CAG expansion induces nucleolar stress in polyglutamine diseases

Ho Tsoi[a,b], Terrence Chi-Kong Lau[c], Suk-Ying Tsang[b,d], Kwok-Fai Lau[b,d,e], and Ho Yin Edwin Chan[a,b,d,e,1]

[a]Laboratory of *Drosophila* Research, [b]Biochemistry Program, [d]Cell and Molecular Biology Program, and [e]Molecular Biotechnology Program, School of Life Sciences, Faculty of Science, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China; and [c]Department of Biology and Chemistry, City University of Hong Kong, Hong Kong SAR, China

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The cell nucleus is a major site for polyglutamine (polyQ) toxicity, but the underlying mechanisms involved have yet been fully elucidated. Here, we report that mutant RNAs that carry an expanded CAG repeat (expanded CAG RNAs) induce apoptosis by activating the nucleolar stress pathway in both polyQ patients and transgenic animal disease models. We showed that expanded CAG RNAs interacted directly with nucleolin (NCL), a protein that regulates *rRNA* transcription. Such RNA–protein interaction deprived NCL of binding to *upstream control element* (*UCE*) of the *rRNA promoter*, which resulted in *UCE* DNA hypermethylation and subsequently perturbation of *rRNA* transcription. The down-regulation of *rRNA* transcription induced nucleolar stress and provoked apoptosis by promoting physical interaction between ribosomal proteins and MDM2. Consequently, p53 protein was found to be stabilized in cells and became concentrated in the mitochondria. Finally, we showed that mitochondrial p53 disrupted the interaction between the antiapoptotic protein, Bcl-xL, and the proapoptotic protein, Bak, which then caused cytochrome *c* release and caspase activation. Our work provides in vivo evidence that expanded CAG RNAs trigger nucleolar stress and induce apoptosis via p53 and describes a polyQ pathogenic mechanism that involves the nucleolus.

Drosophila | Machado-Joseph disease | RNA toxicity | spinocerebellar ataxia

The nucleolus is the site for ribosome biogenesis including *ribosomal RNA* (*rRNA*) transcription, and dysregulation of nucleolar function has been shown to cause diseases (1). Upstream binding factor (UBF) is a nucleolar-specific, high-mobility group-box–containing protein (2) that binds to *upstream control element* (*UCE*) of the *rRNA promoter* to trigger the formation of preinitiation complex for RNA polymerase I-mediated rRNA transcription (3). It has been reported that inhibition of *rRNA* transcription results in apoptosis in neurons (4), and perturbation of *rRNA* transcription has recently been observed in patients with Alzheimer's disease (5). However, the cause of *rRNA* transcription down-regulation in neurodegeneration and how such dysregulation is related to neurodegeneration still remain largely undefined. "Nucleolar stress" is a term used to describe a stress response pathway through which the nucleolus signals to the cytosol to elicit apoptosis. When *rRNA* transcription is halted, the unassembled free ribosomal proteins, such as RpL5 (6), RpL11 (7), and RpL23 (8), accumulate in cells and associate with the MDM2 E3 ubiquitin ligase. The ribosomal protein/MDM2 interaction down-regulates MDM2-mediated ubiquitination of p53, which subsequently causes accumulation of p53 and activation of the downstream nucleolar stress signaling cascade (9), including mitochondrial cytochrome *c* release and caspase activation (10). This process is an effective mechanism to eliminate cells that are incapable of performing protein synthesis efficiently due to ribosome biogenesis defects. It has recently been reported that p53 mediates neuronal death upon nucleolar disruption in an in vivo Parkinson disease model (11).

Polyglutamine (polyQ) toxicity is attributed to the toxic gain-of-function nature of the disease proteins that harbor the expanded polyQ domain (12). The contribution of expanded CAG RNA toxicity to the pathogenesis of polyQ diseases, including Machado–Joseph disease (MJD), which is a dominant form of spinocerebellar ataxia (13), has recently been described. When the expanded CAG repeat sequence was isolated from the *MJD* disease gene and expressed as an untranslated RNA in vivo (ensuring that no expanded polyQ domain would be translated), progressive neural degeneration was observed (13). This finding clearly indicates that expanded CAG RNAs per se are neurotoxic (14). In recent years, increasing attention has been directed toward the understanding of pathogenic mechanisms exploited by expanded CAG RNAs (14–20). In this study, we provide evidence that expanded CAG RNAs induce nucleolus stress and trigger apoptosis via p53 stabilization in both polyQ patient cell and transgenic animal disease models. We demonstrated that expanded CAG RNAs deprive the nucleolar protein nucleolin (NCL) of binding onto *ribosomal RNA* (*rRNA*) *promoter*, which then down-regulates *rRNA* transcription. In addition, we revealed a role of the p53-mitochondrial nucleolar stress pathway in expanded CAG RNA-mediated toxicity of polyQ degeneration.

Results and Discussion

Expanded CAG RNAs Perturbed *Ribosomal RNA* Transcription. We previously reported that the cell nucleus is a toxic site for polyQ toxicity (17, 21). From a gene expression study, we observed that the *pre-rRNA* expression level was down-regulated in our *Drosophila* polyQ disease models (Fig. 1). Compared with the unexpanded MJD control transgenic flies (MJD_{CAG27}), a reduction of *pre-rRNA* levels was observed in degenerative expanded MJD_{CAG78} transgenic flies (Fig. 1*A* and Fig. S1*A*) (17). We also detected a similar reduction in a mouse model of polyQ disease (22). A down-regulation of *pre-45s rRNA* level was observed in 12-wk-old symptomatic R6/2 polyQ transgenic mice, but not in the 2-wk-old asymptomatic ones (Fig. S1*B*). Both cellular pathologies (17, 21, 22) and neuropathological symptoms (22) have been reported in the 12-wk-old transgenic mouse brains. Further, a reduction of *pre-45s rRNA* level was consistently observed in cell lines of patients with MJD (Fig. S1*C*), which indicates that the down-regulation of *rRNA* transcription we observed in the transgenic animal models (Fig. 1*A* and Fig. S1*B*) was not due to transgene overexpression. In addition, no transcription defect was observed in genes mediated by RNA polymerases II and III (Fig. S1*C*). Our data thus indicate that expanded CAG RNAs particularly perturb *rRNA* transcription.

As mutant RNA and protein species both contribute to polyQ toxicity in the cell nucleus (17, 21), we next investigated whether both of these species contributed to the *rRNA* transcription dysregulation in our models. In contrast to that in the expanded MJD_{CAG78} transgenic flies, expression of an expanded but discontinuous CAG repeat construct $MJD_{CAA/G78}$ did not cause any perturbation of *pre-rRNA* gene expression (Fig. 1*A*) (13, 17). As both MJD_{CAG78} and $MJD_{CAA/G78}$ transgenic flies expressed the MJD disease protein at a similar level (13), our data thus indicate that polyQ protein does not have a significant contribution to

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[1]To whom correspondence should be addressed. E-mail: hyechan@cuhk.edu.hk.

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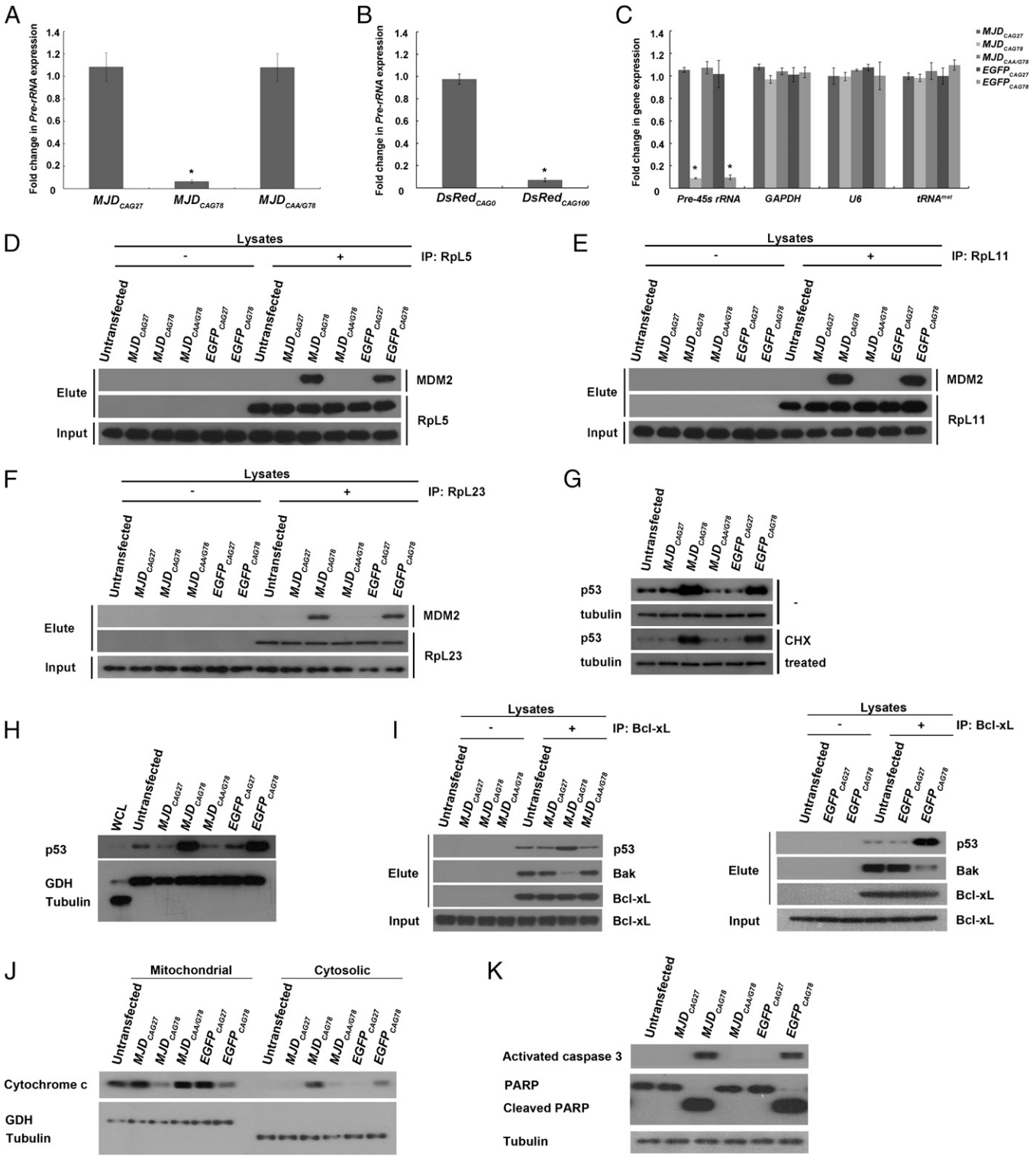

Fig. 1. Expanded CAG RNAs perturbed *rRNA* transcription and induced nucleolar stress. (*A*–*C*) Real-time PCR analysis of *pre-rRNA* expression in *Drosophila* (*A* and *B*) and *pre-45s rRNA*, *GAPDH*, *U6*, and *tRNA^{met}* expression in cell (*C*) models expressing *CAG* constructs. Error bars represent ±SD. This experiment was repeated three times. (*D*–*F*) Coimmunoprecipitation of ribosomal proteins [RpL5 (*D*), RpL11 (*E*), and RpL23 (*F*)] and MDM2 E3 ubiquitin ligase in CAG RNA-expressing cells. (*G*) Effect of expanded CAG RNA expression on p53 protein stability. CHX represents cycloheximide and was used to inhibit de novo protein synthesis. Tubulin was used as a loading control. (*H*) Effect of expanded CAG RNA expression on mitochondrial accumulation of p53. Tubulin and glutamate dehydrogenase (GDH) were used, respectively, as cytosolic and mitochondrial fractionation controls. (*I*) Coimmunoprecipitation of Bcl-xL with p53 and Bak. (*J*) Cytosolic and mitochondrial fractionations of cytochrome *c* in CAG RNA-expressing cells. Tubulin and GDH were used as cytosolic and mitochondrial fractionation controls, respectively. (*K*) Effect of expanded CAG RNA expression on cleavage of caspase 3 and poly(ADP ribose) polymerase. Tubulin was used as a loading control. "+" indicates that antibody was present in the immunoprecipitation reactions and "−" indicates that no antibody was included in the reactions. All experiments were repeated three times, and representative blots or gels are shown.

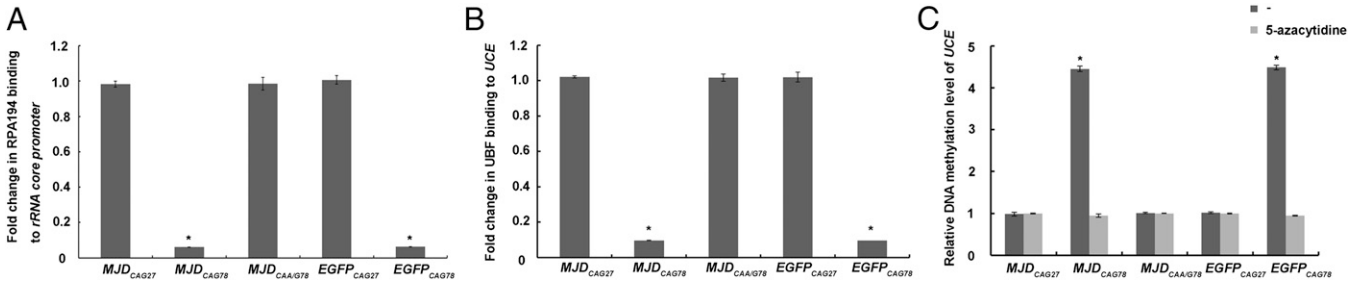

Fig. 2. Expanded CAG RNAs interfered with the formation of *rRNA* transcription preinitiation complex and promoted hypermethylation of *rRNA promoter*. (*A* and *B*) Chromatin immunoprecipitation of the largest subunit of RNA polymerase I (RPA194) and *rRNA promoter* (*A*) and upstream binding factor (UBF) and *upstream control element* (*UCE*) (*B*) in CAG RNA-expressing cells. "+" indicates that antibody was present in the immunoprecipitation reactions and "−" indicates that antibody was not included in the reactions. (*C*) The effect of DNA methyltransferase inhibitor 5-azacytidine on expanded CAG RNA-induced *UCE* CpG DNA hypermethylation. Real-time PCR analysis of *UCE* DNA methylation status is shown. Error bars represent ±SD. All experiments were repeated three times.

perturbation of *rRNA* transcription. The only difference between the MJD_{CAG78} and $MJD_{CAA/G78}$ transgenes lies in the CAG repeat region, where the CAG continuity of the $MJD_{CAA/G78}$ transgene is disrupted by another glutamine-coding triplet CAA (13); this result indicates that the continuity of CAG repeat is crucial for the pathogenic RNA to initiate an inhibitory effect on *rRNA* transcription. A similar perturbation effect was further observed in an independent untranslated CAG RNA fly model, $DsRed_{CAG}$ (13); this finding thus consolidates the deleterious effect of expanded CAG RNAs on *rRNA* transcription (Fig. 1*A*).

We next investigated the effect of expanded CAG RNAs on *rRNA* transcription in mammalian cell models (Fig. S2). In contrast to the controls, including cells transfected with unexpanded CAG (MJD_{CAG27} and $EGFP_{CAG27}$) and interrupted expanded CAG ($MJD_{CAA/G78}$) constructs, a lower level of *pre-45s rRNA* was observed in cells that expressed MJD_{CAG78} or $EGFP_{CAG78}$ RNA (Fig. 1*C*). We also performed fluorescence in situ hybridization to determine the subcellular localization of expanded MJD_{CAG} and $EGFP_{CAG}$ RNAs and found that both expanded MJD_{CAG78} and $EGFP_{CAG78}$ RNAs formed microscopic

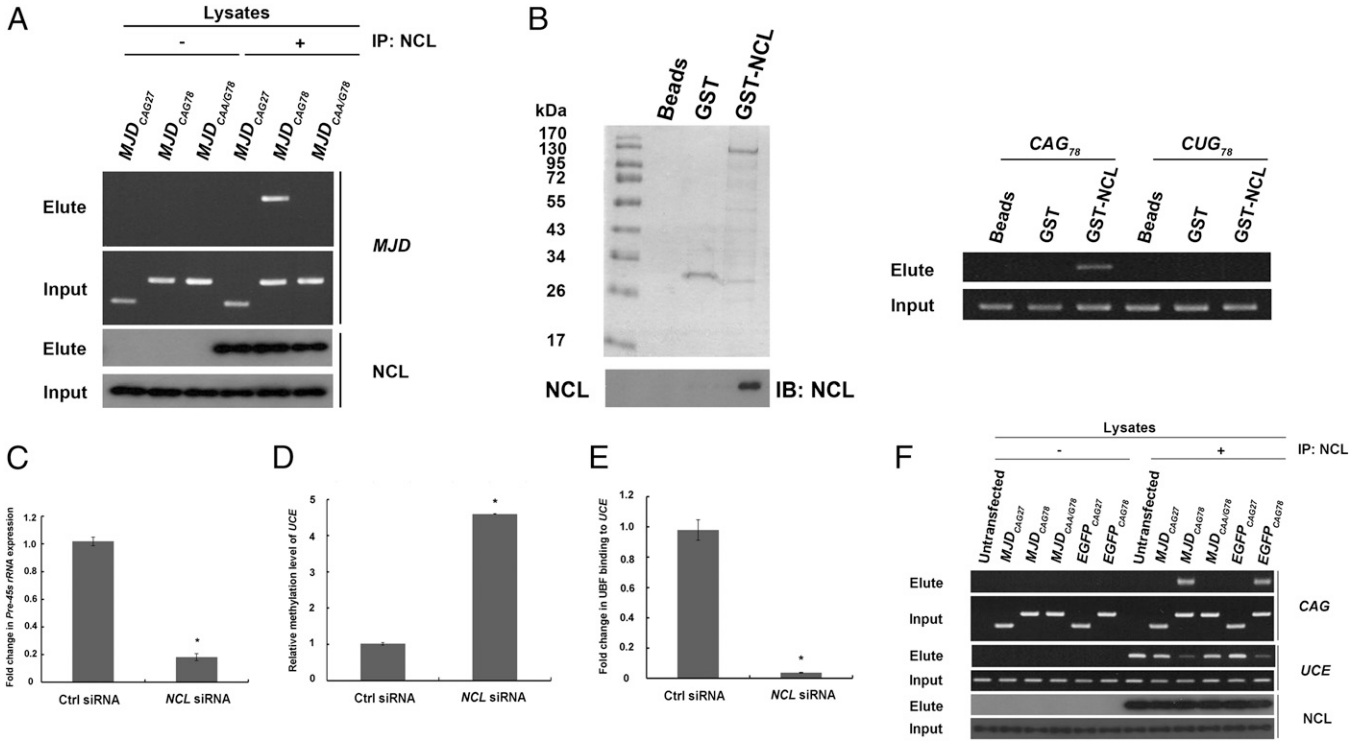

Fig. 3. Physical interaction between expanded CAG RNAs and nucleolin compromised the binding of nucleolin to *upstream control element*. (*A*) Pull-down of MJD_{CAG} RNA by means of NCL immunoprecipitation. "+" indicates that antibody was present in the immunoprecipitation reactions and "−" indicates that no antibody was included in the reactions. This experiment was repeated three times, and a representative gel is shown. (*B*) Direct physical interaction between NCL and expanded CAG RNA. Purified GST-NCL protein and in vitro transcribed RNAs (CAG_{78} and CUG_{78}) were used in the binding reactions. Nonfusion GST protein was used as a negative control. Western blotting was performed to confirm the expression of the GST-NCL protein. This experiment was repeated four times, and a representative blot is shown. (*C*) Real-time PCR analysis of *pre-45s rRNA* expression levels in *NCL* siRNA-treated cells. Error bars represent ±SD. This experiment was repeated three times. (*D*) Real-time PCR analysis of *UCE* DNA methylation status in *NCL* siRNA-treated cells. Error bars represent ±SD. This experiment was repeated three times. (*E*) Coimmunoprecipitation of upstream binding factor (UBF) and *upstream control element* (*UCE*) in *NCL* siRNA-treated cells. Real-time PCR analysis was performed to determine UBF–*UCE* interaction. Error bars represent ±SD. This experiment was repeated three times. (*F*) Chromatin immunoprecipitation of NCL with expanded CAG RNAs and *UCE* in cells. "+" indicates that antibody was present in the immunoprecipitation reactions and "−" indicates that no antibody was included in the reactions. This experiment was repeated three times, and a representative gel is shown.

foci in the nucleolar region (23, 24) (Fig. S3). As *rRNA* transcription takes place in the nucleolus (1), our fluorescence in situ hybridization data are thus in support of a *rRNA* transcription inhibitory property of expanded CAG RNAs.

Expanded CAG RNAs Induced Stabilization and Mitochondrial Accumulation of p53. Inhibition of *pre-rRNA* gene expression has been reported to elevate p53 protein level (25) and trigger neuronal apoptosis (4) via the ribosomal protein–MDM2-p53 nucleolar stress pathway (26). We thus investigated this pathway in cells expressing MJD_{CAG78} or $EGFP_{CAG78}$ RNA. In addition to the observation of a reduction in *rRNA* transcription (Fig. 1 *A–C* and Fig. S1 *B* and *C*), we detected an intense physical interaction between ribosomal proteins (RpL5, RpL11, and RpL23) and E3 ubiquitin ligase MDM2 (Fig. 1 *D–F*). In contrast, no such protein–protein interaction was observed in the controls. One cellular function of MDM2 is to mediate proteasomal degradation of p53 (9). We further showed that the ribosomal protein–MDM2 interaction compromised cellular p53 degradation, and as a result an elevated level of cellular p53 protein was detected in expanded CAG RNA-expressing cells (Fig. 1*G*). We further found that the stabilized cellular p53 protein was enriched in the mitochondrial fraction of these cells (Fig. 1*H*).

Expanded CAG RNAs Induced Mitochondrial Cytochrome *c* Release and Caspase Activation. Cellular accumulation of p53 has been reported to disrupt the interactions between pro- and antiapoptotic Bcl-2 family proteins (10, 27). Under nonstress conditions, the antiapoptotic protein Bcl-xL interacts with the proapoptotic protein Bak. This interaction prevents the oligomerization of Bak. When p53 accumulates in the mitochondria, it interacts with Bcl-xL and allows Bak to oligomerize on mitochondrial outer membrane to form pores. Such pore formation subsequently mediates cytochrome *c* release from the mitochondria (27). We observed an enhanced association between p53 and Bcl-xL in cells that expressed MJD_{CAG78} and $EGFP_{CAG78}$ RNAs and a concomitant reduction in the interaction between Bcl-xL and Bak (Fig. 1*I*). Further, the detection of cytochrome *c* in the cytoplasm in these cells (Fig. 1*J*) indicates a leakage of cytochrome *c* from the mitochondria. Cytochrome *c* release from mitochondria is a universal inducer of caspase cleavage. As predicted, we observed prominent cleavage of caspase 3 in cells expressing MJD_{CAG78} and $EGFP_{CAG78}$ RNAs (Fig. 1*K*). In addition, cleavage of poly(ADP ribose) polymerase (PARP), a caspase 3 substrate, was detected (Fig. 1*K*), which confirms the functionality of the cleaved caspase 3 in expanded CAG RNA-expressing cells. Altogether, our findings indicate that expanded CAG RNAs induce cell death by activating the ribosomal protein–MDM2-p53 nucleolar stress pathway. We also detected a physical interaction between RpL5, L11 and L23 and MDM2 (Fig. S4 *A–C*), and p53 protein accumulation (Fig. S4*D*) in 12-wk-old symptomatic R6/2 transgenic mice, but not in the 2-wk-old asymptomatic ones and the nontransgenic control. This result further confirms that the ribosomal protein–MDM2-p53 nucleolar stress pathway is involved in polyQ neurotoxicity in vivo.

Expanded CAG RNAs Interfered with RNA Polymerase I Preinitiation Complex Formation. After confirming the role of nucleolar stress in polyQ degeneration, we next carried out chromatin immunoprecipitation (ChIP) to determine whether the *rRNA* transcription machinery was perturbed by expanded CAG RNAs. We detected reduced binding of the largest subunit of RNA polymerase I (RPA194) (28) to the *rRNA promoter* in both MJD_{CAG78} and $EGFP_{CAG78}$ RNA-expressing cells (Fig. 2*A* and Fig. S5*A*); this result suggests that the activity of the *rRNA promoter* is compromised in these cells. UBF is a transcription regulatory protein that binds to *UCE* of the *rRNA promoter* and triggers the formation of preinitiation complex (PIC) for RNA polymerase I-mediated *rRNA* transcription (3); we thus investigated whether UBF–*UCE* interaction was affected in cells expressing expanded CAG RNAs. It was found that less UBF was able to bind to *UCE* in

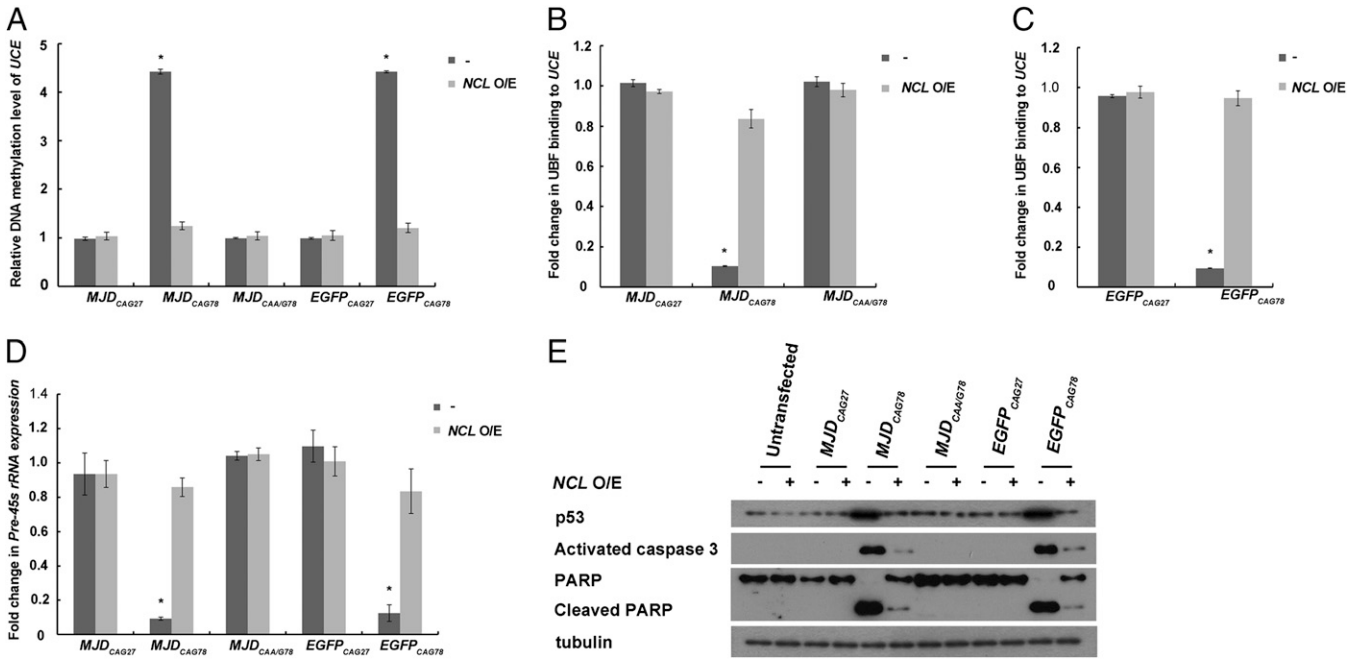

Fig. 4. *Nucleolin* overexpression restored *rRNA* transcription and suppressed nucleolar stress. (*A*) Real-time PCR analysis of *upstream control element* (*UCE*) DNA methylation status in cells coexpressing CAG RNAs and *NCL*. Error bars represent ±SD. This experiment was repeated three times. (*B* and *C*) Chromatin immunoprecipitation of upstream binding factor (UBF) and *UCE* in CAG RNA-expressing cells with or without *NCL* coexpression. Real-time PCR analysis was performed to determine UBF–*UCE* interaction. Error bars represent ±SD. This experiment was repeated three times. (*D*) Real-time PCR analysis of *pre-45s rRNA* expression levels in cells cotransfected with *CAG* and *NCL* constructs. Error bars represent ±SD. This experiment was repeated three times. (*E*) The effect of *NCL* overexpression on p53 protein stabilization and cleavage of caspase 3 and poly(ADP ribose) polymerase in expanded CAG RNA-expressing cells. "+" indicates *NCL* overexpression and "−" indicates no *NCL* overexpression. This experiment was repeated three times, and a representative blot is shown.

both MJD_{CAG78}- and $EGFP_{CAG78}$-expressing cells compared with the MJD_{CAG27}, $EGFP_{CAG27}$, and $MJD_{CAA/G78}$ controls (Fig. 2*B* and Fig. S5*B*). Our data clearly demonstrate that expanded CAG RNAs inhibit *rRNA* transcription by interfering with the binding of UBF to *UCE*, which consequently compromises the association between RNA polymerase I and *rRNA promoter*.

Expanded CAG RNAs Induced Hypermethylation of *rRNA Promoter*. Cytosine methylation at CpG dinucleotide sites on *UCE* has been shown to affect *rRNA* transcription (29); we therefore performed the HpaII-PCR assay to investigate the overall methylation status of *UCE* (30, 31) in MJD_{CAG78} and $EGFP_{CAG78}$ RNA-expressing cells. Compared with the controls, *UCEs* extracted from cells that expressed MJD_{CAG78} and $EGFP_{CAG78}$ RNAs were found to be hypermethylated (Fig. 2*C*). In contrast, we did not observe such a hypermethylation-inducing effect on non-*rRNA promoters* (32–34) (Fig. S6). This result illustrates that expanded CAG RNA specifically modifies the methylation status of *UCE*. When we treated cells that expressed MJD_{CAG78} and $EGFP_{CAG78}$ RNAs with a DNA methyltransferase inhibitor, 5-azacytidine (30), *UCE* hypermethylation was restored to control levels (Fig. 2*C*). This result indicates the involvement of DNA methyltransferase activity in expanded CAG RNA-induced *UCE* hypermethylation.

Expanded CAG RNAs Interacted with Nucleolin. An affinity pull-down experiment using in vitro transcribed *S1* aptamer-tagged (35) MJD_{CAG78} RNAs (*S1*-MJD_{CAG78}) was performed with an aim to identify expanded CAG RNA-interacting proteins. We found that the nucleolar protein, NCL (36), specifically associated with expanded CAG RNAs (Fig. S7). We further showed that endogenous NCL interacted specifically with MJD_{CAG78} RNA, but not with the unexpanded CAG and discontinuous expanded CAG control RNAs in our cell models (Fig. 3*A*). This result indicates that CAG continuity is necessary to mediate the interaction between expanded CAG RNAs and NCL. By means of deletion mapping, we found that both the RRM2 and RRM3 domains of NCL are involved in the expanded CAG RNAs interaction (Fig. S8). To determine whether NCL interacts directly with expanded CAG RNAs, we incubated purified glutathione (GST)-NCL fusion protein with in vitro transcribed RNAs that contain either an expanded *CAG78* or a *CUG78* trinucleotide repeat. We found that GST-NCL interacted with *CAG78* but not with *CUG78* RNA (Fig. 3*B*). This result clearly shows that NCL interacts directly and specifically with expanded CAG RNAs.

Expanded CAG RNAs Reduced the Binding of Nucleolin on *rRNA Promoter*. Nucleolin has previously been reported to regulate RNA polymerase I-mediated transcription (37); we thus further examined its role in *rRNA* transcription in MJD_{CAG78} and $EGFP_{CAG78}$ RNA-expressing cells. As previously reported (37), we detected a reduction in *pre-45s rRNA* levels when *NCL* expression was knocked down in normal cells (Fig. 3*C* and Fig. S9) and also observed that *UCE* in the *rRNA promoter* was hypermethylated in these cells (Fig. 3*D*). The above results indicate the involvement of NCL in regulating *UCE* methylation status. Further, we demonstrated that knockdown of *NCL* expression reduced the binding of UBF to *UCE* (Fig. 3*E* and Fig. S5*C*). Taken together, our data indicate that NCL regulates *rRNA* transcription via modulating the methylation status of *UCE* (Fig. 3*D*) and UBF–*UCE* binding (Fig. 3*E*). We next investigated whether expanded CAG RNAs interfere with NCL function in cells that expressed MJD_{CAG78} and $EGFP_{CAG78}$ RNAs and found that the expression of expanded CAG RNAs compromised NCL/*UCE* interaction (Fig. 3*F*). Intriguingly, the reduced level of binding of NCL to *UCE* was accompanied by an enhanced association between NCL and expanded CAG RNAs. Our findings indicate that expanded CAG RNAs prevent NCL from interacting with *UCE* by directly binding to NCL, and the RNA–protein interaction between expanded CAG RNAs and NCL consequently causes down-regulation of *rRNA* transcription.

Overexpression of *Nucleolin* Rescued *rRNA* Transcription and Suppressed Nucleolar Stress Caused by Expanded CAG RNAs. Our study showed that expanded CAG RNA expression impairs nucleolus function by depriving cellular NCL of binding onto *UCE* (Fig. 3*F*), and such an effect is reminiscent of *NCL* knockdown (Fig. 3 *C*–*E*). We next determined whether overexpression of *NCL* could rescue expanded CAG RNA-mediated dysregulation of *rRNA* transcription. Our results showed that *NCL* overexpression alleviated *UCE* hypermethylation in cells that expressed MJD_{CAG78} or $EGFP_{CAG78}$ RNA (Fig. 4*A*) in a dose-dependent manner (Fig. S10). We further observed that *NCL* overexpression restored UBF–*UCE* interaction (Fig. 4 *B* and *C*) and rescued *rRNA* transcription in expanded CAG RNA-expressing cells (Fig. 4*D*). Intriguingly, *NCL* overexpression also suppressed the expanded CAG RNA-mediated nucleolar stress response, including cellular p53 accumulation, caspase 3 activation, and cleavage of PARP (Fig. 4*E*). To further investigate the relationship between *UCE* hypermethylation and p53 accumulation, we treated expanded CAG RNA-expressing cells with 5-azacytidine and found that inhibition of *UCE* DNA methylation reduced cellular accumulation of p53 in MJD_{CAG78} and $EGFP_{CAG78}$ RNA-expressing cells (Fig. S11). This finding indicates an involvement of DNA hypermethylation in the induction of expanded CAG RNA-mediated nucleolar stress. Moreover, we showed that exogenous NCL did not alter the expression levels of expanded CAG RNAs (Fig. S12), illustrating that the suppressive effect of *NCL* overexpression we observed was not due to a reduction of expanded CAG transgene expression. In summary, our findings show that NCL binds to *UCE* of *rRNA promoter* and regulates *rRNA* transcription. We further performed coimmunoprecipitation to determine whether NCL interacts with expanded polyQ protein and failed to detect any interaction (soluble or insoluble) between the two proteins (Fig. S13). This result indicates that expanded polyQ protein does not affect the cellular availability of NCL, which is therefore in line with our results generated from the "RNA-only" models (Fig. 1 *B* and *C*) and again illustrates that expression of expanded CAG RNAs per se perturbs *rRNA* transcription. Activation of the p53 pathway has previously been reported in polyQ degeneration, including MJD (38). Although we demonstrated that expanded CAG RNAs trigger nucleolar stress by perturbing *rRNA*

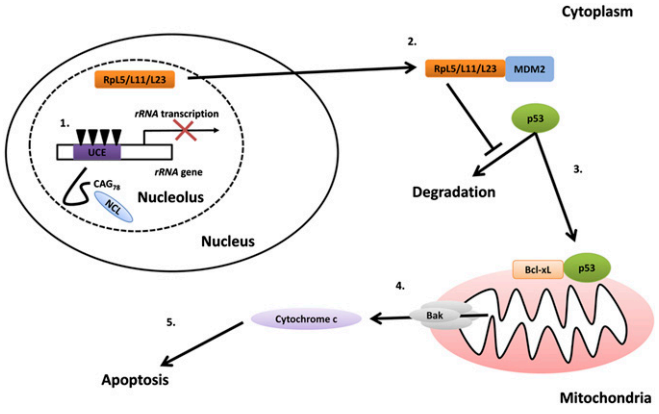

Fig. 5. A proposed model for the activation of nucleolar stress signaling by expanded CAG RNAs. The expression of expanded CAG RNA interacts directly with nucleolin (NCL, in light blue). The expanded CAG-specific RNA–protein interaction causes CpG hypermethylation (inverted triangles) of the *upstream control element* (*UCE*, in purple) in the *rRNA promoter* and results in reduction of *rRNA* transcription. A functional ribosome consists of both *rRNAs* and ribosomal proteins. A reduced level of *rRNAs* causes accumulation of free ribosomal proteins (such as RpL5, RpL11, and RpL23, in orange). The interaction between free ribosomal proteins and E3 ubiquitin ligase MDM2 (in blue) leads to mitochondrial accumulation of p53. The interaction between p53 and antiapoptotic proteins (Bcl-xL, in orange) causes oligomerization of proapoptotic proteins (Bak, in gray) on the mitochondrial membrane and results in cytochrome *c* (in light purple) release. Cytosolic cytochrome *c* in turn activates the caspase cascade and induces apoptosis.

transcription and activating the p53 pathway in polyQ toxicity, our findings do not exclude any contribution of toxic polyQ protein species to the induction of nucleolar stress via other pathways. For instance, it has been reported that expanded polyQ protein induces apoptosis by promoting phosphorylation of p53 on Ser46 (39), and p53 may trigger cell death by inducing the expression of apoptosis-inducing genes (40).

Inhibition of *pre-rRNA* synthesis has been reported to cause neurodegeneration in vivo (25), and dysregulation of CpG methylation of the *rRNA promoter* has been observed in patients with neurodegenerative disease, such as Alzheimer's disease (5). We recently reported that the cell nucleus is a toxic subcellular compartment for expanded CAG RNA to mediate toxicity in polyQ degeneration (17). In this study, we showed that CAG repeat expansion confers a pathogenic property to the mutant RNA molecules that enables them to directly interact with the nucleolar protein NCL. Such RNA–protein interaction causes DNA hypermethylation in the *rRNA promoter* and results in a reduction in *rRNA* transcription. Consequently, the nucleolar stress pathway is activated followed by apoptosis induction (Fig. 5). Our study unequivocally demonstrates that the nucleolus is a major site for expanded CAG RNA toxicity in polyQ diseases. In addition to the induction of DNA hypermethylation in *rRNA promoter*, dysregulation of UBF1 acetylation has also been shown to cause impairment of *rRNA* transcription in R6/2 polyQ transgenic mice (41). These observations suggest that perturbation of any component of the *rRNA* transcription machinery would lead to neuronal cell dysfunction or death. In this study, we report that the pathogenic biomolecule expanded CAG RNA is capable of inducing a nucleolar stress response in neurodegeneration. Our findings thus deliver mechanistic insights into the RNA-mediated toxicity of polyQ pathogenesis and may help develop alternative therapeutic directions. The identification of an additional biomolecular inducer(s) that causes *rRNA* transcriptional dysregulation in individual neurodegenerative diseases is key to our further understanding of the role of the nucleolar stress pathway in neurodegeneration.

Materials and Methods

Real-time PCR Taqman gene expression assays were performed on an ABI 7500 Real-time PCR system. In situ hybridization and immunostaining were performed according to refs. 42 and 21 respectively with minor modifications. Mitochondrial fractionation was performed according to ref. 43. Other materials and methods are described in *SI Materials and Methods*.

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