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Dysregulation of upstream binding factor-1 acetylation at K352 is linked to impaired ribosomal DNA transcription in Huntington's disease

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Huntington's disease (HD) is an autosomal-dominant neurological disorder caused by expanded CAG repeats in the *Huntingtin* (*Htt*) gene, but it is not known how this mutation causes neurodegeneration. Herein, we found that dysfunction of upstream binding factor-1 (UBF-1) is linked to reduced ribosomal DNA (rDNA) transcription in HD. We identified that UBF1 acetylation at Lys (K) 352 by CREB binding protein (CBP) is crucial for the transcriptional activity of rDNA. UBF1 mutation (K352A, K352Q, and K352R) decreased rDNA transcriptional activity. Moreover, both CBP–dHAT mutant and knockdown of CBP by siRNA reduced acetylation of UBF1 and resulted in the decreased transcription of rDNA into rRNA. ChIP analysis showed a significant reduction of UBF1 occupancy in the promoter of rDNA in ST*Hdh*^{Q111} cell line model of HD. These results demonstrate that abnormal activity of UBF1 and its acetylation by CBP are linked to impaired rDNA transcription in HD. This novel mechanism suggests that modulation of UBF-mediated rDNA synthesis by CBP may be a therapeutic target for improving neuronal rDNA transcription in HD.
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Huntington's disease (HD) is an incurable and fatal autosomal dominant neurodegenerative disorder, typically of mid-life onset, characterized by a triad of chorea, cognitive abnormalities, and progressive dementia. The motor symptoms of HD are largely a consequence of profound neurodegeneration affecting the striatum.[1] HD is caused by an expansion of CAG repeats coding for glutamine (Q) in exon 1 of the *Huntingtin* (*Htt*) gene. One mechanism by which mutant Htt (mtHtt) contributes to neurodegeneration is through transcriptional dysregulation and chromatin remodeling.[2] Indeed, polyQ stretches in mtHtt interact physically with CBP (CREB binding protein), a transcriptional coactivator, and block the intrinsic histone acetyltransferase (HAT) activity of CBP.[3] These specific interactions show that mtHtt modulates transcriptional signaling cascades that may initiate a number of downstream pathophysiological mechanisms relevant to HD.

The nucleolus is an important subnuclear component of the transcription machinery of ribosomal genes.[4] The ribosomal DNA (rDNA) encoding ribosomal RNA (rRNA) is organized as tandem repeats, and is transcribed into 47S precursor rRNA by a specialized transcription complex, consisting of RNA polymerase I and other co-regulatory factors.[5] Neurons are postmitotic cells that have prominent nucleoli, but the role of this structure and the regulatory mechanism of rDNA remain unknown.[6] RNA polymerase I and upstream binding factor (UBF) have critical roles in the formation of active nucleolar organizer regions, and maintenance of rRNA transcriptional activity.[7] UBF is a member of the high mobility group (HMG) protein family and contains six HMG box DNA binding motifs. It is a highly conserved protein and there is UBF1 and UBF2 polypeptides, 97 and 94 kDa, respectively.[8] Not only UBF is a RNA polymerase I factor essential for rDNA (genes encoding rRNAs in eukaryotes) transcription by inducing remodeling of ribosomal gene chromatin, but it also serves a structural role by binding to other sequences across the entire rDNA repeat.[4] Interestingly, nucleolar accessory bodies (Cajal bodies) are associated with disorders caused by expansions of CAG repeats within genes, including HD, and the neuronal nuclear inclusions characteristic of polyQ diseases show a marked association with Cajal bodies.[9] In our study, we show for the first time that transcriptional modulation of rDNA, a key process in ribosome biogenesis, is altered in HD and that UBF and CBP have a role in altered rRNA expression. We found that UBF1 protein levels and UBF-mediated transcriptional activity of rDNA are impaired in cellular and animal model of HD. In parallel, we determined that CBP interacts with UBF1, acetylates UBF1 at Lys (K) 352, and modulates rDNA transcription.

Results

UBF1 is dysregulated in an animal model of HD. We examined UBF1 immunoreactivity, protein, and mRNA levels in the R6/2 transgenic mouse model of HD (Figure 1). The R6/2 line is a transgenic mouse line expressing exon 1 of the

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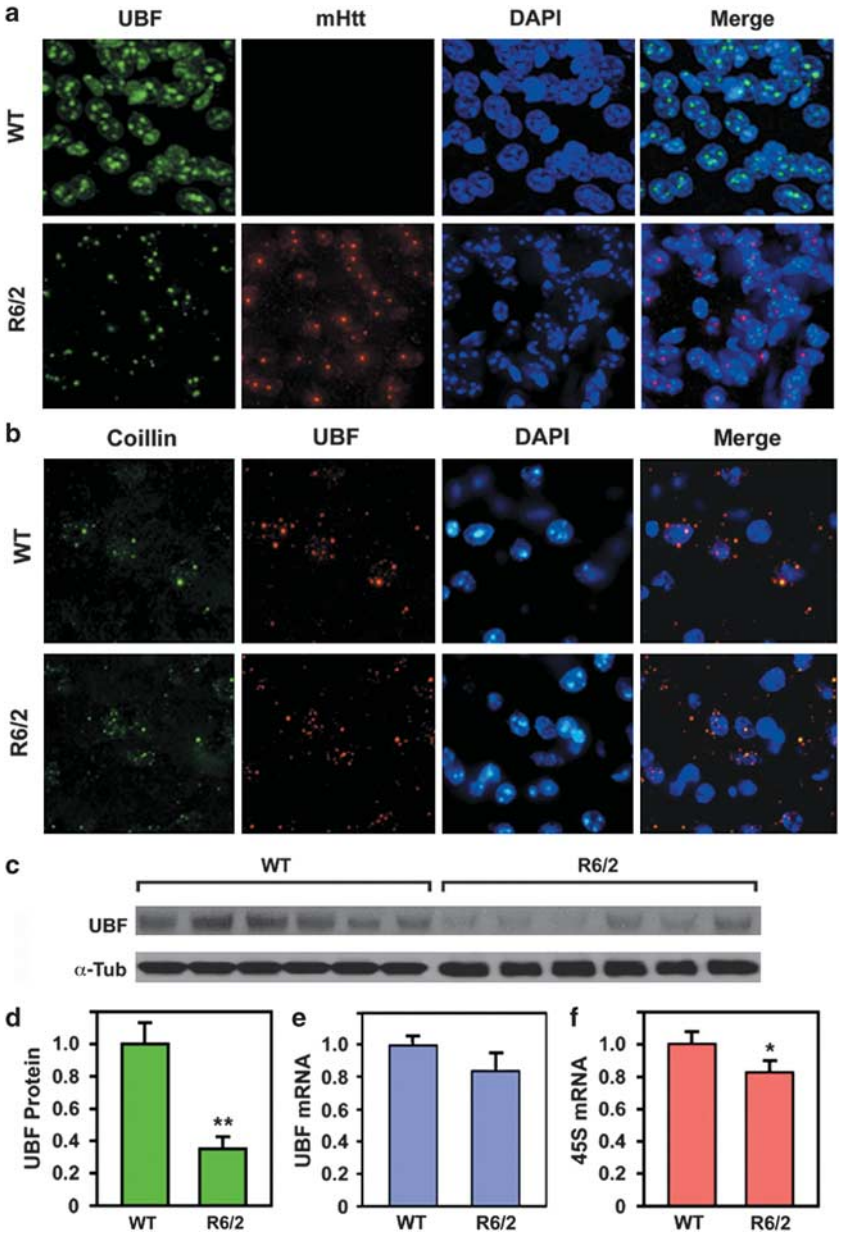

Figure 1 UBF1 is decreased in an animal model of HD (R6/2 line). (**a**) The immunoreactivity of UBF1 is markedly reduced in striatal neurons of R6/2 mice in comparison with wild type littermate control mice at 9 weeks of age. UBF1 and mtHtt are not co-localized. Scale bar: 10 μm. (**b**) Confocal microscopic analysis for UBF1 and coillin co-localization in the striatal tissue. Co-localization of coilin with UBF is decreased in striatal neurons of R6/2 mice in comparison with control mice. (**c**) Immunoblot assay for UBF-1 in the striatal tissue of WT and R6/2 mice. (**d**) Densitometry analysis of UBF1 protein level normalized to the levels of α–tubulin (α–Tub). (**e**) qRT-PCR analysis of UBF1 mRNA shows no significant difference between WT and R6/2 mice. Densitometry analysis from WT ($n=6$) and R6/2 ($n=6$). UBF1 mRNA is normalized to GAPDH mRNA. (**f**) qRT-PCR analysis of 45S mRNA shows a difference between WT and R6/2 mice. *Significantly from wild type mice at $P<0.05$; **$P<0.01$. Scale bar: 10 μm

human HD gene with an expanded CAG repeat.[9] R6/2 mice develop brain and body weight loss at 6 weeks of age and a gait and movement disorder by 9–11 weeks. These clinical features, along with striatal atrophy and neuronal intranuclear inclusions, are similar to changes found in humans with HD. At 10 weeks of age, UBF1 levels in the striatum of R6/2 mice were found to be significantly decreased compared with littermate controls as measured by immunofluorescence staining and confocal microscopy (Figures 1a and b) and Western blot analysis (Figure 1c). In addition, UBF1 levels in the striatum of human HD victims were markedly reduced compared with controls (Supplementary Figure 1A). The level of 18S RNA correlatively reduced in HD (Supplementary Figures 1B and C). In contrast to protein level (Figure 1d), UBF1 mRNA levels in striatum were not significantly decreased in R6/2 mice compared with WT controls (Figure 1e). We also found that 48S levels were downregulated in R6/2 mice in comparison with controls using quantitative real-time PCR (qRT-PCR) (Figure 1f). These results indicate that the UBF1 protein levels are reduced in HD mice and are linked to reduced-ribosomal transcription *in vivo.*

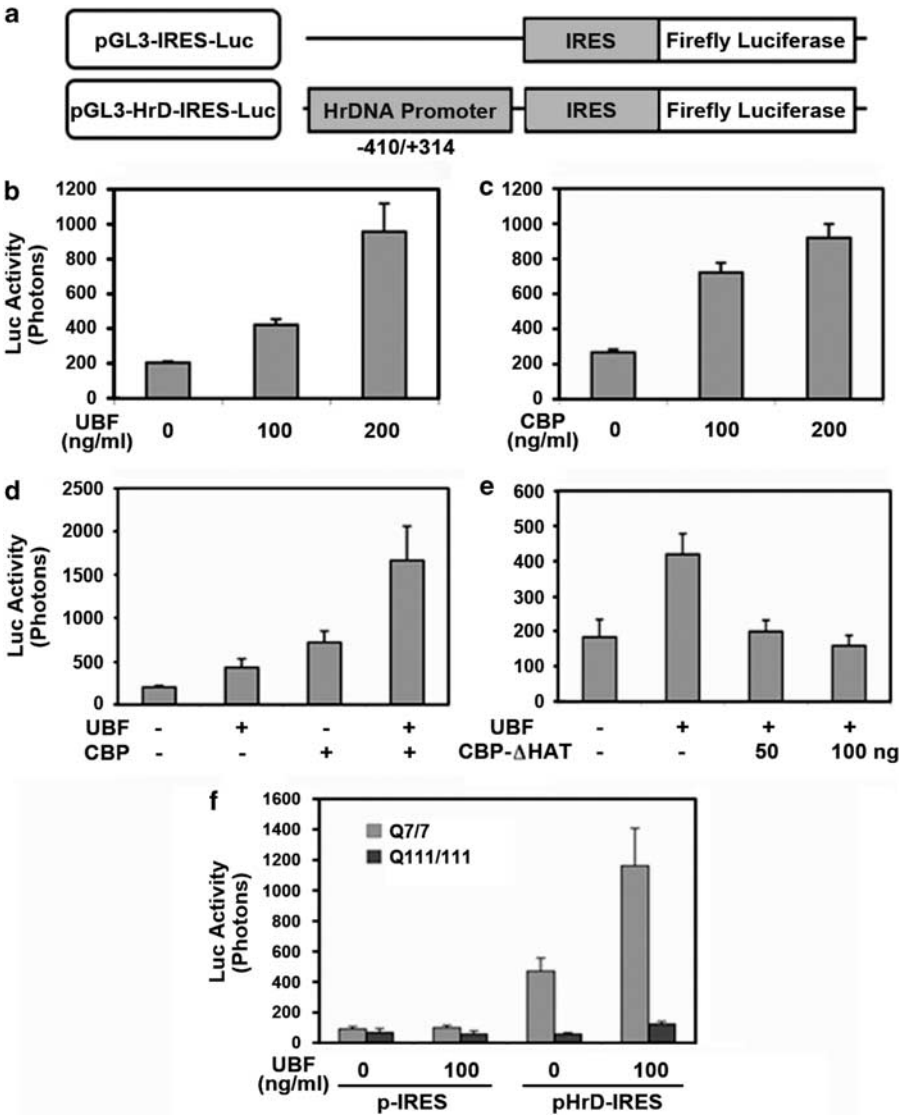

Figure 2 UBF1-dependent rDNA transcription is impaired in mutant HD cells. (**a**) The scheme of HrDNA IRES-reporter constructs. (**b**) UBF1 increases pHrD–IRES promoter activity in a dose-dependent manner. ST*Hdh*$^{Q7/7}$ (Q7) cells were transiently cotransfected with 400 ng of pHrD–IRES luciferase reporter plasmid and the other indicated constructs. Cells were incubated for 48 h and luciferase activity was measured. Transactivation of pHrD–IRES by UBF1 is increased in a dose-dependent manner. (**c**) CBP increases pHrD–IRES promoter activity in a dose-dependent manner. (**d**) UBF1 and CBP synergistically stimulate pHrD–IRES promoter activity. (**e**) The loss of acetyltransferase activity of CBP reduces UBF1-dependent transactivation of pHrD–IRES promoter. UBF1 was cotransfected with a CBP mutant with HAT domain deletion (CBP–dHAT). (**f**) The basal rDNA transcriptional activity was downregulated in ST*Hdh*$^{Q111/111}$ (Q111) cells. The data represent an average of three independent experiments

UBF1-induced rDNA transcription is impaired in a cellular model of HD. In order to examine how the transcriptional activity of rDNA is modulated by UBF1, we transiently transfected luciferase reporter vectors containing pIRES (internal ribosome entry segment) or pHrD (human rDNA)-IRES with UBF1, and measured the change of luciferase activity (Figure 2a). As expected, UBF1 increased in pHrD–IRES promoter activity in a dose-dependent manner in ST*Hdh*$^{Q7/7}$ (Q7) cells (Figure 2b). As CBP can modulate UBF1 activity, we further elucidated how the interplay between UBF1 and CBP affects the regulation of rDNA transcription. We found that CBP enhanced rDNA transcription in a dose-dependent manner (Figure 2c). When UBF and CBP constructs were co-transfected, a synergistic effect on rDNA transcription was found (Figure 2d). To confirm whether CBP-dependent and UBF1-induced rDNA transcription was due to the HAT activity of CBP, we used a CBP mutant construct with a mutant HAT site (CBP–dHAT). As we predicted, co-transfection of UBF1 and mutant CBP–dHAT lead to the loss of the synergistic effect on the rDNA transcription (Figure 2e). The loss of CBP–HAT activity reduced the promoter activity of pHrD–IRES to basal levels. UBF knockdown by shRNA reduced CBP-induced rDNA transcription (Supplementary Figure 2). We then examined transcriptional activity of rDNA in a cell line model of HD using wild type (ST*Hdh*$^{Q7/7}$) and mutant (STH*dh*$^{Q111/111}$) Huntingtin-expressing cells of striatal origin (Figure 2f). Basal rDNA transcriptional activity was higher in cells expressing ST*Hdh*$^{Q7/7}$ (Q7) and downregulated in STH*dh*$^{Q111/111}$ (Q111) cells. UBF1-dependent rDNA transcription was

dramatically impaired in mutant Q111 cells in contrast to Q7 cells, where UBF1 led to increased pHrD–IRES promoter activity in a dose-dependent manner. Using a dual luciferase assay (HrD–IRES fire fly luciferase *versus* Renilla luciferase), we found that the transcriptional system is dysregulated in Q111 cells compared with Q7 cells (Supplementary Figure 3). UBF1 knockdown using shRNA only reduced basal pHrD–IRES promoter activity in WT cells.

CBP dysregulation is linked to UBF1 dysfunction in HD. As CBP is known to modulate UBF1 and CBP and is dysregulated in HD,[10] we sought to determine the relationship between CBP and UBF1 activity in HD. First, we confirmed that CBP levels are affected in HD mice and HD cell lines. CBP levels in the striatum and hippocampus of R6/2 mice were significantly reduced compared with littermate controls at 10 weeks of age, as monitored by western blot analysis (Figure 3a). Punctate CBP

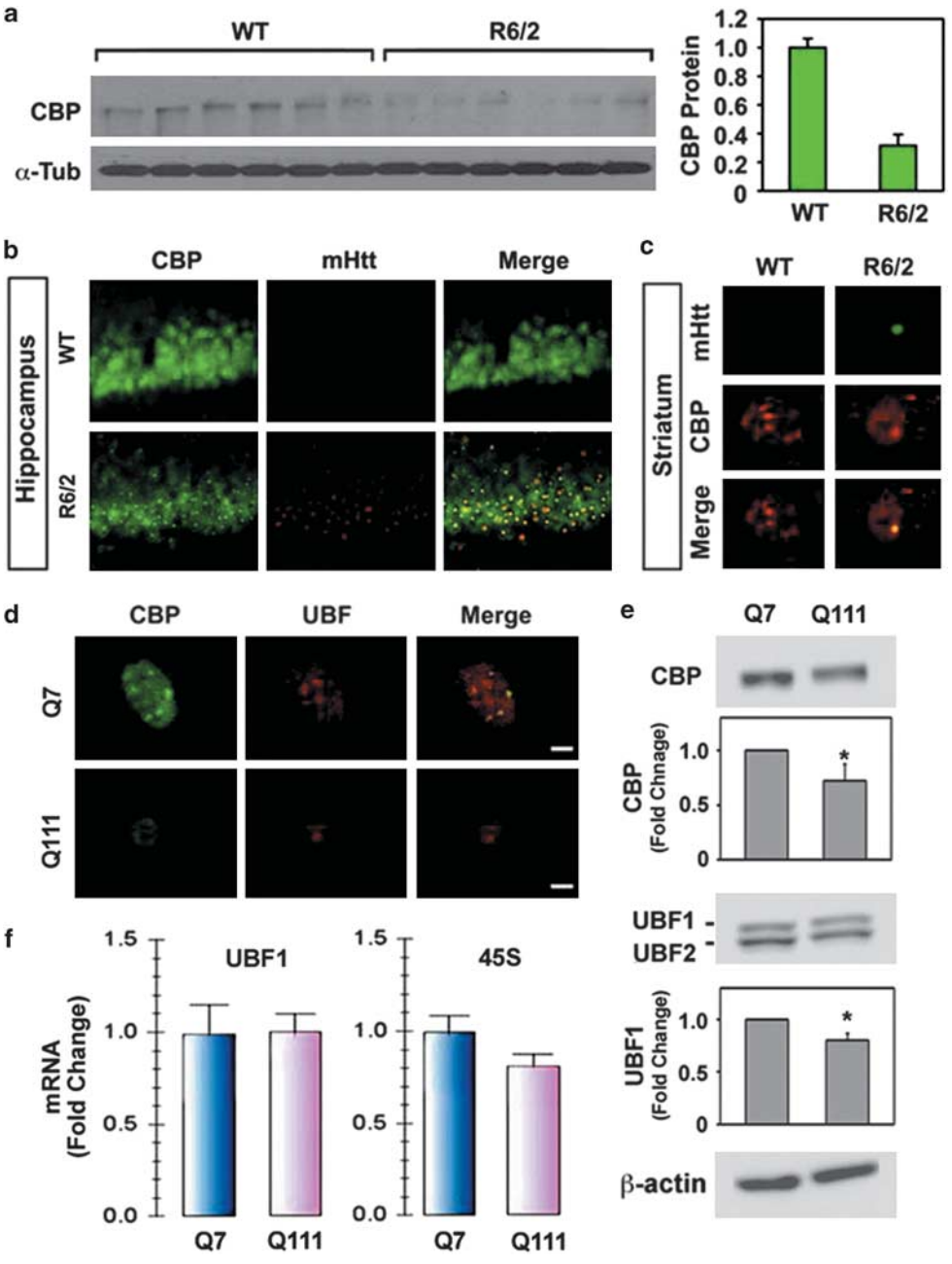

Figure 3 CBP is impaired in HD mice and cell line (Q111). (**a**) The protein level of CBP is markedly reduced in striatal neurons of R6/2 mice compared with wild type littermate control mice at 9 weeks of age. (**b**) CBP and mtHtt are co-localized in hippocampal neurons of R6/2 mice, but not in WT mice. (**c**) Confocal microscopic analysis for mtHtt and CBP co-localization in striatal neurons of R6/2 mice. Co-localization of mtHtt with CBP is increased in striatal neurons of R6/2 mice in comparison to control mice. (**d**) CBP and mtHtt are co-localized in Q7 cells and its colocalization is decreased in Q111 cells. (**e**) Immunoblot assay for CBP, UBF1 and β-actin in Q7 and Q111 cells. The data represent an average of 10 independent experiments. *Significantly from the control at $P<0.05$. (**f**) qRT-PCR analysis of UBF1 mRNA and 45S in Q7 and Q111 cells. UBF1 mRNA and 45S were normalized to GAPDH mRNA. The data represent an average of three independent experiments

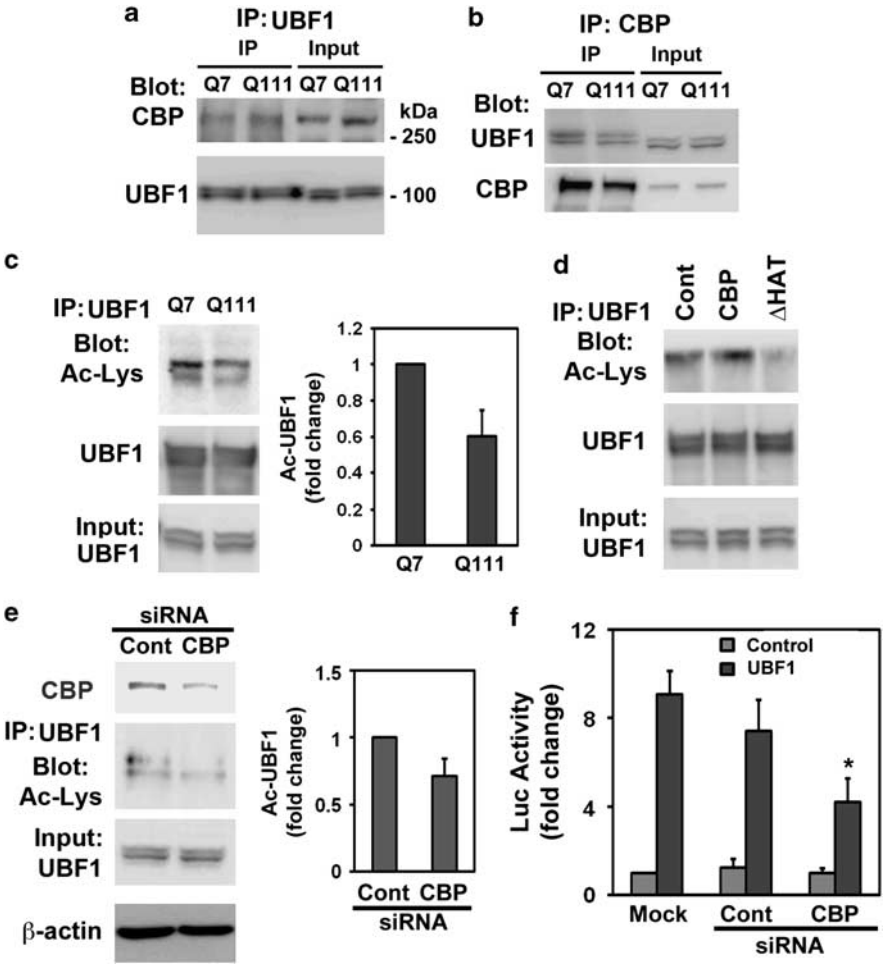

Figure 4 UBF1 interacts with CBP and its acetylation is modulated by CBP–HAT activity. (**a**) UBF1 interacts with CBP in intact cells. Cell lysates were immunoprecipitated with UBF1 and subsequently blots were probed with anti-CBP antibody. The same blots were then stripped and reprobed with anti-UBF1 antibody. (**b**) CBP is also immunoprecipiated with UBF1. (**c**) The acetylated level of UBF1 is reduced in Q111 cells in comparison with Q7 cells. (**d**) HAT deletion in CBP abrogates the acetylation of UBF1. Cells were transiently transfected with pCMV–WT–CBP or pCMV–CBP–dHAT mutant. The UBF1 was immunoprecipitaed and the blot was probed with anti-Ac–Lys antibody. (**e**) Knockdown of CBP by siRNA reduces the acetylation of UBF1. (**f**) Knockdown of CBP reduces UBF1-induced transcriptional activity of rDNA. *Significantly from the control at $P<0.05$

immunoreactivity colocalized with mtHtt in neuronal nuclear inclusions in R6/2 mice (Figures 3b and c). CBP and UBF1 protein levels were reduced in mutant Q111 cells compared with WT Q7 cells (Figures 3d and e) paralleling our *in vivo* findings. We examined the basal stability of UBF1 and CBP in intact cells, and determined whether CBP modulates UBF1 stability or not (Supplementary Figure 4). There was no difference in the stability of UBF1 and CBP in Q7 and Q111 cells, respectively. We did find, however, that ectopic expression of CBP increases the stability of UBF1. qRT-PCR analysis showed decreased 45S mRNA in mutant Q111 cells (Figure 3f), indicating impairment of ribosomal biosynthesis similar to that we found in R6/2 mice.

Next, we further characterized the *in vivo* association of UBF1 and CBP by performing immunoprecipitations (IPs) on neuronal lysates, using either anti-UBF1 or anti-CBP antibodies. A prominent 300 kDa band of CBP protein was present in UBF1 IPs (Figure 4a). The association of UBF1 and CBP was apparent in both Q7 and Q111 cells (Figure 4a). We also confirmed the association between CBP and UBF1 using reverse IP with CBP antibody (Figure 4b). These results indicate that both UBF and CBP constitutively interact to generate rDNA transcription complex formation in neurons. Immunoblotting with anti-UBF1 antibody of anti-UBF1 IP verified that the same amounts of UBF1 were recovered by IP under different conditions. To test whether the acetylation status of UBF1 was altered in HD cells, we detected acetylated UBF1 (Ac-UBF1) using an anti-Ac-Lys antibody on UBF1 IPs. Interestingly, we found that Ac-UBF1 levels were reduced ($>40\%$) in mutant Q111 cells compared with WT Q7 cells (Figure 4c). We then addressed whether acetylation of UBF1 by CBP–HAT activity is responsible for CBP-dependent UBF1 transcriptional activation. Indeed, UBF1 acetylation was significantly augmented by WT CBP in Q7 cells, but not by the CBP–dHAT mutant, as determined by IP using a UBF1 antibody, followed by immunoblotting using acetyl lysine antibody (Ac-UBF1) or UBF1 antibody alone (Figure 4d). CBP knockdown using siRNA CBP reduced

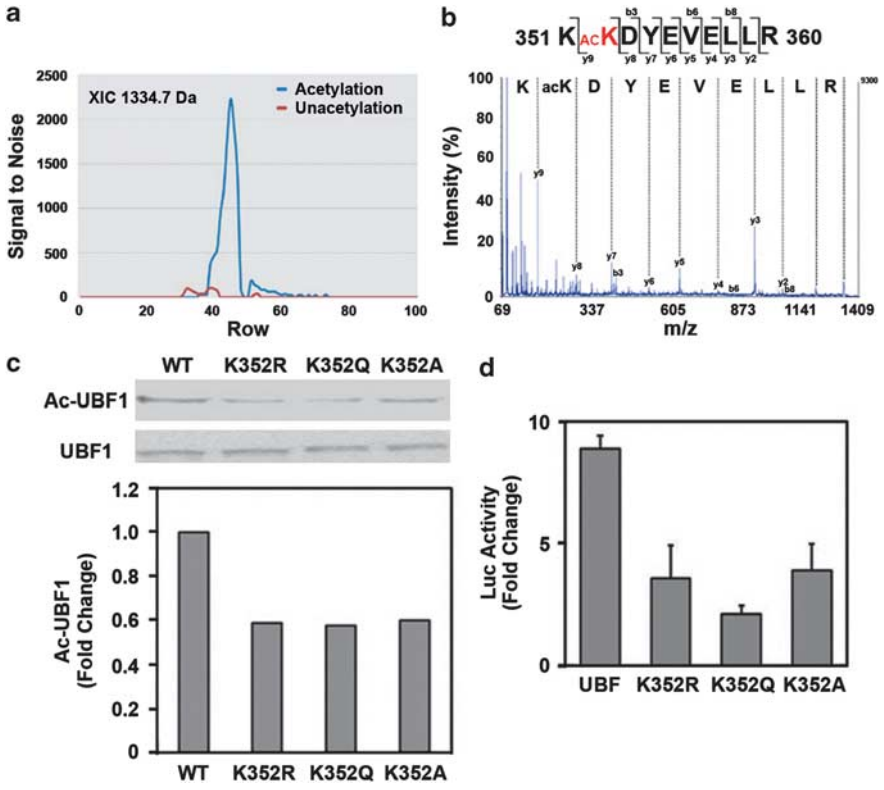

Figure 5 UBF1 is acetylated at K352 residue by CBP, and UBF1 acetylation site mutants (K352A, K352Q, and K352R) decreases the transcriptional activity of rDNA. (**a**) Comparison of LC-MALDI-MS/MS extracted ion chromatogram between *in vitro*-acetylated UBF1–HMG3 domain peptide (acetylation) and untreated control UBF1–HMG3 domain peptide (unacetylation). The extracted ion chromatogram (XIC) of the precursor ion at *m/z* 1334.7 Da was plotted as signal to nose (vertical) *versus* spot number (horizontal). (**b**) MALDI-TOF/TOF MS/MS spectrum of acetylated peptide KacKDYEVELLR (1334.7 m/z) from human UBF1 HMG3 domain (accession number: P17480). The chart represents *m/z* (horizontal) *versus* intensity (vertical). (**c**) Mutation of UBF1 at K352 to A, Q, and R reduced its acetylation level in neuronal cells. pCMV–Flag–UBF1 s (WT, K352A, K352Q, and K352R) were transiently transfected, immunoprecipitaed with anti-Flag antibody and blotted with anti-Ac-Lys antibody. (**d**) UBF1 acetylation site mutants (K352A, K352Q, and K352R) decreases the transcriptional activity of rDNA in response to CBP

UBF1 acetylation levels, but the basal acetylation levels of UBF1 were not changed by siRNA control treatment (Figure 4e). In addition, CBP knockdown by siRNA markedly decreased UBF1-induced transcriptional activity of rDNA, compared with the siRNA control (Figure 4f).

UBF1 is acetylated at K352 by CBP. To identify which lysine (Lys) residue of UBF1 is directly acetylated by CBP, we performed an *in vitro* acetylation assay using GST–UBF1–HMG1-6 proteins and GST–CBP protein. As we found that GST–UBF1–HMG3 domain is specifically acetylated by CBP (Supplementary Figure 5), acetylated GST–UBF1–HMG3 protein was pooled and cut by prescission enzyme for LC-MS/MS analysis. As shown in Figure 5, we identified that the Lys (K) 352 of UBF1 was specifically acetylated by CBP (Figures 5a and b). To further confirm whether the acetylation of UBF1 at K352 is critical for the transcriptional activation of rDNA, we generated K352 acetylation site mutants of UBF1 using site-directed mutagenesis. We co-transfected UBF1 acetylation site mutants (K352A, K352Q, K352R) with or without CBP, and checked the acetylation status of UBF1 using IP and Ac-lys blot analysis. Three acetylation site mutants of UBF1 (K352A, K352Q, K352R) showed a marked reduction of acetylation by CBP (Figure 5c). These results suggest that UBF1 can be directly acetylated at K352 by CBP in intact neuronal cells. Concurrent with the reduced level of UBF1 acetylation in western blot analysis (Figure 5c), acetylation site mutants (K352A, K352Q, K352R) decreased the transcriptional activity of rDNA as determined by reporter assay (Figure 5d).

The occupancy of UBF1 to the rDNA and ribosomal biosynthesis is altered in HD. Chromatin immunoprecipitation (ChIP) assays were performed to detect whether the UBF1 occupancy of DNA was altered within the rDNA gene promoter.[11] Q7 and Q111 cells were cross linked with formaldehyde, sonicated, and immunoprecipitated with anti-UBF1 and normal rabbit IgG (as a control for normalization) antibodies. Eluted DNAs were amplified with specific primer sets for mouse rDNA promoter region such as ENH, UCE, CORE, and ETS3 (Figure 6a and Supplementary Table 1). As we expected, the occupancy of UBF1 to ENH, UCE, CORE, and ETS3 regions of the rDNA promoter was decreased ($>40\%$) in Q111 cells. In contrast, little or no PCR product was generated in Q7 cells, or with normal rabbit IgG as negative control (Figure 6b). In order to examine whether ribosomal biosynthesis is altered in HD, Q7 and Q111 cells were pulsing labeled for 30 min with

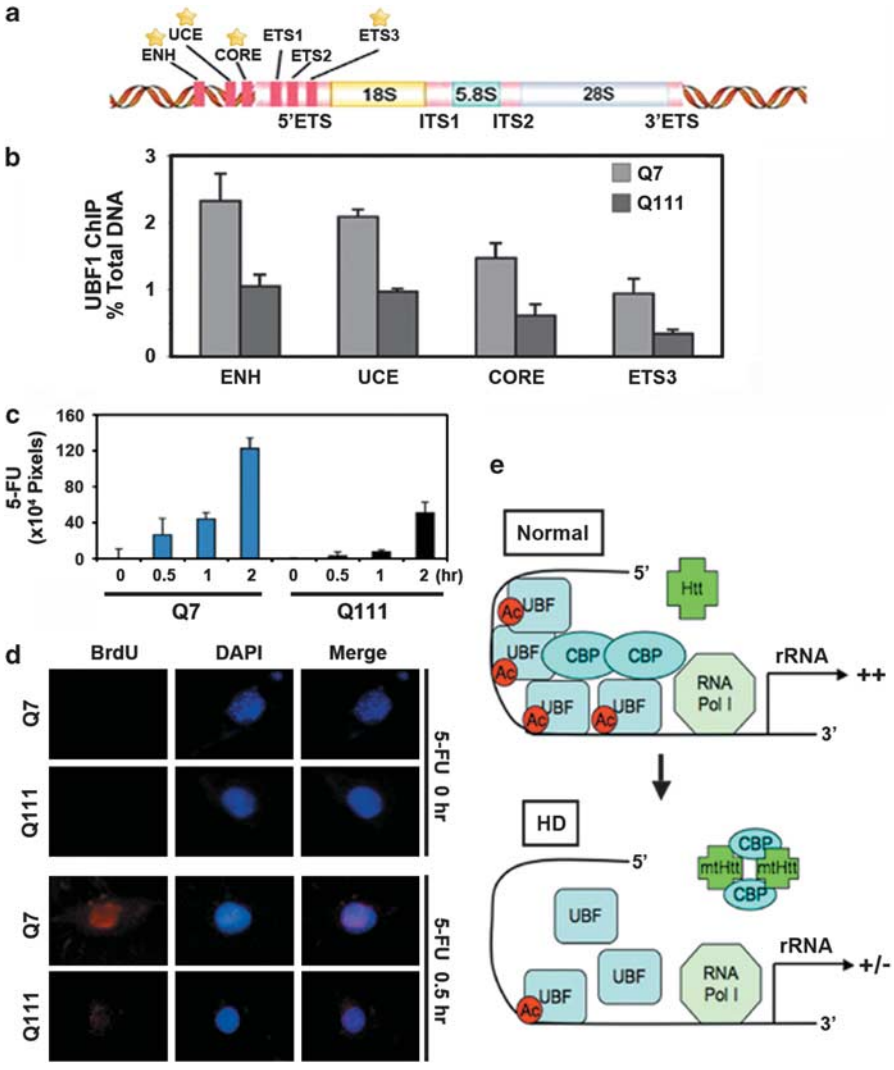

Figure 6 The occupancy of UBF1 to rDNA is changed in HD cell lines. (**a**) Positions of qRT-PCR primer set for detecting UBF1 occupancy in the promoter of mouse rDNA. (**b**) Quantitative ChIP analysis of UBF1 occupancy in the mouse rRNA gene. The UBF1 binding to rDNA was decreased in Q111 HD cells. Quantitative ChIP analysis of UBF1 binding to the rDNA (ENH, UCE, CORE, and ETS3) was performed as described in the 'Materials and Methods' section. (**c**) *In situ* transcription assay of 5′-FU incorporation into nucleolar rRNA in Q7 and Q111 cells. The level of 5′-FU incorporation was lowered in Q111 cells in comparison with Q7 cells. The time-dependent (0.5, 1, and 2 h) incorporation of halogenated nucleotide was detected by anti-BrdU antibody. The density (pixels) was averaged by counting five areas of each 60 cells. (**d**) Immunofluorescence staining images of Q7 and Q111 after 5′-FU incorporation. The nucleus was counterstained with DAPI. (**e**) A scheme of UBF1-dependent rDNA transcription in normal and HD condition. Normal condition (top): CBP interacts and acetylates UBF1 that is important in basal and constitutive transcription of rDNA (top). HD condition (bottom): mtHtt disrupts UBF1 and CBP complexes by sequestering CBP and decreases the acetylation of UBF1 at the proximal promoter of rDNA, subsequently leading to the repression of rDNA transcription

5-fluorouracil (FU) and immunolabeled with a mouse anti-BrdU antibody to visualize nascent transcripts of nucleolus. Incorporation of 5-FU was significantly decreased in mutant HD Q111 cells compared with WT Q7 cells (Figures 6c and d). These results confirm that the occupancy of UBF1 within rDNA gene loci is significantly reduced, which subsequently contributes to the dysregulation of rDNA transcription in HD. Figure 6e summarizes how CBP interacts and acetylates UBF1, which is important in basal constitutive and inducible expression of rDNA. In the context of HD, mtHtt interferes with the ability of CBP to inhibit HAT activity and co-factor function. This in turn disrupts the interaction between UBF1 and CBP, and decreases the acetylation of UBF1 at the proximal promoter of rDNA to repress rDNA transcription.

Discussion

UBF is intimately involved in rDNA transcription as an RNA polymerase I specific transcription factor and is required for maintenance of rRNA genes to be transcriptionally active.[12] UBF appears to be transcriptionally more active in specific neuronal cell type, such as sensory ganglia neurons. UBF consists of two polypeptides (UBF1 and 2), which form hetero- and homodimers and arise from alternative splicing of a single transcript.[13] UBF2 is five-fold less active than UBF1,[14] and

reasons why UBF2 has such poor transcriptional activity are unclear. UBF1 targeting to regions of heterochromatin is sufficient to induce large-scale chromatin decondensation.[15] UBF1 binding throughout the rDNA gene repeat might therefore contribute to the formation of the active chromatin state of rDNA genes.[16] In the current study, we found that UBF1 protein levels in the striatum were significantly decreased in R6/2 transgenic HD mice. We also found that the 45S was downregulated in R6/2 mice in comparison with WT controls. These data indicate that abnormal protein level of UBF1 protein levels correlate with impaired ribosomal transcription in HD mice.

Post-translational modifications of UBF, such as acetylation and phosphorylation, have an important role in the control of rDNA transcription.[17] Transcription factor acetylation is responsible for both activation and subnuclear localization.[18] It has been well established that CBP has HAT activity that contributes to transcriptional activation by nuclear receptors. UBF acetylation is also important for cell cycle-dependent regulation of rRNA expression. It has been proposed that the transcription of rDNA is regulated upon the influence of two opposite processes through UBF acetylation by CBP and deacetylation by HDAC.[10] In this context, CBP-dependent acetylation of UBF is linked to the transcription activation of rDNA.[17] It is noteworthy that reduced HAT activity of CBP is thought to have a role in the toxic, transcriptional, and repressive effects of mutated proteins with expanded polyglutamine repeats such as Htt and atrophin.[19–22] These findings, along with the established ability of UBF1-mediated rDNA transcription to be activated by acetyl transferases,[23] raises the possibility that the neurodegenerative process in HD might be associated with changes in adaptive rDNA transcription caused by altered CBP acetyl transferase that affects the balance of acetylated and deacetylated UBF1. Indeed, we found that the acetylation levels of UBF1 are significantly lowered due to dysregulation of CBP function in both HD cells and HD mice. Our data shows that UBF1 and CBP interact in intact neurons. Reduced levels of acetylated UBF1 correlate with the reduced CBP level and activity in HD. We also directly demonstrated that the HAT activity of CBP controls UBF1 acetylation, using a HAT domain deletion mutant of CBP that caused a marked reduction of UBF1 acetylation.

In order to identify the specific acetylation site of UBF1 by CBP acetyl transferase, we performed LC-MALDI-MS/MS analysis for the *in vitro*-acetylated UBF–HMG protein by CBP. We confirmed the ability of CBP to acetylate UBF1 at K352 that resides in the HMG3 domain. Mutations at K352 to alanine (A), glutamine (Q), and arginine (R) abrogated the acetylation of UBF1, as well as the transcriptional activation of UBF1 in response to CBP. These data prove that the CBP-dependent acetylation of UBF1 has a pivotal role in rDNA gene transcription,[17,24] and that CBP-dependent UBF1-induced rDNA expression is disrupted in HD models. It is unclear, however, whether mtHtt and HD-related cellular changes modulate the association of HDAC to the UBF1 complex. It will be important to define precisely how UBF1 protein is deacetylated in HD in order to more fully understand the role of neuronal rDNA transcription and ribosomal synthesis in HD pathogenesis.

Deacetylase inhibitors, such as trichostatin A, that enhance UBF acetylation have been show to enhance rDNA gene expression.[7,10] In the present study, we found that sodium butyrate triggered dose-dependent stimulation of rDNA transcription in mutant HD Q111 cells and WT Q7 cells in the presence of UBF1 (Supplementary Figure 6). We previously showed that enhanced transcription factors acetylation using three structurally distinct HDAC inhibitors improves neuronal survival in response to oxidative stress.[25] The results of these studies are consistent with a model in which altered rDNA transcription is an additional mechanism contributing to the protective effects of HDAC inhibitors in neurons. Several groups have now reported the neuroprotective effects of HDAC inhibitors in combating the toxicity of mutant huntingtin or mutant androgen receptors, proteins with expanded polyglutamine repeats *in vitro*[26,27] and *in vivo*.[3] Future studies will be needed, however, to clarify the contribution of UBF1 acetylation and rDNA transcription to the neuroprotective effects of HDAC inhibitors.

In summary, our *in vitro* and *in vivo* findings show that CBP acetylates UBF1 at K352 to enhance the transcriptional activity of rDNA. Our results also indicate that dysregulation of UBF1 and CBP is linked to impaired rDNA transcription in HD. An HDAC inhibitor enhanced rDNA transcription in mutant HD cells. These data suggest that modulation of CBP-dependent UBF1-mediated rDNA synthesis may be a therapeutic target to improve neuronal rDNA transcription in HD by blunting the deleterious effects of expanded polyglutamine repeats.

Materials and Methods

Plasmid constructs. pIRES-Luc and human rRNA-luciferase vector (pHrD–IRES–Luc) was generously provided by Dr. Samson T. Jacob (Ohio State University).[28] UBF1 acetylation mutants (K352A, K352Q, and K352R) were generated from pCMV–Flag–UBF1 using site-directed mutagenesis kit (TOYOBO, Osaka, Japan) (Supplementary Table 1). pCMV–WT–CBP and pCMV–CBP–dHAT were previously described.[11]

***In vitro* protein acetylation assay.** GST, GST–CBP, and GST–UBF1 HMG1-6 proteins were synthesized in *Escherichia coli* and purified with glutathione beads. Fusion proteins were eluted by using 20 mM glutathione in 1 × HAT buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM PMSF, 0.1 mM EDTA, 1 mM DTT, 0.05 M NaCl, 0.01 M sodium butyrate (Calbiochem, Darmstadt, Germany)). The acetylation assay was performed as previously described.[25]

Cell culture and transfection assays. ST*Hdh*$^{Q7/7}$ (wild type) and ST*Hdh*$^{Q111/111}$ (HD knock-in striatal cell line expresses mutant huntingtin at endogenous level), were generously provided from Dr. Marcy MacDonald (Harvard Medical School).[29] For transfection assays, 2.5×10^5 cells were plated onto 48-well cell culture plates 24 h before transfection. DMRIE-C reagent (Invitrogen, Paisley, UK) has been used as a transfection reagent, and transfections were performed according to the manufacturer's protocol. When NaB was used in the experiments, the calculated amount of the HDAC inhibitor was added directly to the medium 24 h after transfection. The luciferase activity was measured and normalized to protein concentration. The Dual Luciferase Assay kit (Promega, Madison, WI, USA) was used to determine the difference of internal transcriptional activity between Q7 and Q111 cells. Data represent the mean of three or more independent experiments.

Animals. Male R6/2 mice were bred with females from their background strain (B6CBAFI/J), and offspring were genotyped using PCR.[9,30,31] CAG repeat length remained stable within a 147–153 range. Female mice were used in the experimental paradigms.

Quantitative real time-PCR (qRT-PCR). Total RNA was isolated from cells using a commercial extraction system (Qiagen, Crawley, UK). A total of 1 μg

RNA has been used for cDNA preparation with iScript cDNA Synthesis Kit (BioRad) according to manufacturer's protocols. cDNA from each sample was amplified by RT-PCR using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). RNA quantities were normalized using GAPDH mRNA as a reference.[11] PCR cycling conditions were: denaturation for 3 min at 95°C, then 40 cycles of amplification for 15 s at 95°C, 15 s at 60°C, 20 s at 70°C, followed with 30 s at 72°C. For melt curve, data collection has been used 33 cycles, 6 s each, with the temperature increased from 60°C to 92°C (increase set point temperature after cycle 2 by 1°C).

Chromatin immunoprecipitation (ChIP). ChIP for UBF1 binding to rDNA was performed using a CHIP assay kit (Upstate Biotechnology, Billerica, MA, USA) as described previously.[11,25,32] Q7 and Q111 cells were crosslinked with 1% formaldehyde for 20 min at room temperature. The lysates were sonicated six times, with each time for 30 s using Bioruptor (Diogenode, Denville, NJ, USA). After centrifugation, the supernatant was diluted in ChIP dilution buffer, and then incubated overnight at 4°C with anti-UBF1 antibody. qPCR primers were shown on the Supplementary Table 2.

In-gel trypsin digestion and off-line 2 dimensional LC-MALDI-MS/MS analysis. In-gel trypsin digestion and Nano LC separations were performed as previously reported, with some minor modifications.[32,33,34] The details of method are on line Supplementary Materials and Methods.

Confocal microscopy. Indirect labeling methods were used to determine the UBF-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1 : 200), coillin (Chemicon) (1 : 200), mtHtt (Chemicon, Temecula, CA, USA) (1 : 1000), and CBP (Santa Cruz Biotechnology) (1 : 200). The procedures were performed as previously described.[31] Images were analyzed using a spinning disk confocal microscope (Olympus DSU, Tokyo, Japan). Deconvolution and 3-dimensional construction of the confocal image was performed by AQI-X-COMBO-CWF program (Media cybernetics Bethesda, MD, USA). Control experiments were performed in the absence of primary antibody or in the presence of blocking peptide.[31]

Western blot analysis. Western blot was performed as previously described.[30,35,36] A total of 30 μg of protein was subjected to SDS-PAGE (10%), and blotted with anti-UBF1 (Upstate Biotechnology), anti-CBP (A-22: sc-369 and C-20: sc-583) (Santa Cruz Biotechnology), and anti-huntingtin (C-20: sc-351) (Santa Cruz Biotechnology) antibody. Protein loading was controlled by probing for α-tubulin (Sigma, Gillingham, UK) or beta-actin (Santa Cruz Bitotechnology) on the same membrane.

RNA interference experiment. Q7 and Q111 (1×10^5 cells/ml) or SH-SY5Y (2×10^5 cells/ml) were transiently transfected with 100 to 400 nM of Stealth control RNAi and CBP RNAi (Invitrogen Life Technology, Carlsbad, CA, USA) and control shRNA and UBF1 shRNA using DMRIE-C transfection reagent (Invitrogen Life Technology) in the presence or absence of HrD-IRES reporter for 48 h.[11]

Run-on transcription assay and immunofluorescence. Nascent RNA was detected using a method previously described, with slight modifications.[4] Briefly, cells were incubated with 5 mM 5-FU (Sigma) for indicated time, and fixed with 4% paraformaldehyde for 30 min at 4°C. Incorporated 5-FU was labeled with anti-BrdU antibody (Serotec, Oxford, UK) for overnight at 4°C, and with DyLight-594-conjugated anti-mouse IgG antibody (Jackson Laboratories, Bar Harbor, ME, USA) for 1 h. The nuclei were counterstained with DAPI, and labeled signals were visualized using fluorescent microscopy (Olympus, Tokyo, Japan). For the quantitative analysis, emitted signals for 5-FU was captured and their intensities were analyzed using multigauge program (Fuji, Tokyo, Japan).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)