SIRT7-dependent deacetylation of the U3-55k protein controls pre-rRNA processing

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SIRT7 is an NAD\(^+\)-dependent protein deacetylase with important roles in ribosome biogenesis and cell proliferation. Previous studies have established that SIRT7 is associated with RNA polymerase I, interacts with pre-ribosomal RNA (rRNA) and promotes rRNA synthesis. Here we show that SIRT7 is also associated with small nucleolar RNP (snoRNPs) that are involved in pre-rRNA processing and rRNA maturation. Knockdown of SIRT7 impairs U3 snoRNA dependent early cleavage steps that are necessary for generation of 18S rRNA. Mechanistically, SIRT7 deacetylates U3-55k, a core component of the U3 snoRNP complex, and reversible acetylation of U3-55k modulates the association of U3-55k with U3 snoRNA. Deacetylation by SIRT7 enhances U3-55k binding to U3 snoRNA, which is a prerequisite for pre-rRNA processing. Under stress conditions, SIRT7 is released from nucleoli, leading to hyperacetylation of U3-55k and attenuation of pre-rRNA processing. The results reveal a multifaceted role of SIRT7 in ribosome biogenesis, regulating both transcription and processing of rRNA.
Ribosome biogenesis is a highly regulated process that requires the coordinated activity of all three nuclear DNA-dependent RNA polymerases (Pol I, II and III) along with more than 200 trans-acting factors, including transcription factors, small nucleolar RNPs (snoRNPs), ribosomal proteins, and proteins that promote processing and modification of ribosomal RNA (rRNA)1–3. The initial 47S ribosomal precursor RNA (pre-rRNA) is posttranscriptionally cleaved to form the mature 28S, 18S and 5.8S rRNAs. During the maturation process, the pre-rRNA and its processing intermediates undergo numerous posttranscriptional modifications, which are guided and catalysed by snoRNPs (ref. 4).

In eukaryotes, the U3 snoRNA-containing snoRNP is essential for processing of pre-rRNA (refs 4, 5). U3 snoRNA is associated with four common box C/D core snoRNP proteins, that is, 15.5k, Nop56, Nop58, and fibrillarin and the U3-specific protein U3-55k (refs 4, 6). The 12S U3 snoRNP particle constitutes a subcomplex of the phylogenetically conserved 80S/220MDa small subunit (SSU) processome, a large ribonucleoprotein complex that assembles on nascent pre-rRNA and is indispensable for ribosome biogenesis7–10. The yeast SSU processome contains as many as 72 proteins, including endonucleases, RNA helicases, ATPases, GTPases, protein kinases and other regulatory proteins11. The U3 snoRNA was implicated in pre-rRNA processing by chemical cross-linking and mutational studies, showing that regions of complementarity allow base pairing of U3 snoRNA with the 5'-ETS and pre-18S rRNA, thus directing pre-rRNA cleavage12–16. Conditional knockout of the U3 snoRNA genes in yeast abolished pre-rRNA processing at specific sites, leading to accumulation of unprocessed 35S pre-rRNA and loss of mature 18S rRNA (ref. 17).

For many years, research on mammalian pre-rRNA processing lagged behind that on budding yeast, mainly because of the power of yeast genetics. A recent screen in human cells identified 286 proteins involved in pre-rRNA synthesis and pre-rRNA maturation, 74 of them having no yeast homologue2. Among the identified genes was SIRT7, which encodes a member of the sirtuin family of NAD+-dependent deacetylases. Sirtuins constitute a phylogenetically conserved protein family with central roles in the cellular response to oxidative, metabolic and genotoxic stress18–20. Sirtuins are regarded as intracellular sensors of the metabolic environment, as the enzymatic activity is dependent on the cosubstrate NAD+21. Little is known about SIRT7 function and only a few molecular substrates of SIRT7 have been identified. SIRT7 is enriched in nucleoli, where it facilitates RNA polymerase I (Pol I)-dependent transcription by interacting with the upstream binding factor (UBF; ref. 22) and the Pol I subunit PAF53 (ref. 24). Interaction with and deacetylation of PAF53 by SIRT7 drives ribosomal DNA (rDNA) transcription and ribosome biogenesis, and consequently, cell growth and proliferation24,25. SIRT7 expression correlates with cell growth and proliferation, being abundant in metabolically active cells, and low or even absent in non-proliferating cells25. Consistent with a functional link between SIRT7, ribosome biogenesis and cell proliferation, SIRT7 is overexpressed in several types of tumours26–28.

In accord with previous studies showing that pre-rRNA transcription and processing are functionally coupled25,29,30, we report here that SIRT7 plays an essential role not only in rDNA transcription but also in specific cleavage of pre-rRNA. We show that SIRT7 is associated with U3 snoRNPs and promotes pre-rRNA cleavage at the 5'-terminal processing site by deacetylating U3-55k (Rrp9), a core subunit of the U3 snoRNP complex, deacetylation of U3-55k being required for all subsequent processing events. On exposure to hyperosmotic stress, SIRT7 is released from nucleoli, leading to hyperacetylation of U3-55k and processing defects. The results uncover a SIRT7-dependent mechanism that links rDNA transcription to pre-rRNA processing, reinforcing the pivotal role of SIRT7 in ribosome biogenesis, cell metabolism and homeostasis.

**Results**

**SIRT7 is associated with snoRNAs.** To characterize the repertoire of nuclear RNAs that are associated with SIRT7, we performed cross-linking and immunoprecipitation (CLIP)-seq, that is, immunoprecipitated Flag-tagged SIRT7 from UV-cross-linked HEK293T cells and sequenced the co-precipitated RNA. Deep sequencing of SIRT7-associated RNAs and subsequent bioinformatic analysis identified pre-rRNA, numerous messenger RNAs (mRNAs) and non-coding RNAs (Supplementary Fig. 1a). Consistent with SIRT7 being associated with Pol I and activating rDNA transcription24, the majority of SIRT7-associated RNA reads (87.8%) covered the entire transcribed region of rDNA, supporting that SIRT7 binds to nascent pre-rRNA (Fig. 1a and Supplementary Fig. 1b,c). About 9.7% of reads mapped to transcripts synthesized by Pol II and 0.8% to RNAs synthesized by Pol III. Among the SIRT7-associated Pol II and Pol III transcripts, RNAs implicated in RNA metabolism were enriched, including mRNAs encoding ribosomal proteins and snoRNAs (Fig. 1b). We identified a total of 43 snoRNAs, comprising all three classes of snoRNA, that is, box C/D, box H/ACA and small Cajal body-specific (sca) RNAs (Fig. 1c and Supplementary Table 1). Two of them, U3 and U13 snoRNA, are transcribed from their own promoter, U3 RNA being the most abundant one (Fig. 1d and Supplementary Fig. 1d). In addition, numerous intron-encoded snoRNAs were associated with SIRT7, including SNORA73A, SNORA73B, SNORA74A, SCARNA6, SCARNA13 and SCARNA10, the distribution of reads at the coding region of these snoRNA being markedly higher than at adjacent genomic regions, indicating that SIRT7 is associated with mature snoRNAs (Supplementary Fig. 2a). To validate that SIRT7 binds to mature U3 snoRNA rather than the U3 snoRNA precursor (pre-U3 snoRNA), we performed reverse transcription–quantitative polymerase chain reaction (RT–qPCR) of U3 snoRNA associated with Flag-tagged SIRT7 using primers covering the 8 nt extension at the 3′ end of pre-U3 snoRNA. This analysis, together with alignment of the U3 snoRNA CLIP-seq reads, revealed that SIRT7 predominantly interacts with mature and not with pre-U3 snoRNA (Supplementary Fig. 1e,f).

To prove that the interaction of SIRT7 with snoRNAs is direct rather than mediated by auxiliary proteins, we repeated the CLIP experiments under native and denaturing conditions, under which only direct protein/RNA interactions are preserved. Analysis of recovered RNA by RT–qPCR confirmed direct binding of SIRT7 to pre-rRNA and to snoRNAs identified by CLIP-seq (Fig. 1e and Supplementary Fig. 2b,d). Moreover, we validated the association of SIRT7 with several snoRNAs identified by CLIP-seq in HEK293T cells that stably or transiently express Flag/HA-or Flag-tagged SIRT7 (Supplementary Fig. 2c–f). Moreover, chromatin immunoprecipitation (ChIP) assays revealed that SIRT7 is associated with rDNA and some snoRNA gene loci, for example, U3 and U13 snoRNA genes, but not with intron-encoded snoRNA genes, for example, U14, SNORA73A, SNORA74A, SCARNA10 and SCARNA13 (Fig. 1f). Together with the observation that expression of U3 snoRNA was decreased by 50% in SIRT7-deficient cells (Supplementary Fig. 2g), this result suggests that SIRT7 affects transcription or stability of U3 snoRNA.

**SIRT7 promotes U3 snoRNA-dependent pre-rRNA processing.** The finding that SIRT7 is associated with both pre-rRNA and...
**Figure 1 | SIRT7 is associated with pre-rRNA and snoRNAs.** (a) SIRT7 CLIP-seq reads mapped to a custom annotation file of a human rDNA repeat (middle) or the transcribed region (bottom). The region encoding 18S, 5.8S and 28S rRNA is highlighted. SIRT7 reads after subtraction of IgG reads were normalized to input reads (y axis). (b) Gene ontology categories of SIRT7 CLIP-seq peaks. The most representative clusters are shown according to the adjusted P value (–log_{10} P). (c) SIRT7-bound snoRNAs comprise C/D box, H/ACA box snoRNAs and scaRNAs. The number (n) and relative abundance (%) of each snoRNA class associated with SIRT7 is presented. (d) U3, SNORA73A and 73B snoRNAs are overrepresented among SIRT7-associated snoRNAs. SIRT7 reads mapped to corresponding snoRNAs are indicated as percentage of all snoRNAs identified by CLIP-seq. (e) Comparison of SIRT7-associated RNAs under native and denaturing conditions. His/V5-tagged SIRT7 expressed in HEK293T cells was affinity-purified on Ni-NTA-agarose under native or denaturing conditions, and associated RNAs were detected by RT–qPCR. Lysates from non-transfected HEK293T cells were used for control (Ctrl). Associated pre-RNA was monitored by RT–qPCR using primer H1 (Supplementary Table 3). Bars represent means ± s.d. from three experiments. See also Supplementary Fig. 2b.d. (f) ChIP assays showing association of endogenous SIRT7 (left panel) or transiently overexpressed Flag-SIRT7 (right panel) with the indicated gene loci in HEK293T cells. rDNA was amplified using primers H4 (coding) and H18 (IGS; Supplementary Table 3). Bars represent means ± s.d. from three experiments. See also Supplementary Fig. 2d.
snoRNAs suggests that beyond its function in rDNA transcription SIRT7 may also be involved in snoRNP-dependent processing of pre-rRNA. To test this, RNA was metabolically labelled in control and SIRT7-deficient cells, and pre-rRNA and processing intermediates were analysed by gel electrophoresis and fluorography (Fig. 2a). Consistent with SIRT7 activating Pol I transcription, depletion of SIRT7 led to roughly 50% reduction in 47/45S pre-rRNA and 28S rRNA. Notably, the level of nascent 18S rRNA was even more decreased, suggesting that SIRT7 plays a role in 18S rRNA processing.

To examine whether SIRT7 promotes U3 snoRNA-dependent cleavage of pre-rRNA within the external transcribed spacer (5′ETS), we performed in vitro processing assays using 32P-labelled RNA covering the first processing site at position +650. After incubation with extracts from mouse L1210 cells, transcripts were cleaved in a time-dependent fashion, yielding shorter RNAs that were cut at the 5′ETS processing site (Fig. 2b and Supplementary Fig. 3a). A control transcript comprising nucleotides from +709 to +1290 was not cleaved, underscoring the requirement of sequences around the 5′-terminal processing site at +650 for specific RNA cleavage. In support of SIRT7 serving a role in pre-rRNA processing, in vitro cleavage of the template RNA was inhibited if extracts were prepared from cells that were treated with nicotinamide (NAM), a competitive inhibitor of sirtuins (Fig. 2c). Conversely, processing activity increased if the reactions were supplemented with NAD⁺ (Fig. 2d), corroborating that the enzymatic activity of sirtuin(s) is beneficial for 5′-terminal processing of pre-rRNA. To prove that SIRT7 is the NAD⁺-dependent enzyme that promotes processing, the assays were performed in the absence or presence of recombinant SIRT7. In accord with SIRT7 promoting pre-rRNA processing, exogenous SIRT7, but not the enzymatically inactive mutant SIRT7/H187Y, enhanced specific cleavage of the template RNA (Fig. 2e and Supplementary Fig. 3b). Moreover, 5′ETS processing was attenuated in extracts from SIRT7-depleted cells, processing being restored after addition of wild-type SIRT7.

![Figure 2](image-url)  
**Figure 2 | SIRT7 is involved in pre-rRNA processing.** (a) Knockdown of SIRT7 impairs pre-rRNA synthesis and processing in vivo. U2OS cells transfected with control (siCtrl) or SIRT7-specific siRNAs (siSIRT7) were metabolically labelled with [3H]uridine. RNA was analysed by agarose gel electrophoresis and fluorography. The bar diagram shows quantification of the processing intermediates, values from siCtrl cells being set to 1. (b) In vitro processing assay. Extracts from L1210 cells were incubated with 32P-labelled RNA comprising the 5′ETS and 18S rRNA. Processing intermediates were analysed by gel electrophoresis and PhosphorImaging. See also Supplementary Fig. 3a. (c) 5′ETS processing is inhibited by NAM. The assay contained radiolabelled RNA (+ 541/1,060) and extracts from L1210 cells cultured for 6h in the absence or presence of NAM. (d) Processing is enhanced by NAD⁺. Processing assays containing radiolabelled RNA (+ 541/1,060) were substituted with NAD⁺ as indicated. (e) The catalytic activity of SIRT7 is required for pre-rRNA cleavage. Assays were supplemented with 15 or 30 ng of purified wildtype (WT) or mutant (H187Y) Flag-SIRT7 (Supplementary Fig. 3b). (f) Depletion of SIRT7 impairs processing. SIRT7 was depleted from L1210 cells by shRNAs (shSIRT7-1, shSIRT7-2, Supplementary Fig. 3c). Extracts from non-infected cells (−) or cells expressing control shRNA (shCtrl) served as control (left). To rescue impaired cleavage, 15 ng of wild-type Flag-SIRT7 (WT) or mutant H187Y (HY) were added to SIRT7-depleted extracts (right). (g) Depletion of U3 snoRNA abolishes processing. U3 snoRNA was depleted by preincubating extracts with U3-specific antisense oligos (ASO, 50 ng μl⁻¹) and 2U of RNase H (Supplementary Fig. 3d). In vitro processing was performed with undepleted (−) or depleted extracts in the absence or presence of 15 ng Flag-SIRT7. Bar diagrams in c-g show quantification of the ratio of cleaved versus uncleaved transcripts, presented as mean ± s.d. from three independent experiments (*P < 0.05, **P < 0.01, analysis of variance with Bonferroni’s test).
but not the enzymatically inactive mutant SIRT7/H187Y (Fig. 2f and Supplementary Fig. 3c). Depletion of U3 snoRNA from the cell extract by antisense oligonucleotides abolished cleavage at position +650 regardless of whether SIRT7 was added or not, confirming that in vitro processing was dependent on U3 snoRNP (Fig. 2g and Supplementary Fig. 3d). Together, these data demonstrate that both U3 snoRNA and SIRT7 are required for 5′ETS pre-rRNA cleavage, the initial step in 18S rRNA processing.

SIRT7 counteracts PCAF-dependent acetylation of U3-55k. Mammalian U3 snoRNPs are composed of the five core subunits U3-55k, NOP56, NOP58, fibrillarin and 15.5k (ref. 4). As the catalytic activity of SIRT7 is required for processing in vitro, SIRT7-dependent deacetylation of any core subunit might be required for efficient processing. Previous acetylome studies revealed that the U3 snoRNP-specific protein U3-55k is acetylated in the N-terminal domain31. We therefore reasoned that reversible acetylation of this subunit might regulate U3 snoRNA-dependent pre-rRNA processing. To test this, we monitored acetylation of U3-55k protein in cells treated with trichostatin A (TSA), an inhibitor of class III HDACs, or with NAM, which specifically inhibits sirtuins. In the absence of any inhibitor or on treatment with TSA, acetylation was barely detectable on western blots using an antibody that recognizes acetylated proteins. However, acetylation was markedly increased if cells were treated with NAM (Fig. 3a) or in cells overexpressing PCAF, suggesting that PCAF is the acetyltransferase that acetylates U3-55k (Supplementary Fig. 4a). In support of this view, short interfering RNA (siRNA)-mediated depletion of PCAF abolished acetylation of U3-55k regardless whether or not cells were treated with NAM (Fig. 3b).

To investigate whether SIRT7 or another nuclear sirtuin deacetylates U3-55k, we monitored the association of U3-55k with SIRT1, SIRT6 and SIRT7. The immunoprecipitation experiments in Fig. 3c,d show that endogenous or Flag-U3-55k was associated with endogenous and ectopic SIRT7, but not with SIRT1 or SIRT6. No binding of U3-55k to SIRT7 was observed if SIRT7 was affinity-purified under denaturing conditions (Supplementary Fig. 4b). In addition, U3-55k acetylation was compromised in cells overexpressing green fluorescent protein-human sirtuin 7 (GFP-SIRT7), but not in cells overexpressing SIRT1, underscoring that SIRT7 targets U3-55k for deacetylation (Fig. 3e). In support of this view, depletion of SIRT7 by short hairpin RNAs (shRNAs) or genetic knockout of SIRT7 led to hyperacetylation of U3-55k without affecting expression of U3-55k (Fig. 3f and Supplementary Fig. 4c,d). Significantly, recombinant SIRT7 efficiently deacetylated U3-55k in vitro, reinforcing that PCAF-mediated acetylation of U3-55k is counteracted by SIRT7 (Fig. 3g and Supplementary Fig. 4e).

Figure 3 | SIRT7 interacts with and deacetylates U3-55k. (a) Acetylation of U3-55k is increased on NAM treatment. HEK293T cells expressing Flag-U3-55k were treated for 6 h with 500 nM of TSA, 10 mM of NAM, or were left untreated (—). Acetylation of immunopurified Flag-U3-55k was analysed on western blots using an antibody that recognizes acetylated proteins. However, acetylation was markedly increased if cells were treated with NAM (Fig. 3a) or in cells overexpressing PCAF, suggesting that PCAF is the acetyltransferase that acetylates U3-55k (Supplementary Fig. 4a). In support of this view, short interfering RNA (siRNA)-mediated depletion of PCAF abolished acetylation of U3-55k regardless whether or not cells were treated with NAM (Fig. 3b).

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Deacetylation of U3-55k promotes pre-rRNA processing. To investigate whether reversible acetylation of U3-55k affects its interaction with U3 snoRNA, we monitored the association of U3-55k with U3 snoRNA by CLIP assays. These experiments revealed that binding of U3-55k to U3 snoRNA inversely correlated with U3-55k acetylation, the interaction with U3 snoRNA being decreased if cells were treated with NAM to inhibit SIRT7 activity (Fig. 4a, left). Consistent with acetylation impairing the interaction between U3-55k and U3 snoRNA, the association of U3-55k with U3 snoRNA was decreased in SIRT7 knockout cells (Fig. 4a, right). These findings emphasize that deacetylation of U3-55k by SIRT7 is required for efficient binding of U3-55k to U3 snoRNA.

To decipher the functional significance of U3-55k acetylation, we replaced two lysine residues (K12, K25) within the N-terminal domain of U3-55k that were reported to be acetylated31 by either arginine or glutamine, and monitored the impact of wild-type and mutant U3-55k on pre-rRNA processing. Overexpression of PCAF increased acetylation of wild-type U3-55k but did not augment acetylation of the K12/K25 mutants, supporting that...
To address this issue, we performed northern blot analysis revealing that the 47S precursor was barely detectable on hypertonic stress. The level of 47S pre-rRNA was restored on reversal to normo-osmotic conditions, implying that stress-induced transcriptional repression is reversible (Fig. 5a and Supplementary Fig. 6a). Notably, in hypertonic cells the precursors of 18S rRNA, that is, 21S and 18S rRNA, were barely detectable, indicating that exposure to hypertonic stress impaired the production of mature 18S rRNA (Fig. 5a).

To decipher the molecular mechanism underlying stress-dependent inhibition of pre-rRNA processing, we monitored U3-55k acetylation under different stress conditions that inhibit nucleolar transcription such as hypertonic stress, treatment with actinomycin D or treatment with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Both physiological and drug-induced stress caused robust hyperacetylation of U3-55k, whereas acetylation of mutant K12/25R (2KR) was not affected (Fig. 5b and Supplementary Fig. 6b). Stress-induced hyperacetylation had little effect on the nucleolar localization of U3-55k, whereas both endogenous and ectopic SIRT7 translocated from nucleoli to the nucleoplasm (Fig. 5c and Supplementary Fig. 6c). These results imply that translocation into the nucleoplasm separates SIRT7 from nucleolar U3-55k, which in turn leads to hyperacetylation of U3-55k and processing defects.

To examine whether stress-induced redistribution of SIRT7 from the nucleolus into the nucleoplasm correlates with compromised pre-rRNA processing, we monitored 18S rRNA processing intermediates in parental U2OS cells and in U2OS cells which stably express GFP-SIRT7. Overall, 47S rRNA levels and relevant processing intermediates were elevated in U2OS/GFP-SIRT7 cells compared with parental U2OS cells (Fig. 5d). On exposure to hypertonic stress, pre-rRNA synthesis and processing were suppressed in both cell lines. However, attenuation of processing was less pronounced in cells expressing GFP-SIRT7. This is probably due to the fact that a fraction of ectopic GFP-SIRT7 remained in nucleoli (Supplementary Fig. 6c), which alleviates processing defects. This result underscores the impact of SIRT7 on pre-rRNA processing, release from nucleoli under stress conditions leading to hyperacetylation of U3-55k and impaired pre-rRNA cleavage.

As U3 snRNAs are required for 18S rRNA processing and acetylation of U3-55k compromises its interaction with U3 snRNA, the association of U3-55k with U3 snRNA should be decreased in cells exposed to hypertonicity. Indeed, CLIP experiments revealed a significant decrease in U3-55k-associated U3 snoRNA in stressed cells, while the overall level of U3 snoRNA remained unaffected (Fig. 5e and Supplementary Fig. 6d). Consistent with SIRT7 being released from nucleoli on transcriptional or energetic stress, binding of SIRT7 to snoRNAs and pre-rRNA was no longer detected under hyperosmotic conditions, supporting that the interaction between SIRT7 and U3 snoRNA was abolished (Fig. 5f). Notably, overexpression of the acetylation-deficient mutant U3-55k/2KR, but not the acetylation-mimicking mutant U3-55k/2KQ, rescued the stress-induced processing defect, emphasizing the functional importance of hypoacetylated U3-55k in pre-rRNA processing (Fig. 5g).
Figure 5 | Pre-rRNA transcription and processing are attenuated under stress. (a) Northern blot of pre-rRNA and processing intermediates from HEK293T cells that were untreated, exposed to hypertonic stress for 90 min (hypertonic), or recovered to regular medium for 60 min (hypertonic rel.). Membranes were probed with 32P-labelled antisense riboprobe specific to 47S pre-rRNA (5’ETS, top) or with ITS1 oligos hybridizing to pre-rRNA intermediates (middle panel). (b) Acetylation of U3-55k is increased on different cellular stress conditions. HEK293T cells expressing Flag-U3-55k were treated with actinomycin D (Act D, 0.1 μg ml⁻¹, 4 h), AICAR (0.5 mM, 12 h) or exposed to hypertonic stress. Acetylation of immunopurified Flag-U3-55k and equal loading was monitored on western blots using anti-pan-AcK and anti-Flag antibodies. (c) Cellular localization of SIRT7 and U3-55k on hypertonic stress. Images showing localization of GFP-U3-55k and SIRT7 in normal conditions and on exposure to hyperosmotic stress for 90 min. Nuclei were stained with Hoechst 33342. Scale bars, 10 μm. (d) Overexpression of SIRT7 alleviates processing defects on hypertonic stress. Northern blot of RNA from parental U2OS cells and from cells which stably express GFP-SIRT7 (U2OS-GFP-SIRT7) using 5’ETS and ITS1 probes as in a. (e) CLIP-RT-qPCR monitoring binding of Flag-U3-55k to pre-rRNA, U3 snoRNA and U2 snRNA in HEK293T cells cultured in normo-osmotic medium or exposed to hypertonic stress for 90 min. Precipitated RNA was analysed by RT-qPCR using the indicated primers. Bars represent the means ± s.d. from three biological repeats (*P < 0.05, analysis of variance (ANOVA) with Bonferroni’s test). (f) CLIP-RT-qPCR of Flag-SIRT7 from HEK293T cells as described under e. Co-precipitated RNA was analysed by RT-qPCR using the indicated primers. Bars represent the means ± s.d. from three biological repeats (**P < 0.01, ANOVA with Bonferroni’s test). (g) Overexpression of mutant U3-55k-K12/25R rescues processing defects on stress. Northern blot of RNA from HEK293T cells expressing wild-type or mutant Flag-U3-55k exposed to hypertonic stress for 90 min. RNA was hybridized to 32P-labelled 5’ETS and ITS1 probes.

Discussion
Recent studies have linked expression of SIRT7 to cell proliferation and oncogenic activity, connecting SIRT7-dependent regulation of ribosome biogenesis with checkpoints controlling cell cycle progression, tumorigenesis and formation of metastatic phenotypes. Despite this important functional link, the enzymatic activity, the molecular targets and physiological functions of SIRT7 are poorly defined. Similar to other sirtuins, SIRT7 depletion did not globally change the acetylation levels of histones and nuclear proteomes, indicating that sirtuins exhibit a weak or substrate-specific deacetylase activity. SIRT7 was shown to target lysine 18 of histone H3 (H3K18), hypoacetylation of H3K18 compromising transcription of target genes. No measurable deacetylase activity of SIRT7 has been detected on other histone peptide substrates in vitro, supporting that SIRT7 functions as a highly specific deacetylase. In vivo, SIRT7 has been shown to deacetylate GABPβ1, a key regulator of mitochondrial functions, deacetylation of GABPβ1 promoting mitochondrial gene expression. Moreover, SIRT7 deacetylates the Pol I subunit PAF53, which enhances recruitment of Pol I to the rDNA promoter and activates transcription.

In this study, we have identified the nucleolar protein U3-55k as a novel substrate of SIRT7, deacetylation of U3-55k being required for 18S rRNA maturation. U3-55k is associated with U3 snoRNA, the core of the SSU processome, which promotes 5’ETS processing. Consistent with pre-rRNA transcription and processing being intertwined, the SSU processome binds to the 5’ETS of pre-rRNA enabling endonucleolytic cleavage of the primary transcript 650 nucleotides downstream of the 5’ETS end of pre-rRNA (refs 4,9,11,16). The SSU processome was proposed to act as a pre-rRNA chaperone enabling pre-18S rRNA folding and processing through base pairing of U3 snoRNA with pre-rRNA and acetylation of the rDNA promoter and activates transcription.

Although the importance of snoRNAs in pre-rRNA processing and posttranscriptional 2’-O-methylation and pseudouridylation of the rRNAs has been known for decades, our understanding of the molecular mechanisms by which mammalian pre-rRNA processing is regulated is very limited, owing mainly to the lack of in vitro systems that respond to regulatory factors. Processing of the 5’ETS was first linked to snoRNAs in vertebrates when a crude in vitro system was shown to faithfully cleave the 5’ETS in a U3 snoRNA-dependent fashion. Using this system, we found that cleavage of a template RNA bearing the first processing site...
of mouse pre-rRNA was compromised if SIRT7 activity was inhibited by NAM, or if extracts from SIRT7-depleted cells were used in the processing reactions. Supplementation with NAD+ and ectopic SIRT7 stimulated RNA cleavage, whereas an enzymatically inactive SIRT7 mutant failed to rescue processing activity. Moreover, in SIRT7-deficient cells the level of metabolically labelled 18S rRNA was markedly reduced, reinforcing the functional importance of SIRT7-dependent deacetylation of component(s) of the U3 snorNP in early pre-rRNA processing events.

Our results reveal that SIRT7 regulates pre-rRNA processing by deacetylation of U3-55k, a U3 snoRNA-associated protein that is not present in other box C/D snoRNPs. Consistent with acetylation affecting protein functions, we found that acetylation by PCAF impairs binding of U3-55k to U3 snoRNA, the association of U3-55k with U3 snoRNA being required for processing. Deacetylation by SIRT7, on the other hand, facilitates the interaction of U3-55k with U3 snoRNA, thus promoting pre-rRNA processing. The intimate link between SIRT7 activity and acetylation-dependent U3-55k function underscores the finding that knockdown of U3-55k leads to the same defects in pre-rRNA processing as those observed on knockout of SIRT7. Overexpression of wild-type U3-55k but not the acetylation-mimicking mutant 2KQ overcame the processing defects, emphasizing that SIRT7 and U3-55k are functionally connected.

The link between U3-55k acetylation and its removal by SIRT7 not only highlights the central role of SIRT7 in ribosome biogenesis but also points to an active role of SIRT7 as a pro-survival adaptor molecule in conditions of cellular stress. Previous studies have demonstrated that SIRT7 is released from nucleoli in response to transcriptional or metabolic stress, leading to hyperacyetylation of the Pol I-associated factor PAF53 and downregulation of rDNA transcription. The present study shows that translocation of SIRT7 on short exposure to hyperosmotic stress leads also to hyperacetylation of U3-55k and hence inhibition of pre-rRNA processing. Nucleolar retention of overexpressed SIRT7 under hypertonic stress attenuates processing defects corroborating the stress tolerance conferred by SIRT7 to the cells. Hyperosmosality impaired the generation of 21S and 18S pre-rRNA, whereas 30S pre-rRNA was hardly affected. Interestingly, RNAi-mediated knockdown of U3-55k or other U3 snoRNA-specific proteins, such as NOP56, NOP58 or fibrillarin, led to the same processing defects as U3-55k or other U3 snoRNA-specific proteins, such as NOP56, which in turn inhibits Pol I transcription and processing. Given that SIRT7 interacts with many RNAs that are transcribed by Pol II, it is tempting to speculate that SIRT7 function is not restricted to rDNA but might also prompt other genes to promote cellular survival and stress resistance, thus regulating a variety of important cellular processes.

**Methods**

**Transfections and cell treatments.** U2OS and HEK293T cells (ATCC) cultured in DMEM/10% fetal bovine serum were transfected with expression vectors encoding epitope-tagged proteins using the calcium phosphate precipitation technique or Fugene (Life Technologies). Cells were harvested 36–48 h post transfection. To generate clonal cell lines that stably express FLAG-HA- or GFP-tagged SIRT7, cells were selected in the presence of G418 (750 μg/ml) and G418 or GFP-resistant colonies were isolated. 

**Plasmids and antibodies.** Plasmids encoding hSIRT7, sh-hSIRT7, Flag-PCAF, CBP-HA and p300-HA have been described. To generate GFP- and Flag-tagged U3-55k, cDNA encoding human U3-55k (NCBI reference sequence: NM_004704.4) was inserted in pEGFP-C (Clontech) or pCMV-Tag2 (Stratagene). Point mutations converting K12 and K25 of U3-55k into arginine (2KR) and glutamine (2KQ) were introduced by PCR. To generate FLAG/HA-tagged hSIRT7, the sequence of the Flag/HA-tag was amplified by PCR and cloned into the plasmid pCMV-hSIRT7. Plasmids encoding mSIRT7-specific siRNAs were generated by cloning the corresponding sequences into plK.O.1. The Flag/M2 Affinity Gel (Sigma, F1804) was used for precipitation of Flag-tagged proteins and the GFP-Trap (Chromotek, gta) for purification of GFP-tagged proteins.

**Immunoprecipitation of cross-linked RNA.** RNA immunoprecipitation from UV-cross-linked HEK293T cells expressing Flag-SIRT7 or Flag-U3-55k was essentially done as follows. Nuclei from UV-irradiated (254 nm, 0.15 J cm⁻²) cells were lysed in 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, 0.5% sodium deoxycholate and 1% SDS, before cross-linking with 1 M DTE. After lysis, the lysates were cooled on ice, and immunoprecipitation was performed with mouse IgG control immunoprecipitations was purified with TRIZOL, and treated with DNase I. IgG control immunoprecipitations was purified with TRIZOL, and treated with DNase I. 

**Library preparation.** Libraries were prepared using the Illumina TruSeq Stranded mRNA kit (Illumina) with random hexamers. Libraries were purified using AMPure XP and quantified using the Qubit fluorometer (Life Technologies) with the appropriate standards. Libraries were pooled and sequenced on an Illumina HiSeq 2000 with paired-end 100bp reads. 

**RT-qPCR.** For reverse transcription, total RNA was treated with DNase I. The RNA-seq libraries were created using the NEBNext Ultra RNA Library Prep kit for Illumina (E7350). 50 cycles of sequencing were performed on the Illumina HiSeq 2000 Instrument. cDNA libraries from total RNA were used as Input. The reads were mapped to human hg19 reference genome (including human rRNA sequence) using TopHat with default parameters. The reads with mapping quality > 0 were extracted for analysis. The RPKM (reads per kilobase per million mapped reads) values were calculated for all genes with annotation in Gencode v17 including mRNAs from Dharmacon (ThermoFisher Scientific) and siRNAs against U3-55k (Supplementary Table 2) from Life Technologies. After reverse transfection with Lipofectamine 2000 or RNAiMAX (Invitrogen), cells were harvested 60–68 h. 

**Plasmids encoding hSIRT7, sh-hSIRT7, Flag-PCAF, CBP-HA and p300-HA have been described.** To generate GFP- and Flag-tagged U3-55k, cDNA encoding human U3-55k was amplified by PCR and cloned into the plasmid pCMV-hSIRT7. Plasmids encoding mSIRT7-specific siRNAs were generated by cloning the corresponding sequences into plK.O.1. The Flag/M2 Affinity Gel (Sigma, F1804) was used for precipitation of Flag-tagged proteins and the GFP-Trap (Chromotek, gta) for purification of GFP-tagged proteins.
and snoRNAs. The SIRT7 reads distributions on genes transcribed by different polymerases (Pol I, II, and III) were calculated and normalized to the total number of maps generated. By normalized enrichment analysis (NEA), corresponding to the sgRNAs were cloned into the lentiCRISPRv1 vector and the corresponding epitope peptides or with SDS sample buffer and visualized with the respective antibodies. Precipitated DNA was calculated as the percentage of input DNA. Primers are listed in the Supplementary Table 3.

**In vitro processing assay.** Processing of pre-rRNA in vitro was performed as follows5. Extract proteins (60–80 μg) from L1210 cells were incubated with 20 moles of 32P-labelled synthetic transcripts in 20 mM Hepes pH 7.9, 2 mM MgCl2, 0.14 mM EDTA, 1.5 mM ATP, 120 mM KCl, 2 mM EDTA, transferred to Hybond-N nylon membranes (GE Healthcare), and labelled RNA was visualized by fluorography using ENHANCE (PerkinElmer).

**Chromatin immunoprecipitation.** ChIP experiments were performed as follows5. In brief, nuclei were isolated from HEK293T or U2OS cells fixed with 1% formaldehyde (10 min, room temperature) and quenched with 0.125 M glycine. After sonication (Bioruptor, Diagenode) to yield 250–500 bp fragments chromatin was diluted fivefold with IP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0 and 167 mM NaCl), preclared with protein A and G Sepharose (GE Healthcare) in the presence of 200 μg/ml of sonicated E. coli DNA, and incubated overnight with the respective antibodies. Protein-DNA complexes were captured on protein A and G Sepharose followed by washes in low-salt buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM MgCl2, 1% Triton X-100), high-salt buffer containing 500 mM NaCl, LiCl buffer (250 mM LiCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% Na-deoxycholate and 0.5% Triton X-100) and Tris EDTA buffer. After reversal of the cross-link, DNA was purified and amplified by qPCR. Precipitated DNA was calculated as the percentage of input DNA. Primers are listed in the Supplementary Table 3.

**In vitro deacylation assay.** HEK293T cells co-expressing GFP-U3-55k and Flag-PCAF were harvested and lysed in buffer AM-400 (400 mM KCl, 20 mM Tris-HCl pH 8.0, 0.2 mM EDTA pH 8.0, 5 mM MgCl2, 0.2 mM EDTA, 10% glycerol and 0.5 mM DTT) containing 1% NP-40, 0.8–1 μg of Flag-SIRT7 was immunopurified from HEK293T cells. In brief, cells were lysed in buffer AM-300 (300 mM KCl, 20 mM Tris-HCl pH 7.9, 5 mM MgCl2, 0.2 mM EDTA, 10% glycerol and 0.5 mM DTT) containing 1% NP-40, incubated for 1 h at 4 °C with 0.8 μg of Flag-SIRT7 in 10 mM Tris-HCl pH 8.0, 4 mM MgCl2, 0.2 mM DTT, 10% glycerol in the presence or absence of 2 mM NAD+ (Sigma). Acetylation of U3-55k was monitored on immunoblots using anti-pan-AcK antibodies.

**RNA pull-down experiments.** Immobilized GFP-tagged U3-55k was incubated for 3 h at 4 °C with radiolabelled U3 snoRNA or mutI RNA (ref. 34) in buffer AM-200 (200 mM KCl, 20 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 5 mM MgCl2, 10% (v/v) glycerol, 0.1% NP-40, 1 mM EGTA and 10 mM β-mercaptoethanol). After stringent washing, bound RNA was extracted, subjected to gel electrophoresis and visualized by PhosphorImaging.

**Northwestern blot.** Northwestern assays were performed as described24. Briefly, purified proteins were separated by SDS-polyacrylamide electrophoresis transferred to nitrocellulose membranes (GE Healthcare) and proteins were re-natured in 10 mM Tris-HCl pH 6.8, 25 mM NaCl, 1 mM EDTA, 0.04% BSA and 0.04% NP-40 at 4 °C (16 h) and incubated for 2 h at room temperature in the same buffer supplemented with 6.0 mM of 32P-labelled in vitro transcribed U3 snoRNA. After washing in buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8.0 and 0.1% Tween-20, bound RNA was detected by PhosphorImaging.

**Statistics and quantitative analyses.** The values in the graphs show means of three independent experiments with error bars representing s.d. SPSS 16.0 software was used for statistical analysis. Analyses of variance were performed using analysis of variance with Bonferroni’s test. The significance level was set at P < 0.05 or P < 0.01. Quantification of western blot signals and radioactive signals was performed using ImageJ and Image Gauge software, respectively.

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