Introduction

The silent information regulator (Sir) genes are required for transcriptional silencing in the budding yeast *S. cerevisiae*, and among them, Sir2 is highly conserved from prokaryotes to eukaryotes.1 As an NAD+--dependent histone deacetylase, Sir2 has been found to be necessary for transcriptional silencing at the silent mating loci and at telomeres and in rDNA (rDNA);2 Sir2 also regulates recombination, genomic stability and cellular longevity.3-5 Among the seven mammalian Sir2 homologs (sirtuin 1-7, Sirt1-7),2 Sirt1 is the closest ortholog to Sir2 and is also the most studied sirtuin. It deacetylates histones, as well as a broad range of transcription factors, and has roles in many cellular processes, such as metabolism, cell differentiation and stress response; it has also been implicated in the development of cancer.6 Interestingly, Sirt1 has functions in the cytoplasm, nucleoplasm and even in the nucleolus, where it is involved in the epigenetic regulation of rDNA loci.7,8 Sirt7, the nearest homolog to Sirt6, is a nucleolar protein that functions in the resumption of rDNA transcription upon the cell's exit from mitosis.9,10 Sirt6 is reportedly a chromatin-associated nuclear protein that is involved in transcription, genome and telomere stabilization, DNA repair, and metabolic homeostasis.11-17 Sirt6 knockout cells show genomic instability and hypersensitivity to DNA damage,18,19 and a deficiency of Sir6 in mice leads to an early aging phenotype and severe metabolic problems.14 As a histone deacetylase, Sir6 deacetylates at least two lysines: lysines 9 and 56 of histone H3 (H3K9 and H3K56).13,19,20 It was shown that by acetylating H3K9, Sirt6 modulates telomeric chromatin13 and inhibits the expression of a subset of nuclear factor kappa B (NFκB) target genes.12 In addition to histones, Sirt6 can deacetylate the C-terminal binding protein (CtBP) interacting protein (CtIP) and thus promote DNA repair.11 Sirt6 also promotes mono-ADP ribosylation, an alternative NAD+-dependent reaction that has been reported for some other members of the sirtuin family.21,22 Of the 7 sirtuins, Sir1 and 6 are localized to the nucleoplasm and Sirt7 is localized to the nucleolus.21,23 We noticed that the nucleoli of cultured cells were not free of Sirt6, which was contrary to a previous report of Sirt6 being excluded from the nucleolus.23 In light of the importance of protein localization to its function, we tried to evaluate Sirt6 localization in cultured cells, the underlying mechanisms of its localization and its possible functions, especially in the nucleolus.

Results

Sirt6 nucleolar localization. A rabbit antibody was produced against Sirt6 amino acids 178–250 of the human Sirt6 protein—a region that was not conserved in other members of the sirtuin family.21-23 We noticed that the nucleoli of cultured cells were not free of Sirt6, which was contrary to a previous report of Sirt6 being excluded from the nucleolus.23 In light of the importance of protein localization to its function, we tried to evaluate Sirt6 localization in cultured cells, the underlying mechanisms of its localization and its possible functions, especially in the nucleolus.

Keywords: Sirt6, Sirtuin, nucleolus, cell cycle, mitosis
nucleolus and spread into the nucleoplasm (Fig. 2). Previously, Sirt6 was said to be a nuclear protein that is excluded from the nucleolus,\textsuperscript{23} while Sir2, Sirt1 and Sirt7 have known functions in the nucleolus.\textsuperscript{7,8,24} Our observations indicated a great variation of nucleolar localization of Sirt6 among different cells and under different culture conditions, ranging from no nucleolar Sirt6 in a few cells to a relatively high level of nucleolar Sirt6 in a majority of cells. This suggested that Sirt6 might shuttle rapidly, and its localization might change with cell cycle progression. We thus investigated Sirt6 sub-cellular localization in relation to the different steps of the cell cycle.

On a separate note, Sirt6 was reported to be linked to heterochromatic regions,\textsuperscript{23} but interestingly, our study in HeLa cells transfected with FLAG-tagged wild-type Sirt6 (FLAG-Sirt6), staining for this antibody co-localized with FLAG tag staining in the nucleus, and our antibody did not show cross-reactivity with other nuclear sirtuins (Fig. 1A). By western blot, the Sirt6 antibody specifically recognized FLAG-Sirt6, a 49-kDa polypeptide (Fig. 1B).

When immunostained with the above Sirt6 antibody, most asynchronous HeLa cells showed both nucleoplasmic and nucleolar immunoreactivity of endogenous Sirt6, and among them, ~60% showed enrichment of the protein in the nucleolus (Fig. 2A). Sirt6 was also enriched in the nucleoli of cells subjected to serum deprivation, whereas treatment of HeLa cells with low doses of actinomycin D caused Sirt6 to be released from the nucleus and spread into the nucleoplasm (Fig. 2B). Previously, Sirt6 was said to be a nuclear protein that is excluded from the nucleus,\textsuperscript{21,22} while Sir2, Sirt1 and Sirt7 have known functions in the nucleolus.\textsuperscript{14,15} Our observations indicated a great variation of nucleolar localization of Sirt6 among different cells and under different culture conditions, ranging from no nucleolar Sirt6 in a few cells to a relatively high level of nucleolar Sirt6 in a majority of cells. This suggested that Sirt6 might shuttle rapidly, and its localization might change with cell cycle progression. We thus investigated Sirt6 sub-cellular localization in relation to the different steps of the cell cycle.

On a separate note, Sirt6 was reported to be linked to heterochromatic regions,\textsuperscript{23} but interestingly, our study in HeLa cells...
showed that SirT6 was enriched in areas with weak DAPI staining, which represent euchromatin. Meanwhile, areas with condensed chromatin, including the nucleolar periphery, showed less SirT6 staining (Fig. 2C).

Nuclear localization sequence (NLS) of SirT6. Nuc-PLoc software, a package of computational tools, predicted that SirT6 would localize to the nucleus. The Nucleolar Localization Sequence Detector, NOD, predicted one NLS in the C-terminal 28 aa sequence (between aa 328 and 355, PAS PKR ERP TSP APF RPV KR KAK AVP S) of the SirT6 protein (Fig. 3A). The 7 amino acid sequence from residue 345 to 351 (PKR VKA K) was previously reported as a nucleolar localization signal (NLS) in SirT6 and is partially responsible for the translocation of this protein to the nucleus.

Our results also showed that a SirT6 mutant lacking the last 35 aa (SirT6ΔC) was partially delocalized from the nucleus to the cytoplasm. In HeLa cells, overexpressed FLAG-SirT6ΔC was found in the cytoplasm and nucleus but not in the nucleolus. Thus, although the C-terminus is not sufficient for the nuclear localization of SirT6, the lack of the C-terminus seemed sufficient to completely exclude SirT6 from the nucleus (Fig. 3B). Hence, the SirT6 C-terminus most likely serves both as a partial NLS and a NoLS.

SirT6 nuclear localization is independent of its phosphorylation (at sites 294 and 303) and enzymatic activity. In HeLa cells that have been arrested in the G1 and M phases of the cell cycle, an evaluation of protein phosphorylation has shown that SirT6 is phosphorylated at Thr294 and Ser303 in the G1 phase, but not during mitosis.28 Phosphorylation/dephosphorylation of SirT6 is phosphorylated at Thr294 and Ser303 in the G1 phase, cycle, an evaluation of protein phosphorylation has shown that cells that have been arrested in the G1 and M phases of the cell cycle-dependent expression of genes has known roles in the cell cycle.26,27 Phosphorylation/dephosphorylation has known roles in the cell cycle-dependent expression of genes and in the localization of proteins.28 To evaluate if this phosphorylation/dephosphorylation of SirT6 has any role in its nuclear localization, the following mutants of SirT6 were constructed: T294A, S303A (mimicking dephosphorylated SirT6), T294E and S303E (mimicking phosphorylated SirT6). G1-synchronized HeLa cells transfected with these constructs showed a nuclear localization of all four mutants. Thus, the phosphorylation/dephosphorylation of SirT6 at these sites seemed to not be important to its nuclear translocation (Fig. 3C).

The H133Y mutation abrogates the catalytic activity of SirT6 and alters the dynamics of SirT6 association with chromatin in cells.29 To determine if the enzymatic activity of SirT6 is needed for its nuclear localization, HeLa cells were transfected with FLAG-SirT6 (H133Y) and synchronized at the G1 phase. The cells showed nuclear localization of mutant SirT6 (Fig. 3C). Thus, to be localized to the nucleus, SirT6 does not need to be enzymatically active and associated with chromatin.

Antibodies against the C-terminus of SirT6 do not detect nuclear SirT6. Previous studies, most of which used antibodies against the C-terminus, reported SirT6 localization to the nucleus, but not to the nucleolus. Our immunohostaining of endogenous SirT6 in HeLa cells used an antibody against the SirT6 C-terminus and also failed to detect nuclear SirT6; instead, a diffuse nucleoplasmic staining with perinucleolar enrichment was observed (Fig. 4A). To demonstrate that this is not a non-specific staining artifact, the localization of FLAG-SirT6-HA (FLAG at the N-terminus and HA at the C-terminus of SirT6) was compared with that of SirT6-FLAG (FLAG at the C-terminus) (Fig. 4B). In transfected HeLa cells, the C-terminal antibody and the anti-FLAG and anti-HA antibodies did not show a nuclear signal when SirT6 was tagged at its C-terminus, whereas the signal was very obvious in the nucleus for N-terminally tagged SirT6.

The nucleolar localization of SirT6 is cell cycle dependent. To investigate the sub-nuclear localization of SirT6 during the cell cycle, HeLa cells were synchronized in the G1/S phase by a double thymidine block, released and fixed at different time points. During interphase, both overexpressed FLAG-SirT6 and endogenous SirT6 were mostly localized to the nucleus, but the ratio of nucleoplasmic/nucleolar SirT6 changed as the cell cycle progressed. Two hours after releasing the cells from the double thymidine block, when most of the cells were in S phase, nucleoli were almost free of SirT6, while in cells that were synchronized in G1/G0 (using either sodium butyrate (NaBt) or serum deprivation), SirT6 showed an obvious enrichment in the nucleoli. The distribution of FLAG-SirT6 in the nucleus is shown in Figure 5A. Examples of intensity plots of FLAG, DAPI and nucleolin signals are shown on the right to better illustrate the sub-nuclear localization of SirT6. The distribution of endogenous SirT6 is
shown in Figure 5B. Nucleolar Sirt6 was also detected using electron microscopy (EM) in HeLa cells synchronized in the G3 phase using NaBt (Fig. 5C). The EM results showed that Sirt6 is enriched in G1 phase cells, mostly in the granular components (GCs) of nucleoli, and is almost absent in the fibrillar centers (FCs).

In mitosis, Sirt6 expression increases. The nucleolus is disassembled in prophase and re-assembled at the end of mitosis. To have a more complete view of Sirt6 subcellular localization, we evaluated its localization during mitosis as well. Twelve hours after release from the double thymidine block, cells were fixed to evaluate Sirt6 localization in mitotic cells. In these cells, Sirt6 was spread out in the cytoplasm and was not associated with chromosomes (Fig. 6A). Additionally, Sirt6 was partially co-localized with α-tubulin at the mitotic spindles. Both endogenous and overexpressed FLAG-Sirt6 showed a similar distribution in the mitotic cells. An antibody against the Sirt6 C-terminus also confirmed that during mitosis, Sirt6 is not associated with chromosomes (Fig. 6A). To have a more complete view of Sirt6 subcellular localization, we evaluated its localization during mitosis as well. Twelve hours after release from the double thymidine block, cells were fixed to evaluate Sirt6 localization in mitotic cells. In these cells, Sirt6 was spread out in the cytoplasm and was not associated with chromosomes (Fig. 6A). Additionally, Sirt6 was partially co-localized with α-tubulin at the mitotic spindles. Both endogenous and overexpressed FLAG-Sirt6 showed a similar distribution in the mitotic cells. An antibody against the Sirt6 C-terminus also confirmed that during mitosis, Sirt6 is not associated with chromosomes (Fig. 6A).

This difference was also significant when mitotic cells were categorized into prophase/prometaphase, metaphase, anaphase and telophase, according to DAPI and α-tubulin staining, and each step was compared with interphase cells (p < 0.0001). Metaphase cells showed the highest level of Sirt6 (p < 0.005). Levels of endogenous Sirt6 in mitotic cells were also compared with those in asynchronous cells and cells arrested in G1, S and G2 using a western blot. The results showed that Sirt6 levels increased in mitotic cells, confirming the immunostaining results (Fig. 6C).

Cells with high expression of FLAG-Sirt6 showed a delay in entering mitosis. At 12 h after synchronization in G1/S, immunolabeling of cells transfected with FLAG-Sirt6 showed a lower percentage of mitotic cells (p = 0.02, Student’s t-test) compared with cells transfected by FLAG-H133Y (Fig. 7A). This difference was much stronger when the percentage of mitotic cells was compared in cells with high expression of exogenous proteins (Sirt6 vs. H133Y) (p < 0.005, Student’s t-test). This result suggested a delay in entering mitosis among cells with a high expression of Sirt6. We also assessed the proliferation rate of cultured cells transfected by wild type and mutant Sirt6 by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The result showed that cells transfected with FLAG-Sirt6 had a lower proliferation rate (Fig. 7B). This ruled out the possibility of a fast asynchronous entrance of Sirt6-transfected cells into mitosis. To evaluate the possibility of apoptosis induced by a high level of exogenous Sirt6, we compared apoptosis rate in Sirt6 overexpressing cells with the cells expressing a mutant form of Sirt6, W42R, which is toxic to the cells and increased apoptosis rate in host cells (unpublished data). To measure apoptosis rate, nuclear staining using DAPI together with antibody against cleaved caspase-3 (CC3) was used and the level of CC3 staining was measured as OD using ImageJ software.

Our results showed that Sirt6 overexpression does not have an apoptosis-inducing effect in the cells cultured under normal conditions (Fig. S1). Furthermore, flow cytometric analysis of cell cycle distribution of HeLa cells overexpressing Sirt6 and Sirt6 nonfunctional mutant (H133Y) with PI staining showed that the percentage of S phase cells is higher in Sirt6 group compared with H133Y group (data not shown). These results confirm that Sirt6 overexpression causes S phase arrest, delay in entering mitosis and a lower proliferation rate.
Discussion

Sirt6 is believed to be a nuclear protein that is excluded from nucleoli and associated with heterochromatin. Our results showed, however, that although Sirt6 is mainly in the nucleus, its sub-nuclear localization changes during the cell cycle. While Sirt6 is largely excluded from the nucleolus during S phase, it can be detected there during the rest of interphase and is enriched in the nucleolus during the \( G_1 \) phase. We found that the Sirt6 C-terminus, which is partially responsible for its nuclear localization, also serves as a NoLS. Additionally, Sirt6 mutants could still localize to the nucleolus, regardless of the state of their phosphorylation and catalytic activity. Therefore, we did not have any construct that showed complete nuclear but no nucleolar localization with which to evaluate the results of preventing nuclear localization. However, it is possible that other modifications or protein interactions are involved in this shuttling. The difference in the detection of nucleolar Sirt6 by antibodies against its C-terminus or against C-terminal tags could be accounted for by the cleavage of the Sirt6 C-terminus after its arrival at the nucleolus, or perhaps Sirt6 might interact with some nucleolar proteins that could mask the C-terminal epitope of Sirt6. Theoretically, some post-translational modifications in the nucleolus may alter the C-terminal reactivity of Sirt6 to the antibodies against different domains. We also thought about different isoforms of Sirt6 in the cell (for example some nucleolar isoforms lacking C-terminal), but using Flag-Sirt6-HA we showed that even for a single Sirt6 molecule the antibody against the N-terminal tag detects Sirt6 in the nucleus while antibody against the tag in the C-terminal cannot detect the same molecule.

We showed that Sirt6 does not colocalize with high density DAPI staining, which represents heterochromatin, especially in the perinucleolar region. A large fraction of Sirt1 is associated with euchromatin, and Sirt1 interacts with histone H1 and promotes heterochromatin formation. Thus, further studies are needed to evaluate the exact role of Sirt6 on chromatin regulation.

The nucleolus plays important roles in ribosome biogenesis, as well as in other recently found functions in cell cycle control and stress response. In yeast, only half of rDNA repeats are transcribed, and the rest are repressed by a mechanism that involves Sir2. The yeast Sir2 protein binds to Net1, which associates with rDNA and recruits Sir2 to the nucleolus. Net1, Sir2 and Cdc14 are components of a multifunctional complex, RENT (regulator of nucleolar silencing and telophase) which is involved in nucleolar silencing and exit from mitosis. The nucleolar proteome is largely conserved, and proteomic studies have shown that more than 90 percent of human nucleolar proteins have yeast homologs. The mammalian rDNA locus is also under epigenetic regulation by protein complexes, such as NoRC (nucleolar remodeling complex) and eNoSC (energy-dependent nucleolar silencing complex), which involve histone deacetylases and methyltransferases. A notable exception is Sirt1, the key component of the eNoSC complex, which is essentially nucleoplasmic but shows a faint nucleolar location. Epigenetic mechanisms, such as DNA methylation and histone modification, are major mechanisms in silencing mammalian rRNA genes. Among mammalian sirtuins, Sirt7 is exclusively nucleolar and is a component of the RNA polymerase I (Pol I) transcriptional machinery, where it functions by stimulating Pol I transcription. Recently, Sirt7 was found to be involved in the resumption of rDNA transcription upon exit from mitosis. Here, we report for the first time that another member of the sirtuin family, Sirt6, also shows nucleolar localization, and its nucleolar level changes during the cell cycle. Based on the role of Sir2 in rDNA repression and the functions of other sirtuin members (Sirt1 and 7) in the nucleolus, it is very possible that Sirt6 plays a role in rRNA
transcription. Low doses of actinomycin D selectively inhibit transcription by RNA Pol I and causes the release of proteins such as Sirt7 which are involved in rDNA transcription from the nucleolus to the nucleoplasm. The treatment of HeLa cells with actinomycin D caused the release of endogenous Sirt6 from the nucleus. This result first confirms nucleolar localization of Sirt6 and highlights the signal intensity that we see in normal cell, serum deprived cell and G1 arrested cell. It also gives preliminary information suggesting a role for Sirt6 in rDNA transcription system and suggests possible interacting partners.

Figure 5. Sirt6 is enriched in the nucleolus in the G1 phase of the cell cycle. (A) Distribution of Sirt6 in the nucleus. Comparison with nucleolin and DAPI shows that Sirt6 is enriched in the nucleolus during the G1 phase of the cell cycle. In S phase, the nucleolus is almost free of Sirt6. The framed cells in the panels of the third column were analyzed, as shown in the photomicrographs in the last column, and the quantitative results are shown on the right. Scale bars, 10 μm. (B) Endogenous Sirt6 is also enriched in the nucleolus in G1, while in S phase, the Sirt6 signal is stronger in the nucleoplasm than in the nucleolus. (C) Immunoperoxidase electron microscopy of G1 arrested HeLa cells using anti-Sirt6 confirms nucleolar Sirt6 and shows that it is mostly in GCs. (D) shows the negative control staining with no primary antibody. N, nucleus; Nucl, nucleolus; F, fibrillar center; D, dense fibrillar component; G, granular component. Scale bars, 1 μm.
for Sirt6 in the nucleolus, which is discussed below. However, none of available Sirt6 antibodies which are against N-terminal or middle part of Sirt6 are suitable for immunoprecipitation and our ChIP assay failed to detect a direct association of Sirt6 with rDNA, which might be due either to technical problems or to the possibility of an indirect association. Furthermore, EM studies showed that in G0-arrested cells, Sirt6 localized mostly to the GCs of the nucleolus, which raises the possibility that Sirt6 plays a role in the modification of later steps of rRNA processing and the maturation of ribosomal structures. Transcription of rDNA is modulated during the cell cycle, from low levels in the early G1 phase to its highest levels in the S and G2 phases.54 G0 enrichment of nucleolar Sirt6 suggests that Sirt6 plays a repressor role with regards to ribosome biogenesis. Furthermore, Sirt6 impacts DNA repair, and interestingly, both ribosome biogenesis and rDNA stability are linked to aging.41,42 rDNA is the most unstable part of the genome because of its repetitive nature. Thus, in a manner similar to telomere shortening, rDNA instability may trigger DNA damage response (DDR), causing cell cycle arrest and the activation of DNA damage repair pathways.43 Therefore, it is possible that the Sirt6 role in the nucleolus has a direct impact on the process of aging due to its possible effects on ribosome biogenesis or rDNA damage repair. Beside all of these possibilities, for some proteins, the nucleolus is a place of sequestration, which prevents proteins from reaching their targets, such as those in the nucleoplasm.43

It was previously reported that GFP-tagged Sirt6 and Sirt7 remain associated with condensed chromosomes in mitosis.23 However, it was later shown that in contrast to GFP-Sirt7, endogenous Sirt7 remains associated with nucleolar organizer regions (NORs) and co-localizes with the upstream binding factor (UBF) during mitosis.10 In this study, we showed that mitotic HeLa cells show a high level of Sirt6, with the highest level in metaphase, and this suggests that Sirt6 may play a role in mitosis, which would require Sirt6 to be regulated during the cell cycle as well as during the mitosis steps themselves. We also showed that endogenous Sirt6 did not associate with condensed chromatin during mitosis, but showed a diffuse staining in the cytoplasm, was excluded from chromatin, and was partially localized to mitotic spindles. Interestingly, the other nucleolar protein, nucleolar phosphoprotein B23 (or nucleophosmin), which is also present in GCs and involved in rRNA processing, shows a similar localization in mitosis.34 B23 is also released from nucleoli to the nucleoplasm after actinomycin D treatment, which is comparable to Sirt6 relocation. B23, however, is a known substrate of Sirt1,44 and we suggest that its possible interaction with Sirt6...
should also be evaluated. The fact that antibodies against the Sirt6 C-terminus could not detect nuclear Sirt6, along with the fact that Sirt6 localizes to the mitotic spindles, suggests that the spindle-localized Sirt6 had previously been released from the nucleus, and for some reason, its C-terminus is not detectable.

According to our results, in cells with a high expression of exogenous Sirt6, mitosis was delayed or inhibited relative to cells with a high level of mutant Sirt6. Previous studies did not show any change in cell cycle checkpoints in Sirt6-deficient neurons and for some reason, its C-terminus is not detectable. The percentage of mitotic cells in neighboring untransfected cells was not different between the two groups (Untreated). (B) An MTT assay showed a slower proliferation rate in Sirt6-overexpressing cells compared with cells coexpressing the Sirt6 mutant. Scale bar, 50 μm.

Figure 7. Sirt6 delays host cell mitosis. (A) The left panel shows that 12 h after transfected cells were synchronized in early S phase, the percentage of transfected cells that were in mitosis was much less for Sirt6 than for the nonfunctional Sirt6 mutant (H133Y). The difference is significant (Trans (p = 0.02), especially when we compared the mitotic cell percentage in cells expressing Sirt6 or the mutant at a high level (Trans high (p < 0.005). The percentage of mitotic cells in neighboring untransfected cells was not different between the two groups (Untreated). (B) An MTT assay showed a lower proliferation rate in Sirt6-overexpressing cells compared with cells overexpressing the Sirt6 mutant. Scale bar, 50 μm.

Materials and Methods

Cloning of Sirt6 constructs. The open reading frame (ORF) of human Sirt6 (Accession: BC005026, protein ID: AAH05026) and nucleotides 1–960 (used to construct Sirt6ΔC) were amplified by PCR using a cDNA template purchased from the MGC (cDNA clone MCG: 12574; IMAGE: 2828472) (Invitrogen-FL1002). The fragment was inserted between the BamHI and HindIII sites of the FLAG tag pXJ40 vector to produce FLAG-tagged Sirt6 mammalian expression plasmids. Sirt6 was also tagged at both the N-terminus and C-terminus with FLAG and HA, respectively, by designing a reverse primer that contains the HA tag sequence. Point mutants of Sirt6 were generated by the GeneTailor Site-Directed Mutagenesis Kit (Invitrogen).

Cell culture and transfection. Cells were grown in the presence of 5% CO₂ at 37°C, cultured in Dulbecco's modified Eagle’s medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% Pen-Strep. The cells were transfected with Sirt6 plasmids using electroporation (MP-100 Microporator, Digital Bio Technology) and cultured on cover-slips for appropriate times (48 h in asynchronous cultures, and different time points after releasing synchronous cultures) before being fixed. For the inhibition of RNA polymerase I transcription, cells were treated with 50 ng/ml actinomycin D (Sigma) for 2 h before being fixed.

Immunocytochemistry assays. The cells were fixed using paraformaldehyde (3%) for 20 min at room temperature, washed with PBS and blocked with PBS-T-NGS (0.3% Triton X-100 and 6% normal goat serum in 0.1 M PBS). Then, the cells were stained with primary antibodies and corresponding secondary antibodies. DAPI was used to stain the nuclei. Stained cells were observed and analyzed using a fluorescence microscope or a laser scanning confocal microscope (Olympus Fluoview FV 1000, Olympus). Anti-α-tubulin B-5-1-2 (T5168), anti-FLAG M2 (F1804), Anti-HA (H 4908), anti-Sirt6 antibodies (N-terminal (S4322) and C-terminal (S4197)), anti-nucleolin (N6288) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. The Sirt6 antibody that was generated for this study was raised in rabbit against amino acids 178–250 of the human Sirt6 protein.

To compare the signal intensity (fluorescent signal) of Sirt6 in the nucleoplasm and nucleolus and to compare that signal with DAPI intensity, confocal images were merged and the signals were analyzed by plot profile using ImageJ software.
For apoptosis assay, cells were subjected to immunostaining using anti-cleaved caspase 3 (CC3) antibody, and signal intensity of CC3 was measured as optical density (OD) using image software. After subtracting background signal, intensity of signal was compared between transfected and untransfected cells.

**Immunoperoxidase staining.** For immun EM, immunoperoxidase (Avidin-biotin complex method) was performed using an ABC kit (Vectorlabs), and sections were sliced (~100 nm thickness) and observed under TEM. For details, refer to protocols described previously.²⁶ A Philips EM 208S electron microscope was used for the EM observation.

**Cell synchronization.** For the double thymidine block (early S-phase block), HeLa cells were cultured in 24-well or 6-well plates. At 30–40% confluency, the cells were washed two times with PBS, and DMEM + 10% FBS + 1% Pen-Strep and 2 mM thymidine added to them for 18 h (first block). The thymidine was removed by washing the cells with PBS, and fresh media was added for the next 9 h to release the cells. For the second block, DMEM + 10% FBS + 1% Pen-Strep and 2 mM thymidine were added to them for 24 h (second block). The thymidine was removed, the cells were washed with PBS and fresh media was added. Cells progressed synchronously through the G2 and mitotic phases and therefore could be fixed at different time points to have cells in different phases of cell cycle.

For the thymidine-nocodazole block (mitotic block), HeLa cells at 40% confluency were washed with PBS, and DMEM + 10% FBS + 1% Pen-Strep and 2 mM thymidine were added for 4 h (first block). The thymidine was removed by washing the cells with PBS, and fresh media (DMEM + 10% FBS + 1% Pen-Strep) was added for the next 3 h to release the cells. 100 ng/ml nocodazole was then added to the media for 12 h (mitotic block). Nocodazole was removed by washing with PBS, and fresh medium was added to release the cells. To prepare a lysate of mitotic cells after nocodazole treatment, cells were collected using a shake-off method and seeded on another plate with fresh media. They were incubated for 1 h and then lysed.

To block the cells in G1, HeLa cells were cultured in normal media, and for 24 h, 6 mM NaBt was added to the media. To synchronize the cells in G2, HeLa cells were cultured in serum-deprived media (DMEM + 0.5% FBS) for 72 h.

**Immunoblotting.** Protein was extracted from the cells using an M-PER extraction kit (Pierce, Rockford, IL), resolved by SDS-PAGE gel, and transferred to PVDF membrane. After washing and blocking, membranes were incubated with primary antibody overnight at 4°C. Then, the membranes were incubated for one more hour with the secondary antibody (alkaline phosphatase-conjugated antibodies), and the signal was detected using CDAP-Star reagent (Roche) on Kodak light films (Kodak). Anti-β-actin (AS16) was purchased from Sigma.

**MTT assay.** Transfected cells were seeded in 96-well plates (2000 cell/well). For measurement, a MTT (Sigma) stock solution, which was prepared in PBS (5 mg/ml), was added to cells in fresh medium for 1 h under cell culture conditions. The precipitates were dissolved by adding dimethylsulfoxide to the wells, and the absorbance was read by an Elisa reader (SpectraMax M5, Molecular Devices) at 595 nm.

**Disclosure of Potential Conflicts of Interest**

The authors do not have any conflict of interest.

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**Supplemental Materials**

Supplemental materials may be found at: www.landesbioscience.com/journals/nucleus/article/21134/
Okuwaki M, Tephouré N, Zhou C, Scott MS, Boisvert FM, Li LH, Shen HB, Chou Straight AF, Mi- chishita E, McCord RA, Boxer LD, Barber MF, PseAA composition and PsePSSM. Protein Eng Des Nucl. 2.3.16246.

19. Mekhitarian E, McCune RA, Broude LD, Barbee ME, Hong T, Gurart O, et al. Cell cycle-dependent deacetylation of silence nucleosome H3K18Ac by human SIRT1. Mol Cell 2009; 32:666-410. PMID:19425759; http://dx.doi.org/10.1016/j.molcel.2009.06.018.

20. Yang B, Zissis BM, Eckhoff DJ, Lobidie DB. The stringent SHIFTs associate H3K4me3 in vivo to promote genomic stability. Cell Cycle 2009; 8:2062-3. PMID:19973893; http://dx.doi.org/10.4161/cc.8.16.9298.

21. Lim T, Fung E, Kinner M, Giamaretta M, Solaiman S. Human SET binding SHIHT is a nuclear ADP-ribosyltransferase. J Biol Chem 2005; 280:21313-20; PMID:15795229; http://dx.doi.org/10.1074/jbc.E05-01-0033.

22. Straughn AM, Shaw W, Devad GC, Tschirn CW, Duhoux W, Jellis AM, et al. Sirtuin 2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell 1999; 97:276-50. PMID:10323924; http://dx.doi.org/10.1016/S0092-8674(00)00197-5.

23. Shaw HB, Oka KE. Nu-Plac-a new in-frame for producing protein subunit localization by fusing Perch part composition and PerPSCM Protein Eng Des Sel 2007; 20:9-17. PMID:17999069; http://dx.doi.org/10.1007/s10142-006-9103-x.

24. Lf I, Wu I, Ida T, Takeda T, Ueda K, Uekimi I, Hayma T. Activation of the SHIHT1 pathway and nucleo-cytoplasmic shuttling of the tumor suppressor protein phosphatase 5 against CCN dependent activation of NF-kappaB. Mol Biol Cell 2009; 20:561-7; PMID:17993650; http://dx.doi.org/10.1016/j.cell.2009.04.002.

25. Daphna N, Zhou Z, Villeneuve J, Quinlivan RE, Tanaka H, Bannister AJ, et al. Acetylation and deacetylation of histone H3 lysine residues 14, 18, and 23 by the Histone deacetylase complex. Mol Cell 2006; 24:203-15. PMID:17207015; http://dx.doi.org/10.1016/j.molcel.2006.10.014.

26. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

27. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

28. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

29. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

30. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

31. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

32. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

33. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

34. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

35. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

36. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

37. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

38. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

39. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.

40. Ofek E, Neubauer M, Cattoretti G, Acetylation and deacetylation of H3K9ac in silico. Nucleic Acids Res 2011; 39:5270-8. PMID:21731836; http://dx.doi.org/10.1093/nar/gkr010.