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Polyglutamine Expansion Induces a Protein-damaging Stress Connecting Heat Shock Protein 70 to the JNK Pathway*

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Polyglutamine diseases, including Huntington's disease, designate a group of nine neurodegenerative disorders characterized by the presence of a toxic polyglutamine expansion in specific target proteins. Using cell and mouse models, we have shown that expanded polyglutamine led to activation of the stress kinase JNK and the transcription factor AP-1, which are implicated in neuronal death. Polyglutamine expansion-induced stress shared common features with protein-damaging stress such as heat shock, because activation of JNK involved inhibition of JNK phosphatase activities. Indeed, expanded polyglutamine impaired the solubility of the dual-specificity JNK phosphatase M3/6. Aggregation of M3/6 by polyglutamine expansion appeared to be indirect, because M3/6 was not recruited into polyglutamine inclusions. The heat shock protein HSP70, which is known to inhibit JNK during the heat shock response, suppressed polyglutamine-mediated aggregation of M3/6 and activation of JNK. Interestingly, levels of HSP70 were down-regulated by polyglutamine expansion. We suggest that reduction of HSP70 by expanded polyglutamine is implicated in aggregation and inhibition of M3/6 and in activation of JNK and AP-1.

Nine neurodegenerative disorders, including Huntington's disease and spinocerebellar ataxia type 7 (SCA7)¹ are caused by expansion of a CAG trinucleotide repeat coding for a polyglutamine tract in unrelated target proteins (1). Progressive neuronal loss characterizes all polyglutamine diseases, although the affected brain regions are variable between disorders (2). An inverse correlation between age of onset of the disease and the length of the expansion is observed.

Polyglutamine expansion (polyQ) confers a gain of toxic func-

tion to the respective proteins, which results in neurodegeneration (3, 4). Why and how polyQ is toxic has still to be determined, although recent findings have shown that it impairs different critical cellular processes. PolyQ is highly prone to aggregation, as shown *in vitro* (5) and *in vivo*, where it forms nuclear inclusions (NIs) (6). NIs are believed to impair major cellular processes through sequestration of chaperones, proteasomal components, and transcription factors (7, 8). Indeed, overexpression of chaperones in *Drosophila* and mice is able to rescue the phenotypes induced by expression of polyQ (9, 10), and there is evidence showing that protein degradation (11) and transcription (12) are impaired by polyQ. However, other studies suggest that NIs are not required to initiate neurodegeneration (13) and that soluble polyQ can directly interfere with cellular processes such as transcription (14, 15).

Protein aggregation is also induced by protein-damaging stress such as heat shock (16). In contrast to UV irradiation, osmotic stress and certain cytokines that activate the c-Jun N-terminal Kinase (JNK) via a signal transduction pathway that involves small GTP-binding proteins (17) and a cascade of protein kinases (18, 19), protein-damaging stresses, including heat shock and arsenite, lead to activation of JNK primarily through inhibition of JNK phosphatase(s) (16, 20). Recent findings suggest that the dual-specificity JNK phosphatase M3/6 is inactivated by protein-damaging stress (21, 22). M3/6 solubility properties would change after heat shock, resulting in inhibition of phosphatase activity. The heat shock protein HSP70 negatively regulates JNK activity by promoting JNK dephosphorylation (16, 23). It is believed that redistribution of HSP70 at the sites of damaged proteins affects its JNK regulatory function (16).

Members of the JNK family, which are encoded by three different genes, phosphorylate and activate the transcription factor c-Jun (24). C-Jun homo- or heterodimerizes with proteins of the Fos family, forming AP-1 complexes. AP-1 activity is implicated in numerous functions, including proliferation, survival, and programmed cell death (25). In the nervous system, excitotoxic stress such as stimulation with kainic acid induces apoptosis in a manner dependent on both JNK3 (the brain-specific isoform) and c-Jun (26, 27). In addition, withdrawal of NGF in sympathetic neurons triggers a cell death program that is inhibited by dominant negative mutants of c-Jun (28).

As discussed above, there is evidence that the toxicity of polyQ results in part from its propensity to misfold, a situation that might be equivalent to the one induced by a protein-damaging stress. Thus, to get insight into polyQ toxicity, we have investigated the possibility that polyQ could lead to a cellular stress comparable to a protein-damaging stress. Here we have shown that polyQ expression resulted in activation of

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¹ The abbreviations used are: SCA7, spinocerebellar ataxia type 7; TRE, TPA-responsive element; polyQ, polyglutamine expansion; NIs, nuclear inclusions; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; CAT, chloramphenicol acetyltransferase; SAPK, stress-activated protein kinase; P-JNK, anti-activated JNK antibody; MAPK, mitogen-activated protein kinase; P-c-Jun, anti-phospho c-Jun antibody; ER, endoplasmic reticulum; SEK, SAPK/ERK kinase; MKK, MAP kinase kinase.

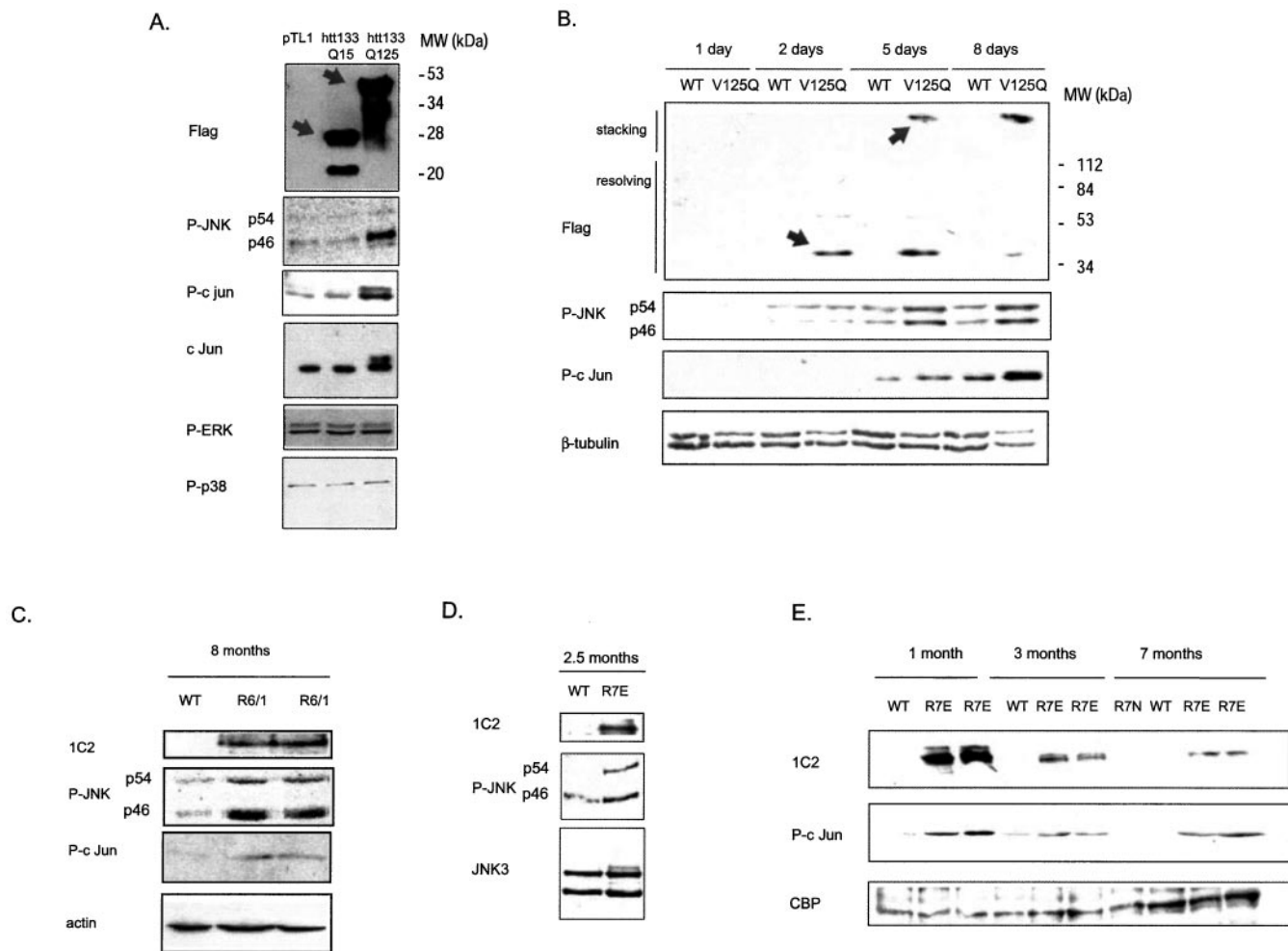


FIG. 1. Expression of polyglutamine expansion results in hyperphosphorylation of JNK and c-Jun. *A*, JNK and c-Jun are hyperphosphorylated in COS cells expressing ectopic htt133Q125. COS cells were transfected either with htt133Q15 or htt133Q125 constructs. Whole cell extracts were analyzed by Western blot with the different anti-phospho-MAPK antibodies, including anti-P-JNK, anti-P-p38, and anti-P-ERK antibodies. Membranes were also revealed with anti-P-c Jun and anti-c-Jun antibodies. Anti-FLAG antibody was used to control transfection efficiencies. *B*, JNK and c-Jun hyperphosphorylation is concomitant to aggregation of mutated htt in the inducible neuronal NG108 V125 cell line (V125Q). Control cells (WT) are clones that only express the tetracycline-inducible transactivator. A time course over 8 days was performed after differentiation and induction of mutated htt expression. Whole cell extracts were prepared at the indicated periods and analyzed by Western blot with anti-P-JNK and anti-P-c Jun antibodies. Anti-FLAG and anti- β -tubulin antibodies were used to control htt expression and total protein levels, respectively. The *arrows* indicate soluble (in the resolving) and aggregated (in the stacking) htt V125Q. *C*, basal phosphorylation levels of JNK and c-Jun are increased in retinas of R6/1 mice. Retinas of R6/1 and wild-type littermate mice aged 8 months were dissected, and total protein extracts were prepared and analyzed by Western blot. Expression of expanded exon 1 of htt and total protein levels were controlled with 1C2 and anti-actin antibodies, respectively. *D* and *E*, JNK3 and c-Jun are hyperphosphorylated in retinas of R7E mice. Retinas of R7E, R7N, and wild-type littermate mice at different ages (1, 2.5, 3, and 7 months) were dissected, and total proteins were extracted. Western blot analysis was performed subsequently with anti-P-JNK and anti-JNK3 and antibodies (*D*) and with anti-P-c-Jun (*E*). 1C2 and anti-CBP antibodies were used to control expression of mutated ataxin-7 and total protein levels, respectively.

JNK and c-Jun, through inhibition of JNK phosphatase activities. In particular, the solubility of the JNK phosphatase M3/6 was impaired by polyQ. M3/6 was not recruited into NIs, suggesting that its aggregation was indirectly induced by polyQ. Expression of HSP70 suppressed polyQ-mediated aggregation of M3/6 and activation of JNK, suggesting that HSP70 is a critical regulator of polyQ-induced stress. In addition, expression of polyQ in cells resulted in down-regulation of HSP70 levels and in its recruitment into NIs. Altogether, our results indicate that impairment of HSP70 by polyQ is responsible for inactivation of JNK phosphatase(s). As a result, activation of JNK and c-Jun, which are involved in neuronal death, might be critical in the triggering of polyQ-mediated neurodegeneration.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal 1C2 and 2A10 antibodies and polyclonal 1261 and M3/6 antibodies have been previously described (21, 29, 30). Anti-phosphorylated SEK-1, JNK, p38, ERK, and c-Jun

(ser63) antibodies were from Cell Signaling. Anti-JNK3, anti-c-Jun (sc-45), anti-ERK (K23), and anti-CBP (A22) were from Santa Cruz Biotechnology. Anti-FLAG (M2), anti-actin, and anti- β -tubulin antibodies were from Sigma. Anti-HSP70 and anti-HSC70 antibodies were from Stressgen. Secondary antibodies (goat anti-mouse or goat anti-rabbit immunoglobulins) conjugated to peroxidase or CY3 or Oregon Green were from Jackson Laboratories.

Transient Transfection and Treatments of COS-1 Cells—pTL1-based expression vectors encoding the first 133 amino acids of the human huntingtin protein with either 15 or 125 glutamines and FLAG-tagged at the N-terminal extremity have been previously described (29). The expression vector encoding murine c-Jun and reporter constructs were a gift from P. Sassone-Corsi. Expression vector encoding murine M3/6 has been described previously (31). A pcDNA-based expression vector encoding HSP70 was generated from a pH2.1 plasmid containing the sequence of a human *hsp70* gene, which was given by R. I. Morimoto. Transient transfections were performed by the phosphate calcium method as follows: COS-1 cells were cultured in DMEM/5% fetal calf serum (FCS) and plated at 30% confluence before transfection. After transfection (15 h), medium was changed and cells were grown for additional 48 h.

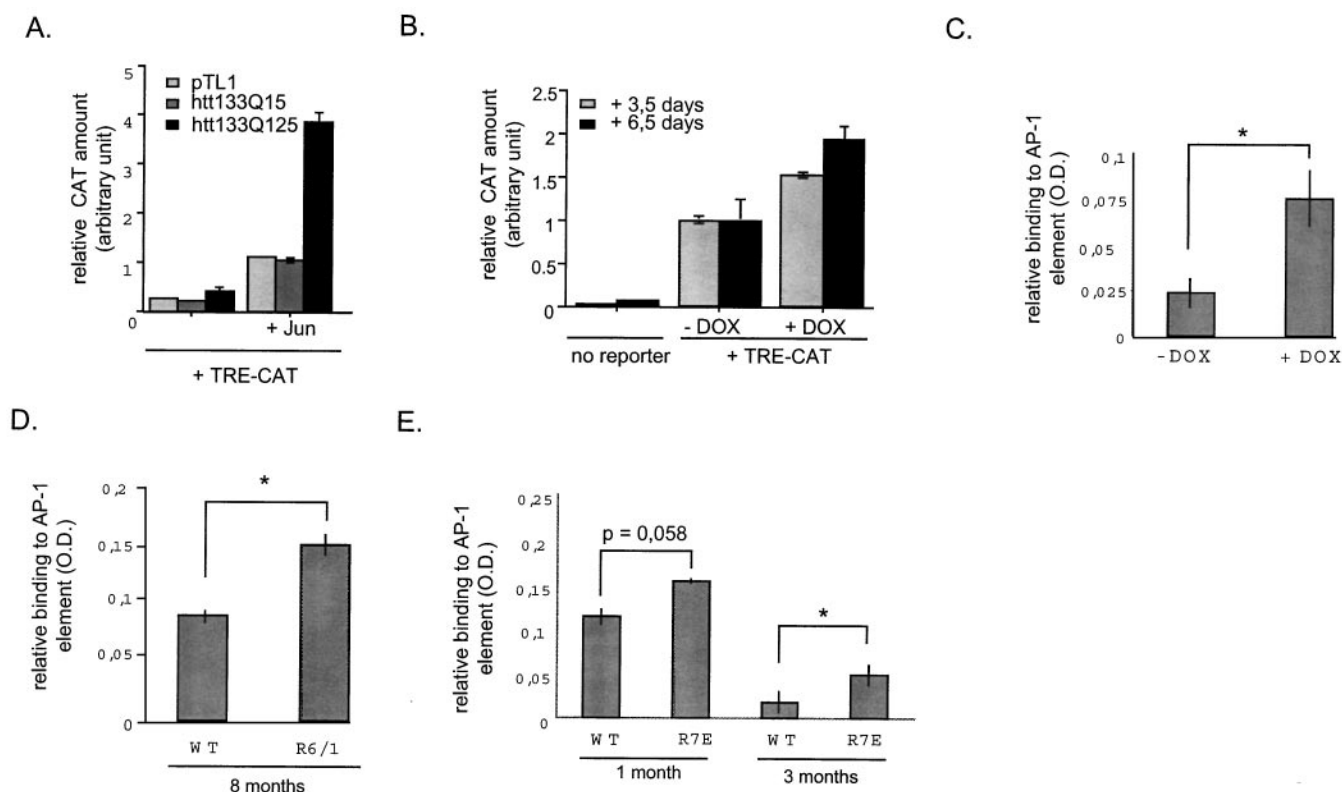


FIG. 2. Expression of polyglutamine expansion results in transcriptional activation of AP-1 complex. *A*, ectopic expression of htt133Q125 in COS cells induces a 4-fold increase in AP-1-dependent gene expression of CAT. COS cells were transfected with both TRE-CAT and lacZ reporter plasmids together with either the empty expression vector, htt133Q15, or htt133Q125. Protein extracts were prepared 65 h post-transfection, and the CAT amount present in the extracts was assessed using a CAT ELISA kit (Roche Molecular Biochemicals). Results were normalized with respect to β -galactosidase activities and are expressed as arbitrary units. A value of 1 was assigned to the sample corresponding to cells cotransfected with empty pTL1 and TRE-CAT plasmids. Experiments were repeated three times, and for each repetition samples were duplicated. *B*, induction of htt V125Q expression in neuronal NG108 V125 cells results in activation of AP-1-dependent expression of CAT. NG108 V125 cells were transfected with the TRE-CAT construct or not (no reporter). Cells were then differentiated and, concomitantly, expression of htt V125Q was induced or not with doxycycline. Protein extracts were prepared at the indicated periods, and the levels of CAT within the extracts were determined using the CAT ELISA method. Results were normalized with respect to β -galactosidase activities and are expressed as arbitrary units. Values obtained at 3.5 and 6.5 days cannot be compared, because measurements were not performed the same day. Values of 1 were given for the samples transfected with the TRE-CAT plasmid, but not treated with doxycycline. The experiment was repeated twice, and each repetition duplicates were performed. *C*, binding of AP-1 complexes to TRE is increased in NG108 cells expressing htt V125Q. NG108 V125 cells were differentiated for 7 days and, concomitantly, expression of htt V125Q was induced or not. Nuclear extracts were prepared and tested for binding to the AP-1 element, using the Trans-AM AP-1 kit (Active Motif). Results are expressed as optical density values. Two independent experiments were realized, with duplicates in each of it. The asterisk indicates that the difference between both values is significant with a $p < 0.05$ according to Student's *t* test. *D*, binding of AP-1 complexes to TRE is increased in R6/1 mice. Retinas of R6/1 and wild-type littermate mice of 8 months were dissected and nuclear proteins were prepared. The extracts were tested for binding to the AP-1 element as in *C*. The experiment was repeated twice, and for each repetition, duplicates corresponding to two different mice were performed. Results are significant at $p < 0.05$. *E*, binding of AP-1 proteins to TRE is augmented in R7E mice. Retinas of R7E and wild-type littermate mice of both 1 and 3 months were dissected and nuclear proteins were extracted. Protein extracts were tested for binding to AP-1 as in *C*. Experiments were done twice, and values are duplicated as described for *D*, i.e. two R7E and two WT mice were used for each age, in each experiment. The data are significant at $p < 0.05$ for mice of 3 months of age.

Cell Culture and Differentiation of NG108 Cell Lines—Generation, culture, and differentiation of reverse tetracycline-inducible NG108 cell lines expressing an N-terminal FLAG-tagged version of the first 80 amino acids of the human huntingtin protein with either 15 or 125Q have been previously described (32). Briefly, clones were maintained in DMEM/10% FCS, antibiotics, 0.5 mg/ml G418, and 250 μ g/ml hygromycin B. For neuronal differentiation and induction of expression, medium was replaced by DMEM/1% FCS, antibiotics, G418, hygromycin B, 10 μ M forskolin, 100 μ M isobutylmethylxanthine, and 1 μ g/ml doxycycline. Transient transfections with CAT- and lacZ-reporter constructs were performed as followed. Cells maintained in the DMEM/10% FCS medium were plated at 30% confluence and transfected with the phosphate calcium method. After transfection (15 h), medium was replaced by the differentiation and induction medium for the indicated period.

Proteins: Extraction, Fractionation, and Immunoprecipitation—COS-1 and NG108 cell extracts were prepared as follows: cells were washed twice in ice-cold PBS and directly lysed in boiling Laemmli buffer. Mice retinas were dissected and homogenized in TGEK buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM EDTA, 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, a mixture of protease inhibitors, 1 mM

sodium orthovanadate, and 20 mM sodium fluoride). They were incubated for 15 min on ice, sonicated, and centrifuged for 20 min at 13,000 rpm and 4 $^{\circ}$ C. Supernatants were collected.

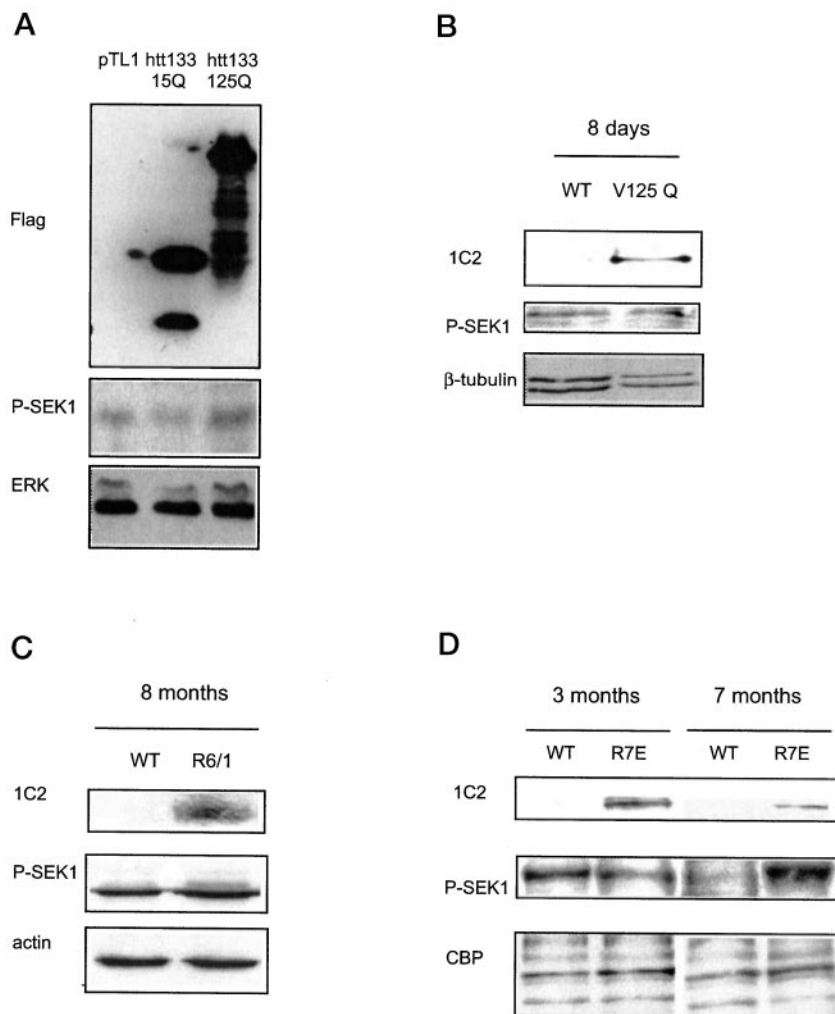
For fractionation experiments, proteins were prepared as described previously (33). Briefly, the soluble SN1 fraction of cells or retinas was recovered in TGEK buffer, as described above. After centrifugation, the pellet was conserved and solubilized in an SDS buffer (final concentration of 2% SDS, 5% β -mercaptoethanol, 15% glycerol) by boiling for 10 min. The preparation was centrifuged for 20 min at 13,000 rpm and room temperature, and the supernatant was recovered, giving the SN2 fraction. The pellet (fraction P) was solubilized by incubation in pure formic acid at 37 $^{\circ}$ C for 30 min then lyophilized overnight and resuspended in SDS buffer.

For immunoprecipitation experiments, protein extracts were prepared as described above, by lysing cells or retinas in TGEK buffer. Equal amounts of whole cell extracts were incubated with anti-M3/6 antibody overnight at 4 $^{\circ}$ C, and with protein A-Sepharose for additional 30 min. Immunoprecipitates were washed three times and resuspended in TGEK buffer.

Western Blotting—Protein extracts were resolved by standard SDS-PAGE. Samples were electroblotted onto Protan nitrocellulose

FIG. 3. Hyperphosphorylation of JNK by polyglutamine expansion results from inactivation of JNK phosphatases.

A, SEK-1 is slightly hyperphosphorylated in COS cells expressing ectopic htt133Q125. COS cells were transfected with htt133Q15 or htt133Q125. Phosphorylation levels of SEK-1 were detected with P-SEK-1 antibody. **B**, SEK-1 is not hyperphosphorylated in neuronal NG108 V125 cells expressing htt V125Q. NG108 V125 cells were differentiated and induced for 8 days. The parental cell line was used as control, as in Fig. 1B. The P-SEK-1 antibody was used to check phosphorylation levels of SEK-1, as in A. In C and D phosphorylation levels of SEK-1 are normal in retinas of R6/1 and R7E mice. Protein extracts from retinas of R6/1 mice of 8 months and of R7E mice of 3 and 7 months were analyzed with the P-SEK-1 antibody.



(Schleicher and Schuell). Membranes were incubated in PBS/5% low fat milk overnight at 4 °C with non-phosphospecific antibodies, and in TBS/5% bovine serum albumin with phosphospecific antibodies. Immunocomplexes were revealed by chemiluminescence with anti-mouse or anti-rabbit antibodies.

CAT ELISA Assays—Quantitative determination of chloramphenicol acetyltransferase (CAT) in transfected cells was performed using a CAT ELISA kit (Roche Molecular Biochemicals). Briefly, cells were lysed and proteins extracts were prepared according to the manufacturer's instructions. The extracts were incubated on ELISA wells, which were precoated with a polyclonal antibody to CAT. A sandwich ELISA was then realized, by incubating an anti-CAT antibody coupled to digoxigenin, followed by an anti-digoxigenin antibody coupled to peroxidase. A colorimetric substrate was added, and the reaction was quantitated by measuring the optical density at 405 nm. Results were normalized with respect to protein concentration and transfection efficiency. Cells were cotransfected with a lacZ-reporter construct and the β -galactosidase activity was dosed to assess the efficacy of transfection.

AP-1 Binding Assays—AP-1 binding assays were realized using the kit Trans-AM AP-1 (Active Motif) and following the instructions given by the manufacturer. Briefly, nuclear extracts from NG108 cells or mice retinas were prepared by standard methods using the kit reagents. 10 μ g of nuclear extracts was incubated on ELISA wells, where oligonucleotides containing the TRE had been precoated. Binding of AP-1 complexes to TRE was determined using a phospho c-Jun antibody, followed by a secondary antibody coupled to peroxidase. A colorimetric substrate was then incubated, and the reaction was quantified by measuring the optical density at 450 nm.

Immunofluorescence—Fixed eyes were incubated overnight in PBS/30% sucrose and snap frozen on dry ice. 50- μ m free-floating sections were blocked in PBS/0.3% Triton X-100/5% bovine serum albumin, washed in PBS, and incubated with primary and secondary antibodies (Cy3- or Oregon Green-conjugated) diluted in PBS/5% bovine serum albumin. M3/6, HSP70, 1261, and 1C2 antibodies were diluted to 1/200 each.

RESULTS

Expression of Expanded Polyglutamine Results in Moderate but Sustained Hyperphosphorylation of JNK and c-Jun—We asked whether expression of polyQ could result in a toxic cellular stress. To investigate this hypothesis, we tested whether stress-activated protein kinases (SAPKs) were activated in different polyQ models. COS cells were transiently transfected with expression vectors encoding a FLAG-tagged version of the first 133 amino acids of huntingtin protein (htt133) with either a normal (15) or mutated (125) number of glutamines. Western blot analysis performed on whole cell extracts with anti-activated JNK antibody (P-JNK) revealed that JNK was moderately phosphorylated in presence of mutated htt (Fig. 1A). The activation was specific, because basal phosphorylation levels of other related kinases, p38/SAPK and ERK/MAPK, were not affected (Fig. 1A). The downstream target of JNK, c-Jun transcription factor, was also hyperphosphorylated in cells expressing mutated htt, as detected with an anti-phospho c-Jun antibody (P-c-Jun), which reveals N-terminal phosphorylation of c-Jun (Fig. 1A).

We then wished to extend these results to a more physiological cell system. For this purpose, we expressed a FLAG-tagged truncated version of mutated htt (V125Q) in stable and inducible NG108 cell lines, which we differentiated into neurons for various days (32). Whole cell extracts analyzed by Western blot showed that both JNK and c-Jun were hyperphosphorylated in NG108 V125 cells (Fig. 1B). In contrast, in parental cells and in NG108 cells expressing the same truncated form of htt, but with 15Q, JNK and c-Jun were not hyperphosphorylated (Fig.

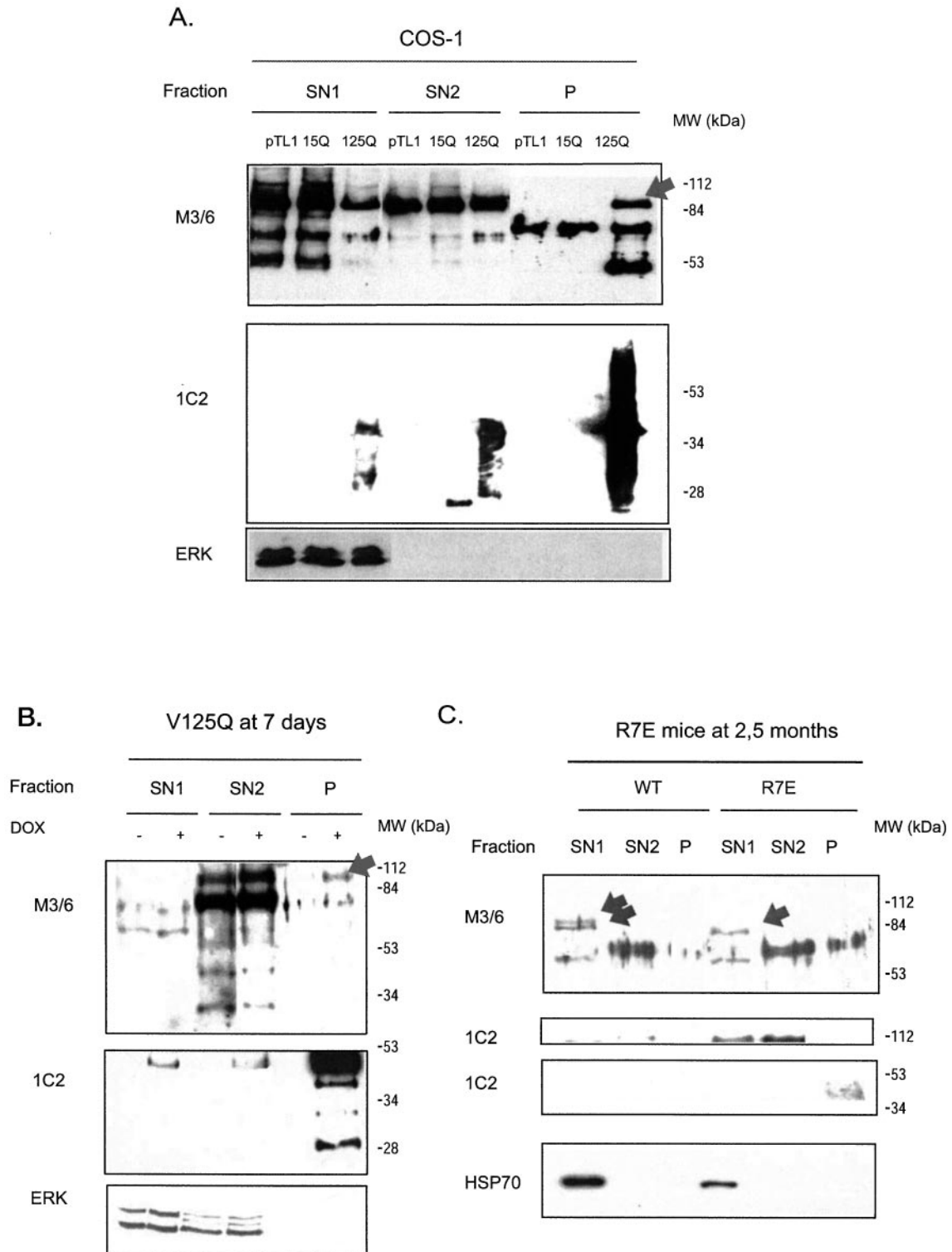


FIG. 4. The solubility of the JNK phosphatase M3/6 is impaired by polyglutamine expansion. *A*, expression of expanded htt in COS cells promotes aggregation of ectopic M3/6. COS cells were transfected with M3/6 together with htt133Q15 or htt133Q125. Soluble and insoluble proteins were prepared and recovered in fractions SN1 (soluble), SN2 (rather insoluble), and P (very insoluble). Analysis of the content of M3/6 in the different fractions was realized by Western blot. 1C2 and anti-ERK antibodies were used to control the fractionation procedure. The full-length M3/6 is indicated by the *arrow*. *B*, aggregation of endogenous M3/6 is increased in neuronal NG108 V125 cells expressing htt V125Q. NG108 V125 cells were differentiated for 7 days and at the same time induced or not with doxycycline. Proteins were submitted to the fractionation protocol to recover soluble and insoluble materials. The anti-M3/6 antibody was used to analyze the different fractions. *C*, expression of mutated ataxin-7 augments the aggregation potential of M3/6 in R7E mice. Retinas of R7E and wild-type littermate animals of 2.5 months were dissected and subjected to fractionation. Western blot analysis of the different fractions was performed as for (*A*) and (*B*) with anti-M3/6 antibody. Detection with 1C2 antibody revealed the presence of the full-length mutated ataxin-7 in SN1 and SN2 and of a fragment of about 40 kDa in P. In between, cross-reacting bands were detected (not shown).

R7E mice at 2.5 months

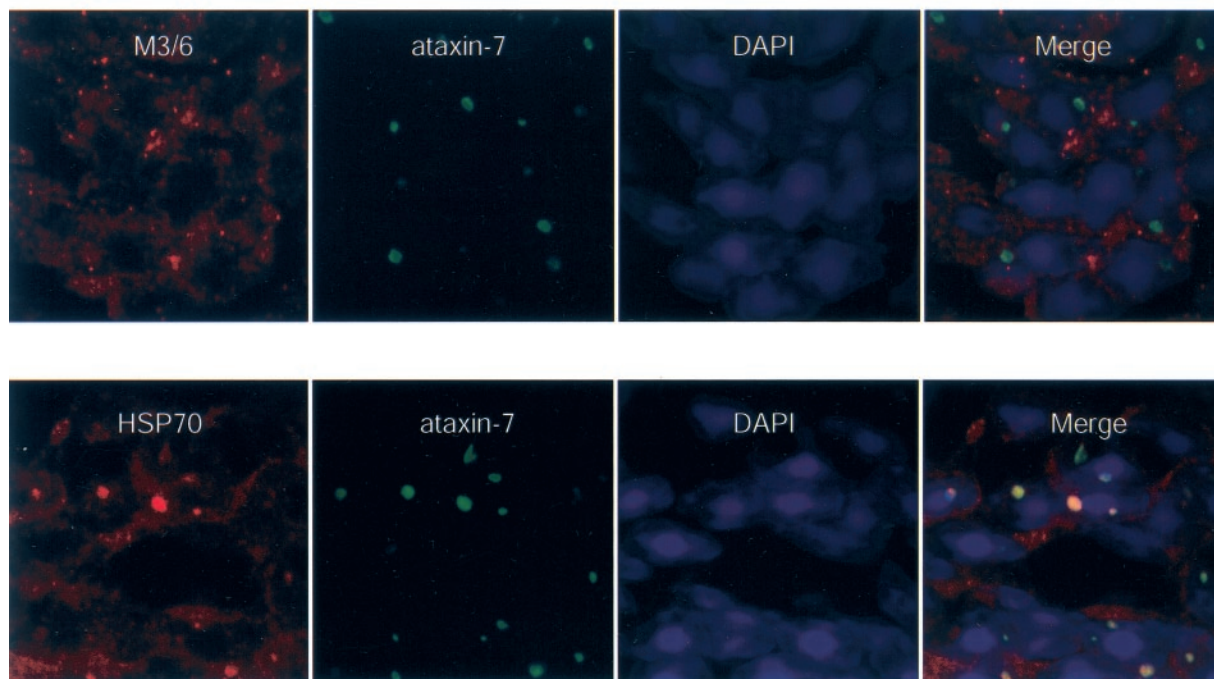


FIG. 5. **NIs do not recruit M3/6 phosphatase but HSP70.** HSP70, but not M3/6, is recruited into NIs in the R7E model. Colocalization studies of M3/6 and ataxin-7 (*upper panel*) and of HSP70 and ataxin-7 (*lower panel*) were performed by double-immunofluorescent confocal analysis. Sections of retinas from R7E animals of 2.5 months were stained with anti-ataxin-7 antibodies (monoclonal 2A10 in the *upper panel* and polyclonal 1261 in the *lower panel*) and anti-M3/6 or anti-HSP70 antibodies. Colocalization in NIs was systematically observed for HSP70, but never for M3/6.

1B and not shown). Interestingly, JNK and c-Jun activation was concomitant to aggregation of mutated htt, which was detectable 5 days after induction (Fig. 1B). From day 5, aggregated htt remained in the stacking part of the SDS-PAGE gel.

We then asked whether expression of polyQ could also lead to sustained activation of JNK *in vivo*. To address this, we analyzed R6/1 transgenic mice, which express expanded-exon 1 of htt in brain (4). To establish whether the molecular process of JNK activation could be common to other polyQ diseases, we included in the study transgenic mice expressing mutated ataxin-7, the protein involved in SCA7. The R7E mice we used express mutated ataxin-7 specifically in photoreceptors and have been shown to represent a good model of SCA7 retinal pathology (30). Interestingly, we found that mutated exon 1 of htt is also expressed in photoreceptors of R6/1 animals (34). Taking advantage of this observation and to compare both pathologies at the molecular level, we analyzed the phosphorylation status of JNK and c-Jun in retinas of R6/1 and R7E animals. R6/1 mice were sacrificed at 8 months, *i.e.* a few weeks before death, and R7E mice, for which disease progression is more severe, were sacrificed at both early and late time points after the onset of retinopathy (1 and 3 months). Control wild-type littermates as well as transgenic mice expressing the normal ataxin-7 (with 10Q, referred to as R7N) were included in the study (30). Expression of mutated htt or ataxin-7 was controlled with the 1C2 antibody, which recognizes specifically expanded polyglutamine (29). JNK was hyperphosphorylated in retinas expressing mutated htt exon 1 and ataxin-7 (Fig. 1, C and D). Migration of part of the neuronal-specific JNK3 isoform was shifted to higher molecular weights, suggesting that JNK3 was itself a target of activation (Fig. 1D). c-Jun was also found hyperphosphorylated in R6/1 and R7E transgenic mice (Fig. 1, C and E). Hyperphosphorylation of c-Jun was already detectable in R7E mice of 1 month, which corresponds

to the beginning of the pathology. This suggested that activation of the JNK pathway by polyQ is an early and persistent event. In conclusion, polyglutamine-expanded htt and ataxin-7 both induce activation of JNK and c-Jun proteins, indicating that the polyQ tract is sufficient to mediate this effect. In addition, we show that activation of JNK by polyQ occurs *in vivo* in animal models. Thus, these data extend previous observations showing that overexpression of mutated htt in PC12 cells differentiated into neurons results in a cellular stress that leads to activation of JNK (35).

Hyperphosphorylation of c-Jun by Polyglutamine Expansion Leads to Activation of AP-1-dependent Gene Expression—N-terminal phosphorylation of c-Jun regulates AP-1 activity (25) and is involved in neuronal cell death (27, 28, 36). Thus, we asked whether expression of polyQ in our different models would result in enhancement of AP-1 transcriptional activity. To test this, we coexpressed in cells mutated htt, c-Jun, and a reporter gene under the control of a promoter containing the AP-1 element (also named the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element or TRE). Expression of mutated htt in COS cells induced a 4-fold activation of the TRE reporter (Fig. 2A). Similarly, stable expression of mutated htt in NG108 V125 cells differentiated for 3.5 and 6.5 days resulted in modest but progressive activation of the expression of the CAT gene (up to 2-fold, Fig. 2B). PolyQ did not increase transcription from other responsive elements, such as the serum-responsive element (not shown).

Binding assays that allow testing the amount of active (*i.e.* phosphorylated) c-Jun bound to the TRE further supported these results. The principle of the new and sensitive technology we used is based on an ELISA (see “Experimental Procedures”). Nuclear extracts prepared from NG108 V125 cells differentiated for 7 days into neurons were tested by this method. Binding of AP-1 to TRE was significantly increased (about 3-fold) in

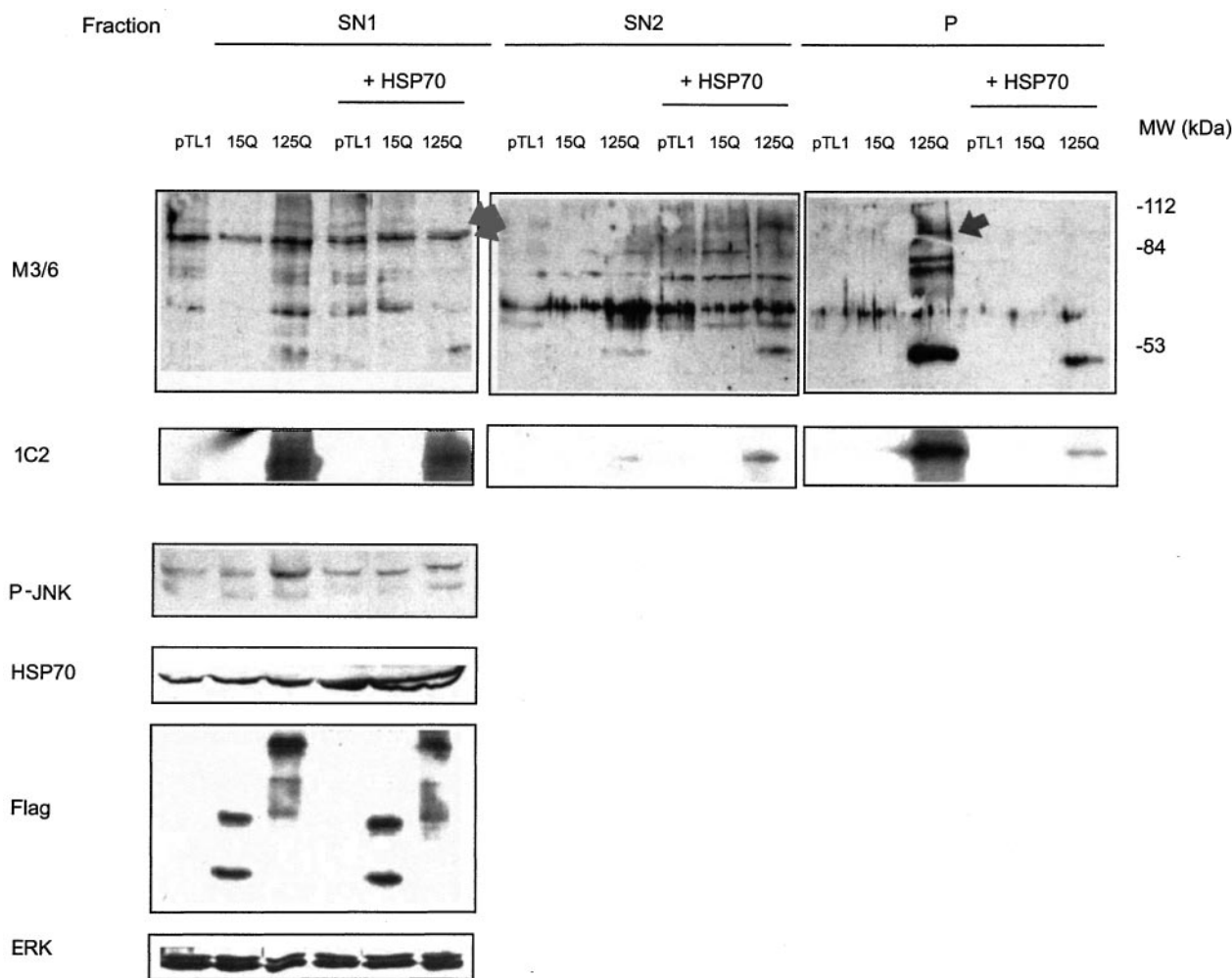


FIG. 6. Overexpression of HSP70 inhibits polyQ-mediated aggregation of M3/6 and activation of JNK. COS cells were transfected with htt133Q15 or htt133Q125 together or not with HSP70. Fractionation experiments were performed to recover soluble and insoluble proteins, as SN1, SN2, and P fractions. The content of the different fractions was analyzed by Western blotting, using anti-M3/6 and 1C2 antibody. The soluble SN1 fraction was controlled with anti-ERK, anti-HSP70, and anti-FLAG antibody. The P-JNK antibody was also used to test the activation level of JNK in this fraction.

extracts prepared from cells induced for expression of htt V125Q, compared with non-induced cells (Fig. 2C). The binding was specific because the presence of free TRE-oligonucleotides abolished the ELISA signal when incubated with nuclear extracts (not shown). Next, we performed nuclear preparations from retinas of R6/1 mice of 8 months. Fig. 2D shows that binding of AP-1 to TRE was significantly augmented when extracts were from transgenic, *versus* normal mice. We also measured the binding capacity of AP-1 complexes derived from retinas of R7E and wild-type mice of 1 and 3 months (Fig. 2E). AP-1 binding was significantly increased in transgenic mice of 3 months. Thus, binding of active AP-1 to TRE is increased by polyQ. In conclusion, these results suggest that polyQ induces activation of AP-1-dependent gene expression.

Activation of JNK by Polyglutamine Expansion Involves Inactivation of the Dual-specificity JNK Phosphatase M3/6—JNK is activated through phosphorylation by upstream kinases, including SEK-1/MKK4 and MKK7 (18, 19). The phosphorylation of JNK is a reversible event, involving the action of JNK phosphatases. Whereas stresses such as UV irradiation or osmotic stress induce strong activation of kinases upstream of JNK (37), protein-damaging stress such as heat shock induces an activation of JNK that is mainly mediated by inhibition of JNK phosphatases (16). We wished to investigate which mechanism was primarily implicated in polyQ-mediated activation of JNK.

To test this, we examined the phosphorylation status of the JNK activator SEK-1, using an anti-phospho SEK-1 antibody. Expression of mutated htt in COS cells slightly induced the phosphorylation of SEK-1 (Fig. 3A). In retinas of R7E mice at 7 months of age, which corresponds to a very late pathological stage, SEK-1 was also moderately hyperphosphorylated (Fig. 3D). However, the phosphorylation levels of SEK-1 were normal in neuronal NG108 V125 cells and in R6/1 and R7E mice of, respectively, 8 and 3 months (Fig. 3, B–D). These data suggest that early activation of JNK by polyQ mainly results from inactivation of JNK phosphatases and that a secondary event might further activate JNK through upstream kinases.

To further investigate this hypothesis, we asked whether we could identify a JNK phosphatase that would be inactivated by polyQ. We focused on the mouse M3/6 dual-specificity JNK phosphatase, whose orthologue hVH5 in the human is highly expressed in the nervous system (38). In addition, upon protein-damaging stress, M3/6 solubility is decreased, which leads to its inactivation (21, 22). Thus, we wished to test whether M3/6 solubility was impaired in cells expressing polyQ. COS cells were cotransfected with expression vectors encoding M3/6 and mutated or normal htt. Fractionation experiments were conducted as described (33) to isolate insoluble proteins from cell extracts. Fraction SN1 contained soluble proteins, which represent most of the proteins. Indeed, Western blot analysis

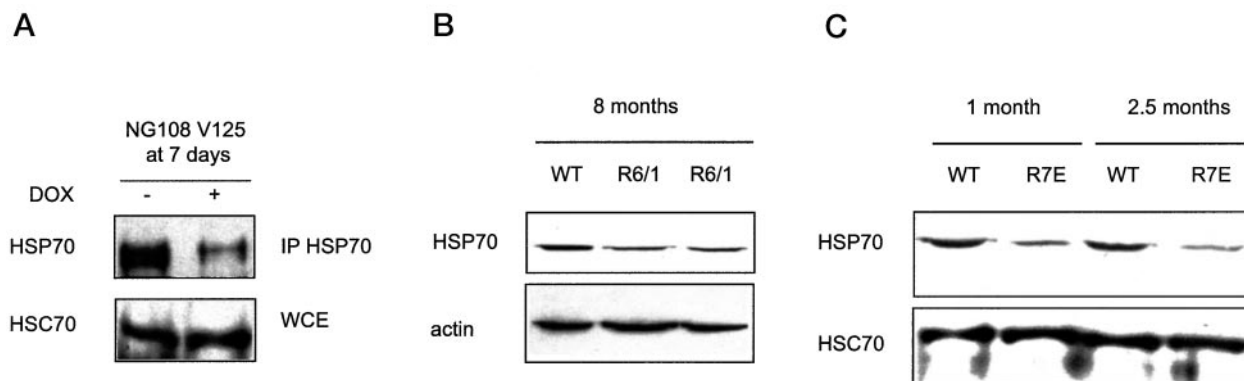


FIG. 7. HSP70 protein levels are down-regulated by polyglutamine expansion. *A*, HSP70 is reduced in neuronal NG108 V125 cells expressing mutated htt. NG108 V125 cells were differentiated for 7 days and induced or not with doxycycline. Whole cell extracts were prepared and, because HSP70 is expressed at low levels in NG108 cells, immunoprecipitated with anti-HSP70 antibody. Detection of HSP70 was then performed with anti-HSP70 antibody. Protein levels were controlled with an anti-HSC70 antibody, before immunoprecipitation. *B* and *C*, HSP70 is down-regulated in R6/1 and R7E mice. Retinas of transgenic and wild-type animals of 8 months (for R6/1) and of 1 and 3 months (for R7E) were dissected. Proteins were extracted and directly analyzed with the anti-HSP70 antibody by Western blot. Indeed, the constitutive expression level of HSP70 is rather elevated in mouse retinas.

shows that ERK proteins are primarily localized in this cell fraction (Fig. 4A). Insoluble material was recovered in two different fractions referred to as SN2 and P, which correspond respectively to SDS-soluble and SDS-resistant but formic acid-soluble material (33). Thus, fraction P contained the most insoluble material. As shown in Fig. 4A, mutated htt, which aggregates in cells, was significantly detected in this fraction with 1C2 antibody. The M3/6 content present in the three fractions was analyzed by Western blot analysis, using an anti-M3/6 antibody (21). Fig. 4A shows that this antibody detects the full-length 90-kDa M3/6 protein as well as two other major breakdown products. Remarkably, in cells that were left untreated or transfected with normal htt133, large amounts of M3/6 were detected in fractions SN2 (mainly as a full-length protein) and P (as a breakdown product). This indicated that M3/6 might be prone to aggregation. As shown on Fig. 4A, expression of mutated but not of normal htt increased this intrinsic tendency. Indeed, the content of M3/6 was significantly decreased in fraction SN1 and increased in fraction P.

We then performed the same kind of experiments with neuronal NG108 V125 cells, expressing or not mutated htt. The content of the different fractions was controlled with anti-ERK and 1C2 antibodies, as in COS cells (Fig. 4B). The complete htt V125Q as well as a cleaved form were detected in fraction P. In cells that did not express mutated htt, endogenous M3/6 was barely visible in SN1 but was present in SN2 as a full-length protein and as a major breakdown product. Interestingly, fraction P of cells expressing mutated htt was clearly enriched in the full-length M3/6 protein and the breakdown product.

We then examined the solubility of M3/6 in retinas of R7E and wild-type mice at 2.5 months of age. In fraction SN1 of wild-type animals, we detected two bands at 90 kDa (Fig. 4C), possibly corresponding to phosphorylated and dephosphorylated forms of M3/6 protein (31). SN2 and P fractions contained only a breakdown product of the phosphatase. In R7E mice, the upper band of fraction SN1 disappeared, and fraction P was enriched in the cleaved form of M3/6. 1C2 antibody, which was used as a control, revealed the presence of the full-length mutated ataxin-7 in fractions SN1 and SN2, but only of a fragment of mutated ataxin-7 in fraction P (Fig. 4C), as previously described (30).

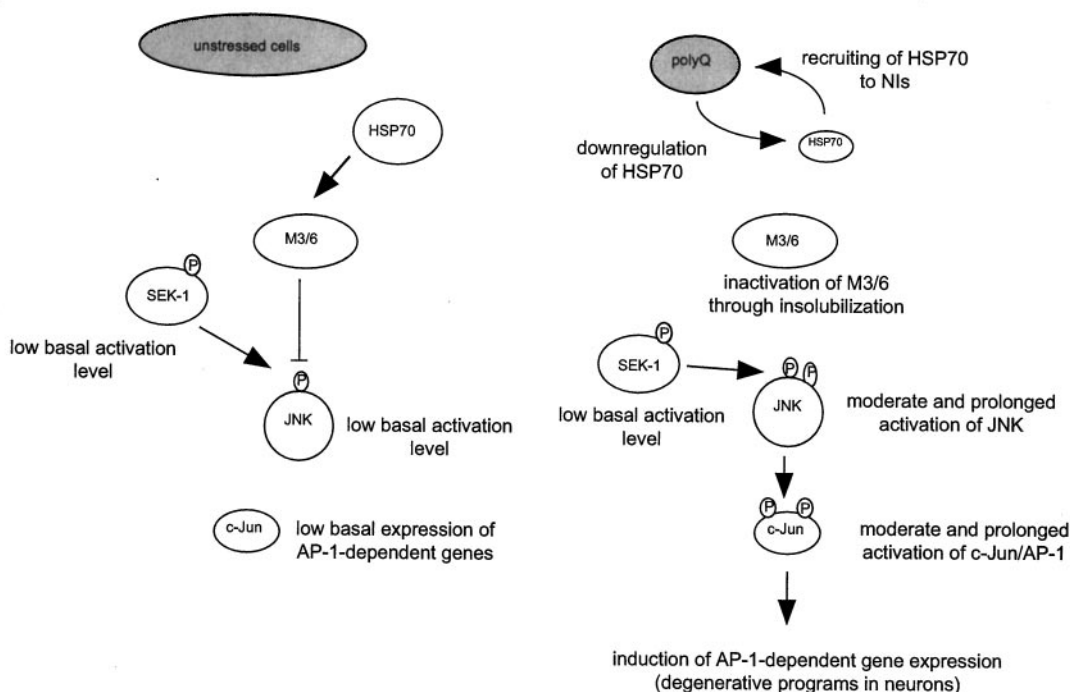
Altogether, these data show that M3/6 is intrinsically prone to degradation and aggregation, indicating that these two processes are essential for the regulation of the phosphatase activity. We also provide evidence that polyglutamine-expanded proteins promote aggregation and degradation of M3/6. Thus,

although our results do not exclude that other JNK phosphatases than M3/6 might be affected by polyQ, they show that M3/6 at least is impaired in cells expressing polyQ.

Aggregation of M3/6 by PolyQ Is Not Direct—Many proteins, including chaperones, proteasomal components, and transcription factors have been shown recruited into NIs (7, 8). Thus, we asked whether polyQ-induced aggregation of M3/6 could be the consequence of the sequestration of the phosphatase by NIs. To test this, we performed immunofluorescence-based experiments. Fixed retinas of R7E animals of 2.5 months were double-labeled with the anti-ataxin-7 2A10 and anti-M3/6 antibodies using confocal laser microscopy and counterstaining with 4',6-diamidino-2-phenylindole. As described before (30) and shown Fig. 5A (*upper panel*), the immunoreactivity of mutated ataxin-7 was primarily concentrated in large nuclear inclusions (NIs), corresponding to aggregated ataxin-7. M3/6 immunoreactivity was cytoplasmic and never colocalized with that of ataxin-7. Similar results were obtained with NG108 V125 cells (not shown). This indicates that polyQ does not sequester M3/6 into NIs and thus indirectly affects the solubility of M3/6.

HSP70 Suppresses PolyQ-induced Aggregation of M3/6 and Activation of JNK—As discussed above, our data show that polyQ-mediated and protein-damaging stresses have similar molecular features. The heat shock protein HSP70 was shown to suppress the activation of JNK caused by protein-damaging stresses by reverting the inhibition of JNK dephosphorylation (16). One possible mechanism linking HSP70 to JNK involves the control of JNK phosphatases activities by HSP70. We investigated the possibility that HSP70 could suppress polyQ-induced activation of JNK, through inhibition of the M3/6 JNK phosphatase. COS cells were transfected with expression vectors encoding HSP70 together with mutated or normal htt. The solubility of M3/6, which likely reflects its activity, was examined by fractionation experiments. The fractionation procedure was controlled with the 1C2 antibody (Fig. 6). As expected, overexpression of HSP70 in cells improved the solubility of polyQ aggregates. Fig. 6 shows that the amount of polyQ is decreased by HSP70 in fraction P, which contains the most insoluble materials, and increased in fraction SN2. Endogenous M3/6 was detected in the SN1-soluble fraction mainly as full-length proteins. In contrast, fractions SN2 and P contained mostly breakdown products. Interestingly, similar fragments were generated from both ectopic and endogenous M3/6 (Figs. 4A and 6). In response to polyQ-induced stress, full-length form and breakdown products of M3/6 accumulated in fraction P of both conditions. As shown in Fig. 6, the solubility of M3/6 was

A.



B.

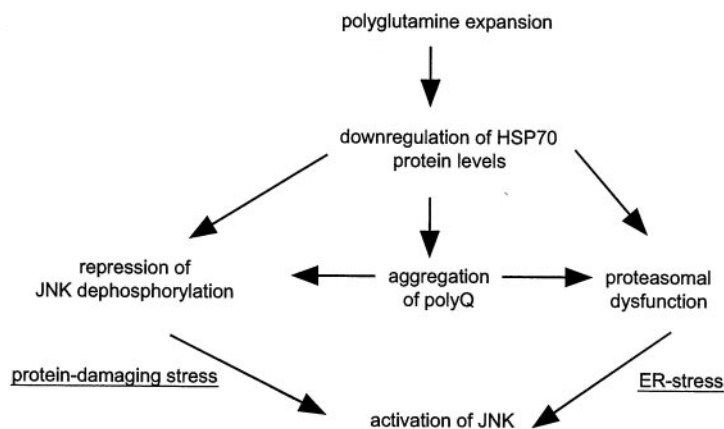


FIG. 8. **Model for the JNK-dependent toxicity of polyglutamine expansion.** *A*, proposed model of the sequence of events, by which polyQ might trigger activation of JNK and AP-1. In the absence of polyQ and stress, HSP70 might repress JNK dephosphorylation, through activation of phosphatases such as M3/6. Kinases upstream of JNK, such as SEK-1, have a low basal phosphorylation level. In consequence, the basal activation levels of JNK and c-Jun are very low. When polyQ are expressed, HSP70 is both down-regulated and recruited into NIs, which might result in the aggregation of M3/6 and in the increase of the basal phosphorylation and activation levels of JNK and c-Jun. In neurons, prolonged activation of c-Jun has been shown to trigger activation of toxic or death transcriptional programs. *B*, schematic representation of the different pathways that might converge to activation of JNK by polyQ. See text for details.

clearly improved in cells expressing mutated htt together with HSP70. Indeed, expression of ectopic HSP70 resulted in the reduction of M3/6 the immunoreactivity of M3/6 in fraction P. Conversely, the content of M3/6 in fraction SN2 was enriched upon expression of ectopic HSP70. In addition, overexpression of HSP70 led to the appearance of an additional form of full-length M3/6, characterized by a higher mobility shift on Western blot, which presumably corresponds to the phosphorylated form of M3/6 (31). We then asked whether HSP70-mediated solubilization of M3/6 was correlated with the suppression of JNK signaling, in polyQ-expressing cells. Fig. 6 shows that the phosphorylation of JNK is significantly reduced by HSP70 in

cells expressing mutated htt. Altogether, our results suggest that HSP70 promotes the stabilization of a soluble form of the JNK phosphatase M3/6, which is phosphorylated and active. Thus, HSP70 appears to be a critical regulator of polyQ-induced activation of JNK.

HSP70 Protein Levels Are Down-regulated by Polyglutamine Expansion—As discussed above, our data show that polyQ-mediated and protein-damaging stresses have similar molecular features. Redistribution of the heat shock protein HSP70 at the sites of damaged proteins is believed to prevent its capacity to activate JNK dephosphorylation (16). Thus, we asked whether such an effect could lead to polyQ-induced aggregation

of M3/6. Indeed, heat shock proteins are recruited into NIs of polyQ models (7), and in particular, NIs of R7E mice are positive for HSP40/HDJ-2 proteins (30). Therefore, we asked whether HSP70 was also recruited into NIs of R7E mice. Fixed retinas of mice at 3 months of age were double-labeled with the anti-ataxin-7 1261 and anti-HSP70 antibodies and analyzed by confocal microscopy (Fig. 5A, lower panel). Most NIs, if not all, were labeled with HSP70. This suggested that recruitment of HSP70 into NIs could be responsible for M3/6 aggregation. To test this idea, we asked whether HSP70 was depleted from the soluble cell fraction. Surprisingly, HSP70 proteins expressed in R7E mice were completely recovered in the soluble SN1 fraction (Fig. 4C). No HSP70 was detected in the insoluble SN2 and P fractions. However, HSP70 total protein levels were clearly reduced in transgenic, compared with wild-type mice (Figs. 4C and 7C). These results were further confirmed in R6/1 mice and in neuronal NG108 V125 cells (Fig. 7, A and B). Reduction of HSP70 by polyQ appeared to be specific, because the constitutive heat shock 70 chaperone, HSC70, was not affected (Fig. 7, A and C). To conclude, these data show that polyQ leads to down-regulation of HSP70 proteins and suggest that this effect, enhanced by the recruitment of HSP70 into NIs, might be implicated in the inactivation of the JNK phosphatase M3/6.

DISCUSSION

PolyQ results in neuronal dysfunction and death. Thus, deciphering the molecular events implicated in polyQ toxicity is critical for the design of therapeutic routes. Here we show that polyQ induces a protein-damaging stress that triggers activation of JNK and AP-1 transcription factor, through inhibition of JNK phosphatase(s). Our data show that the JNK phosphatase M3/6 is a target for JNK activation by polyQ and that HSP70, which promotes JNK dephosphorylation, might be involved in this process. Indeed, we show that polyQ impairs HSP70 levels and localization, and M3/6 solubility. The sequence of events we propose is schematized in Fig. 8A.

Whether aggregation of polyQ causes activation of JNK is an important outcome, because the toxicity of NIs is still debated. We observe that both events are concomitant, suggesting they are correlated (Fig. 1B). We also show that polyQ-induced stress and protein-damaging stress such as heat shock, which causes locally protein aggregation, have common features, as both induce activation of JNK through inhibition of JNK phosphatase(s). However, in response to heat shock, hsp70 gene expression is induced, resulting in a secondary inactivation of JNK (16). Strikingly, polyQ-induced stress does not lead to activation of such an auto-regulatory feedback loop. In contrast, we observe a significant and sustained reduction of HSP70 total proteins amounts, indicating that polyQ impairs HSP70 either at the level of gene expression or at the level of protein turnover. This might suggest that soluble polyQ affects some critical cellular processes implicated in the production and maintenance of HSP70 and that down-regulation of HSP70 is not caused by polyQ aggregation. According to this view, reduction of HSP70 proteins by soluble polyQ could be a primary event resulting independently in activation of JNK, via inhibition of its JNK-inactivating function, and in polyQ aggregation, as a consequence of the lack of HSP70-chaperoning activity (Fig. 7B). Indeed, HSP70 is a molecular chaperone essential for *de novo* protein folding and prevention of protein aggregation (39). Thus, reduction of HSP70 protein levels might enhance the intrinsic tendency to aggregation of polyQ-containing proteins as well as other proteins such as M3/6 for instance. Aggregated-polyQ might recruit HSP70 in a secondary step, thus enhancing repression of JNK dephosphorylation (Fig. 8B) (40).

Down-regulation of HSP70 by polyQ might also trigger acti-

vation of JNK by impairing processes involved in intracellular protein breakdown. Aggregated proteins that cannot be refolded by chaperones are targeted to the degradation machinery, which is mainly represented by the proteasome in eukaryotes (41). Chaperones such as HSP70 can promote this situation, by linking physically aggregated proteins and proteasomal components such as the ubiquitin-ligase CHIP or the ubiquitin-related BAG-1 (42, 43). As a result, down-regulation of HSP70 by polyQ might also trigger proteasomal dysfunction and impair degradation of polyQ-containing proteins, which are ubiquitinated (6), as well as other proteins targeted to the proteasome. Interestingly, very recently, polyQ has been shown to trigger endoplasmic reticulum (ER) stress and activation of JNK through proteasomal dysfunction (44, 45). PolyQ-mediated ER stress involves activation of ASK-1, a kinase upstream of SEK-1. In conclusion, reduction of HSP70 proteins by polyQ might induce both protein-damaging and ER stresses. Aggregation of polyQ could enhance these stresses by recruiting chaperones and proteasomal components (Fig. 8B) (7).

In response to stress, activation of JNK and AP-1 results in regulation of pathways of both cell life and death (25). Interestingly, prolonged activation of JNK3 and c-Jun has been associated with neuronal cell death. Indeed, *jnk3*-deficient mice and *c-junala63/73* mice, which express a version of c-Jun that cannot be phosphorylated by JNK, are protected against the neuronal death triggered by excitotoxic stress such as stimulation with kainic acid (26, 27). We show here that polyQ induces prolonged activation of JNK and c-Jun/AP-1 in neurons. Thus polyQ-mediated neuronal toxicity might critically involve activation of c-Jun/AP-1-dependent programs. Although AP-1 complexes have been extensively studied, target genes involved in neurodegeneration are not well defined. C-Jun-dependent induction of pro-apoptotic molecules such as Bim or FasL has, however, been documented (28, 36).

Down-regulation of HSP70 is also susceptible to facilitate induction of apoptosis via JNK-independent mechanisms. HSP70 exerts a cytoprotective function by inhibiting the action of key components of the apoptotic machinery. HSP70 directly interacts with Apaf-1, thus preventing the recruitment of procaspase 9 to the apoptosome (46, 47). The action of apoptosis-inducing factor, a caspase-independent death effector, is also antagonized by HSP70 (48). In addition, depletion of HSP70 has been shown to trigger cell death of tumor cells through activation of apoptotic pathways (49). To what extent this can be true in neurons is now being investigated.

In conclusion, by revealing the deficiency of HSP70 proteins in various polyQ models, this study gives clues to understanding polyQ toxicity and confirms the relevance to develop therapeutic protocols aimed to activate chaperone expression. In addition, our findings underscore the importance of JNK and AP-1 in polyQ diseases, which offers new therapeutic perspectives. Consequently, it might be of benefit to develop strategies for JNK and AP-1 inhibition.

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REFERENCES

- Zoghbi, H. Y., and Orr, H. T. (2000) *Annu. Rev. Neurosci.* **23**, 217–247
- Ross, C. (1995) *Neuron* **15**, 493–496
- Burright, E. N., Clark, H. B., Servadio, A., Matilla, T., Feddersen, R. M., Yunis, W. S., Duvick, L. A., Zoghbi, H. Y., and Orr, H. T. (1995) *Cell* **82**, 937–948
- Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trotter, Y., Leach, H., Davies, S. W., and Bates, G. P. (1996) *Cell* **87**, 493–506
- Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Leach, H., and Wanker, E. E.

- (1997) *Cell* **90**, 549–558
6. Ross, C. A. (1997) *Neuron* **19**, 1147–1150
 7. Cummings, C. J., Mancini, M. A., Antalfy, B., DeFranco, D. B., Orr, H. T., and Zoghbi, H. Y. (1998) *Nat. Genet.* **19**, 148–154
 8. Nucifora, F. C., Jr., Sasaki, M., Peters, M. F., Huang, H., Cooper, J. K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V. L., Dawson, T. M., and Ross, C. A. (2001) *Science* **291**, 2423–2428
 9. Warrick, J. M., Chan, H. Y. E., Gray-Board, G. L., Chai, Y., Paulson, H. L., and Bonini, N. M. (1999) *Nat. Genet.* **23**, 425–428
 10. Cummings, C. J., Sun, Y., Opal, P., Antalfy, B., Mestrl, R., Orr, H. T., Dillmann, W. H., and Zoghbi, H. Y. (2001) *Hum. Mol. Genet.* **10**, 1511–1518
 11. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* **292**, 1552–1555
 12. Luthi-Carter, R., Strand, A., Peters, N. L., Solano, S. M., Hollingsworth, Z. R., Menon, A. S., Frey, A. S., Spektor, B. S., Penney, E. B., Schilling, G., Ross, C. A., Borchelt, D. R., Tapsco, S. J., Young, A. B., Cha, J. H., and Olson, J. M. (2000) *Hum. Mol. Genet.* **9**, 1259–1271
 13. Klement, I. A., Skinner, P. J., Kaytor, M. D., Yi, H., Hersch, S. M., Clark, H. B., Zoghbi, H. Y., and Orr, H. T. (1998) *Cell* **95**, 41–53
 14. McCampbell, A., Taye, A. A., Whitty, L., Penney, E., Steffan, J. S., and Fischbeck, K. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 15179–15184
 15. Steffan, J. S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B. L., Kazantsev, A., Schmidt, E., Zhu, Y. Z., Greenwald, M., Kurokawa, R., Housman, D. E., Jackson, G. R., Marsh, J. L., and Thompson, L. M. (2001) *Nature* **413**, 739–743
 16. Meriin, A. B., Yaglom, J. A., Gabai, V. L., Zon, L., Ganiatsas, S., Mosser, D. D., and Sherman, M. Y. (1999) *Mol. Cell. Biol.* **19**, 2547–2555
 17. Derjard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* **76**, 1025–1037
 18. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372**, 798–800
 19. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7337–7342
 20. Cavigelli, M., Li, W. W., Lin, A., Su, B., Yoshioka, K., and Karin, M. (1996) *EMBO J.* **15**, 6269–6279
 21. Palacios, C., Collins, M. K., and Perkins, G. R. (2001) *Curr. Biol.* **11**, 1439–1443
 22. Theodosiou, A., and Ashworth, A. (2002) *Oncogene* **21**, 2387–2397
 23. Park, H. S., Lee, J. S., Huh, S. H., Seo, J. S., and Choi, E. J. (2001) *EMBO J.* **20**, 446–456
 24. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7**, 2135–2148
 25. Shaulian, E., and Karin, M. (2002) *Nat. Cell Biol.* **4**, E131–E136
 26. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) *Nature* **389**, 865–870
 27. Behrens, A., Sibia, M., and Wagner, E. F. (1999) *Nat. Genet.* **21**, 326–329
 28. Whitfield, J., Neame, S. J., Paquet, L., Bernard, O., and Ham, J. (2001) *Neuron* **29**, 629–643
 29. Trotter, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E. C., and Mandel, J. L. (1995) *Nat. Genet.* **10**, 104–110
 30. Yvert, G., Lindenberg, K. S., Picaud, S., Landwehrmeyer, G. B., Sahel, J. A., and Mandel, J. L. (2000) *Hum. Mol. Genet.* **9**, 2491–2506
 31. Johnson, T. R., Biggs, J. R., Winbourn, S. E., and Kraft, A. S. (2000) *J. Biol. Chem.* **275**, 31755–31762
 32. Lunke, A., and Mandel, J.-L. (1998) *Hum. Mol. Genet.* **7**, 1355–1361
 33. Lunke, A., Lindenberg, K. S., Ben-Haiem, L., Weber, C., Devys, D., Landwehrmeyer, G. B., Mandel, J. L., and Trotter, Y. (2002) *Mol. Cell* **10**, 259–269
 34. Helmlinger, D., Yvert, G., Picaud, S., Merienne, K., Sahel, J., and Devys, D. (2002) *Hum. Mol. Genet.* **11**, 3351–3359
 35. Liu, Y. F. (1998) *J. Biol. Chem.* **273**, 28873–28877
 36. Le-Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F. X., Green, D. R., and Karin, M. (1999) *Mol. Cell. Biol.* **19**, 751–763
 37. Davis, R. J. (2000) *Cell* **103**, 239–252
 38. Martell, K. J., Seasholtz, A. F., Kwak, S. P., Clemens, K. K., and Dixon, J. E. (1995) *J. Neurochem.* **65**, 1823–1833
 39. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science* **295**, 1852–1858
 40. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) *J. Biol. Chem.* **272**, 18033–18037
 41. Hohfeld, J., Cyr, D. M., and Patterson, C. (2001) *EMBO Rep.* **2**, 885–890
 42. Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Hohfeld, J., and Patterson, C. (2001) *Nat. Cell Biol.* **3**, 93–96
 43. Luders, J., Demand, J., and Hohfeld, J. (2000) *J. Biol. Chem.* **275**, 4613–4617
 44. Kouroku, Y., Fujita, E., Jimbo, A., Kikuchi, T., Yamagata, T., Momoi, M. Y., Kominami, E., Kuida, K., Sakamaki, K., Yonehara, S., and Momoi, T. (2002) *Hum. Mol. Genet.* **11**, 1505–1515
 45. Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002) *Genes Dev.* **16**, 1345–1355
 46. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Taylor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000) *Nat. Cell Biol.* **2**, 469–475
 47. Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D., and Alnemri, E. S. (2000) *Nat. Cell Biol.* **2**, 476–483
 48. Ravagnan, L., Gurbuxani, S., Susin, S. A., Maise, C., Daugas, E., Zamzami, N., Mak, T., Jaattela, M., Penninger, J. M., Garrido, C., and Kroemer, G. (2001) *Nat. Cell Biol.* **3**, 839–843
 49. Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F., and Jaattela, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7871–7876