Tudor Staphylococcal Nuclease (Tudor-SN), a Novel Regulator Facilitating G_{1}/S Phase Transition, Acting as a Co-activator of E2F-1 in Cell Cycle Regulation*

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Background: Tudor staphylococcal nuclease (Tudor-SN) is an RNA-binding protein that has been linked to stress responses. Tudor-SN is a potential substrate of G_{1}/S phase Cdns, and promotes cell cycle progression by facilitating E2F-1-mediated gene transcription. Tudor-SN is a new regulator of the G_{1}/S transition. This study reveals a new function of Tudor-SN and elucidates a novel mechanism for cell cycle regulation.

Significance: Tudor-SN is a potential substrate of Cdk2/4/6, supportively, Tudor-SN is identified as a novel co-activator of E2F-1, which could facilitate E2F-1-mediated gene transcriptional activation of target genes, which play essential roles in G_{1}/S transition.

Tudor staphylococcal nuclease (Tudor-SN) is a multifunctional protein implicated in a variety of cellular processes. In the present study, we identified Tudor-SN as a novel regulator in cell cycle. Tudor-SN was abundant in proliferating cells whereas barely expressed in terminally differentiated cells. Functional analysis indicated that ectopic overexpression of Tudor-SN promoted the G_{1}/S transition, whereas knockdown of Tudor-SN caused G_{1} arrest. Moreover, the live-cell time-lapse experiment demonstrated that the cell cycle of MEF^{+/−} (knock-out of Tudor-SN in mouse embryonic fibroblasts) was prolonged compared with wild-type MEF^{++/+}. We noticed that Tudor-SN was constantly expressed in every cell cycle phase, but was highly phosphorylated in the G_{1}/S border. Further study revealed that Tudor-SN was a potential substrate of Cdk2/4/6, supportively, we found the physical interaction of endogenous Tudor-SN with Cdk4/6 in G_{1} and the G_{1}/S border, and with Cdk2 in the G_{1}/S border and S phase. In addition, roscovitine (Cdk1/2/5 inhibitor) or CINK4 (Cdk4/6 inhibitor) could inhibit the phosphorylation of Tudor-SN, whereas ectopic overexpression of Cdk2/4/6 increased the Tudor-SN phosphorylation. The underlying molecular mechanisms indicated that Tudor-SN could physically interact with E2F-1 in vivo, and could enhance the physical association of E2F-1 with GCN5 (a cofactor of E2F-1), which possesses histone acetyltransferase activity, and promote the binding ability of E2F-1 to the promoter region of its target genes CYCLIN A and E2F-1, and as a result, facilitate the gene transcriptional activation. Taken together, Tudor-SN is identified as a novel co-activator of E2F-1, which could facilitate E2F-1-mediated gene transcriptional activation of target genes, which play essential roles in G_{1}/S transition.

Cell cycle is a well orchestrated process consists of four distinct phases. G_{1}, S, and G_{2} phases are collectively known as interphase, during which cells accumulate nutrients for mitosis and duplication of DNA. M phase is the process by which a mother cell divides into two daughter cells. Cells that have temporarily or reversibly stopped dividing are in the state of quiescence, which was called the G_{0} phase or resting phase. A series of biochemical transitions control the switches between various phases of cell cycle. The first essential transition is the G_{1}/S transition in which a cell decides its fate, such as division, G_{1} arrest, quiescence, or differentiation, in response to diverse signals. G_{2}/M transition is the other transition in which cells trigger the early mitotic events that lead to chromosome alignment on the spindle in metaphase (1). Many regulators are involved in cell cycle transitions. Cdns interact with specific cyclin regulatory subunits and are activated by phosphorylation. Then the activated Cdk-cyclin complexes phosphorylate a number of specific substrates that were involved in cell cycle progression. The transcription factor E2F-1 plays an essential role in the G_{1}/S transition through regulating the timing and expression levels of target genes in the S phase. A number of factors regulate the activity of E2F-1. For example, the retinoblastoma pro-
tein (Rb) interacts with E2F-1 and suppresses E2F-1-mediated gene transcriptional activation in G0 and early G1 phases (2, 3). Cyclin A/Cdk2 could phosphorylate and activate E2F-1 in S phase (4).

A series of classical signal transduction pathways are also involved in cell cycle regulation. For example, the Ras/MAPK pathway controls G1/S progression through regulating the expression of cyclin D and p27 (5), affecting Cdns/cyclins assembly and catalytic activities (6). The tumor suppressor protein p53 is also essential in cell cycle regulation through con-

EXPERIMENTAL PROCEDURES

Cell Cycle Synchronization and Analysis—

Cell Cycle Synchronization and Analysis—Cells were plated in standard growth medium to achieve approximate 40% confluence. The following day, the standard growth medium was replaced with medium containing 2 mM thymidine and the cells were incubated for 16 h under normal conditions. After washing the cells three times with PBS, the cells were re-fed with standard growth medium for 8 h. Then the standard growth medium was replaced with medium containing 2 mM thymidine and incubated for 16 h again. After the double thymidine block, cells were synchronized to the G1/S border. Specific phase cells can be collected at various time points following the second exposure to thymidine.

The cells were collected in the usual way, and the cell pellets were washed with PBS. Approximately 1 × 10^6 cells were fixed in 70% ethanol overnight at 4 °C. The cells were centrifuged at 400 × g for 5 min to remove the ethanol. The cell pellets were then resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.4) and incubated for 30 min at 37 °C. Then the cells were stained with 50 μg/ml of propidium iodide (15 μg/ml, Sigma) in 50 mM sodium citrate at 4 °C for 20 min. Cell cycle distribution was detected by a flow cytometer (Guava easyCyte, Millipore) and analyzed by Modfit software.

4 The abbreviations used are: Rb, retinoblastoma; Tudor-SN, Tudor staphylococcal nuclease; MEF, mouse embryonic fibroblast; CREB, cAMP-response element-binding protein; HDAC, histone deacetylase.

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Tudor-SN Promotes G₁/S Transition as a Co-activator of E2F-1

Co-immunoprecipitation—Total cell lysates were collected with Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 20% glycerol, 0.1 mM sodium orthovanadate, 1 mM sodium butyrate) supplemented with PMSF and protease inhibitor mixture (04693124001, Roche Applied Science). Protein concentrations of the lysates were measured using the Pierce BCA Protein Assay Kit (number 23227, Thermo Scientific). Total cell lysates were incubated with the interesting antibodies, mouse polyclonal anti-IgG antibody (Santa Cruz Biotechnology) as a negative control, followed by incubation with Pierce Protein A/G-agarose (20422, Thermo Pierce) overnight at 4 °C in a roller. 10% of the total cell lysates were used as input. The bound proteins were subjected to SDS-PAGE and blotted with the corresponding antibodies.

Western Blotting and Antibodies—Total cell lysates or the immunoprecipitated proteins were finally analyzed by 8 or 10% SDS-PAGE. Antibodies against Cdk2 (rabbit mAb number 2546, Cell Signaling Technology), Cdk4 (mouse mAb number 2906, Cell Signaling Technology), Cdk6 (mouse mAb number 3136, Cell Signaling Technology), cyclin A (mouse mAb number 4656, Cell Signaling Technology), cyclin D1 (mouse mAb number 2926, Cell Signaling Technology), cyclin E (mouse mAb number 4129, Cell Signaling Technology), E2F-1 (mouse mAb E8901, Sigma), phospho-Thr/Ser 42H4 (mouse mAb number 4129, Cell Signaling Technology), and β-actin (Mouse mAb sc-69879, Santa Cruz Biotechnology) were used for Western blotting analysis according to the manufacturer’s recommended concentrations. The mouse monoclonal anti-Tudor-SN antibody was generated against the SN4 domain (amino acids 507–674) of Tudor-SN at the Institute of Medical Technology, University of Tampere, Finland.

Live-cell Time-lapse Analysis—MEF⁻/⁻ and MEF⁺/+ cells were cultured in 20-mm glass-bottomed Petri dishes (NEST Biotechnology) in DMEM supplemented with 15% FBS. The OLIMPIUS IX81-CSU living cell system was used for live-cell imaging. Time-lapse images were captured at 10-min intervals from a separate 950/11003 region per well using a Carl Zeiss) and run in quadruplicate.

Prediction of the Phosphorylation Sites and the Potential Kinase—The Phospho.ELM database was used to predict the potential phosphorylation sites of Tudor-SN. Phosphorylation sites in Phospho.ELM were extracted from scientific literature and phosphoproteomic analyses. After querying the database (keyword SND1) or sequence identifier (Ensembl ID: ENSG00000197157; UniprotKB/SwissProt: Q7KZF4), the detailed information of all potential phosphorylation sites was obtained. The PhosphoNET database was used to predict the kinase of the potential phosphorylation sites. The kinase predictions are based on deduced consensus phosphorylation site amino acid frequency scoring matrices determined for each of ~500 different human protein kinases. The database was searched by the protein name (SND1) or Uniprot number (Q7KZF4), all possible phosphorylation sites were determined first and then by using the orange buttons under the “Kinase Pred.” of the interesting sites, the potential kinases of these sites were acquired. The higher the Kinase Predictor V2 score, the better the prospect that the kinase will phosphorylate the given site.

Relative Quantitative Two-step Real-time RT-PCR—Total RNA was isolated using TRIzol reagent (15596, Invitrogen). The reverse transcriptase reaction was carried out using a RevertAid™ First Strand cDNA Synthesis Kit (K1621, Fermentas) according to the manufacturer’s protocol. All primers were designed and synthesized by Takara Biotechnology (Dalian, China). The primer sequences were as follows: CDK1, forward: TTCACGAGACCTTAAACCTCA, reverse: TCGGTACCACAGGGTCA; CYCLIN A, forward: CGGAGATCTGTCCCTGATCTT, reverse: GGTCCTCCCAGCTGAGATACTCT; E2F-1, forward: ACCATCAGTACTGGCGAGAG, reverse: TTGGTGTCAGATTCTAGGTGC; CYCLIN E, forward: ACCGGTATATGCGACACAGAA, reverse: TCACTACGCAAACCTGTCAGCA; c-MYC, forward: CCTGCGTGCCTCACGAGAGAG, reverse: CTCCAGCGAAAGCTGTCAGCAGA; TUDOR-SN, forward: CCCACAGACATTCTTACACAC, reverse: GCTGAAACCTGTGGCCTATG; and GAPDH, forward: GCACGGTCAAGGCTGAGAC, reverse: TGTGGAAGACGCCAGTGGA. Real-time quantitative PCR were performed in the StepOne™ Real-time PCR System (ABI) using the FastStart Universal SYBR Green Master (Roche Applied Science). The PCR conditions were 95 °C for 2 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min, fold-changes were calculated using the ΔΔCt method. The results were normalized to GAPDH levels.

Reporter Gene Assay—HeLa cells were plated in a 12-well plate at a density of 3 × 10⁴ cells/well and grown to 60–80% confluence. Then the cells were co-transfected with 2 μg of reporter plasmids, 500 ng of pCMV-β-galactosidase plasmids, and 1 or 2 μg of pSG5-Tudor-SN-Flag or pSG5-vector plasmids as control. 48 h after transfection, cells were lysed with Reporter Lysis Buffer (E3971, Promega), and luciferase activity was measured using the Luciferase Assay System (E1483, Promega) through a GLOMA 96 Microplate Luminometer (Promega). The luciferase values were normalized against β-galactosidase activity of the lysates.

Chromatin Immunoprecipitation (ChIP)—HeLa cells were transfected with pSG5-Tudor-SN-Flag plasmids or pSG5 vectors as negative control. 48 h after transfection, formaldehyde was added to the cells at a final concentration of 1% for 10 min. Cross-linking was stopped by the addition of glycine to a final concentration of 100 mM. The cells were washed with PBS and lysed in SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS with phosphatase and protease inhibitors) on ice for 20 min. The chromatin was sheared to an average size of 500–1000 base pairs under identical conditions using a Vibra Cell 500 watt sonicator (Sonics and Materials) on high power for 6 cycles (15 s on/15 s off). After centrifugation, the sheared chromatin was diluted 1:10 in dilution buffer and pre-cleared with 60 μl of protein A/G beads for 1 h at 4 °C. A fraction of the diluted chromatin lysate for immunoprecipitation was saved as input. Immunoprecipitation was performed with the anti-E2F-1 antibody (E8901, Sigma) or anti-IgG antibody (Sigma) overnight at 4 °C with rotation. The immunocomplexes were then incubated with 60 μl of protein G beads for 6 h. The beads were washed with 0.5 ml of the following cold buffers in the
order listed: low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer. The DNA-protein complexes were eluted from the beads with 100 μl of 1% Proteinase K in ChIP elution buffer for 3 h at 65 °C. DNA was extracted with phenol/chloroform, ethanol precipitated, and analyzed for the presence of the E2F-1 and CYCLIN A promoter by PCR, which was performed with FastStart Universal SYBR Green Master (Rox) (04913914001, Roche). The primers targeting the promoter regions were designed by NCBI/Primer-BLAST and synthesized by Takara Biotechnology (Dalian, China): E2F-1, forward, AAGCCCATAGGAACCGCG, reverse, AGTCCCGGCGCACTTTTACG; CYCLIN A, forward, CCACCTCTTAAACCGCGATCC, reverse, CAGACAGAGGCCCAAAAAC. The PCR conditions were 95 °C for 2 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min, which were controlled using a StepOne Real-time PCR System (ABI). The normalized immunoprecipitated DNA concentrations represent the relative levels of transcription factors binding to the promoters and are presented as fold-changes compared with the control group.

RESULTS

The Expression Level of Tudor-SN Varies in Different Tissues and Cells—In our previous study, we noticed that Tudor-SN protein could affect cell proliferation (19), we therefore detected the expression of Tudor-SN in different kinds of tissues isolated from BALB/c mice (16 weeks). The result (Fig. 1A, upper panel) indicated that Tudor-SN protein was highly expressed in kidney, liver, and spleen tissues (lanes 2–4), which possess potential proliferation capacity, whereas only a small amount of Tudor-SN was detected in terminally differentiated cells, such as cardiac and skeletal muscle (lanes 1 and 5).

We further detected the protein level of Tudor-SN in human granulocytes and lymphocytes separated from the heparin anti-coagulated human peripheral blood by Ficoll density gradient centrifugation. As shown in Fig. 1B, Tudor-SN was highly expressed in lymphocytes that have proliferating ability (first panel, lane 1), but undetectable in granulocytes, which are terminally differentiated cells (first panel, lane 2). It is well established that CdkS are present throughout the cell cycle, whereas the cyclins are expressed at specific phases in the cell cycle. In the cells permanently withdraw from the cell cycle, many of the CdkS and cyclins are down-regulated or diminished. To our expectation, Cdk2, Cdk4, Cdk6, and cyclin D1 proteins were undetectable or at very low levels in granulocytes (Fig. 1B, lane 2) compared with lymphocytes (Fig. 1B, lane 1), but not cyclin A and cyclin E. These data demonstrate that the expression pattern of Tudor-SN is the same as Cdk2/4/6, which are G1/S regulators, indicating Tudor-SN is potentially involved in cell cycle regulation.

Knockdown of Endogenous Tudor-SN Protein Arrests the HeLa Cells in G1 Phase and Affects the G1/S Transition—HeLa cells can be easily synchronized to different phases. We thus utilized HeLa cells to investigate the potential role of Tudor-SN in cell cycle regulation. We first analyzed the cell cycle distribution of parental HeLa cells, HeLa cells with knockdown of endogenous Tudor-SN by siRNA, or cells transfected with scramble siRNA as negative control. 48 h after transfection, the cells were collected and analyzed by flow cytometry. The knockdown efficiency was detected by Western blot (Fig. 2A). The results showed that (Fig. 2B and C) compared to scramble control cells (G1: 60.14%, S: 26.44%) or parental cells (G1: 58.10%; S: 30.21%), more Tudor-SN knockdown cells were distributed in the G1 phase (71.79%) but less in the S phase (18.29%). These data demonstrate that Tudor-SN is likely to be involved in the G1/S phase transition. To investigate the effect of Tudor-SN protein on G1/S phase transition, HeLa cells were first synchronized at the G1/S border by blocking using a double thymidine treatment, then released from synchronization by incubating with normal cell culture media for different time points, and the cell cycle distribution was analyzed by flow cytometry. The results showed that (Fig. 2D and E) most of the parental cells (75.62%), Tudor-SN knockdown cells (85.87%), or scrambled control cells (80.78%) were synchronized at the G1 phase (upper panel). When the cells were released from the synchronization for 3 h (middle panel), most of the parental cells (93.53%) entered S phase, as well as the scrambled control cells (98.38%), whereas only 60.58% of Tudor-SN knockdown cells entered S phase, and more cells were retained in

FIGURE 1. The expression level of Tudor-SN in different tissues and cells. A, the protein level of Tudor-SN in cardiac and skeletal muscle, liver, spleen, and kidney tissues separated from the BALB/c mouse (16 weeks). B, the protein level of Tudor-SN in lymphocytes and granulocytes separated from the heparin anti-coagulated human peripheral blood.
the G1 phase (39.42%). After releasing the cells for 9 h (lower panel), most of the parental cells (82.58%) or scramble cells (78.73%) entered the G2/M phase, whereas only 65.13% of Tudor-SN knockdown cells were in the G2/M phase, but more cells were in the G1 phase (17.63%). These results further verify that temporary knockdown of Tudor-SN could cause G1 phase arrest, and delay cell cycle transition.

Knock-out of Tudor-SN in MEF Cells Prolongs the Cell Cycle—To further improve the influence of Tudor-SN on the cell cycle, we detected the cell cycle distribution of the mouse embryonic fibroblasts (MEF) with knock-out of Tudor-SN protein (MEF^−/−), and the wild-type MEF (MEF^+/+) cells were used as control. As shown in Fig. 3, A and B, more MEF^−/− cells were distributed in the G1 phase (72.32%) than MEF^+/+.
Tudor-SN Promotes G\textsubscript{1}/S Transition as a Co-activator of E2F-1

FIGURE 3. Knock-out of Tudor-SN in MEF cells prolongs the cell cycle. A, the cell cycle distribution of wild type MEF (MEF\textsuperscript{+/+}) and Tudor-SN knock-out MEF (MEF\textsuperscript{−/−}) cells was analyzed by FACS. B, the percentage of MEF\textsuperscript{+/+} and MEF\textsuperscript{−/−} cells in each phase are shown in the histogram. C, the OLYMPUS IX81-CSU living cell system was used to observe living cell imaging of MEF\textsuperscript{+/+} and MEF\textsuperscript{−/−} cells. Time-lapse images were captured at 10-min intervals from a separate 950 × 760 μm\textsuperscript{2} region per well using a ×20 objective. D, independent samples of Student’s t test was performed using SPSS 16.0. Significant difference was indicated as *\textsubscript{p} < 0.01 (n = 10).

(52.22%), but less in S phase (16.02 to 29.13%) and G\textsubscript{s}/M phase (10.86 to 18.66%). Then, live-cell time-lapse analysis was performed to observe the cell cycle progression of live MEF\textsuperscript{+/+} and MEF\textsuperscript{−/−} cells. The results showed that the duration of the cell cycle in MEF\textsuperscript{−/−} cells (420 ± 20 min, n = 10) was longer than that of MEF\textsuperscript{+/+} cells (340 ± 15 min, n = 10) (Fig. 3, C and D). It indicates that knock-out of Tudor-SN in MEF cells prolongs the cell cycle, which is consistent with the previous data that Tudor-SN regulates G\textsubscript{s}/S phase transition.

Tudor-SN Is a Potential Substrate of Cdk2/4/6—To characterize the underlying mechanisms of Tudor-SN protein being involved in the cell cycle, we detected the expression pattern of Tudor-SN in different phases of cell cycle by synchronizing the cells at different cell cycle phases. The G\textsubscript{s}/S border cells were obtained from the double thymidine block. The cells in S, G\textsubscript{s}/M, or G\textsubscript{s} (middle G\textsubscript{s}) phases were collected after being released from the G\textsubscript{s}/S border for 3, 9, or 16 h, respectively. The results showed that Tudor-SN was expressed constantly throughout the cell cycle (Fig. 4A, first panel), which was the same as Cdk2/4/6. The expression level of cyclins (cyclin A, E, and D1) varied dramatically, which was consistent with the distribution of cell cycle phases. Equal amounts of endogenous β-actin was present in different samples (last panel). These data demonstrate that the expression profile of the Tudor-SN protein is the same as G\textsubscript{s}/S phase Cdkks, which is present throughout the cell cycle in proliferation cells but at significantly lower levels in terminally differentiated cells.

Many regulators control cell cycle progression through sequential threonine or serine phosphorylation and dephosphorylation. We therefore detected the threonine/serine phosphorylation level of Tudor-SN in different phases of the cell cycle. Lysates of cells synchronized at different phases were immunoprecipitated with anti-Tudor-SN antibody, the phosphorylation level of Tudor-SN was detected by anti-phosphothreonine/serine immunoblotting. The results showed that the same amount of Tudor-SN protein was precipitated from different samples, but the phosphorylation level of Tudor-SN (Fig. 4, B and C, upper panel) was varied in different cell cycle phases, which was much higher in the G\textsubscript{s}/S border (lane 2) and G\textsubscript{s}/M (lane 4) phase than G\textsubscript{s} (lane 1) and S (lane 3) phases (p < 0.01, n = 3).

Cdkks are threonine/serine kinases that could phosphorylate substrates, which are crucial in cell cycle regulation (21). Tudor-SN regulates the G\textsubscript{s} to S phase transition and is hyperphosphorylated in the G\textsubscript{s}/S border, we wonder whether Tudor-SN is phosphorylated by the G\textsubscript{s} and S phase Cdkks. We first detected the threonine/serine phosphorylation state of Tudor-SN after inhibiting the Cdkks activities by treating cells with roscovitine (Cdk1/2/5 inhibitor) or CINK4 (Cdk4/6 inhibitor), respectively. The results showed that both roscovitine (Fig. 4, D and E, upper panel, lane 2) and CINK4 (upper panel, lane 3) obviously attenuated the threonine/serine phosphorylation level of Tudor-SN (p < 0.01, n = 3). Then, we ectopically overexpressed Cdk2/4/6 in HeLa cells by transfecting with plasmids containing full-length cDNA of Cdkks (pCMV-Cdk2/4/6-HA), respectively, and detected the phosphorylation state of Tudor-SN. After transfection for 48 h, the lysates of different samples were immunoprecipitated with anti-Tudor-SN antibody, and the phosphorylation level of Tudor-SN was detected by anti-phosphothreonine/serine immunoblotting. The results showed that ectopic overexpression of Cdk2/4/6 could enhance the phosphorylation state of Tudor-SN (Fig. 4F, upper panel). The statistical analysis (Fig. 4G) further indicated that Cdk2/4/6 increased the Tudor-SN phosphorylation level (p < 0.05,
**FIGURE 4. Tudor-SN in a potential substrate of Cdk2/4/6.** A, HeLa cells were synchronized in different phases, and then the protein level of Tudor-SN and other cell cycle regulators (Cdk2, 4, and 6, cyclin A, D1, and E) were detected by immunoblotting with the antibodies. B, Tudor-SN protein was first immunoprecipitated with anti-Tudor-SN antibody from the cell lysate of synchronized cells in different phases, then the Thr/Ser phosphorylation level of Tudor-SN was detected. C, the graph represents densitometric units normalized to Tudor-SN. The gray scale value was measured by ImageJ2X. One-way analysis of variance was performed using SPSS 16.0. Significant difference was indicated as *, p < 0.01 (n = 3). D, the Thr/Ser phosphorylation level of Tudor-SN was detected after treating the HeLa cells with roscovitine and CINK4. E, band intensity was quantified using densitometry and normalized to Tudor-SN. Independent samples of Student’s t test was performed using SPSS 16.0. Significant difference was indicated as *, p < 0.01 (n = 3). F, Tudor-SN protein was first immunoprecipitated with anti-Tudor-SN antibody from the lysate of cells that were transfected with different plasmids (pCMV-Cdk2, Cdk4, and Cdk6-HA plasmids and pCMV-HA plasmid), then the Thr/Ser phosphorylation level of Tudor-SN was detected. G, the gray scale value was measured by the ImageJ2X. Independent samples of Student’s t test was performed using SPSS 16.0. Significant difference was indicated as #, p < 0.05 (n = 3). H, the Cdks in HeLa cells were efficiently overexpressed. I, total cell lysates of HeLa cells were immunoprecipitated with anti-Tudor-SN antibody or anti-IgG as control, then Cdk2, Cdk4, and Cdk6 were detected by immunoblotting according to the antibodies. J, Tudor-SN protein was immunoprecipitated with anti-Tudor-SN antibody from the cell lysates of synchronized cells in different phases, then Cdk2, Cdk4, and Cdk6 were detected with according to the antibodies. K, the efficiency of the synchronization was analyzed by FACS.
n = 3). The overexpression efficiency of Cdks was detected by Western blot (Fig. 4H).

We also performed co-immunoprecipitation to detect the physical interaction between Tudor-SN and Cdk2/4/6. The endogenous Tudor-SN protein was immunoprecipitated with anti-Tudor-SN antibody, or anti-IgG antibody as negative control. The results showed that Cdk2/4/6 were efficiently co-immunoprecipitated with the endogenous Tudor-SN (Fig. 4I, lane 3) but not IgG (lane 2). It has been reported that different Cdks are activated in specific phases, therefore the physical interaction of Tudor-SN and Cdk2/4/6 may also depend on cell cycle phases. To verify this hypothesis, a co-immunoprecipitation assay was performed in HeLa cells synchronized at specific cell cycle phases. The results showed (Fig. 4J) that Cdk4 (second panel) and Cdk6 (third panel) mainly formed the complex with Tudor-SN in the G1 (lane 1) phase and G1/S (lane 2) border, whereas Cdk2 (first panel) was associated with Tudor-SN in the G1/S border (lane 2) and S (lane 3) phase. The synchronization efficiency is shown in Fig. 4K. All these data indicate that Tudor-SN is a potential substrate of Cdk2/4/6.

Ser-426 and Thr-429 of Tudor-SN Are Identified as Potential Threonine/Serine Phosphorylation Sites Targeted by Cdk2/4/6 in Cell Cycle Regulation—To identify the potential serine or threonine sites of Tudor-SN, which could be phosphorylated by Cdk2/4/6, we first used the Phospho.ELM database assembled from phosphoproteome analysis data to predict the potential threonine/serine phosphorylation sites of the amino acid residues in Tudor-SN protein. After querying the Phospho.ELM database by keyword (SND1) or sequence identifier (Ensembl ID: ENSG00000197157; UniprotKB/SwissProt: Q7KZF4), all potential threonine/serine phosphorylation sites of Tudor-SN were identified. Table 1 lists the residue code (column 1), position of amino acid sequence (column 2), the peptide sequence (±5) surrounding the phospho-residue (column 3), the PubMed references (column 4), the conservation score (column 5), domain identified by SMART or Pfam (column 6), and Iupred score (column 7). It indicates that 3 residues (Ser-426, Thr-429, and Ser-781) in the Tudor-SN protein are potential threonine/serine phosphorylation sites.

Then the PhosphoNET database was used to identify the protein kinases that may target these three sites. It could identify the top 50 out of 500 human protein kinases that have the highest Kinase Predictor V2 score using amino acid frequency scoring matrices trained with over 22,000 kinase-substrate pairs. The result indicates that (Table 2) Ser-426 and Thr-429 are the potential targets of kinase Cdk2/4/6, whereas Ser-781 is the potential target of Cdk2/6. The higher the Kinase Predictor V2 score, the better the prospect that a kinase will phosphorylate the given site. This information is supportive to our previous results that Cdk2/4/6 could phosphorylate Tudor-SN.

To verify the predicted phosphorylation sites of Tudor-SN, we constructed mammalian expression plasmids containing the full-length cDNA of Tudor-SN with S426A, T429A, or S781A mutations, respectively, and tagged with the FLAG epitope (pCMV-S426A, pCMV-T429A, pCMV-S781A). HeLa cells were transfected with these plasmids, respectively, the plasmid pCMV-Tudor-SN-Flag was used as positive control and pCMV-Flag vector was used as negative control. After 48 h, the transfected cells were collected, and cell cycle distribution was detected by flow cytometry. The results showed that by comparing the negative control cells (S phase: 33.44%), more S781A-Tudor-SN cells (42.84%, p < 0.05) were distributed in the S phase, which was similar to Tudor-SN control cells (47.90%, p < 0.01), but no statistical difference was observed in S426A-Tudor-SN (38.93%) or T429A-Tudor-SN cells (36.82%) (Fig. 5, A–C). Transfection efficiency was shown in Fig. 5D. These results indicate that mutation of either Ser-426 or Thr-429, but not Ser-781 could affect the ability of Tudor-SN to promote G1/S phase transition.

Tudor-SN Enhances the Gene Transcriptional Activation of CYCLIN A and E2F-1—To determine the molecular mechanism of Tudor-SN on promoting G1 to S phase transition, we focused on some target genes that are important in G1 and S phases. We first knocked down the endogenous Tudor-SN in HeLa cells with siRNA, and then carried out quantitative PCR to detect the mRNA levels of CYCLIN A and E2F-1, which play essential roles in G1/S transition. The parental cells or cells transfected with scramble siRNA were used as negative control. The results showed that with knockdown of Tudor-SN, the mRNA level of CYCLIN A and E2F-1 was significantly
decreased, compared with scramble or parental control groups ($p < 0.01$, $n = 3$) (Fig. 6A). Meanwhile, we also detected the mRNA level of some S phase genes ({CDK1, CYCLIN E, and c-MYC}), which did not present statistical changes among the different cells. The Western blot results showed that compared with the parental (Fig. 6B, lane 1) and scramble (lane 3) control cells, knockdown of endogenous Tudor-SN (lane 2) reduced the expression of cyclin A and E2F-1, which is consistent with the quantitative PCR results. The statistical analysis (Fig. 6C) further indicated that knockdown of Tudor-SN decreased the protein level of cyclin A and E2F-1 ($p < 0.01$, $n = 3$). These results demonstrate that Tudor-SN could promote gene transcription and expression of $\text{CYCLIN A}$ and $\text{E2F-1}$.

The luciferase reporter assay was performed to investigate whether Tudor-SN regulated the expression of $\text{CYCLIN A}$ and $\text{E2F-1}$ at the gene transcription level. The luciferase reporter plasmids containing the promoter region of $\text{CYCLIN A}$ (pGL2-cyclinA-luc) or $\text{E2F-1}$ (pGL2-E2F1-luc) were transfected into HeLa cells, together with plasmid containing full-length cDNA of Tudor-SN, or with mutations of Ser-426, Thr-429, and Ser-781, respectively. The results showed that ectopic overexpression of wild type Tudor-SN or S781A-Tudor-SN enhanced the gene transcriptional activation of $\text{CYCLIN A}$ and $\text{E2F-1}$ in a dose-dependent manner, whereas overexpression of S426A-Tudor-SN or T429A-Tudor-SN had no effect on gene transcriptional activation (Fig. 6, D and E). The transfection efficiency was detected by Western blot. These data indicate that Tudor-SN could regulate the expression of $\text{CYCLIN A}$ and $\text{E2F-1}$ at gene transcription levels. Moreover, Ser-426 and Thr-429 are key residues of Tudor-SN in the regulation.

**Tudor-SN Is a Novel Co-activator of E2F-1—CYCLIN A and E2F-1** are direct target genes of transcription factor E2F-1, which plays essential roles in regulating G1/S phase transition. Tudor-SN protein has been reported as a co-activator of many transcription factors, thus it is highly possible that Tudor-SN is also a co-activator of E2F-1 in cell cycle regulation. We first detected the physical association of Tudor-SN and E2F-1 by co-immunoprecipitation assay. As shown in Fig. 7A, the endogenous Tudor-SN protein could precipitate endogenous E2F-1 (upper panel, lane 2) efficiently, reciprocally, Tudor-SN (lower panel, lane 1) was precipitated by E2F-1. This result demonstrates that Tudor-SN could associate with E2F-1 in vivo. Taken together with our earlier data that Tudor-SN could facilitate E2F-1-mediated gene transcriptional activation (Fig. 6, D and E), all the data indicate that Tudor-SN is a potential co-activator of E2F-1 in cell cycle regulation.

Appropriate regulation of gene transcription requires the coordination of activating and repressing signals modulating DNA accessibility. We have reported earlier that Tudor-SN directly interacted with CREB-binding protein and enhanced histone acetyltransferase activity via recruitment of the CREB-binding protein to STAT6 (13). It is possible that Tudor-SN might enhance the gene transcriptional activation of E2F-1-mediated target genes with the same mechanisms. It has been reported that GCN5 (a known cofactor of E2F-1, which possesses histone acetyltransferase activity) (22), and HDAC-1 (a transcriptional repressor of E2F-1) (2), are the proteins that...
Tudor-SN enhances the transcriptional activation of CYCLIN A and E2F-1. A, quantitative PCR-detected S phase gene mRNA expression levels of parental HeLa cells, HeLa cells with knockdown of endogenous Tudor-SN protein, or scramble control cells. One-way analysis of variance was performed using the SPSS 16.0 software. Significant difference was indicated as *, p < 0.01 (n = 3). B, the protein level of S phase regulators was detected by immunoblotting according to the antibodies. C, band intensity was quantified using densitometry and normalized to parental cells. One-way analysis of variance was performed using the SPSS 16.0 software. Significant difference was indicated as *, p < 0.01 (n = 4). D and E, luciferase reporter assay was performed to detect the transcription activities of CYCLIN A and E2F-1 after co-transfecting HeLa cells with reporter plasmids, β-galactosidase vector, and different amounts of Tudor-SN or Tudor-SN mutation plasmids. The luciferase values were normalized against β-galactosidase activity of the lysates. Independent samples of Student’s t test was performed using SPSS 16.0. Significant difference was indicated as: #, p < 0.05; *, p < 0.01 (n = 3). F and G, the transfection efficiency was detected by Western blot.
Tudor-SN Promotes $G_1$/S Transition as a Co-activator of E2F-1

We therefore performed a co-immunoprecipitation assay to investigate in vivo protein complex formation. HeLa cells were transfected with pSG5-Tudor-SN-Flag plasmids, or pSG5 vector as the negative control. After 48 h, the total cell lysate was collected and immunoprecipitated with anti-E2F-1 antibody or anti-IgG as negative control, and then blotted with anti-HDAC-1 or anti-GCN5 antibody. The results showed that HDAC-1, GCN5, and E2F-1 (Fig. 7B, lane 1) could physically form the complex. With ectopic overexpression of Tudor-SN protein, less HDAC-1 (Fig. 7B, lane 2, upper panel) but more GCN5 (Fig. 7B, lane 2, middle panel) was precipitated with E2F-1. The statistical analysis (Fig. 7C) verified this result ($p < 0.01, n = 3$). Meanwhile, we detected the acetylation level of histone 3. It was shown that more histone 3 was acetylated with overexpression of Tudor-SN (Fig. 7D, third panel, lane 2).

To further confirm that Tudor-SN acts as a co-activator affecting E2F-1-mediated gene transcriptional activation, ChIP assays were performed to detect the binding ability of E2F-1 to the DNA promoter region of its target genes with overexpression of Tudor-SN. The result showed (Fig. 7E) that with ectopic overexpression of Tudor-SN protein, more CYCLIN A (first panel, lane 2) or E2F-1 (second panel, lane 2) promoter regions were precipitated with E2F-1, compared with the control group (lane 1). To exclude the unspecific binding in the ChIP assay, the region located outside the promoter region was used as a negative control (lanes 1 and 2, third and fourth panel). The statistical analysis (Fig. 7F) further indicated that overexpression of Tudor-SN could enhance the association of E2F-1 to the promoter region of target genes CYCLIN A and E2F-1 ($p < 0.01, n = 3$). All these data demonstrate that Tudor-SN is a novel co-activator of E2F-1, which could enhance E2F-1-mediated gene transcriptional activation.

DISCUSSION

Cells can be divided into three categories according to the proliferation capacity: proliferating cells, which always keep division activity; terminal differentiation cells, also called end cells, which lose the split ability; and quiescent cells, which enter the G0 state. The cells transit from proliferation to terminal differentiation or quiescent are always accompanied by turnover of cell cycle accelerators via down-regulation of specific cell cycle proteins, such as Cdks. Brooks et al. (23) reported that the expression and activities of Cdks were down-regulated significantly during the development of cardiac myocyte, committed with the loss of proliferative capacity, which is pivotal in...
Tudor-SN Promotes G1/S Transition as a Co-activator of E2F-1

FIGURE 8. Tudor-SN is a novel protein involved in regulating G1/S phase transition through facilitating E2F-1-mediated target gene transcription.

In summary, Tudor-SN is identified as a novel regulator of cell cycle, which is likely to be essential for the G1 to S phase transition (Fig. 8). As a potential substrate of the G1/S phase Cdks, Tudor-SN is likely to be phosphorylated by the Cdk2/4/6 in the G1/S border and S phase. The mutation of either Ser-426 or Thr-429, which are the predicted potential phosphorylation sites in Tudor-SN, reduced the ability of Tudor-SN to promote G1/S phase transition. We further identify Tudor-SN as a co-activator of E2F-1 in cell cycle regulation. Tudor-SN could enhance the association of E2F-1 with DNA-bound E2F-1 and prevents its activity (2). The Rb pathway is disrupted by expression of papillomavirus E6/E7 proteins in many tumor cells (32), including HeLa cells. Even though Rb is functionally inactive in HeLa cells, E2F-1 still plays essential roles in regulating the G1/S phase transition, and many other regulators influence its activity. For example, the nuclear matrix protein (NRP/B) acts as a transcriptional repressor of E2F-1 by recruiting HDAC in HeLa cells (33). The apoptosis inhibitor-5 (Apis5) contributes positively to E2F1 transcriptional activity by promoting the binding ability of E2F1 to the promoter of the target genes (34).

We have reported previously (13) that as a co-activator of STAT6, Tudor-SN could interact directly with CREB-binding protein and recruit histone acetyltransferase activity to STAT6 in vivo, while enhancing acetylation of histone H4 at the IgE promoter. Consistent with this idea, Tudor-SN is identified as a novel co-activator of the transcription factor E2F-1 in cell cycle regulation. Tudor-SN could enhance the association of E2F-1 with GCN5, promote the binding ability of E2F-1 to the promoter region of CYCLIN A and E2F-1, as a result, facilitate the gene transcriptional activation.

In summary, Tudor-SN is identified as a novel regulator of cell cycle, which is likely to be essential for the G1 to S phase transition (Fig. 8). As a potential substrate of the G1/S phase Cdks, Tudor-SN is likely to be phosphorylated by the Cdk2/4/6 in the G1/S border. The mutation of either Ser-426 or Thr-429, which are the predicted potential phosphorylation sites in Tudor-SN, reduced the ability of Tudor-SN to promote G1/S phase transition. We further identify Tudor-SN as a co-activa-
Tudor-SN Promotes G1/S Transition as a Co-activator of E2F-1

Tudor-SN promotes G1/S transition as a co-activator of E2F-1, which could facilitate the gene transcriptional activation of E2F-1-mediated G1/S phase target genes. This novel finding reveals a new function of Tudor-SN and illustrates the mechanism of Tudor-SN in the cell cycle regulation.

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