Proteasomal Regulation of Mammalian SPT16 in Controlling Transcription

Amala Kaja,^a Abhinav Adhikari,^{a*} Saswati Karmakar,^b Wanwei Zhang,^c Gerson Rothschild,^c Uttiya Basu,^c Surinder K. Batra,^b Judith K. Davie,^a Suresh R. Bhaumik^a

^aDepartment of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, Illinois, USA

^bDepartment of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska, USA

^cDepartment of Microbiology and Immunology, Vagelos College of Physicians and Surgeons, Columbia University, New York, New York, USA

Amala Kaja and Abhinav Adhikari contributed equally. They performed initial experiments together, and later on, Amala Kaja completed the project with additional experiments including revision.

ABSTRACT FACT (facilitates chromatin transcription), an essential and evolutionarily conserved heterodimer from yeast to humans, controls transcription and is found to be upregulated in various cancers. However, the basis for such upregulation is not clearly understood. Our recent results deciphering a new ubiquitin-proteasome system regulation of the FACT subunit SPT16 in orchestrating transcription in yeast hint at the involvement of the proteasome in controlling FACT in humans, with a link to cancer. To test this, we carried out experiments in human embryonic kidney (HEK293) cells, which revealed that human SPT16 undergoes ubiquitylation and that its abundance is increased following inhibition of the proteolytic activity of the proteasome, thus implying proteasomal regulation of human SPT16. Furthermore, we find that the increased abundance/expression of SPT16 in HEK293 cells alters the transcription of genes, including ones associated with cancer, and that the proteasomal degradation of SPT16 is impaired in kidney cancer (Caki-2) cells to upregulate SPT16. Like human SPT16, murine SPT16 in C2C12 cells also undergoes ubiquitylation and proteasomal degradation to regulate transcription. Collectively, our results reveal a proteasomal regulation of mammalian SPT16, with physiological relevance in controlling transcription, and implicate such proteasomal control in the upregulation of SPT16 in cancer.

KEYWORDS gene expression, transcription, cancer, FACT, proteasome, SPT16

The eukaryotic genome is organized into chromatin that is an array of nucleosomes. Each nucleosome is composed of a histone H3-H4 tetramer and two histone H2A-H2B dimers forming a histone octamer wrapped by an ~147-bp DNA duplex. Such an organization with higher-order folding/compaction helps long genomic DNA fit inside the nucleus but obstructs other proteins to access DNA and, hence, poses an impediment to transcription (1). Therefore, the chromatin structure needs to be altered to allow transcription factors/machinery to access DNA for transcription. Chromatin alteration can occur via histone covalent modification, breakage of histone-DNA contacts, or histone eviction/nucleosomal disassembly (2–8). Following transcription, genomic DNA is repackaged via nucleosomal reassembly. Thus, nucleosomal disassembly and reassembly play important roles in regulating transcription, and such chromatin dynamics is controlled by histone chaperones. One such chaperone is an essential heterodimeric FACT (facilitates chromatin transcription) containing SPT16 and SSRP1 (9, 10). Although FACT is evolutionarily conserved among eukaryotes (9, 10), SSRP1's yeast homologue, Pob3, does not contain an HMG (high mobility group) domain. However, an HMG protein, Nhp6, associates with FACT in yeast to carry out the function of the HMG domain of SSRP1 (9, 10).

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Address correspondence to Suresh R. Bhaumik, sbhaumik@siu.edu.

* Present address: Abhinav Adhikari, Molecular Pathology Unit, Massachusetts General Hospital Research Institute, Charlestown, Massachusetts, USA.

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FACT was originally identified to be required for transcription elongation on chromatinized DNA templates *in vitro* (11). Consistently, it was found to travel with elongating RNA polymerase II at the coding sequence to facilitate transcription *in vivo* (12). FACT is also involved in transcription initiation through enhancement of nucleosomal disassembly at the promoter in facilitating TBP (TATA-box-binding protein) recruitment (and, hence, preinitiation complex formation) (9, 10, 12–14). In addition to its role in nucleosomal disassembly in promoting transcription, it also enhances nucleosomal reassembly at the coding sequence in the wake of elongating RNA polymerase II in order to suppress interfering cryptic transcription (15–17). Thus, FACT is involved in the regulation of transcription at the levels of nucleosomal disassembly and reassembly, and these functions are mediated via the interactions of both components of FACT with histone H2A-H2B dimers and H3-H4 tetramers (9, 10, 18–25).

Although the loss of FACT function alters transcription (9–17, 26), FACT is intriguingly found to be upregulated in several cancers (27–36). Increased abundances of FACT components have been shown to induce cellular transformation or oncogenesis (30). However, it is unclear how FACT is upregulated and how upregulated FACT affects transcription and induces oncogenesis. Toward addressing these questions, we carried out experiments in yeast (*Saccharomyces cerevisiae*) and found that the Spt16 subunit of FACT undergoes ubiquitylation by an E3 ubiquitin ligase, San1, and degradation by the 26S proteasome, hence deciphering a new proteasomal regulation of FACT (37). Furthermore, we found that increased abundance of Spt16 in the absence of proteasomal degradation alters transcription in yeast (37, 38). Such a mechanism might be present in humans to regulate FACT and alter the transcription of the genes associated with cancer. To test this, here, we analyzed the regulation of FACT by the proteasome, with physiological relevance in transcription in human and murine cells, and results are presented below.

RESULTS

SPT16 is regulated by the 26S proteasome in human embryonic kidney (HEK293) cells. As mentioned above, Spt16, a bona fide component of FACT, undergoes proteasomal degradation to control transcription in yeast (37). Since Spt16 and the 26S proteasome are conserved from yeast to humans, human SPT16 (expressed from the *SUPT16H* gene) is likely to be regulated by the proteasome. To test this, we analyzed the stability or abundance of SPT16 in human embryonic kidney (HEK293) cells in the presence and absence of a peptide aldehyde, MG132 (carbobenzoxymethyl-Leu-Leu-leucinal), which inhibits the proteolytic function of the 26S proteasome. If human SPT16 is targeted for 26S proteasomal degradation, the inhibition of the proteolytic function of the proteasome by MG132 would increase the stability/abundance of SPT16 in HEK293 cells. We found an increased abundance of SPT16 in HEK293 cells following proteasomal inhibition by 20 μ M MG132 for 6 or 10 h (Fig. 1A). However, a similar increase was not observed following treatment with dimethyl sulfoxide (DMSO) that was used to prepare the MG132 solution (Fig. 1A). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level was monitored as a loading control (Fig. 1A). Furthermore, our mRNA analysis in HEK293 cells following MG132 treatment did not reveal an increased *SUPT16H* mRNA level (Fig. 1B). Rather, the SPT16 protein level is increased following MG132 treatment (Fig. 1A). Additionally, we find that SPT16 stability is increased with MG132 treatment following translational inhibition by cycloheximide (Fig. 1C). Thus, our results support the proteasomal regulation of human SPT16. We also find a similar inhibition of the proteasomal degradation of SPT16 in HEK293 cells using 10 μ M MG132 for 6 h (Fig. 1D), and tubulin was used as a loading control (Fig. 1D). However, the other component of FACT (i.e., SSRP1) is not similarly regulated by the proteasome (Fig. 1A and D).

Since ubiquitylated proteins are targeted for proteasomal degradation (39, 40), SPT16 is likely to be ubiquitylated, similar to our results in yeast (37). To test this, we analyzed the ubiquitylation status of SPT16 in HEK293 cells. For this purpose, we introduced a plasmid expressing hemagglutinin (HA)-tagged ubiquitin by transient

FIG 1 Proteasomal regulation of SPT16 in HEK293 cells. (A) Inhibition of the proteolytic activity of the 26S proteasome by 20 μ M MG132 for 6 and 10 h increases the stability/abundance of SPT16. Data from WB analysis of SPT16, SSRP1, and GAPDH with or without treatment with MG132 (Sigma-Aldrich) and DMSO (Fisher) are shown. When cells reached 80% confluence, they were treated with MG132/DMSO for the indicated times before harvesting. Proteins were extracted according to the procedure described in Materials and Methods, and 30 μ g of the protein extract was analyzed by SDS-PAGE and WB using anti-SPT16 (catalogue no. 12191; Cell Signaling Technology), anti-SSRP1 (catalogue no. 60970; BioLegend), and anti-GAPDH (catalogue no. MAB374; Millipore) antibodies. The fold changes of the WB signals following 6- and 10-h treatment with MG132 are shown in (A). (B) *SUPT16H* mRNA levels were not significantly changed by MG132 treatment. (C) Cycloheximide treatment for 3 h reduces SPT16 levels, which is partially rescued by MG132. (D) MG132 treatment for 6 h increases SPT16 and SSRP1 levels. (E) Ubiquitination of SPT16 is inhibited by MG132. (Continued on next page)

transfection in HEK293 cells, grew the cells for 42 h, and then collected the cells following 10 μ M MG132 treatment for 6 h. As a control, HEK293 cells were transfected with an empty plasmid (that does not express HA-tagged ubiquitin) and were similarly grown and treated with MG132. Subsequently, whole-cell extract was prepared, followed by immunoprecipitation using an anti-SPT16 antibody. The immunoprecipitate was analyzed by Western blotting (WB) for the presence of ubiquitylated SPT16 using an anti-HA-horseradish peroxidase (HRP) antibody. We observed ubiquitylated SPT16 in the immunoprecipitate (Fig. 1E). Thus, like in yeast, human SPT16 undergoes ubiquitylation and proteasomal degradation.

Increased expression/abundance of SPT16 alters transcription in HEK293 cells.

Impairment of proteasomal degradation of human SPT16 would increase its level/abundance. Such an increase of human SPT16 abundance might affect transcription, as our previous studies in yeast (37, 38) demonstrated that increased abundance of Spt16 in the absence of proteasomal degradation alters transcription. To test this, we analyzed the effect of the high abundance/expression of SPT16 on transcription in HEK293 cells. In this direction, we transfected HEK293 cells with a plasmid (pCMV-4-SPT16) expressing murine SPT16 (which shows 98.76% similarity to human SPT16) (Fig. 2A) and then analyzed SPT16 and SSRP1 protein and mRNA levels at 48 h posttransfection. We also analyzed the mRNA levels of cell cycle genes under this condition, as an increased abundance of SPT16 might be associated with altered cell cycle in cancer. We find that the level of SPT16, but not SSRP1, is dramatically increased following the expression of SPT16 from a plasmid (Fig. 2B). Tubulin was monitored as a loading control (Fig. 2B). Likewise, the mRNA level of *SUPT16H*, but not *SSRP1*, was increased following transient transfection of the plasmid expressing SPT16 (Fig. 2C). Under this condition, the transcription of the cell cycle genes (e.g., *CCND1*, *CDK2*, *CDK4*, and *PCNA*) is increased (Fig. 2C and D). Thus, increased expression/abundance of human SPT16 alters transcription. Therefore, impaired proteasomal degradation of human SPT16 would change transcription via an increased abundance of SPT16. Hence, our results support the physiological relevance of the proteasomal regulation of human SPT16 in controlling transcription. Since *CCND1*, *CDK2*, *CDK4*, and *PCNA* are involved in cell cycle progression, enhanced expression of these genes following increased expression/abundance of SPT16 could promote cellular growth. Indeed, we find enhanced growth of HEK293 cells following increased expression/abundance of SPT16 under transient-transfection conditions at 48 h (Fig. 2E). Together, our results indicate that impairment of proteasomal degradation enhances SPT16 abundance, which in turn increases the transcription of cell cycle progression genes and, hence, cellular growth.

SPT16 in murine C2C12 cells undergoes proteasomal degradation to regulate transcription. The above-described results (Fig. 1) and previous studies (37) suggest evolutionarily conserved proteasomal regulation of Spt16 from yeast to humans. Thus, murine SPT16 is likely to be regulated by the 26S proteasome in controlling transcrip-

FIG 1 Legend (Continued)

10-h treatments with MG132 in comparison to the corresponding DMSO treatments are presented below the bands. (B) RNA analysis. HEK293 cells were grown to 80% confluence before treatment with 20 μ M MG132 for 10 h. DMSO-treated cells were used as a control by the addition of the equal volume of DMSO as the MG132 solution. Following the treatments, RNA was isolated. A total of 2 μ g RNA was used for cDNA synthesis. Subsequently, RT-PCR was performed to measure relative gene expression as described in Materials and Methods. ns, not significant. (C) SPT16 levels following 3 h of treatment with cycloheximide (100 μ g/ml) in the presence and absence of MG132 (20 μ M). The whole-cell extract was sonicated for 30 s (at an amplitude of 40 for 10-s pulses [with incubation in ice for 2 min between pulses] using a Misonix S-4000 sonicator) prior to collecting the clear lysate following centrifugation at 14,000 rpm for 10 min at 4°C. An anti-SPT16 antibody from BioLegend (catalogue no. 607001) was used in the WB analysis. SPT16 levels are plotted in the form of a histogram in the bottom panel. *, $P < 0.05$. (D) Inhibition of the proteolytic activity of the 26S proteasome by 10 μ M MG132 for 6 h increases the stability/abundance of SPT16. The whole-cell extract was prepared as described above for panel C, and 20 μ g of the sonicated protein extract was used for SDS-PAGE and WB analysis. An antitubulin antibody from the Developmental Studies Hybridoma Bank (DSHB) (catalogue no. E7) was used in the WB analysis. (E) Ubiquitylation analysis of SPT16 in HEK293 cells under high-stringency conditions, as described in Materials and Methods. HEK293 cells were transfected with a plasmid expressing HA-tagged ubiquitin (HA-Ub) or an empty plasmid that does not express HA-tagged ubiquitin and treated with 10 μ M MG132 for 6 h at 42 h posttransfection, and subsequently, the whole-cell extract was prepared for immunoprecipitation (IP) to pull down SPT16 using an anti-SPT16 antibody. Immunoprecipitates were then analyzed by WB using anti-HA-HRP and anti-SPT16 antibodies. +, HEK293 cells with a plasmid expressing HA-tagged SPT16; –, HEK293 cells with an empty plasmid; Ub_n, polyubiquitin. * represents degraded fragments of SPT16.

FIG 2 Increased abundance/expression of SPT16 in HEK293 cells alters transcription. (A) SPT16 identity matrix in yeast, mouse, and human using the ClustalO multiple-sequence alignment tool (EMBL-EBI) with default settings. The amino acid sequences for each species were downloaded from the Ensembl genome browser. *M. musculus*, *Mus musculus*; *H. sapiens*, *Homo sapiens*. (B) Increased abundance of SPT16 in HEK293 cells following transient transfection with a plasmid (pCMV-4-SPT16) expressing murine SPT16 (pSpt16) or an empty plasmid (that does not express SPT16) (Empty or pEmpty). The protein extract was prepared as described in the legend of Fig. 1C, and 2 μ g protein extract was used for SDS-PAGE for WB analysis. (C) Increased expression of *SUPT16H* (which codes for SPT16) and *CCND1*, but not *SSRP1*, in HEK293 cells following transient transfection with a plasmid expressing SPT16. RNA was quantified using NanoDrop One (Thermo Fisher), and 2 μ g RNA was used to prepare a cDNA template for quantitative PCRs. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. (D) Increased expression of *CDK2*, *CDK4*, and *PCNA* in HEK293 cells following transient transfection with a plasmid expressing SPT16. (E) Increased growth of HEK293 cells following increased expression of SPT16 by transient transfection.

tion. To test this, we analyzed the effect of inhibition of the proteolytic function of the 26S proteasome by MG132 (20 μ M for 12 h) on SPT16 in murine C2C12 cells. We found an increased abundance of SPT16 following proteasomal inhibition in C2C12 cells (Fig. 3A). The level of the tubulin loading control was not altered following proteasomal inhibition (Fig. 3A). Furthermore, proteasomal inhibition did not increase the *Supt16h* mRNA level (Fig. 3B) but rather enhanced the SPT16 protein level (Fig. 3A). Thus, our results support the proteasomal regulation of murine SPT16. However, the SSRP1 component of murine FACT is not similarly regulated by the 26S proteasome (Fig. 3A). Thus, like in human and yeast cells, SPT16 is regulated by the proteasome in murine cells. Therefore, murine SPT16 is likely to be ubiquitinated, similar to the results in yeast (37) and humans (Fig. 1E). To test this, we analyzed the ubiquitylation status of SPT16 in C2C12 cells by the introduction of a plasmid expressing HA-tagged ubiquitin and subsequent immunoprecipitation using an anti-SPT16 antibody. The immunoprecipitate was analyzed for ubiquitinated SPT16 by Western blotting using an anti-HA-HRP antibody. Such analysis revealed SPT16 ubiquitylation (Fig. 3C). Thus, murine SPT16 undergoes ubiquitylation and proteasomal degradation, similar to the results in yeast (37) and humans (Fig. 1).

Next, we analyzed the physiological relevance of the proteasomal regulation of murine SPT16 in transcription. In this direction, we increased the abundance/expression of SPT16 in C2C12 cells via transfection of the plasmid (pCMV-4-SPT16) expressing murine SPT16 (as impairment of proteasomal degradation would upregulate SPT16) and then analyzed the effect of enhanced SPT16 abundance/expression on transcription by monitoring the mRNA levels of *Supt16h*, *Ssrp1*, cell cycle genes, and myogenic factor genes (e.g., *Ccnd1*, *Ccne1*, *Myod1*, *Myog*, and *Tnni2* [41–45]) at 48 h posttransfection. We find that the SPT16, but not the SSRP1, protein level was increased following transient transfection of the plasmid expressing SPT16 (Fig. 3D). Likewise, the mRNA level of *Supt16h*, but not *Ssrp1*, was increased following the exogenous expression of SPT16 from the plasmid (Fig. 3E). Under this condition, the transcription of *Ccnd1* and *Ccne1* was increased (Fig. 3F), while other muscle genes such as *Myod1*, *Myog*, and *Tnni2* were downregulated (Fig. 3E). Thus, increased expression/abundance of SPT16 alters transcription in the murine system. Since CCND1 and CCNE1 are involved in the positive regulation of cell cycle progression, enhanced expression of CCND1 and CCNE1 following increased expression/abundance of SPT16 would promote cellular growth. Indeed, we find enhanced growth of C2C12 cells following increased expression/abundance of SPT16 (Fig. 3G). Furthermore, decreased transcription of cell differentiation genes such as *Myod1*, *Myog*, and *Tnni2* in response to increased expression of SPT16 (Fig. 3E) would impede cellular differentiation. Indeed, the SPT16 abundance/expression level is high in the undifferentiated proliferative stage of myoblasts such as C2C12 cells, and expression declines as the cells differentiate into myotubes (41, 42). Thus, our results indicate that the increased abundance of SPT16 keeps the cell in an undifferentiated or poorly differentiated state via the downregulation of cell differentiation genes. Collectively, our results demonstrate the proteasomal regulation of murine SPT16, with physiological relevance in controlling transcription, consistent with the results in yeast (37) and human cells (Fig. 1 and 2).

Proteasomal degradation of SPT16 is impaired in kidney cancer (Caki-2) cells.

Our results in yeast, HEK293, and C2C12 cells suggest that proteasomal degradation of SPT16 might be impaired in cancer cells, thus increasing the abundance of SPT16. To test this, we analyzed the level of SPT16 in kidney cancer (Caki-2) cells following proteasomal inhibition by MG132 and found that proteasomal inhibition did not increase the abundance of SPT16 in Caki-2 cells (Fig. 4A). Likewise, the *SUPT16H* mRNA level was not changed following proteasomal inhibition in Caki-2 cells (Fig. 4B). However, the abundance of SPT16 in HEK293 cells was increased following proteasomal inhibition by MG132 (Fig. 1A to D). It could be possible that Caki-2 cells are not responsive to MG132 due to multidrug resistance. To address this, we analyzed the abundance/stability of p53 (which is known to be regulated by the proteasome [46] but does not

FIG 3 Proteasomal regulation of SPT16 in C2C12 cells in controlling transcription. (A) Inhibition of the proteolytic activity of the 26S proteasome by MG132 increases the stability/abundance of SPT16. Protein
(Continued on next page)

control SPT16 abundance [47]) in Caki-2 cells following MG132 treatment. We find an increased abundance of p53 in Caki-2 cells following MG132 treatment (Fig. 4A). These results support that Caki-2 cells are sensitive to MG132, and the abundance/stability of SPT16 is not increased in Caki-2 cells following proteasomal inhibition by MG132. This could be due to the fact that the Ubiquitin-Proteasome System (UPS) for SPT16 is impaired or downregulated in these kidney cancer cells, and hence, proteasomal inhibition did not further enhance the stability/abundance of SPT16 in Caki-2 cells. Similar results are also found in pancreatic cancer cells (Fig. 4C and D). We find that the SPT16 level is increased in normal pancreatic (human pancreatic nestin-expressing [HPNE]) cells following proteasomal inhibition by MG132 (Fig. 4D). However, proteasomal inhibition did not increase the SPT16 level in pancreatic cancer (Panc1) cells (Fig. 4C) that are not resistant to MG132 (as p53 stability/abundance in Panc1 cells was increased in response to MG132 treatment [Fig. 4C]). Thus, as in kidney cancer cells, proteasomal degradation of SPT16 is impaired in pancreatic cancer cells.

Increased expression/abundance of SPT16 in HEK293 cells alters the transcription of genes associated with cancer. To determine the effect of a high level of SPT16 in the absence of proteasomal degradation on transcription with a link to cancer, we analyzed genome-wide transcription in HEK293 cells following increased abundance/expression of SPT16. In this direction, we isolated RNAs from HEK293 cells after 48 h following the transient transfection of a plasmid (pCMV-4-SPT16) expressing SPT16 or an empty plasmid and then performed RNA-seq (RNA sequencing) analysis. We find an alteration of the transcription of 99 genes (>1.5 -fold) in response to the increased expression (Fig. 2C and Fig. 5A) or abundance (Fig. 2B) of SPT16 in HEK293 cells (Fig. 5B and C; see also Tables S1 to S3 in the supplemental material). Next, we analyzed whether these genes are linked to kidney cancer, using the cancer patient database in cBioPortal (<https://www.cbioportal.org/>) (48–51). Our analysis revealed that up- and downregulated genes in response to increased expression/abundance of SPT16 (Fig. 5B and C; Tables S1 and S2) are linked to clear and non-clear cell kidney cancers (Fig. 6A), and a set of the upregulated genes is correlated with a decrease in the survival of renal clear cell carcinoma patients (Fig. 6B). In agreement, the kidney cancer patient database of UALCAN (<http://ualcan.path.uab.edu/>) (52, 53) also showed up- as well as downregulation of many genes that matched those in our RNA-seq analysis in response to increased SPT16 expression/abundance in HEK293 cells (Table S4). Thus, the increased expression/abundance of SPT16 altered the expression of the genes (Fig. 5B and C; Tables S1 and S2) that are linked to kidney cancer (Fig. 6A and B; Table S4). Hence, the upregulation of SPT16 in the absence of proteasomal degradation would alter the expression of the genes associated with renal cancer. Indeed, proteasomal degradation of SPT16 was found to be impaired in kidney cancer (Caki-2) cells (Fig. 4A and B), and such an impairment would upregulate SPT16 to alter the transcription of the genes associated with renal cancer (Fig. 6A and B). In agreement, a high level of SPT16 is found in renal cancer patients (Fig. 6C).

In addition to the impairment of proteasomal degradation, the SPT16 level can also be increased via gene amplification in renal cancer. However, our cBioPortal analysis

FIG 3 Legend (Continued)

extraction and WB were performed as described in the legend to Fig. 1A. An anti-SPT16 antibody from BioLegend was used in the WB analysis. (B) RNA analysis. C2C12 cells were grown to 80% confluence before treatment with 20 μ M MG132 for 12 h. DMSO-treated cells were used as a control by the addition of an equal volume of DMSO as the MG132 solution. Following the treatments, RNA was isolated. A total of 2 μ g RNA was used for cDNA synthesis. Subsequently, RT-PCR was performed to measure relative gene expression as described in Materials and Methods. (C) Ubiquitylation analysis of SPT16 in C2C12 cells. C2C12 cells were transfected with a plasmid expressing HA-tagged ubiquitin or an empty plasmid. Immunoprecipitation was performed using an anti-SPT16 antibody and analyzed by WB using anti-HA-HRP and anti-SPT16 antibodies. (D) Increased abundance of SPT16 in C2C12 cells following transient transfection with a plasmid (pCMV-4-SPT16) expressing murine SPT16. The whole-cell extract was prepared as described in the legend to Fig. 1C. (E) Increased expression of *Supt16h* but not *Ssrp1* and decreased expression of *Myod1*, *Myog*, and *Tnni2* in C2C12 cells following transient transfection with a plasmid expressing SPT16. (F) Increased expression of *Ccnd1* and *Ccne1* in C2C12 cells following transient transfection with a plasmid expressing SPT16. (G) Increased growth of C2C12 cells following increased expression of SPT16 by transient transfection. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

FIG 4 Analysis of SPT16 in cancer cells in response to MG132 treatment. (A) WB analysis of SPT16, SSRP1, p53, and GAPDH with or without treatment with MG132 and DMSO in renal cancer (Caki-2) cells. "0 h" represents no DMSO or MG132 treatment. (B) RNA analysis in Caki-2 cells. The *SPT16H* mRNA level was normalized to the GAPDH level. (C) WB analysis of SPT16, SSRP1, p53, and GAPDH with or without treatment with MG132 and DMSO in pancreatic cancer (Panc1) cells. (D) WB analysis of SPT16 and GAPDH with or without treatment with MG132 and DMSO in normal pancreatic (HPNE) cells.

FIG 5 RNA-seq analysis in response to increased expression of SPT16 in HEK293 cells. (A) Increased expression of *SUPT16H* in HEK293 cells following transient transfection with a plasmid expressing SPT16 at 48 h. **, $P < 0.01$. (Continued on next page)

did not reveal the amplification of *SUPT16H* in renal cancer but rather showed depletion (see below) (Fig. 7A). Consistently, the *SUPT16H* mRNA level is not increased in renal tumor samples in comparison to normal samples (Fig. 6D). However, the SPT16 protein level is high in renal tumor samples (Fig. 6C). These observations suggest that SPT16 could be regulated at the protein level in renal cancer. Indeed, we find here that the SPT16 protein level is regulated by the proteasome (Fig. 1A to D, Fig. 3A and B, and Fig. 4D), and such regulation is impaired in Caki-2 kidney cancer cells (Fig. 4A and B), thereby leading to its increased abundance. Thus, our results provide a new mechanism of SPT16 upregulation via the proteasome, independently of *SUPT16H* gene amplification.

While renal cancer patients in cBioPortal did not show *SUPT16H* amplification, SPT16 expression was found to be increased via gene amplification in a number of other cancers, as revealed by cBioPortal analysis (Fig. 7). Therefore, differentially expressed genes in response to increased expression of SPT16 are likely to be associated with other cancers. Indeed, the above-mentioned up- and downregulated genes in response to increased SPT16 expression/abundance are also found to be linked to other cancers such as breast, prostate, pancreas, head, neck, and ovarian cancers (cBioPortal, The Human Protein Atlas [THPA], [The Cancer Genome Atlas [TCGA], and UALCAN). Thus, our RNA-seq analysis indicates that the upregulation of SPT16 in HEK293 cells alters the expression of the genes associated with cancer.

Our RNA-seq analysis revealed 99 differentially expressed genes with a 1.5-fold cutoff in response to the increased expression/abundance of SPT16 in HEK293 cells. To identify additional genes, we performed RNA-seq analysis with a 1.2-fold cutoff and found another 85 statistically significant genes with altered expression (Table S5), and a set of these genes is associated with cancer (cBioPortal, THPA, TCGA, and UALCAN). Thus, RNA-seq analysis with a 1.2-fold cutoff in response to the increased expression/abundance of SPT16 in HEK293 cells revealed 184 differentially expressed genes. We next analyzed the involvement of these genes in other biological processes, using the Gene Ontology (GO) Web resource. Our GO analysis revealed that genes with altered transcription in response to increased abundance/expression of SPT16 are associated with tissue development, myotube development, regulation of muscle cell differentiation, and other related organ development processes (Fig. 8A and B). Likewise, our pathway analysis from another Web-based tool, ShinyGO, also yielded pathways involved in development such as muscle cell differentiation, muscle fiber development, myotube differentiation, and other related processes (Fig. 8C). In addition, we observed an enrichment of cellular component morphogenesis, animal organ development, and cell development pathways. These results allude to the role of SPT16 in cellular growth and development and are in line with our observation of enhanced growth following increased expression/abundance of SPT16. Thus, increased expression/abundance of SPT16 would also affect cellular differentiation and development processes, which need to be further elucidated. Nonetheless, our results indicate that the increased expression/abundance of SPT16 alters the transcription of a set of genes associated with cancer, cellular growth, and development.

DISCUSSION

Here, we show that the abundance of SPT16 is increased in human and murine cells when the proteolytic activity of the proteasome is inhibited. Furthermore, SPT16 in these cells is found to be ubiquitinated. These results demonstrate a new regulation of mammalian SPT16 by the UPS. Hence, downregulation or impairment of the proteasomal degradation of SPT16 would increase its abundance. We find that increased abun-

FIG 5 Legend (Continued)

(B) Heat map for altered transcripts of genes by >1.5-fold in response to increased expression of SPT16 in HEK293 cells. SA001, SA003, and SA005 represent biological triplicates of HEK293 cells transfected with an empty plasmid. HEK293 cells were transfected with pCMV-4-SPT16 expressing SPT16 in biological triplicates, SA002, SA004, and SA006. (C) Volcano plot for altered transcripts of genes by >1.5-fold in response to increased expression of SPT16 in HEK293 cells.

FIG 6 Correlation of up- and downregulated genes in response to increased expression/abundance of SPT16 with kidney cancer. (A) The renal cancer patient database in cBioPortal shows that 74.72%, 90.72%, and 95.45% of renal clear cell carcinoma (The Cancer Genome Atlas [TCGA], (Continued on next page)

dance/expression of SPT16 in human and murine cells alters transcription, therefore supporting the physiological relevance of the proteasomal regulation of SPT16 in controlling transcription. Furthermore, we observe that the proteasomal degradation of SPT16 is impaired in cancer cells, and the upregulation of SPT16 alters the transcription of the genes associated with cancer. These results implicate the impairment of the proteasomal degradation of SPT16 to its upregulation in altering the expression of genes linked to cancer. Collectively, our results reveal a novel proteasomal regulation of mammalian gene expression via SPT16, with implications in cancer.

We recently demonstrated that SPT16's yeast homologue undergoes proteasomal degradation (37). Since yeast Spt16 shows 37.63% similarity to human SPT16 (Fig. 2A), it is not clear whether human SPT16 can undergo proteasomal degradation, similar to yeast Spt16. Here, we show that, like in yeast, human SPT16 undergoes proteasomal degradation (Fig. 1A to D). Likewise, murine SPT16 also undergoes proteasomal degradation (Fig. 3A and B). Thus, proteasomal regulation of SPT16 appears to be conserved from yeast to humans. Impairment of the proteasomal degradation of SPT16 would increase its abundance (37), and such an enhanced abundance has been shown to alter transcription in yeast (37). Consistently, we find that increased abundance/expression of SPT16 in human and murine cells alters transcription (Fig. 2B to D, Fig. 3D to F, and Fig. 5A to C). Therefore, the proteasomal regulation of SPT16 in controlling transcription appears to be conserved among eukaryotes.

We find that enhanced abundance/expression of SPT16 increases as well as decreases transcription in both human and murine cells (Fig. 2B to D, Fig. 3D to F, and Fig. 5A to C). However, it is not clear whether both upregulation and downregulation of transcription are the direct effects of the increased abundance/expression of SPT16 or indirectly regulated. It is possible that a set of genes directly regulated by the increased expression of SPT16 controls another set of genes. It is also possible that increased expression of SPT16 contributes to antisense or noncoding RNA transcription, which could affect sense transcription, as antisense transcription antagonizes sense transcription (54, 55). In support of this possibility, a recent study in *Schizosaccharomyces pombe* indicated the role of Spt16 in the regulation of antisense transcription (56). Furthermore, our RNA-seq analysis also revealed alteration of noncoding RNA expression in response to increased expression of SPT16 (see Tables S3 and S5 in the supplemental material). In addition, SPT16 was recently found to interact with noncoding RNA in human cells (57). Moreover, our recent study in *S. cerevisiae* demonstrated the impairment of chromatin reassembly at the coding sequence in the presence of high expression/abundance of Spt16 (37), and such a defect in chromatin reassembly can promote cryptic transcription that can interfere with sense transcription (12, 58–62). Thus, the proteasomal regulation of mammalian SPT16 controls transcription via as-yet-unknown mechanisms. It is possible that excess SPT16 in the absence of proteasomal degradation is working outside FACT or via the formation of a FACT variant containing extra SPT16, which remains to be further elucidated. Nonetheless, our results demonstrate a new regulatory pathway of mammalian gene expression via the proteasomal regulation of SPT16.

Our genome-wide RNA-seq analysis revealed a set of genes with altered transcription in response to increased expression/abundance of SPT16 in HEK293 cells,

FIG 6 Legend (Continued)

Firehose Legacy), renal papillary cell carcinoma (TCGA, Firehose Legacy), and kidney chromophobe (TCGA) (51) patients, respectively, are linked to differentially expressed genes in response to increased expression/abundance of SPT16 in HEK293 cells. Z values of greater and less than 2 were used for upregulated and downregulated genes, respectively, that were obtained from our RNA-seq analysis. The mRNA expression Z-scores relative to diploid samples (RNA Seq V2 RSEM) were used in the cBioPortal analysis. (B) Overall survival analysis of kidney cancer patients in cBioPortal. A set of upregulated genes in response to increased SPT16 expression/abundance in HEK293 cells is correlated with decreased survival of renal clear cell carcinoma patients (TCGA, Firehose Legacy). (C) SPT16 levels in kidney cancer patients were analyzed using the UALCAN Web portal. This Web portal utilizes proteomics data from the National Cancer Institute (NCI)-sponsored Clinical Proteomic Tumor Analysis Consortium (CPTAC) (53). The Z values for SPT16 in normal and clear cell renal cell carcinoma samples (CPTAC) are plotted. n, number of samples. (D) *SUPT16H* mRNA levels were analyzed in kidney cancer patients based on TCGA using the UALCAN Web portal. The *SUPT16H* transcripts in normal and kidney renal clear cell carcinoma samples (TCGA) are plotted.

FIG 7 Analysis of SPT16 in different cancers. (A) Cross-cancer analysis of copy number alterations in *SPT16H* based on data in the cBioPortal database. All patient samples (a total of 70,868 patients/74,204 samples from 239 studies) were analyzed in cBioPortal. CNA, copy number alteration; NOS, not otherwise specified. (B) *SPT16H* mRNA levels in response to copy number alterations in *SPT16H* in cBioPortal. All patient samples (a total of 70,868 patients/74,204 samples from 239 studies) were analyzed in cBioPortal. A putative copy number from GISTIC 2.0 was used. A value of -2 indicates homozygous deletion, -1 indicates hemizygous deletion, 0 indicates neutral/no change, 1 indicates gain, and 2 indicates high-level amplification. (C) SPT16 levels were analyzed in breast, ovarian, and colon cancer patients based on CPTAC samples using the UALCAN Web portal. The Z values for SPT16 in normal and cancer samples (CPTAC) are plotted.

with links to cancer. Since SPT16's abundance is upregulated following impairment of proteasomal degradation, it is likely that proteasomal degradation of SPT16 is impaired in cancer cells to upregulate SPT16 in altering the transcription of genes associated with cancer. Indeed, we find that the proteasomal degradation of SPT16 is impaired in cancer cells (Fig. 4). Thus, our results implicate a new pathway for SPT16 regulation at the level of proteasomal degradation with a link to cancer. Previous studies demonstrated an increased abundance or upregulation of both SPT16 and SSRP1 components of FACT in cancer (27, 30). However, such an increase is mediated by SSRP1 and SPT16 mRNAs (34, 47), and the role of the proteasome in the regulation of SPT16 and SSRP1 was unknown until this study.

Our results reveal that the upregulation of only the SPT16, but not the SSRP1, component of FACT (Fig. 2B and C and Fig. 5A) alters the transcription of genes associated

FIG 8 Pathway analysis in response to increased expression of SPT16 in HEK293 cells. (A and B) *In silico* pathway analysis of differentially expressed genes in response to increased expression of SPT16 in HEK293 cells, using the GO Web resource. (A) Pathway analysis for upregulated genes; (B) pathway analysis for downregulated genes. (C) Pathway analysis of the upregulated genes in response to increased expression of SPT16 in HEK293 cells, using the ShinyGO Web tool.

with cancer (Fig. 5B and C and Fig. 6A to D; Tables S1 to S4). Furthermore, cellular growth is enhanced in response to transiently increased expression of SPT16 in both human and murine cells (Fig. 2E and Fig. 3G). However, such increased growth could be more dramatic over longer time periods following the overexpression of SPT16 under the control of an inducible promoter in a stable cell line. Nonetheless, our results suggest that enhanced expression/abundance of only the SPT16, but not the SSRP1, component of FACT can be associated with cancer. Indeed, the cancer patient database in cBioPortal revealed that SPT16, but not SSRP1, was amplified in a number of cancers such as cervical adenocarcinoma (2 of 46 patients; 4.35%), B lymphoblastic leukemia/lymphoma (26 of 818 patients; 3.18%), colon cancer (1 of 7 patients; 14.29%), stomach adenocarcinoma (4 of 211 patients; 1.9%), adrenocortical carcinoma (1 of 90 patients; 1.11%), leukemia (2 of 441 patients; 0.45%), germ cell tumor (1 of 331 patients; 0.3%), salivary cancer (2 of 902 patients; 0.22%), and cervical squamous cell carcinoma (1 of 247 patients; 0.04%). While SPT16 is upregulated in the above-mentioned cancer patients via gene amplification, it is unknown whether the upregulation of SPT16 in these and other cancer patients can occur via impairment of its proteasomal degradation, which remains to be further investigated toward searching for a new mechanism of cancer.

In summary, our results support the proteasomal regulation of SPT16 in human and murine cells (Fig. 1A to D and Fig. 3A and B). Consistently, SPT16 is found to be ubiquitinated in these cells (Fig. 1E and Fig. 3C). Impairment or downregulation of such UPS regulation of SPT16 would elevate its abundance to change the expression of a number of genes, and altered expression of a set of these genes is associated with cancer. Thus, our results provide a new regulatory pathway of mammalian gene expression via SPT16, with implications in cancer. However, how SPT16 undergoes ubiquitylation and proteasomal degradation remains to be further elucidated. In addition, the factors (e.g., E3 ubiquitin ligase, E2 ubiquitin conjugase, and ubiquitin protease) involved in UPS regulation of SPT16 are yet unknown, which (via mutations/malfunctions) might be involved in cancer by regulating SPT16's proteasomal degradation or abundance. Therefore, our results would open an important avenue of future research in identifying new factors involved in the regulation of SPT16's abundance in controlling transcription, with implications in oncogenesis.

MATERIALS AND METHODS

Cell lines and growth media. HEK293 (human), C2C12 (murine), Caki-2 (human renal cancer), and Panc1 (human pancreatic cancer) cell lines used in this study were obtained from the ATCC. hTERT-HPNE (human pancreatic cell line) was obtained from the Batra laboratory (University of Nebraska Medical Center, Omaha, NE, USA). HEK293, C2C12, and Panc1 cells were grown in Dulbecco's modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (FBS; HyClone) and penicillin (100 U/ml)-streptomycin (100 μ g/ml) antibiotics (Corning) in a humidified CO₂ incubator at 37°C according to standard protocols. hTERT-HPNE cells were grown according to ATCC recommendations in a medium containing 3 parts DMEM without glucose (Sigma) (additional 2 mM L-glutamine and 1.5 g/liter sodium bicarbonate) and 1 part medium M3 base (Incell Corp.) with 5% FBS, 5.5 mM D-glucose, 10 ng/ml human recombinant epidermal growth factor (EGF), 750 ng/ml puromycin, and penicillin (100 U/ml)-streptomycin (100 μ g/ml). Caki-2 cells were cultured in McCoy's 5a medium modified with 10% FBS and penicillin (100 U/ml)-streptomycin (100 μ g/ml). Cells were subcultured using trypsin according to standard procedures. To maintain the culture, cells were split when they reached 80% confluence by treating them with trypsin-EDTA (0.25% and 0.01%, respectively).

Proteasomal inhibition by MG132. For inhibition of the proteolytic function of the 26S proteasome, cells were grown to 80% confluence and subsequently treated with MG132 or an equivalent volume of DMSO at 37°C. For analysis of SPT16 upon translation inhibition with or without MG132 treatment, cells were grown to 80% confluence and subsequently treated with cycloheximide (Sigma-Aldrich) for 3 h at 37°C with or without MG132 treatment.

Plasmids. The plasmid pCMV-4-SPT16 was used to express murine SPT16. This plasmid is a gift of Takanori Kihara and Masayuki Murata (University of Tokyo, Japan) (63). The plasmid expressing HA-tagged ubiquitin was obtained from Manas Santra (National Centre for Cell Science, Pune, India) (64).

Cell transfection. The pCMV-4-SPT16 plasmid was transfected into HEK293 cells using the calcium phosphate method according to standard protocols. C2C12 cells were transfected with the pCMV-4-SPT16 plasmid using TurboFect (Fisher Scientific) according to the manufacturer's protocol. As a control plasmid, pCMV-Empty (Agilent) that does not express Spt16 was used. At 24 h posttransfection, the growth medium was replaced with fresh medium. Cells were harvested for RNA and protein analyses

48 h following transfection. A similar protocol was used for the transfection of a plasmid expressing HA-tagged ubiquitin into HEK293 and C2C12 cells.

Protein extraction. To harvest cells, growth medium was discarded, and cells were washed twice with ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.4]). Finally, cells were scraped in PBS and centrifuged at 6,000 rpm for 2 min at 4°C. The cell pellet was then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% NP-40, 1 mM EDTA) supplemented with protease inhibitors (Complete protease inhibitor; Roche Diagnostics) and dithiothreitol (DTT) (1 mM). Following a 30-min incubation in ice with intermittent vortexing (10 s, every 10 min), the clear supernatant was collected after centrifugation at 14,000 rpm for 10 min at 4°C. The protein concentration was determined by a Bradford assay (Bio-Rad).

Western blot analysis. Protein extracts were resolved on SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, membranes were blocked in 5% nonfat milk in TBST [Tris-buffered saline [10 mM Tris-HCl (pH 7.4), 0.9% NaCl] plus 0.1% [vol/vol] Tween 20] for at least an hour and then incubated with the primary antibody overnight at 4°C. Membranes were then washed with TBST three times prior to incubation with the secondary antibody conjugated with HRP at room temperature for a minimum of 2 h. Membranes were then washed again three times with TBST. The signal was detected with an ECL-Prime chemiluminescence substrate (Amersham) on an autoradiogram.

RNA isolation and RT-PCR. Total RNA isolation was performed using RiboZol RNA extraction reagent (VWR) according to the manufacturer's protocol. Following treatment with DNase (Promega), 2 µg of total RNA was reverse transcribed with MultiScribe murine leukemia virus (MuLV) reverse transcriptase (RT) (Applied Biosystems) to prepare cDNA for qPCR (quantitative PCR) amplification (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems). Samples with no RT were included for each RNA sample. The relative fold expression changes were calculated using the comparative threshold cycle (C_T) method (Applied Biosystems). Standard deviations (SD) of the means of the (Δ) C_T values were calculated from at least three independent RNA samples. Where possible, intron-spanning primers were used. For measurements of relative gene expression, a fold change was calculated for each sample pair and then normalized to the fold change observed for *HPRT1* (hypoxanthine phosphoribosyltransferase 1), 18S rRNA, or *GAPDH*. Primers used for RT-PCR in human and murine cells are as follows: 5'-CGCCGCTAGAGGTGAAATTCT-3' and 5'-CGAACCTCCGACTTTCGTCT-3' for 18S rRNA, 5'-CTGGATGCTGAGGCTCTG-3' and 5'-TCCACTTGAGCTTGTACC-3' for *CCND1*, 5'-CAAAGTGCACCA GTTGC-3' and 5'-ACATTCAGCCAGGACACAAT-3' for *CCNE1*, 5'-CTCTCCGGATCTTCGG-3' and 5'-ATCCTGGAAGAAAGGGTGAG-3' for *CDK2*, 5'-CAAGCTGGCTGACTTTGG-3' and 5'-GTCGGCTTCAGAG TTCCA-3' for *CDK4*, 5'-TGACACTGGCAAAACAATGCA-3' and 5'-GGTCCTTTCCACGCAAGCT-3' for *HPRT1*, 5'-ACCTGCAGAGCATGGACTC-3' and 5'-GCATATACGTGCAAATTCACC-3' for *PCNA*, 5'-GCCG GTGTGCATTCCTCA-3' and 5'-CACTCCGGAACCCCAACAG-3' for *MyoD1*, 5'-GACCTGATGGAGCTGTA TGAG-3' and 5'-CTGAAGGTGGACAGGAAGG-3' for *Myog*, 5'-GCCGCCGAGAATCTGAGA-3' and 5'-GACATGGAGCCTGGGATGTG-3' for *Tnni2*, 5'-AGAAAGCAGCCAGCATCAC-3' and 5'-CACCACCTGTAA TGATAGGAGG-3' for *SUPT16H*, and 5'-GCACCTAGTATACCTTCAGCAGC-3' and 5'-TGTCATTGGCATT CTCTCC-3' for *SSRP1*.

RNA-seq analysis. RNA was prepared under the same conditions as the ones mentioned above, and the RNA quality was confirmed via the Bioanalyzer system with samples showing an RNA integrity number (RIN) of at least 9.30. The total RNA was then depleted of rRNA using the Ribo-Zero removal kit (Epicentre). Libraries were prepared with the Illumina TruSeq stranded total RNA-Gold sample preparation kit and then sequenced with 80 million 2- by 100-bp paired raw passing filter reads on an Illumina Novaseq 6000 instrument at the J. P. Sulzberger Columbia Genome Center (Columbia University). Three biological replicates were performed and sequenced. We use RTA (Illumina) for base calling and bcl2fastq2 (version 2.20) for converting BCL to fastq format, coupled with adaptor trimming. We mapped the sequenced reads to the reference genome (hg38) by using hisat2 (version 2.1.0) (65) with default parameters. StringTie (version 1.3.3b) (66) was used to quantify the expression of genes with the parameter “-rf -e -G Homo_sapiens.GRCh38.91.chr.gtf,” where the gtf file was from Ensembl. The read count table of genes in each sample was estimated by using PrepDE (<http://ccb.jhu.edu/software/stringtie/dl/prepDE.py>). To identify the differentially expressed genes, we use DESeq2 (67), a method using shrinkage estimation for dispersions and fold changes to improve stability and interpretability of estimates. The volcano plot and the heat map of differentially expressed genes were generated using self-designed MATLAB codes. In the heat map, the fragments per kilobase per million (FPKM) values from the StringTie output were normalized to Z-scores for each gene across different samples and were hierarchically clustered using correlation distance. To quantify the isoforms of some genes, we used the same pipeline but a different gtf file (hg38.knownGene.gtf from UCSC).

Proliferation assay. Equal numbers of HEK293 and C2C12 cells were seeded into 60-mm plates. After 24 h, cells were transfected with an empty plasmid or the pCMV-SPT16 plasmid. Following transfection, cells were harvested for counting under a light microscope using a hemocytometer. Trypan blue staining was performed to determine cell viability. Cell counting was done in duplicates, repeated three times, and performed in a blind manner to avoid experimental bias. The average number of cells at the indicated time point was plotted along with the SD.

Ubiquitylation analysis. For the ubiquitination analysis, cells were transfected with either HA-tagged ubiquitin (pHA-Ub) or an empty plasmid (pcDNA3.1 V5/His). Five micrograms of the plasmid was used for transfection in HEK293 cells, while C2C12 cells were transfected with 10 µg of the plasmid. After 42 h, cells were treated with 10 µM MG132 for 6 h and harvested at 48 h. Cells were washed in

chilled PBS, harvested, and lysed in RIPA buffer with 10 mM *N*-ethylmaleimide (Sigma-Aldrich) and protease inhibitors (Roche Diagnostics). The lysate was vortexed for 10 s and placed in ice for 10 min (vortexing a total of 3 times). The lysates were then sonicated for 30 s (3 cycles of 10 s each) at an amplitude of 40. The sonicated lysate was spun down at $16,000 \times g$ at 4°C for 30 min. The supernatant was collected, and 1 mg of the total protein was used for immunoprecipitation with an anti-Spt16 antibody. Prior to immunoprecipitation, the extract was precleared with protein A-agarose beads at 4°C for 30 min. Five micrograms of antibody was used for each immunoprecipitation overnight, followed by incubation with equilibrated protein A-agarose beads. Immunoprecipitation was carried out in the presence of 300 mM NaCl in RIPA buffer, and agarose beads were washed 5 times. The first wash step was done using RIPA buffer containing 300 mM NaCl, and subsequently, the second and third wash steps were done using FA lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% Na-deoxycholate) with 1 M NaCl, the fourth wash step was done using FA-W3 buffer (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, and 1 mM EDTA), and the last wash step was done using RIPA buffer with 150 mM NaCl. Immunoprecipitates were eluted into 1.5× SDS gel loading dye at 95°C for 20 min. Samples were analyzed by SDS-PAGE and Western blotting using anti-HA-HRP or anti-SPT16 antibodies.

Pathway analysis. The GO Web resource (<http://geneontology.org/>) was used to categorize the differentially expressed genes. Differentially expressed genes in response to increased expression of SPT16 in HEK293 cells were used for pathway analyses. Fisher's exact test was used with a correction for the false discovery rate (FDR). The outcomes with an FDR-corrected *P* value of <0.05 are plotted in Fig. 8A and B.

To complement the results obtained from GO pathway analysis, we used ShinyGO, an online Web tool, which is based on a large annotation database derived from the Ensembl and STRING databases (68). Fisher's exact test was used with a correction for FDR. The outcomes with an FDR-corrected *P* value of <0.05 are shown in Fig. 8C. In the table in Fig. 8C, total genes indicate the number of genes annotated as belonging to a particular pathway in the ShinyGO database, whereas the genes in the list indicate the number of upregulated genes obtained from our RNA-seq results that belong to that particular pathway.

Statistical analysis. Data are presented as means $\pm$ SD. Statistical comparisons were performed using unpaired two-tailed Student's *t* tests. A *P* value of <0.05 was taken to indicate significance. All statistical analyses and graphs were made in MS Excel or GraphPad Prism 8.0 software.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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REFERENCES

- Li B, Carey M, Workman JL. 2007. The role of chromatin during transcription. *Cell* 128:707–719. <https://doi.org/10.1016/j.cell.2007.01.015>.
- Zhou K, Gaullier G, Luger K. 2019. Nucleosome structure and dynamics are coming of age. *Nat Struct Mol Biol* 26:3–13. <https://doi.org/10.1038/s41594-018-0166-x>.
- Henikoff S. 2008. Nucleosome destabilization in the epigenetic regulation of gene expression. *Nat Rev Genet* 9:15–26. <https://doi.org/10.1038/nrg2206>.
- Avvakumov N, Nourani A, Côté J. 2011. Histone chaperones: modulators of chromatin marks. *Mol Cell* 41:502–514. <https://doi.org/10.1016/j.molcel.2011.02.013>.
- Bhaumik SR, Smith E, Shilatifard A. 2007. Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol* 14:1008–1016. <https://doi.org/10.1038/nsmb1337>.
- Durairaj G, Malik S, Bhaumik SR. 2017. Eukaryotic gene expression by RNA polymerase II, p 1–28. In Mandal SS (ed), *Gene regulation, epigenetics, and hormone signaling*, vol 1. Wiley-Blackwell, Oxford, United Kingdom.
- Shukla A, Chaurasia P, Bhaumik SR. 2009. Histone methylation and ubiquitination with their cross-talk and roles in gene expression and stability. *Cell Mol Life Sci* 66:1419–1433. <https://doi.org/10.1007/s00018-008-8605-1>.
- Malik S, Bhaumik SR. 2010. Mixed lineage leukemia: histone H3 lysine 4 methyltransferases from yeast to human. *FEBS J* 277:1805–1821. <https://doi.org/10.1111/j.1742-4658.2010.07607.x>.
- Winkler DD, Luger K. 2011. The histone chaperone FACT: structural insights and mechanisms for nucleosome reorganization. *J Biol Chem* 286:18369–18374. <https://doi.org/10.1074/jbc.R110.180778>.
- Formosa T. 2013. The role of FACT in making and breaking nucleosomes. *Biochim Biophys Acta* 1819:247–255.
- Orphanides G, LeRoy G, Chang CH, Luse DS, Reinberg D. 1998. FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell* 92:105–116. [https://doi.org/10.1016/s0092-8674\(00\)80903-4](https://doi.org/10.1016/s0092-8674(00)80903-4).
- Mason PB, Struhl K. 2003. The FACT complex travels with elongating RNA polymerase II and is important for the fidelity of transcriptional initiation in vivo. *Mol Cell Biol* 23:8323–8333. <https://doi.org/10.1128/mcb.23.22.8323-8333.2003>.
- Biswas D, Yu Y, Prall M, Formosa T, Stillman DJ. 2005. The yeast FACT complex has a role in transcriptional initiation. *Mol Cell Biol* 25:5812–5822. <https://doi.org/10.1128/MCB.25.14.5812-5822.2005>.
- Ransom M, Williams SK, Dechassa ML, Das C, Linger J, Adkins M, Liu C, Bartholomew B, Tyler JK. 2009. FACT and the proteasome promote

- promoter chromatin disassembly and transcriptional initiation. *J Biol Chem* 284:23461–23471. <https://doi.org/10.1074/jbc.M109.019562>.
15. Schwabish MA, Struhl K. 2004. Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. *Mol Cell Biol* 24:10111–10117. <https://doi.org/10.1128/MCB.24.23.10111-10117.2004>.
 16. Pathak R, Singh P, Ananthakrishnan S, Adamczyk S, Schimmel O, Govind CK. 2018. Acetylation-dependent recruitment of the FACT complex and its role in regulating Pol II occupancy genome-wide in *Saccharomyces cerevisiae*. *Genetics* 209:743–756. <https://doi.org/10.1534/genetics.118.300943>.
 17. Fleming AB, Kao CF, Hillyer C, Pikaart M, Osley MA. 2008. H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. *Mol Cell* 31:57–66. <https://doi.org/10.1016/j.molcel.2008.04.025>.
 18. Liu Y, Zhou K, Zhang N, Wei H, Tan YZ, Zhang Z, Carragher B, Potter CS, D'Arcy S, Luger K. 2020. FACT caught in the act of manipulating the nucleosome. *Nature* 577:426–431. <https://doi.org/10.1038/s41586-019-1820-0>.
 19. Wang T, Liu Y, Edwards G, Krizike D, Scherman H, Luger K. 2018. The histone chaperone FACT modulates nucleosome structure by tethering its components. *Life Sci Alliance* 1:e201800107. <https://doi.org/10.26508/lsa.201800107>.
 20. Hsieh FK, Kulaeva OI, Patel SS, Dyer PN, Luger K, Reinberg D, Studitsky VM. 2013. Histone chaperone FACT action during transcription through chromatin by RNA polymerase II. *Proc Natl Acad Sci U S A* 110:7654–7659. <https://doi.org/10.1073/pnas.1222198110>.
 21. Winkler DD, Muthurajan UM, Hieb AR, Luger K. 2011. Histone chaperone FACT coordinates nucleosome interaction through multiple synergistic binding events. *J Biol Chem* 286:41883–41892. <https://doi.org/10.1074/jbc.M111.301465>.
 22. Tsunaka Y, Fujiwara Y, Oyama T, Hirose S, Morikawa K. 2016. Integrated molecular mechanism directing nucleosome reorganization by human FACT. *Genes Dev* 30:673–686. <https://doi.org/10.1101/gad.274183.115>.
 23. Valieva ME, Armeev GA, Kudryashova KS, Gerasimova NS, Shaytan AK, Kulaeva OI, McCullough LL, Formosa T, Georgiev PG, Kirpichnikov MP, Studitsky VM, Feofanov AV. 2016. Large-scale ATP-independent nucleosome unfolding by a histone chaperone. *Nat Struct Mol Biol* 23:1111–1116. <https://doi.org/10.1038/nsmb.3321>.
 24. Kemble DJ, McCullough LL, Whitby FG, Formosa T, Hill CP. 2015. FACT disrupts nucleosome structure by binding H2A–H2B with conserved peptide motifs. *Mol Cell* 60:294–306. <https://doi.org/10.1016/j.molcel.2015.09.008>.
 25. Chen P, Dong L, Hu M, Wang Y-Z, Xiao X, Zhao Z, Yan J, Wang P-Y, Reinberg D, Li M, Li W, Li G. 2018. Functions of FACT in breaking the nucleosome and maintaining its integrity at the single-nucleosome level. *Mol Cell* 71:284–293. <https://doi.org/10.1016/j.molcel.2018.06.020>.
 26. Sen R, Kaja A, Ferdoush J, Lahudkar S, Barman P, Bhaumik SR. 2017. An mRNA capping enzyme targets FACT to the active gene to enhance the engagement of RNA polymerase II into transcriptional elongation. *Mol Cell Biol* 37:e00029-17. <https://doi.org/10.1128/MCB.00029-17>.
 27. Garcia H, Fleyshman D, Kolesnikova K, Safina A, Commane M, Paszkiewicz G, Omelian A, Morrison C, Gurova K. 2011. Expression of FACT in mammalian tissues suggests its role in maintaining of undifferentiated state of cells. *Oncotarget* 2:783–796. <https://doi.org/10.18632/oncotarget.340>.
 28. Hsieh FK, Kulaeva OI, Orlovsky IV, Studitsky VM. 2011. FACT in cell differentiation and carcinogenesis. *Oncotarget* 2:830–832. <https://doi.org/10.18632/oncotarget.356>.
 29. Koman IE, Commane M, Paszkiewicz G, Hoonjan B, Pal S, Safina A, Toshkov I, Purmal AA, Wang D, Liu S, Morrison C, Gudkov AV, Gurova KV. 2012. Targeting FACT complex suppresses mammary tumorigenesis in Her2/neu transgenic mice. *Cancer Prev Res (Phila)* 5:1025–1035. <https://doi.org/10.1158/1940-6207.CAPR-11-0529>.
 30. Garcia H, Miecznikowski JC, Safina A, Commane M, Ruusulehto A, Kilpinen S, Leach RW, Attwood K, Li Y, Degan S, Omilian AR, Guryanova O, Papantonopoulou O, Wang J, Buck M, Liu S, Morrison C, Gurova KV. 2013. Facilitates chromatin transcription complex is an “accelerator” of tumor transformation and potential marker and target of aggressive cancers. *Cell Rep* 4:159–173. <https://doi.org/10.1016/j.celrep.2013.06.013>.
 31. Dermawan JK, Gurova K, Pink J, Dowlati A, De S, Narla G, Sharma N, Stark GR. 2014. Quinacrine overcomes resistance to erlotinib by inhibiting FACT, NF- κ B, and cell-cycle progression in non-small cell lung cancer. *Mol Cancer Ther* 13:2203–2214. <https://doi.org/10.1158/1535-7163.MCT-14-0013>.
 32. Burkhart C, Fleyshman D, Kohn R, Commane M, Garrigan J, Kurbatov V, Toshkov I, Ramachandran R, Martello L, Gurova KV. 2014. Curaxin CBL0137 eradicates drug resistant cancer stem cells and potentiates efficacy of gemcitabine in preclinical models of pancreatic cancer. *Oncotarget* 5:11038–11053. <https://doi.org/10.18632/oncotarget.2701>.
 33. Fleyshman D, Prendergast L, Safina A, Paszkiewicz G, Commane M, Morgan K, Attwood K, Gurova K. 2017. Level of FACT defines the transcriptional landscape and aggressive phenotype of breast cancer cells. *Oncotarget* 8:20525–20542. <https://doi.org/10.18632/oncotarget.15656>.
 34. Attwood K, Fleyshman D, Prendergast L, Paszkiewicz G, Omilian AR, Bshara W, Gurova K. 2017. Prognostic value of histone chaperone FACT subunits expression in breast cancer. *Breast Cancer* (Dove Med Press) 9:301–311. <https://doi.org/10.2147/BCTT.S126390>.
 35. Bi L, Xie C, Yao M, Thae Hnit SS, Vignarajan S, Wang Y, Wang Q, Xi Z, Xu H, Li Z, de Souza P, Tee A, Wong M, Liu T, Zhao X, Zhou J, Xu L, Dong Q. 2019. The histone chaperone complex FACT promotes proliferative switch of G₀ cancer cells. *Int J Cancer* 145:164–178. <https://doi.org/10.1002/ijc.32065>.
 36. Hudson ME, Pozdnyakova I, Haines K, Mor G, Snyder M. 2007. Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays. *Proc Natl Acad Sci U S A* 104:17494–17499. <https://doi.org/10.1073/pnas.0708572104>.
 37. Sen R, Ferdoush J, Kaja A, Bhaumik SR. 2016. Fine-tuning of FACT by the ubiquitin proteasome system in regulation of transcriptional elongation. *Mol Cell Biol* 36:1691–1703. <https://doi.org/10.1128/MCB.01112-15>.
 38. Ferdoush J, Sen R, Durairaj G, Barman P, Kaja A, Guha S, Bhaumik SR. 2020. An F-box protein, Mdm30, interacts with TREX subunit Sub2 to regulate cellular abundance cotranscriptionally in orchestrating mRNA export independently of splicing and mitochondrial function. *Mol Cell Biol* 40:e00570-19. <https://doi.org/10.1128/MCB.00570-19>.
 39. Frankland-Searby S, Bhaumik SR. 2012. The 26S proteasome complex: an attractive target for cancer therapy. *Biochim Biophys Acta* 1825:64–76. <https://doi.org/10.1016/j.bbcan.2011.10.003>.
 40. Bhaumik SR, Malik S. 2008. Diverse regulatory mechanisms of eukaryotic transcriptional activation by the proteasome complex. *Crit Rev Biochem Mol Biol* 43:419–433. <https://doi.org/10.1080/10409230802605914>.
 41. Lolis AA, Londhe P, Beggs BC, Byrum SD, Tackett AJ, Davie JK. 2013. Myogenin recruits the histone chaperone facilitates chromatin transcription (FACT) to promote nucleosome disassembly at muscle-specific genes. *J Biol Chem* 288:7676–7687. <https://doi.org/10.1074/jbc.M112.426718>.
 42. LeRoy G, Oksuz O, Descostes N, Aoi Y, Ganai RA, Kara HO, Yu JR, Lee CH, Stafford J, Shilatfard A, Reinberg D. 2019. LEDGF and HDGF2 relieve the nucleosome-induced barrier to transcription in differentiated cells. *Sci Adv* 5:eay3068. <https://doi.org/10.1126/sciadv.aay3068>.
 43. Ishibashi J, Perry RL, Asakura A, Rudnicki MA. 2005. MyoD induces myogenic differentiation through cooperation of its NH₂- and COOH-terminal regions. *J Cell Biol* 171:471–482. <https://doi.org/10.1083/jcb.200502101>.
 44. Walsh K, Perlman H. 1997. Cell cycle exit upon myogenic differentiation. *Curr Opin Genet Dev* 7:597–602. [https://doi.org/10.1016/s0959-437x\(97\)80005-6](https://doi.org/10.1016/s0959-437x(97)80005-6).
 45. Kitzmann M, Fernandez A. 2001. Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. *Cell Mol Life Sci* 58:571–579. <https://doi.org/10.1007/PL00000882>.
 46. Kwon SK, Saundane M, Baek KH. 2017. p53 stability is regulated by diverse deubiquitinating enzymes. *Biochim Biophys Acta* 1868:404–411. <https://doi.org/10.1016/j.bbcan.2017.08.001>.
 47. Safina A, Garcia H, Commane M, Guryanova O, Degan S, Kolesnikova K, Gurova KV. 2013. Complex mutual regulation of facilitates chromatin transcription (FACT) subunits on both mRNA and protein levels in human cells. *Cell Cycle* 12:2423–2434. <https://doi.org/10.4161/cc.25452>.
 48. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. 2012. The cBio Cancer Genomics Portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2:401–404. <https://doi.org/10.1158/2159-8290.CD-12-0095>.
 49. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. 2013. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6:pl1. <https://doi.org/10.1126/scisignal.2004088>.
 50. Cancer Genome Atlas Network. 2012. Comprehensive molecular portraits of human breast tumours. *Nature* 490:61–70. <https://doi.org/10.1038/nature11412>.
 51. Davis CF, Ricketts CJ, Wang M, Yang L, Cherniack AD, Shen H, Buhay C, Kang H, Kim SC, Fahey CC, Hacker KE, Bhanot G, Gordenin DA, Chu A, Gunaratne PH, Biehl M, Seth S, Kaiparettu BA, Bristow CA, Donehower LA, Wallen EM, Smith AB, Tickoo SK, Tamboli P, Reuter V, Schmidt LS,

- Hsieh JJ, Choueiri TK, Hakimi AA, The Cancer Genome Atlas Network, Chin L, Meyerson M, Kucherlapati R, Park W-Y, Robertson AG, Laird PW, Henske EP, Kwiatkowski DJ, Park PJ, Morgan M, Shuch B, Muzny D, Wheeler DA, Linehan WM, Gibbs RA, Rathmell WK, Creighton CJ. 2014. The somatic genomic landscape of chromophobe renal cell carcinoma. *Cancer Cell* 26:319–330. <https://doi.org/10.1016/j.ccr.2014.07.014>.
52. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK, Varambally S. 2017. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia* 19:649–658. <https://doi.org/10.1016/j.neo.2017.05.002>.
53. Chen F, Chandrashekar DS, Varambally S, Creighton CJ. 2019. Pan-cancer molecular subtypes revealed by mass-spectrometry-based proteomic characterization of more than 500 human cancers. *Nat Commun* 10:5679. <https://doi.org/10.1038/s41467-019-13528-0>.
54. Malik S, Durairaj G, Bhaumik SR. 2013. Mechanisms of antisense transcription initiation from the 3' end of the GAL10 coding sequence in vivo. *Mol Cell Biol* 33:3549–3567. <https://doi.org/10.1128/MCB.01715-12>.
55. Barman P, Reddy D, Bhaumik SR. 2019. Mechanisms of antisense transcription initiation with implications in gene expression, genomic integrity and disease pathogenesis. *Noncoding RNA* 5:11. <https://doi.org/10.3390/ncrna5010011>.
56. Murawska M, Schauer T, Matsuda A, Wilson MD, Pysik T, Wojcik F, Muir TW, Hiraoka Y, Straub T, Ladurner AG. 2020. The chaperone FACT and histone H2B ubiquitination maintain *S. pombe* genome architecture through genic and subtelomeric functions. *Mol Cell* 77:501–513. <https://doi.org/10.1016/j.molcel.2019.11.016>.
57. Rothschild G, Zhang W, Lim J, Giri PK, Laffleur B, Chen Y, Fang M, Chen Y, Nair L, Liu ZP, Deng H, Hammarström L, Wang J, Basu U. 2020. Noncoding RNA transcription alters chromosomal topology to promote isotype-specific class switch recombination. *Sci Immunol* 5:eaay5864. <https://doi.org/10.1126/sciimmunol.aay5864>.
58. Kaplan CD, Laprade L, Winston F. 2003. Transcription elongation factors repress transcription initiation from cryptic sites. *Science* 301:1096–1099. <https://doi.org/10.1126/science.1087374>.
59. Pinskaya M, Gourvennec S, Morillon A. 2009. H3 lysine 4 di- and tri-methylation deposited by cryptic transcription attenuates promoter activation. *EMBO J* 28:1697–1707. <https://doi.org/10.1038/emboj.2009.108>.
60. Kim T, Xu Z, Clauder-Münster S, Steinmetz LM, Buratowski S. 2012. Set3 HDAC mediates effects of overlapping noncoding transcription on gene induction kinetics. *Cell* 150:1158–1169. <https://doi.org/10.1016/j.cell.2012.08.016>.
61. McDaniel SL, Hepperla AJ, Huang J, Dronamraju R, Adams AT, Kulkarni VG, Davis IJ, Strahl BD. 2017. H3K36 methylation regulates nutrient stress response in *Saccharomyces cerevisiae* by enforcing transcriptional fidelity. *Cell Rep* 19:2371–2382. <https://doi.org/10.1016/j.celrep.2017.05.057>.
62. Uprety B, Kaja A, Ferdoush J, Sen R, Bhaumik SR. 2016. Regulation of antisense transcription by NuA4 histone acetyltransferase and other chromatin regulatory factors. *Mol Cell Biol* 36:992–1006. <https://doi.org/10.1128/MCB.00808-15>.
63. Kihara T, Kano F, Murata M. 2008. Modulation of SRF-dependent gene expression by association of SPT16 with MKL1. *Exp Cell Res* 314:629–637. <https://doi.org/10.1016/j.yexcr.2007.10.004>.
64. Malonia SK, Dutta P, Santra MK, Green MR. 2015. F-box protein FBXO31 directs degradation of MDM2 to facilitate p53-mediated growth arrest following genotoxic stress. *Proc Natl Acad Sci U S A* 112:8632–8637. <https://doi.org/10.1073/pnas.1510929112>.
65. Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 12:357–360. <https://doi.org/10.1038/nmeth.3317>.
66. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* 33:290–295. <https://doi.org/10.1038/nbt.3122>.
67. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:550. <https://doi.org/10.1186/s13059-014-0550-8>.
68. Ge SX, Jung D, Yao R. 2020. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* 36:2628–2629. <https://doi.org/10.1093/bioinformatics/btz931>.