Supplementary Data

Consisting of:

Supplementary Figures

Supplementary Figures

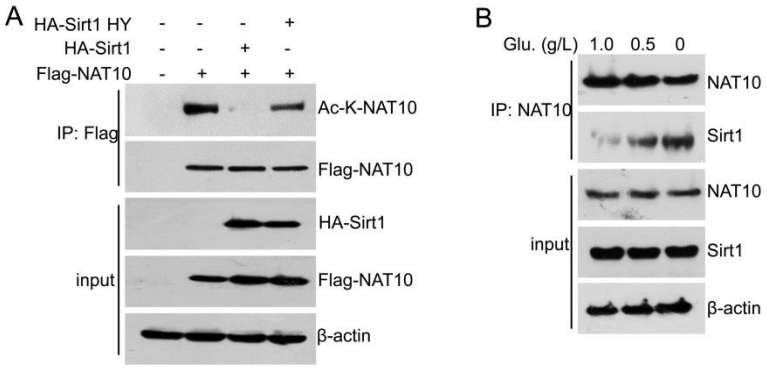

Figure S1. NAT10 is deacetylated by Sirt1 upon energy deprivation

A The indicated plasmids were cotransfected into HEK293T cells. Cell lysates were prepared and subjected to immunoprecipitation using anti-Flag antibody. Acetylation levels of Flag-NAT10 were evaluated by Western blot using an anti-acetyl-lysine antibody.

B HCT116 cells were cultured in medium containing 1.0 g/L, 0.5 g/L or 0 g/L of glucose for 18 hrs. Immunoprecipitation was performed with anti-NAT10 antibody and the existence of Sirt1 in the precipitants was evaluated by immunoblot.

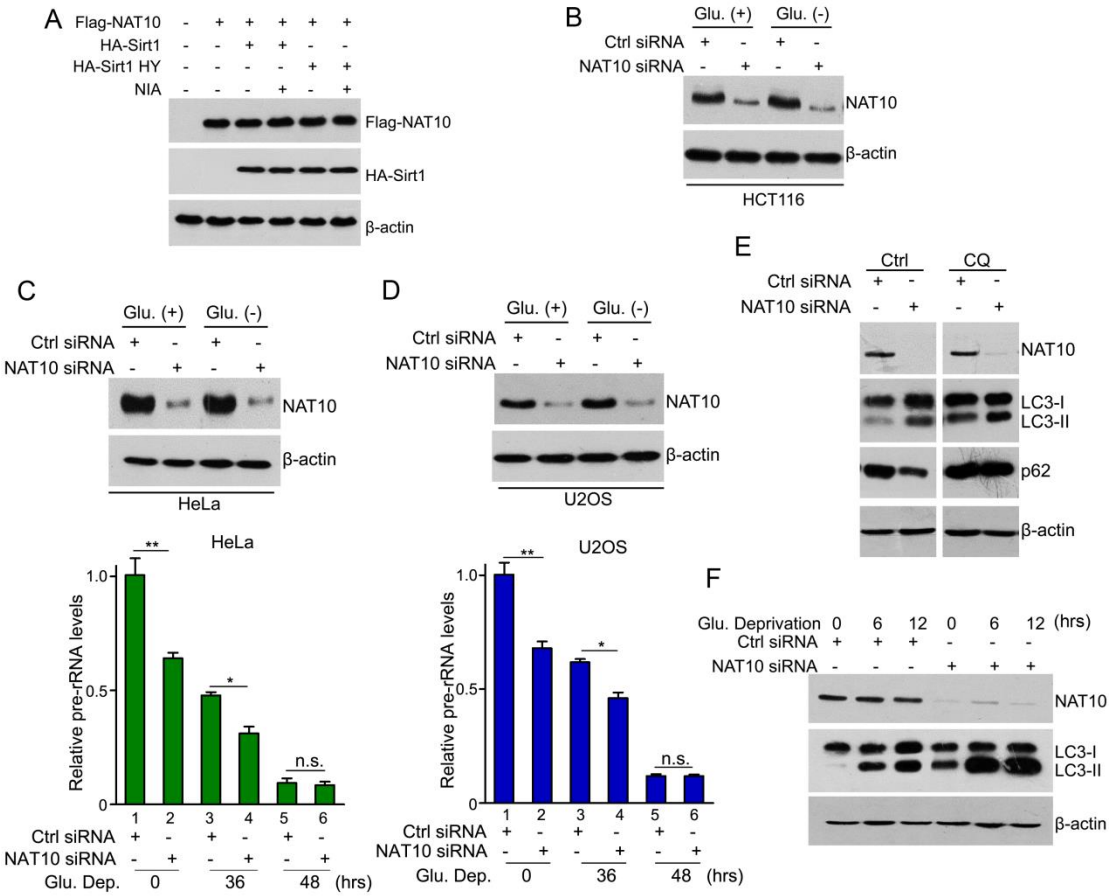

Figure S2. NAT10 regulates rRNA biogenesis and autophagy induction

A HCT116 cells were transfected with the indicated plasmids and treated with NIA. After 24 hrs, cells were harvested and Western blotting was performed to evaluate the indicated proteins.

B HCT116 cells were transfected with the indicated siRNAs and cultured in normal or glucose-free medium for 18 hrs. Cells were harvested and total proteins were subjected to Western blot for evaluating the indicated proteins.

C HeLa cells were transfected with the indicated siRNAs and cultured in normal or glucose-free medium as indicated. Cells were harvested and cellular proteins were subjected to Western blot (upper). Meanwhile, total RNAs were extracted and pre-rRNA levels were determined by RT-qPCR (lower). Error bars indicate SEM (n = 3). $^{**}P < 0.01$. $^{*}P < 0.05$. n.s., no significance. (two-tailed *t*-test).

D U2OS cells were transfected with the indicated siRNAs and cultured in normal or glucose-free medium as indicated. Cells were harvested and total proteins were subjected to Western blot (upper). Meanwhile, total RNAs were extracted and pre-rRNA levels were determined by RT-qPCR (lower). Error bars indicate SEM (n = 3). $^{**}P < 0.01$. $^{*}P < 0.05$. n.s., no significance. (two-tailed *t*-test).

E HCT116 $p53^{-/-}$ cells were transfected with a control siRNA or NAT10 siRNA. After treated

with chloroquine (CQ, 100 μM), cells were harvested and Western blotting was performed to evaluate the indicated proteins.

F HCT116 $p53^{-/-}$ cells were transfected with a control siRNA or NAT10 siRNA. Cells were cultured in glucose-free medium as indicated and Western blotting was performed to evaluate the indicated proteins.

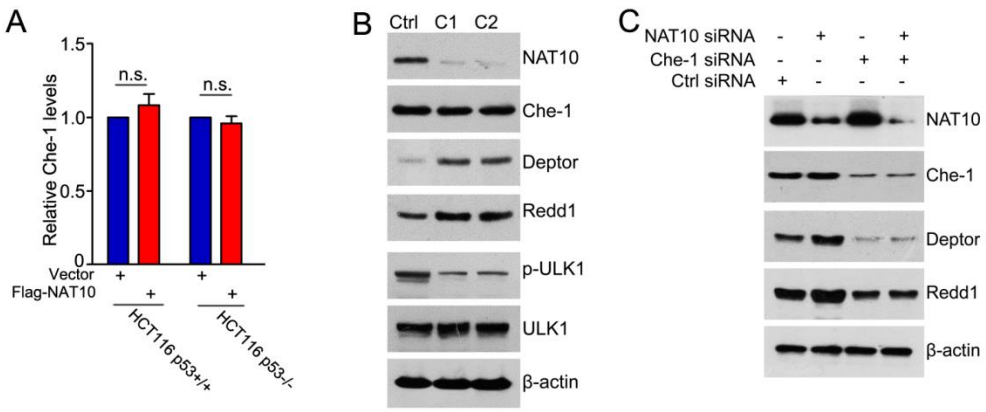

Figure S3. NAT10 inhibits Che-1 transcriptional activation of *Deptor* and *Redd1* under energy-rich condition

A The summarized data of the densitometry scanning of Che-1 bands standardized by that of β-actin from three independent experiments of Figure 4A. Error bars indicate SEM (n = 3). n.s., no significance.

B Cell lysates were prepared from HCT116 NAT10 KO cells (C1 and C2) or the HCT116 Ctrl cells. Western blotting was performed on the cell lysates for evaluating the indicated proteins.

C HCT116 cells were transfected with the indicated siRNAs. After 72 hrs, whole cell lysates were prepared and subjected to Western blot for evaluating the indicated proteins.

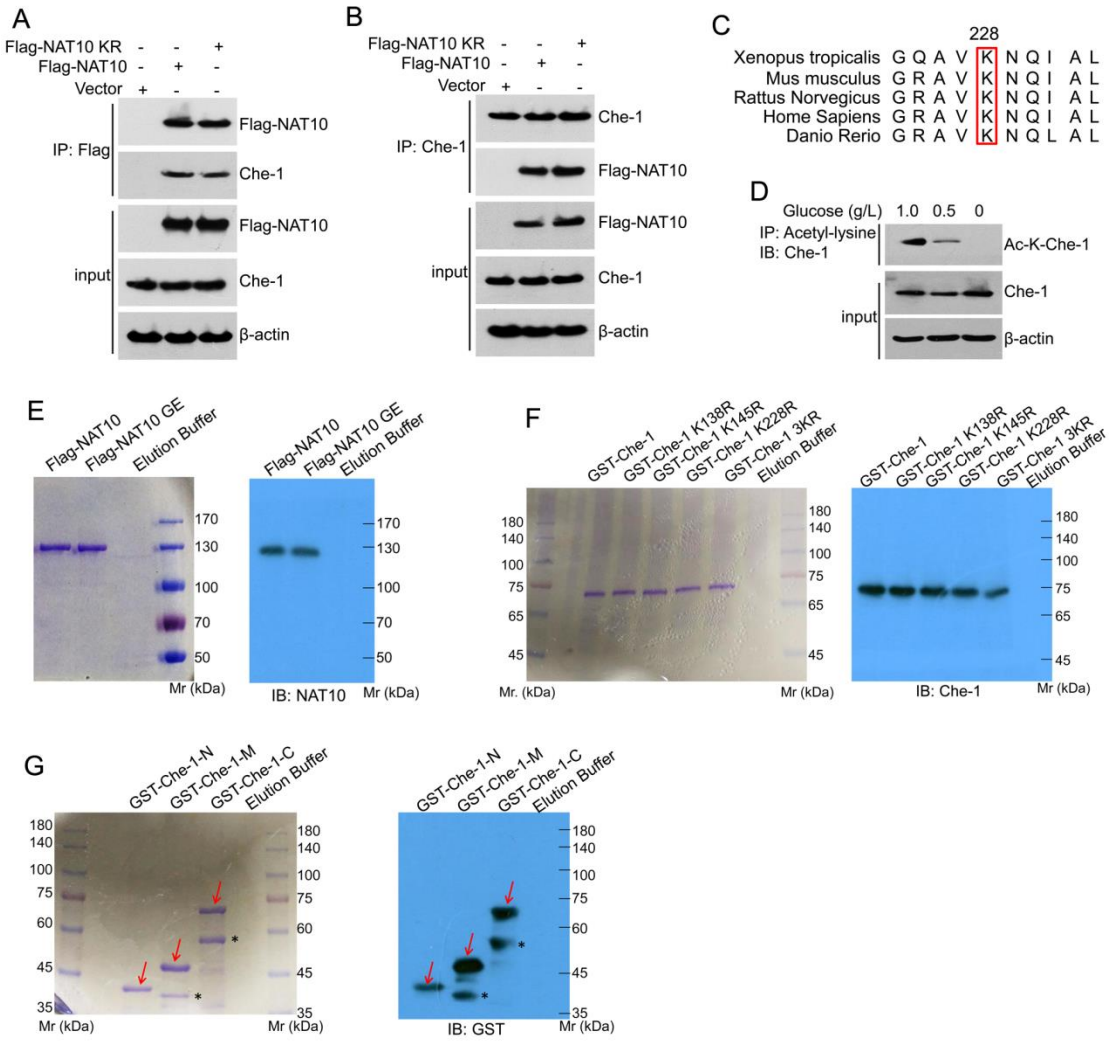

Figure S4. NAT10 acetylates Che-1 at K228

A HCT116 cells were transfected with the indicated plasmids. Twenty-four hours later, immunoprecipitation was performed with anti-Flag antibody on the cell lysates. Immunoprecipitants were immunoblotted to evaluate the indicated proteins.

B HCT116 cells were transfected with the indicated plasmids. Twenty-four hours later, the cell lysates were immunoprecipitated with anti-Che-1 antibody. Immunoprecipitants were immunoblotted with the indicated antibodies.

C The amino acid sequences of Che-1 from different species (homo sapiens, rattus norvegicus, danio reio, xenopus tropicalis and mus musculus) were compared. The conserved acetylation site of Che-1 is indicated by the red box.

D U2OS cells were cultured with medium containing 1.0 g/L, 0.5 g/L or 0 g/L glucose for 36 hrs. Cell lysates were immunoprecipitated with an anti-acetyl-lysine antibody. Immunoprecipitants were immunoblotted with anti-Che-1 antibody.

E Flag-NAT10 or Flag-NAT10 G641E mutant (GE) was purified from Sf9 insect cells as

described in Supplementary Methods and analyzed by Commassie Blue Staining (left) and Western blot (right).

F GST-Che-1, GST-Che-1 K138R, GST-Che-1 K145R, GST-Che-1 K228R or GST-Che-1 3KR was purified from *E. Coli* bacterial cells as described in Supplementary Methods and analyzed by Commassie Blue Staining (left) and Western blot (right).

G GST-Che-1 N, GST-Che-1 M or GST-Che-1 C was purified from *E. Coli* bacterial cells as described in Supplementary Methods and analyzed by Commassie Blue Staining (left) and Western blot (right). Arrows indicate the GST fusion proteins. * presents the fragment of the GST fusion protein.

(**E-G**) These purified proteins were used in the *in vitro* acetylation assays in Figure 5A, 5E and 5G.

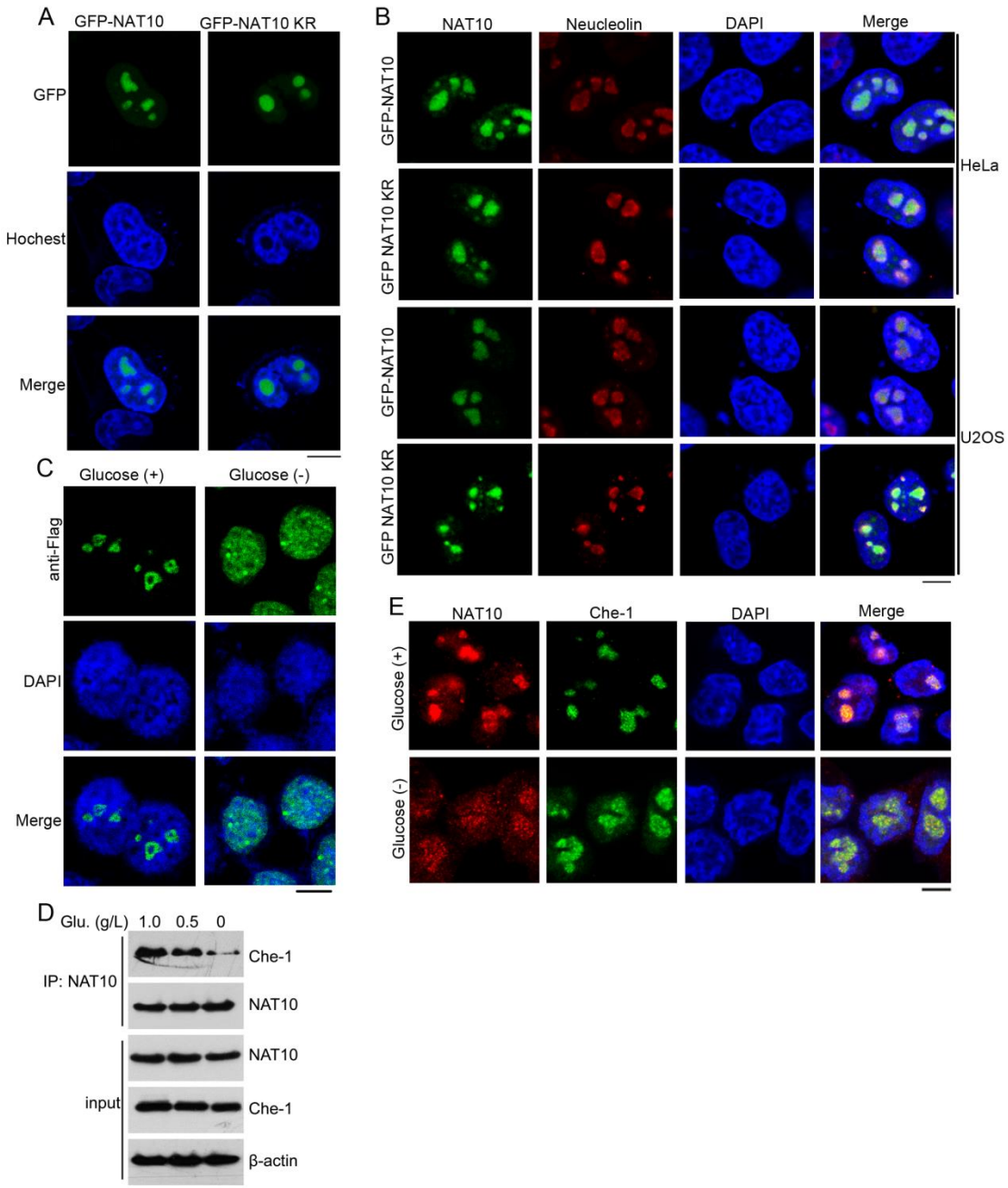

Figure S5. Nucleolar enrichment of NAT10 requires nascent pre-rRNA

A SW480 cells were transfected with the indicated plasmids. The localization of GFP-NAT10 or GFP-NAT10 KR was photographed under confocal microscopy. Nuclei were stained with Hochest33342. Bar represents 10 μM.

B HeLa or U2OS cells were transfected with the indicated plasmids. Immunofluorescence assay was performed using anti-nucleolin antibody. Nuclei were stained with DAPI. Fluorescence images were photographed under confocal microscopy. Bar represents 10 μM.

C HCT116 cells transfected with Flag-NAT10 KR were cultured in the glucose-rich or glucose-free medium for 18 hrs. Indirect immunofluorescence was performed with anti-Flag antibody

for determining the cellular localization of Flag-NAT10 KR (Green). Bar represents 10 μM.

D U2OS cells were cultured in medium containing 1.0 g/L, 0.5 g/L, or 0 g/L of glucose for 24 hrs. Immunoprecipitation was performed with anti-NAT10 antibody and the existence of Che-1 in the precipitants was evaluated by immunoblot.

E HCT116 cells were cultured in the glucose-rich or glucose-free medium for 18 hrs. Indirect immunofluorescence was performed for determining the cellular localization of NAT10 (Red) and Che-1 (Green). Nuclei were stained with DAPI. Bar represents 10 μM.

Supplementary Methods

RNA interference

For silencing NAT10,

siRNA-1 5'-CAGCACCACUGCUGAGAAUAAGA-3',

siRNA-2 5'-CCGAAUCCGGAUUCUCAUU-3' were used (1).

For silencing Sirt1,

siRNA-1 5'-AUUAAUAUCUGAGGUACUUCAUGGG-3'

siRNA-2 5'-AAAGGUUCGAACAUCCUCAAAGCGG-3' was used (2).

Protein Purification

Flag-NAT10-His or its mutants were cloned into pFast-Bac1. The recombinant baculoviruses were generated with the Bac-to-Bac Baculovirus expression system (Invitrogen). Recombinant proteins were purified from baculovirus-infected Sf9 cells using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. GST fusion proteins were purified using the Glutathione Sepharose 4B (GE Healthcare) after protein expression was induced with isopropyl-β-D-thiogalactoside in *E. coli* strain BL21 (DE3, Tiangen) and the bacteria was lysed by sonication. Purified proteins were confirmed by Coomassie blue staining after separated on SDS-PAGE and Western blotting probed with corresponding antibodies.

***In vitro* Acetylation Assay**

In vitro acetylation assay was set in 20 μls of reaction mixture containing 50 mM Tris-Cl (pH 7.9), 10% Glycerol, 0.1 mM EDTA, 1 mM PMSF, 10 mM sodium butyrate, 10 μM acetyl-CoA, 50 ng of purified Flag-NAT10 and 200 ng of purified GST-Che-1. The reaction mixtures were incubated at 30℃ for 1 hr and pulled down with Glutathione sepharose 4B. Western blotting was perfomed using anti-acetyl-lysine antibody for evaluating the acetylation levels of Che-1. Flag-NAT10 and GST-Che-1 fusion proteins used in the experiments were evaluated by Western blotting using the indicated antibodies.

GST Pull-down Assay

GST fusion proteins conjugated to the Glutathione Sepharose 4B (GE Healthcare) were incubated with purified proteins. After washing, the GST-fusion protein-bound proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Chromatin Immunoprecipitation and Quantitative-PCR (qPCR) Detection

Chromatin Immunoprecipitation was performed as described previously (2). Briefly, nuclear proteins were cross-linked to genomic DNA by treating cells with 1% formaldehyde for 10 min. Cross-linking was stopped by treatment with 0.125 M glycine. Cells were collected, centrifugated and resuspended in FA lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors and 50 mM Tris-Cl pH 8.0). Then, genomic DNA was sonicated to result in DNA fragments of

300 to 1,000 bp in length. Cellular debris was removed by centrifugation and lysates were diluted by 1:10 in ChIP dilution buffer (0.01% SDS, 1.0% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors and 16.7 mM Tris-Cl pH 8.0). After incubation with a salmon sperm DNA/protein A agarose slurry (GE Healthcare), the chromatin resuspension was centrifuged and the recovered chromatin solutions were subjected to immunoprecipitation. The immuno-complexes were collected with protein A agarose slurry. The beads were washed sequentially with the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-Cl pH 8.0), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl and 20 mM Tris-Cl pH 8.0) and LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris-Cl pH 8.0). Finally, beads were washed twice with 1 ml of TE buffer (1 mM EDTA and 10 mM Tris-Cl pH 8.0). Cross-linking was reversed by adding 200 mM NaCl and incubating at 65°C overnight. DNA was extracted after the remaining proteins were digested by proteinase K.

The purified DNA was amplified by real-time PCR using the ABI 7500/7500 fast Real-Time PCR systems (Applied Biosystems) and SYBR Green qPCR Mix (Roche). All Real-Time PCR data were analyzed as previously described (2).

RNA immunoprecipitation

RNA immunoprecipitation was performed as described previously (3). Briefly, HEK293T cells expressing Flag-NAT10 or Flag-Sirt1 were UV irradiated and resuspended in hypotonic buffer (5 mM HEPES pH 7.4, 85 mM KCl, 0.5% NP40). After centrifugation, nuclei were resuspended in 20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate in the presence of protease inhibitors. After sonication, lysates were precleared with protein A Sepharose and submitted to immunoprecipitation using anti-Flag M2 beads (Sigma) or protein A Sepharose coated with mouse IgG as control. After washes once with IP buffer and twice with IP buffer containing 400 mM KCl, immuno-complexes were eluted from beads with Flag peptide (20μg/100μl), decrosslinked and digested with proteinase K. RNA was extracted from the immunoprecipitates with TRIzol and incubated with DNase I (Promega). Reverse transcription was performed using random hexamers and MoMLV reverse transcriptase (Roche). Quantitative PCR (qPCR) was performed with the cDNA.

RNA Pull-down Assay

RNA Pull-down Assay was performed as previously described (3,4). Briefly, biotinylated 5' ETS-RNA was prepared using Biotin RNA Labeling Kit (Roche). Nuclear extract from HEK293T cells was percleared by incubating with Streptavidin (Invitrogen) beads. RNA binding was conducted by incubating the biotinylated RNA with the precleared nuclear extract.

Then, pre-washed Streptavidin-beads was added to the RNA binding reaction and further incubated at 4℃ for 1 hr. Beads were washed five times in buffer A (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 1 mM PMSF, 100 U/ml RNase inhibitor and proteinase inhibitor cocktail) and the bound proteins were analyzed by immunoblotting.

Northern Blotting

Northern Blotting for evaluating 47S pre-rRNA was performed as previously described (5). In brief, total RNA was loaded onto 1% glyoxal-agarose (NorthernMax Gly, Ambion) and blotted onto positively charged Nylon membrane (Ambion). The 47S pre-rRNA was hybridized with digoxigenin-labelled RNA probe complementary to the first 155 nucleotides of pre-rRNA using the DIG Northern starter kit (Roche) according to the manufacturer's instructions (6). The hybridized bands were detected by CDP-Star chemiluminescence (Roche) using alkaline phosphatase-conjugated anti-digoxigenin.

Sequential Immunoprecipitation and LC-MS/MS

U2OS cells were transfected with plasmids encoding Flag or Flag-NAT10. Forty-eight hours later, cells were collected and lysed in lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 0.5% Triton X-100, 10% glycerol and fresh protease inhibitor cocktail). Cell extracts were immunoprecipitated using anti-Flag monoclonal antibody M2-conjugated affinity gel (Sigma) followed by elution with Flag peptide. The eluted material was resolved by SDS-PAGE and visualized by silver staining. The bands were cut from SDS-PAGE gel, fully trypsinized and analyzed by Q-Extractive liquid chromatography tandem mass spectrometry (LC-MS/MS) using mass spectrometer (Thermo). Mass spectrometry was carried out at Protein Chemistry Facility at the Center for Biomedical Analysis of Tsinghua University and the data were processed using the Proteome Discoverer software (Version 1.4).

Statistical analysis

All statistical analyses were carried out using SPSS software 17 version (SPSS Inc.) and GraphPad Prism Software (GraphPad). The significance of the difference between two groups was assessed using the Student two-tailed *t*-test. The significance level was set at $P < 0.05$ or $P < 0.01$. Quantification of Western blot signals was performed using ImageJ.

Supplementary Table S1. Sequences of primers used in this study

The sequences of DNA oligonucleotides are shown in 5' to 3' orientation. All primers are specific to human sequences.

	Primer Sequence
pre-RNA (RT-qPCR)[a]	F: GAACGGTGGTGTGTCGTTC R: GCGTCTCGTCTCGTCTCACT
β-actin (RT-qPCR)[a]	F: ATCGTCCACCGCAAATGCTTCTA R: AGCCATGCCAATCTCATCTTGTT
rDNA-H0[b]	F: GGTATATCTTTCGCTCCGAG R: GACGACAGGTCGCCAGAGGA
rDNA-H8[b]	F: AGTCGGGTTGCTTGGGAATGC R: CCCTTACGGTACTTGTTGACT
rDNA-H23[b]	F: CCTTCCACGAGAGTGAGAAGC R: TCGACCTCCCGAAATCGTACA
rDNA-H0.3[b]	F: CGTGCGTGTCAGGCGTTCTCG R: GCCAGCGAGCCGATCGGCTCC
rDNA-H1[b]	F: GGCGGTTTGAGTGAGACGAGA R: ACGTGCGCTCACCGAGAGCAG
rDNA-H4[b]	F: CGACGACCCATTCGAACGTCT R: CTCTCCGGAATCGAACCCTGA
rDNA-ITS2[b]	F: CGGAAGACGGAGAGGGAAAG R: CGAGGGAGGAACCCGGA
rDNA-H13[b]	F: ACCTGGCGCTAAACCATTCGT R: GGACAAACCCTTGTGTCGAGG
rDNA-H18[b]	F: GTTGACGTACAGGGTGGACTG R: GGAAGTTGTCTTCACGCCTGA
Deptor (RT-qPCR)[c]	F: AGCTTTGCCACCGGCTTAT R: GGCAGAAGGGACTGTCATGAG
Redd1 (RT-qPCR)[c]	F: CACCCCAAAAGTTCAGTCGT R: TGTTTAGCTCCGCCAACTCT
Che-1 (RT-qPCR)[c]	F: AGCGCTTTGCCGACTTTACA R: GCTTGGTCTGTGTCCTTCGAA
Deptor (ChIP)[c]	F: ATGGCTGAGGTCTTGGTCAC R: GTACACGGGAAGACGGACAG
Redd1 (ChIP)[c]	F: CAGGAGAGAACGTTGCTTACG R: AGCCGCTGTAAGACAAGAGG

[a](Murayama A*, et al*, 2008)

[b](Chen S, et al, 2013)

[c](Agata Desantis, et al, 2015)

Supplementary Table S2. Summary of cell lines used in the study

Figure	Cell lines	Conclusions
Figure 1A, 1B	HCT116 cells	Sirt1 deacetylates NAT10
Figure S1A	HEK293T cells	
Figure 1D, 1E	HCT116 cells	Sirt1 interacts with NAT10
Figure 1H, 1I, 1J, S1B	HCT116 cells	Glucose deprivation promotes deacetylation of NAT10 by Sirt1
Figure 2A, 2B, S2A, S2B	HCT116 cells	Energy stress inhibits NAT10-mediated rRNA transcription
Figure 2C, 2D	HEK293T cells	
Figure S2C, S2D	Hela cells U2OS cells	
Figure 2E, 2F, 2G, 2H, 2I, 2J, 2K, S2E, S2F	HCT116 $p53^{+/+}$ cells HCT116 $p53^{-/-}$ cells	NAT10 regulates autophagy
Figure 3A	U2OS cells	NAT10 interacts with Che-1
Figure 3B, 3C	HCT116 cells	
Figure 3F, 3G	HCT116 cells	NAT10 has no effect on Che-1 stability
Figure 4A-4E, 4G, S3C	HCT116 cells	NAT10 inhibits transcriptional activation of Che-1 on *Deptor* and *Redd1*
Figure S3B	HCT116 NAT10 KO cells	
Figure 4F	U2OS cells	
Figure 5B, 5C, 5H, 5J, 5K, S4A, S4B	HCT116 cells	NAT10 acetylates Che-1
Figure 5I, S4D	U2OS cells	
Figure 6A	U2OS, HEK293T cells	Nucleolar enrichment of NAT10 requires nascent pre-rRNA
Figure 6B, 6F, 6G, 6H, S5C, S5E	HCT116 cells	
Figgure 6C, 6D	HEK293T cells	
Figure S5A	SW480 cells	
Figure S5B, S5D,	HeLa, U2OS cells	
Figure 7A-7D	HCT116 cells	NAT10 regulates Che-1 activity *in vivo*

Supplemental References

1. Liu, X., Tan, Y., Zhang, C., Zhang, Y., Zhang, L., Ren, P., Deng, H., Luo, J., Ke, Y. and Du, X. (2016) NAT10 regulates p53 activation through acetylating p53 at K120 and ubiquitinating Mdm2. *EMBO Rep*, **17**, 349-366.
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3. Chen, S., Seiler, J., Santiago-Reichelt, M., Felbel, K., Grummt, I. and Voit, R. (2013) Repression of RNA polymerase I upon stress is caused by inhibition of RNA-dependent deacetylation of PAF53 by SIRT7. *Mol Cell*, **52**, 303-313.
4. Marin-Bejar, O. and Huarte, M. (2015) RNA pulldown protocol for in vitro detection and identification of RNA-associated proteins. *Methods Mol Biol*, **1206**, 87-95.
5. Ito, S., Horikawa, S., Suzuki, T., Kawauchi, H. and Tanaka, Y. (2014) Human NAT10 is an ATP-dependent RNA acetyltransferase responsible for N4-acetylcytidine formation in 18 S ribosomal RNA (rRNA). *J Biol Chem*, **289**, 35724-35730.
6. Chen, S., Blank, M.F., Iyer, A., Huang, B., Wang, L., Grummt, I. and Voit, R. (2016) SIRT7-dependent deacetylation of the U3-55k protein controls pre-rRNA processing. *Nat Commun*, **7**, 10734.