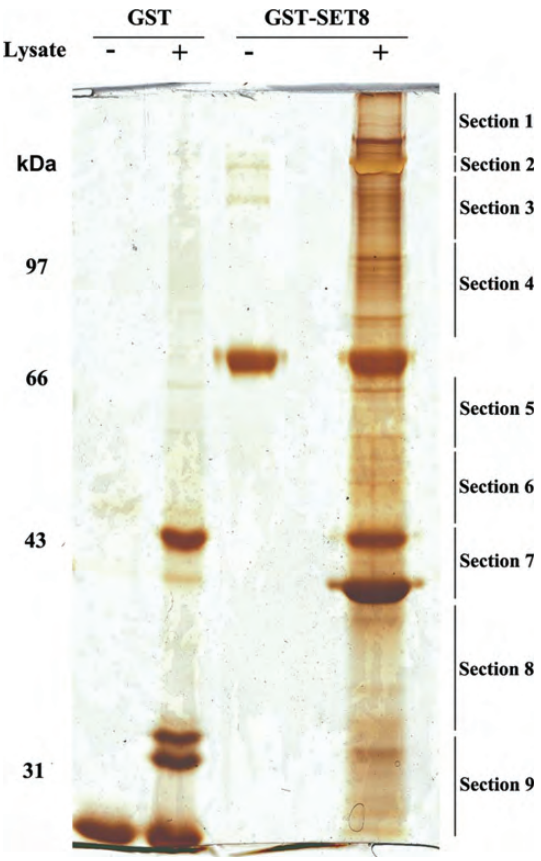

Figure 1 GST-pull-down to purify proteins that potentially interact with SET8 GST-SET8-bound glutathione beads incubated with HeLa cell lysate were boiled in SDS-PAGE loading buffer. GST-bound glutathione beads were processed in parallel and used as control. The supernatants of boiled samples were separated using SDS-PAGE and proteins were visualized by silver staining. The visible protein bands were grouped into nine sections, and each section was analyzed by MOLDI-TOF mass spectrometry.

Table 1 Proteins potentially interact with SET8 identified using MS

Protein	Cellular function
	DNA replication and cell cycle control
APC7	Cell cycle control
BUB1B	Mitotic checkpoint
CDK6	Cell cycle control
hCdh1	Cell cycle control
hROD	Cell cycle control, cell division, spindle pole, kinetochore microtubule
MAD1	Mitotic spindle assembly checkpoint
ORC3	DNA replication, cell cycle control
PCNA	DNA replication, cell cycle control, DNA damage response
PURA	Cell cycle control, transcription regulation
RecQ4	DNA repair, helicase activity, DNA recombination
SOX2	Cell cycle control, cell fate specification, chromatin organization, development
	Chromatin modification and remodeling
FXL11	Histone demethylase, chromatin remodeling
RBBP2	Histone demethylase, chromatin remodeling
SKB1	Protein arginine methyltransferase, chromatin remodeling, rRNA transcription regulation
SIRT2	NAD-dependent deacetylase, chromatin remodeling
SNF2h	Chromatin remodeling
SUZ12	PcG protein, component of polycomb repressive complex, methyltransferase activity
UTX	histone demethylase, chromatin remodeling
	RNA processing
DDX19	RNA processing
DDX21	RNA processing
DDX41	RNA processing
	Transcription factor
CNBP1	Inhibitor of beta-catenin and TCF4
COBRA1	Cofactor of BRCA1
E2F6	Transcription factor
ERCC3	TFIIH base transcription factor complex helicase XBP subunit
HIF1N	HIF1 alpha inhibitor
KBF2	NF kappa B p100/p49 subunit
MED31	Mediator of RNA polymerase II
RAR beta	Nuclear receptor
RORA	Nuclear receptor
RXR beta	Nuclear receptor
TWIST1	Regulation of transcription
	Signal transduction
AKT1	signal transduction
Beta-TrCP	Proteasome-dependent degradation
CK1AL	Casein kinase I alpha like isoform
CKSII	Casein kinase II alpha chain
EGLN2	Hypoxia regulation
FMR1	Negative regulation of translation, mRNA export
S6K1	mTOR
STAT5A	Signal transduction

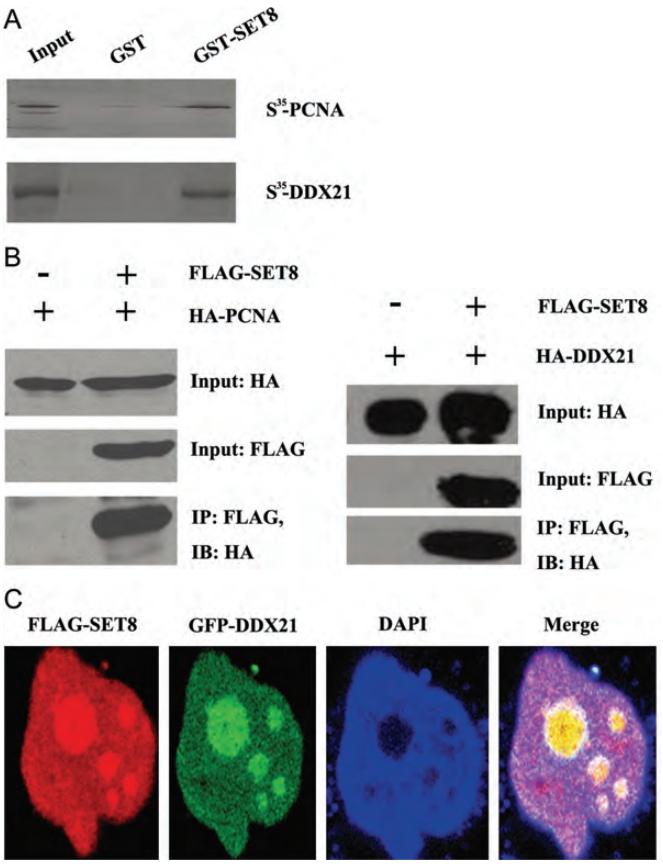

Figure 2 Verification of the interaction between SET8 with PCNA and DDX21 (A) Direct binding between SET8 with PCNA and DDX21 were validated by GST-pull-down assay with GST-SET8 fusion protein and *in vivo* translated HA-tagged PCNA and DDX21. (B) Association of SET8 with PCNA and DDX21 in cells were confirmed by Co-IP assays. (C) Co-localization of SET8 and DDX21, FLAG-SET8, and GFP-DDX21 were found to exhibit a strong co-localization in the nucleolus. IP, immunoprecipitation; IB, immunoblot.

PCNA has been reported to associate with SET8 [3,5,21] and therefore served as a positive control. ^{35}S-methionine-labeled HA-tagged PCNA and DDX21 were *in vitro* translated and used, respectively, in incubation with GST-SET8 in pull-down assays. Both HA-PCNA and HA-DDX21 could specifically bind to SET8 [**Fig. 2(A)**]. To test whether SET8 is associated with PCNA and DDX21 in cells, FLAG-tagged SET8 was co-expressed with HA-PCNA or HA-DDX21 in HEK293T cells. FLAG-SET8 was able to co-immunoprecipitate with HA-PCNA and HA-DDX21 [**Fig. 2(B)**]. The results indicated that the protein identification process is effective.

DDX21 (also known as RH-II/GU) has been reported as a RNA helicase involved in RNA processing and has a mainly nucleolar localization [22]. The interaction between SET8 and DDX21 suggests that SET8 may co-localize with DDX21 in nucleolus. This hypothesis was confirmed in an immunofluorescence experiment with co-expression of GFP-DDX21 and FLAG-SET8 [**Fig. 2(C)**].

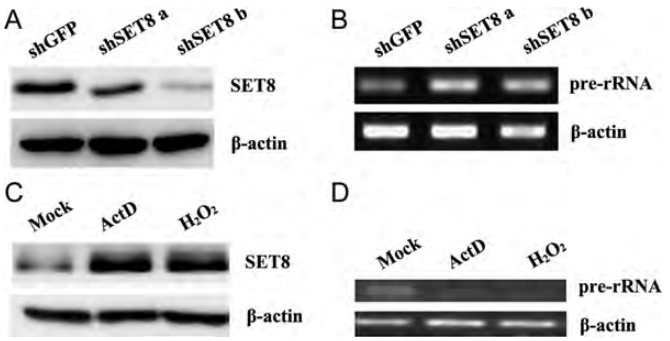

Figure 3 SET8 is involved in the regulation of pre-rRNA transcription (A) Lentivirus-mediated knockdown of SET8 expression in HeLa cells. Silencing efficiencies were confirmed by immunoblotting on SET8 proteins. shGFP was used as negative control. (B) pre-rRNA level increased in SET8 knockdown HeLa cells. (C) Endogenous SET8 level was increased in HeLa cells treated with ActD (1 μg/ml, 30 min) and H_2O_2 (200 μM, 1 h). (D) pre-rRNA level was decreased in HeLa cells treated with ActD (1 μg/ml, 30 min) and H_2O_2 (200 μM, 1 h).

SET8 is involved in pre-rRNA transcriptional regulation

The finding that SET8 co-localized with DDX21 in the nucleolus where the transcription of pre-rRNA took place raised the possibility that SET8 might participate in the regulation of pre-RNA transcription. To address this issue, we knocked down SET8 expression in HeLa cells by infection of lentivirus expressing SET8 shRNA. Two siRNA target sites on SET8 coding sequence were validated using immunoblotting on SET8 [**Fig. 3(A)**]. SET8 knockdown in HeLa cells markedly increased pre-rRNA transcription as shown by the results of semi-quantitative RT-PCR [**Fig. 3(B)**].

Actinomycin D (ActD) and H_2O_2 were reported as stress-inducing agents that down-regulate pre-rRNA transcription [23]. We then examined whether SET8 protein level changed when HeLa cells were treated with ActD and H_2O_2. Immunoblotting results indicated that SET8 level increased after stimulation of cells with ActD and H_2O_2 [**Fig. 3(C)**]. Meanwhile, pre-rRNA level decreased significantly [**Fig. 3(D)**]. These results suggest that SET8 is involved in the regulation of pre-rRNA transcription.

Enrichment of SET8 at rDNA promoter region

SET8 can associate with silent heterochromatins, but its association with heterochromatic marker HP1γ has not been reported. Here, we demonstrated that SET8 co-immunoprecipitated with HP1γ [**Fig. 4(A)**]. Furthermore, ChIP results indicated that occupancy of SET8 and H4K20me1 at rDNA promoter increased when cells were treated with ActD, meanwhile, HP1γ enrichment at rDNA promoter increased accordingly [**Fig. 4(B)**]. These results suggest that SET8 down-regulates pre-rRNA transcription by promoting formation of heterochromatin on rDNA promoter region in association with HP1γ.

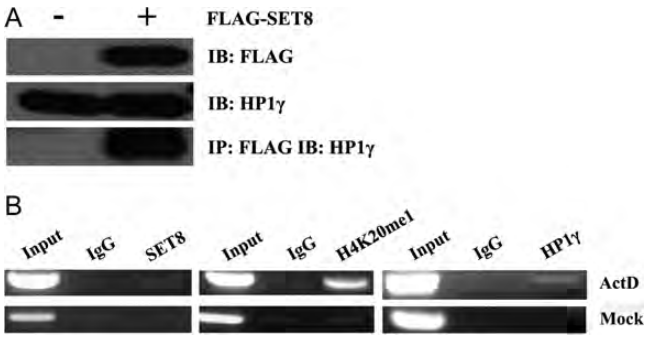

Figure 4 Enrichment of SET8, H4K20me1, and HP1γ on rDNA promoter after treatment with ActD (A) SET8 co-immunoprecipitates with the heterochromatic marker HP1γ. (B) ChIP experiment was used to examine SET8, H4K20me1, and HP1γ on rDNA promoter after cells were treated with ActD (1 μg/ml, 30 min).

Discussion

There are many strategies and techniques to systematically investigate protein–protein interaction. Among these, the most convenient and efficient way is to employ GST-pull-down or immunoprecipitation coupled with MS technique. Using this strategy, we identified several novel potential SET8-interacting proteins. The validation of the interactions of SET8 with PCNA and DDX21 proved effectiveness of this strategy. Besides, interaction between SET8 and nucleolar protein DDX21 prompts us to raise the question whether SET8 functions in the nucleolus? Our results indicate that SET8 participates in pre-rRNA transcription regulation.

PCNA has been reported to interact with SET8 and the interaction is important in regulation of replication origin, cell cycle progression, and epigenetic information inheritance [3–5,8,24]. In addition, interaction of SET8 with PCNA can cause deregulated expression of PCNA, which may promote the carcinogenesis process [25]. Given the important role of PCNA plays in genome replication [26] and multiple chromatin modifiers we identified, we speculate that SET8–PCNA interaction may offer a platform for recruiting these chromatin modifiers to the replication fork to maintain fidelity of inheritance of the epigenetic information.

Our results indicated that knockdown of SET8 expression in HeLa cells results in up-regulation of pre-rRNA transcription. Moreover, in cells treated with stress-inducing agents, we could observe down-regulation of pre-rRNA and up-regulation of SET8. Further, ChIP experiments demonstrated that occupancy of SET8, H4K20me1, and HP1γ increased upon treatment of cells with stress-inducing agents that could result in heterochromatin formation at rDNA promoter region. Endogenous rRNA has to be effectively transcribed to keep up with the cell's metabolic activity and demand for ribosome. Alterations in cell cycle progression and proliferation are accompanied by changes in the transcriptional rate of rRNA genes [23]. Our data may stimulate future efforts to elucidate the role of SET8 plays in rRNA transcriptional regulation. The exact mechanism and the role of the interaction between SET8 and DDX21 in this process remain to be elucidated.

Among the identified proteins, HIF1AN and TWIST1 are closely related to carcinogenesis and cancer metastasis. The interaction of TWIST with SET8 was also observed in pull-down assay in combination with MS by another group [14], and was reported to promote metastasis. HIF1AN is an interacting partner of HIF1α that is a key player in cellular hypoxia response. It is interesting to see whether SET8 is involved in HIF1α-related pathways, such as carcinogenesis, metastasis, stem cell maintenance, and glycolysis [27–29].

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