DNA damage induced by CAG-expanded proteins

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ABSTRACT

We have developed stable cell lines expressing GFP fusion proteins containing the CAG repeats of various lengths under tetracycline control. The expression of the expanded (43Q) repeat protein resulted in aggregate formation in a time–dependent fashion. The accumulation of aggregates did not induce apoptosis, although the survival of these cells was critically dependent on the presence of serum and growth factors. However, the expression of 43 CAG expanded protein strongly activated the ATM/ATR dependent DNA damage response, as shown by selective phosphorylation of ATM substrates. This activation was dependent on 43 CAG protein expression, was reversible and sensitive to caffeine and reducing agents. Similarly, we found phosphorylated ATM substrates in fibroblasts from HD or SCA-2 patients. Oxidative stress induced accumulation of ATM/ATR phosphorylated protein in HD and SCA-2 patients, but not in normal controls. Furthermore, a significant phosphorylation of H2AX was shown by fibroblasts from patients.

We conclude that polyglutamine induce ATM/ATR dependent DNA damage response through accumulation of ROS. ATM activation can be used to monitor the disease in vivo.

Keyword : CAG expanded proteins; DNA damage; ATM
INTRODUCTION

Polyglutamine diseases are late-onset, neurodegenerative disorders arising from the expansion of an unstable CAG repeat in the coding DNA sequence, which is translated in a series of glutamine residues in the protein. Growing number of inherited neurodegenerative disorders, caused by polyQ expansion, includes spinobulbar muscular atrophy (SBMA) (1,2), the Huntington’s disease (HD) (3), dentatorubral pallidolusian atrophy (DRPLA) (4,5), and six spinocerebellar ataxias (SCA-1, 2, 3, 6, 7, 17) (6-14). Although the proteins harbouring the polyglutamine tracts are unrelated to each other and the neurons affected are different, it is likely that a common pathogenic mechanism might be responsible for the pathology of this group of diseases (15). Evidences have been provided that mitochondrial dysfunction plays a role in polyQ-induced cell death (16-18).

Recently two neurodegenerative diseases such as the spinocerebellar ataxia with axonal neuropathy (SCAN1) and the ataxia ocular apraxia (AOA1) have been linked to defects in DNA repair (19-21). The genes mutated are DNA repair genes. These disorders share several neurological features with Ataxia Telangiectasia (AT) syndrome, including early onset ataxia and cerebellar atrophy (22,23).

The aims of our study are: 1. set up an inducible cellular model, which recapitulates the features of expanded polyQ-containing proteins and 2. analyze the biological effects of polyQ protein expression. To this end, we have constructed PC12 cell lines, conditionally expressing a synthetic protein containing the green fluorescence protein, GFP, fused to a stretch of 17 or 43 glutamine and to the HA (hemoagglutinin influenza human virus) epitope.

We report here that polyQ expanded protein induced a severe stress response of the cells. We also found that polyQ protein expression induced a robust and reversible activation of ATM/ATR dependent DNA damage response. This response was also detected \textit{ex vivo} in fibroblasts derived from patients with HD or SCA-2 diseases.

RESULTS
The expression of polyQ protein causes time-dependent formation of aggregates
GFP fusion proteins were generated by linking GFP and the HA epitope with 17 or 43 glutamine residues (24). These proteins were expressed under the control of TRE (tetacycline responsive element) promoter in a rat pheochromocytoma cell line, PC12 cells, carrying an engineered version of tetacycline-controlled transactivator (tTa). Single clones were characterized for induced-expression of the fusion proteins, following removal of the tetacycline analogue doxycycline (Dox). The best responders were further analyzed. Very low basal levels of the fusions proteins were detected in the cells grown in the presence of doxycycline. Upon removal of doxycycline, the TRE promoter was activated and an intense GFP fluorescence was detected in cells transfected with the two expression vectors encoding the 17Q or 43Q fusion proteins. HA-17Q-GFP expressing cells showed a diffuse signal throughout the cytoplasm and nucleus and did not form aggregates (Fig. 1A). The cells expressing the expanded poly-Q protein (HA-43Q-GFP) showed initially (1-2 days following doxycycline removal) a diffuse GFP fluorescence, followed by a progressive formation of aggregates. Three days after the induction, intranuclear and perinuclear aggregates were visible in 30-40% of the cells. After 6 days, they were present in 100% of the cells (Fig. 1A). Immunoblot analysis with an anti-HA antibody confirmed the fluorescence data (Fig. 1B). In the presence of doxycycline, a faint band HA-positive could be detected in HA-17Q-GFP and HA-43Q-GFP cells. The signal increased approximately 50 fold following the removal of doxycycline. Both HA-17Q-GFP and HA-43Q-GFP proteins of the expected molecular weight were present in the soluble fraction (Fig. 1B). We noticed that only in 43Q expressing cell extracts there was a high molecular weight HA positive band at the top of the gel. This band appeared 3 days after removal of the drug and steadily increased after 6 days (Fig. 1B). These bands reacted with both the anti HA and anti GFP antibody, indicating that the proteins present in the aggregates were not cleaved (data not shown) (24). However, we cannot rule out that a mixture of cleaved and uncleaved fragments constitutes the nuclear aggregates.

Expression of expanded polyQ proteins severely impairs cell response to stress
The data reported above indicate that the expression of the 43Q containing protein induces the formation of nuclear aggregates, which are critically dependent on the length and the time of expression of CAG repeats (25). Aggregates have been linked to neuronal cell death in vivo and in vitro (26-28). However, under normal growth conditions, we did not appreciate differences in the growth rate and DNA synthesis of 43Q expressing cells (data not shown). To find out if expanded polyQ proteins influenced the response of the cells to stress, we have investigated the sensitivity of these cells to hormone and serum starvation. Fig. 2A shows that PC12 cells in low serum undergo cell death in a time-dependent manner. No difference in cell death was noted among different cell lines in the presence of doxycycline. When the HA-43Q-GFP protein was induced, a greater and significant fraction of cells underwent apoptosis compared to un-induced cells or HA-17Q-GFP expressing cells. 25% of HA-43Q-GFP expressing cells underwent apoptosis after 48 hours of serum starvation. 7-8% of control PC12 cells or lines expressing the HA-17Q-GFP protein underwent apoptosis. Under these conditions, treatment of the cells with a caspase inhibitor, Z-VAD-FMK, completely blocked cell death induced by serum withdrawal (data not shown).

To get an insight on the cell signaling pathways involved, we measured the activation of stress activated protein kinases. JNK (c-jun N-terminal kinase) activation has been implicated in neuronal cell death induced by environmental stress and by glutamate-mediated excitotoxicity (29,30). Furthermore, the activation of JNK has been reported in a neuronal cell line expressing full-length huntingtin with 48Q repeats (31). Fig. 2B shows that serum deprivation induced a sustained activation of JNK only in 43Q expressing cells. 43Q un-induced cells and 17Q expressing cells (control cells), under the same conditions, did not activate JNK.

Since the mitochondria are the main effectors of stress-induced cell death (32), we have measured reactive oxygen species (ROS) levels and cytochrome C release in 43Q transfected cells. Higher ROS levels were found in growing HA-43Q-GFP expressing cells compared to parental cells or HA-17Q-GFP expressing cells. A
sustained and further increase of ROS levels was found after 6 hrs of serum deprivation (Fig. 2C). Under the same conditions, 17Q cells or 43Q cells treated with doxycycline did not show any increase in cellular ROS (Fig. 2C and data not shown). ROS accumulation was associated to cytochrome c release in the cytosol. Fig. 2D shows that cytochrome c was released in the cytosol of cells expressing the 43Q protein as early as 12 hours following serum withdrawal while in doxycycline treated cells the release of cytochrome C was late and scant. Inhibition of ROS elevation even at earlier times with NAC prevented cytochrome release later (data not shown).

Taken together these findings suggest an increased sensitivity to stress-induced cell death by expanded polyQ containing proteins and indicate a specific mitochondrial involvement.

**Activation of DNA damage checkpoint by polyQ proteins**

Oxidative stress is the principal source of DNA lesions. ATM (ataxia telangiectasia mutated kinase), ATR (ATM and Rad3-related kinase) and DNA-PK (DNA-dependent protein kinase) are serine/threonine protein kinases belonging to phosphatidylinositol (3) kinase family that are involved in DNA repair and recombination. These three related enzymes have similar target sequences and a general consensus motif has been defined (23, 33-35). Using a phospho-Ser/Thr antibody generated with the peptide L-S/T-Q (specific for phosphorylated ATM/ATR substrates) we have analyzed the accumulation of ATM phosphorylated substrates. Fig. 3A shows an immunoblot of nuclear proteins of PC12 cells. A 32 kD band appears very early (1 day) following induction of the expanded polyQ protein. After 4 days in the absence of doxycycline, a new band of 60 kD appeared (Fig. 3A, 4 and 6 days). At this time point the addition of doxycycline for 1 day to repress polyQ protein expression resulted in disappearance of both bands (Fig. 3A, lane indicated by 6/1). These bands disappeared when the cells were treated with caffeine, an inhibitor of ATM and ATR kinases (Fig. 3C). The bands were differentially sensitive to caffeine. The 32 kD band was highly sensitive since it disappeared after 2 hours of...
treatment with 2 mM caffeine. On the other hand, the 60 kD band appeared to be more resistant to caffeine treatment. Cells expressing the 17Q protein did not accumulate any specific band recognized by the antibody above indicated after 6 days of induction (Fig. 3A).

To further investigate if oxidative stress indeed contributed to polyQ-induced DNA damage, we have tested the effect of a reducing agent, the N-Acetyl-L-Cysteine (NAC) on the accumulation of ATM/ATR substrates. We found (Fig. 3D) that the phosphorylation status of two bands was greatly dependent on ROS levels, because two hours of NAC treatment inhibited the accumulation of both bands.

The data shown above indicate a robust activation of the ATM/ATR dependent DNA damage response following expanded polyQ proteins. Since expanded polyQ proteins are ubiquitously expressed, we asked whether fibroblasts from HD and SCA-2 patients showed ATM/ATR activation as PC12 cells expressing expanded polyQ proteins. We have extracted nuclear proteins from control, HD and SCA-2 fibroblasts stressed with 50 µm H2O2 for 15 min and immunoblotted them with the anti ATM/ATR substrate antibody. Fig. 4A shows that primary fibroblasts from healthy subjects stressed with H2O2 accumulated a 32 kD band, which reacts with anti-ATM/ATR substrate antibody. The fibroblasts derived from HD and SCA-2 patients contain a band of the same size in basal conditions. Treatment with H2O2 significantly stimulated the 32 kD band in cells derived from HD and SCA-2 patients. The 32 kD band was also caffeine and NAC sensitive (data not shown), similarly to the 32 kD band detected in PC12 cells (Fig. 3). Taken together these data strongly suggest that the 32 kD band is the same protein found in PC12 cells, induced by expanded polyQ protein. We have analyzed two additional fibroblasts cell lines derived from one HD and one SCA-2 patient. The 32 kD band was present in the nuclear extracts and was significantly stimulated by 50 µm H2O2 (data not shown).

A very early step in the cellular response to DNA double strand breaks is represented by phosphorylation of histone H2AX at the sites of DNA damage by ATM kinase (36). To obtain an independent evidence of ATM activation following to DNA damage, fibroblasts from HD and SCA-2 patients were H2O2 treated and the
presence of phosphorylated H2AX was monitored by immunofluorescence with an anti-γ-H2AX antibody (Fig. 4B). A low and diffuse signal was detected in basal conditions in all fibroblasts with small appreciable differences between patients and controls (data not shown), but upon the stimulation with H$_2$O$_2$, many foci appeared in response to DNA damage induced by H$_2$O$_2$. In normal controls the signal was diffuse with small foci, while in the cells derived from the patients an intense fluorescence with larger foci was present. These data indicate that in fibroblasts derived from polyglutamine disease patients, there is a strong ATM/ATR dependent DNA damage response compared to normal and healthy cells.

DISCUSSION

Polyglutamine neurodegenerative diseases are inherited disorders with an insidious onset in middle-age (mean ~40 years), with a strong inverse correlation between age of onset and length of CAG repeat. The clinical features result from accumulation of cell damage, caused by mutated protein (37).

To better define the early events, we created an inducible system of PC12 cell line expressing HA-43Q-GFP fusion protein. By modulating the time of protein expression, we were able to identify and dissect the short and long term biological consequences following expanded polyQ expression.

DNA damage

Cells expressing expanded polyQ repeats accumulate intranuclear aggregates and suppress transcription of NGF- and cAMP-induced genes (38-40 and data not shown). These cells under standard culture conditions grow and proliferate as well as wild type cells. The accumulation of expanded polyQ proteins induces a significant stress to the cells (41). Mitochondria and the nucleus appear to be the primary sites. Recently, calcium defects have been found in lymphoblast mitochondria from HD patients. N-terminal mutant huntingtin has also been found in neuronal mitochondrial membranes (18). Under normal growth conditions, we find that polyQ expressing cells accumulate ROS. ROS can be efficiently scavenged in the mitochondria,
cytoplasm and membrane by SOD enzymes (42). The only location where SOD enzymes are not present is the nucleus. This is an important site of stress in cells expressing expanded polyQ proteins, as shown by the formation of nuclear aggregates. The data reported above indicate a robust activation of ATM in these cells. Two different substrates are found phosphorylated in HA-43Q-GFP expressing cells. Both bands are caffeine sensitive (Fig. 3C) (ATM and ATR dependent) (43), although we cannot discriminate between these two kinases. The cells that accumulate these bands are not apoptotic cells, since we do not find any sign of cell death under these conditions, or gross impairment of the growth. The time course of phosphorylation of the bands shown in Fig. 3 allows a clear discrimination because the 32 kD substrate is rapidly induced and downregulated, while the 60 kD band disappears slowly. The different turnover of phosphorylated ATM bands might be dependent on phosphatase activity. The sensitivity of these band to N-acetylcysteine indicates that the primary cause of damage is the accumulation of ROS.

We have shown that ATM/ATR activation is a specific and reliable marker of polyQ protein expression in PC12 cells (Fig. 3). Similarly, fibroblasts derived from HD and SCA-2 patients shows a robust activation of ATM/ATR as shown by accumulation of a 32 KD band, which reacts with anti ATM/ATR substrate antibody. We suggest that this band is the same protein found in PC12 cells expressing the polyQ expanded protein, because 1. Is dependent on polyQ protein expression; 2. is caffeine and NAC sensitive. This band is stimulated by H$_2$O$_2$ also in cells derived from healthy subjects (Fig. 4). In several normal controls we have screened, the accumulation of the 32kD band was never comparable to that found in the cells derived from the HD or SCA-2 patients. It is worth noting that primary fibroblasts do not activate the 60 kD band visible in PC12 cells. At present we cannot discriminate if the 60 kD band is tissue specific, i.e., present only in neuronal cells, or it is less sensitive to oxidative stress. Activation of ATM/ATR can be triggered by several stimuli that do not involve DNA damage (44). In vivo, at the sites of DNA lesions, accumulate nuclear foci, containing more than 20 proteins involved in DNA repair (45). The phosphorylation of H2AX and the presence of nuclear foci have always been found linked to DNA damage.
checkpoint dependent on ATM (46). To determine whether the cells derived from the HD and SCA-2 patients accumulate phosphorylated H2AX at DNA lesions (foci), we have stained normal and HD or SCA-2 fibroblasts with phospho-H2AX antibody and we have analyzed the localization by fluorescence microscopy. The foci on the DNA of fibroblasts from HD or SCA-2 patients are significantly larger relative to normal healthy cells. Although \( \text{H}_2\text{O}_2 \) induces foci in healthy cells, the immunofluorescence signal in HD and SCA-2 fibroblasts is quantitatively and qualitatively different from that found in normal cells. The presence of phosphorylated H2AX and the accumulation of the 32 kD band strongly indicate the presence of DNA damage in fibroblasts derived from the HD and SCA-2 patients.

The repair of DNA lesions is critical for the survival and genetic stability of mammalian cells. Several neurodegenerative disorders arise from mutations in DNA repair genes, but only the ataxia-telangiectasia disorder shows an increased genetic instability and a cancer predisposition (22, 47). We note, however, that CAG-expressing cells efficiently repaired the DNA lesions, because they were not seriously impaired in short term growth or survival. We cannot predict at the present the long term effects of repaired DNA lesions on survival of post-mitotic cells. We did not find significant differences in the ATM response between NGF-differentiated or undifferentiated PC12 cells (data not shown). A deficiency of some components (for example tyrosyl phosphodiesterase, TDP1) of the DNA repair system in humans, does not predispose to neoplasia or dysfunctions in rapidly replicating tissues, but instead causes spinocerebellar ataxia with axonal neuropathy (21).

Our results indicate that in PC12 expressing expanded polyQ proteins or in fibroblasts derived from HD or SCA-2 patients, there is a significant activation of DNA damage response, which may represent a specific marker of polyQ diseases. We suggest that the accumulation of polyQ containing proteins imposes a considerable stress with an increased ROS accumulation (41). In the long term cultures or in vivo in post-mitotic neuronal cells, these events might seriously compromise transcription. Under these conditions, apoptosis could contribute to neurodegeneration. The extent and the rate of apoptosis may be different between
neuronal and non-neuronal cells and among various types of neurons subjected to the same type of stress. For example, cerebellar granule or Purkinje cells are extremely sensitive to $\text{H}_2\text{O}_2$-induced apoptosis compared to cortical neurons (48). We suggest that ROS-induced DNA damage is amplified in specific neurons and might result in apoptosis later in the life of these cells. Moreover, a practical consequence of our finding is the possibility to monitor the progression of the disease by determining ATM/ATR activation in fibroblasts.
MATERIAL AND METHODS

Plasmid construction
HA-17Q-GFP and HA-43Q-GFP cDNAs were excised from pCMV-HA-17Q-GFP and pCMV-HA-43Q-GFP constructs (24) using SacII/BamHI restriction enzymes. cDNAs were sub-cloned into pTRE-vector (Clontech) pre-digested with the same restriction enzymes. Nucleotide sequencing was performed as described (49) by using CEQ 2000 DNA Analysis System, Beckman automated sequencer, according to the manufacturer’s protocol. cDNA vectors encoding GFP-fusion proteins were purified using QIAGEN Plasmid Maxi kit tips columns (Qiagen, Chatsworth, CA).

Cell Culture and transfection
Tet-Off PC12 cell line (Clontech, Palo Alto, CA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL) supplemented with 10% heat-inactivated horse serum (HS, Gibco-BRL), 5% foetal bovine serum (FBS, Gibco-BRL), 1 mM glutamine (Gibco-BRL), 100 µg/ml streptomycin (Gibco BRL), 100 U/ml penicillin (Gibco-BRL) and 100 µg/ml G418 (Gibco-BRL) in a humidified 5% CO₂ atmosphere at 37 °C. PC12 cells were transfected at 40-60% confluence with 10 µg DNA using liposome-mediated transfection according to the manufacturer’s instructions (LIPOFECTIN Reagent, Gibco-BRL). Positive clones were selected in complete medium containing: G-418, 100 µg/ml, hygromycin, 200 µg/ml (Gibco-BRL) and doxycycline hydrochloride 10 ng/ml (Gibco-BRL).

Cell death analysis
Cell death was analysed by staining with propidium iodide (PI). Briefly, cells were harvested at indicated times after treatment, washed twice with PBS 1X and incubated for 10 minutes with propidium iodide (50 ng/ml in PBS 1X, SIGMA). Cells were washed and analyzed by fluorescence microscopy using an Axiover microscope IX70 (Olympus). The data are presented as percent of propidium iodide
positive cells on total number of cells (700 cells were considered in each of three independent experiments).

**Western blot analysis**

Cells were homogenized in lysis buffer (40 mM Hepes pH 7.5, 120 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM EDTA, 0.6% Triton X-100) containing protease (Complete Tablets- EDTA-free, Roche) and phosphatase (20 mM β-glycerolphosphate, 2.5 mM Na-pyrophosphate) inhibitors. Total lysate was cleared by centrifugation at 15,000 x g for 20 min. Protein concentration of supernatant was measured using Bio-Rad protein assay. The remaining insoluble pellet was dissolved in lysis buffer with 100U Dnase for 15 min. For ATM/ATR substrates analysis, nuclear extracts were loaded according to supernatant concentration. Extracts were denatured and resolved by SDS-PAGE (12%) gel electrophoresis, transferred to a Hybond ECL nitrocellulose membrane (Amersham) by electroblotting at 250 mA for 2-3 hours. For cytochrome C, cytosolic fractions were prepared as described (50). The following primary antibodies were used: anti-HA (Roche), anti-SAPK/JNK and anti-P (Thr183/Tyr185)-JNK (Cell Signaling Technology), anti-cytochrome c (BD PharMingen), anti-α-tubulin (Sigma), anti-P(Ser139)-H2AX (Upstate Biotecnology), anti-P(Ser/Thr) ATM/ATR substrate (Cell Signaling Technology), anti-H2A (Cell Signaling Technology). Anti-mouse IgG or anti-rabbit IgG coupled to peroxidase were used as secondary antibodies (Sigma). Chemioluminescent (ECL) signals were quantified by scanning densitometry using “Scion Image” software.

**Measurement of Reactive Oxygen Species**

The generation of reactive oxygen intermediates in cells was detected using dichlorodihydrofluorescein diacetate (H₂DCFDA). After each treatment, cell pellets were washed three times with PBS and then with PBS containing H₂DCFDA (5 µg/µl) (Molecular Probes). Fluorescence was determined by FACS analysis (fluorescence-
activated cell sorter) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The actual ROS levels were determined by subtracting GFP fluorescence in samples not treated with H$_2$DCFDA. Under these conditions fluorescence was linearly dependent on the presence of H2DCFDA. Treatment of the cells with N-acetylcysteine (1 mM) completely suppressed H2DCFDA fluorescence. The values reported are the mean of at least three experiments.

**Immunofluorescence**

Primary fibroblasts grown on glass slides were fixed in 50% methanol/50% aceton for 2 hours at –20 °C. Following fixation, slides were washed three times for 5 min each in PBS and blocked in 10% FBS-PBS for 1 hr at room temperature. Primary antibody (γ-H2AX) was diluted at 1:500 in 1% bovine serum albumin-PBS and was incubated overnight at +4°C. After washing, secondary antibody (fluorescein isothiocyanate anti-rabbit) was incubated at 1:200 in 10% FBS-PBS for 1 hr at room temperature. All images were captured with Axiocam microscopy (Zeiss) in the same conditions of brightness and contrast.
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Legends to Figures

**Figure 1.** Time-dependent expression of polyglutamine-GFP fusion proteins in inducible PC12 cell lines. (A) Phase contrast (left) and fluorescence images (right) of PC12 cells stably transfected with either HA-17Q-GFP or HA-43Q-GFP and grown in the absence of Doxycycline for the indicated times. The localization of GFP-fusion proteins was examined by fluorescence microscopy. (B) Western blot of total cell extracts with anti-HA antibody, which recognizes the epitope-tagged fusion proteins. 50µg of extracts were fractionated on a 10% polyacrylamide gel, blotted and incubated with the antibody (1:5000). The arrows indicate the apparent molecular weight of fusion proteins. The high molecular weight bands visible in the top of the gel are only seen in extracts of cells induced for 6 days.
Figure 2. PolyQ-expressing cells are more susceptible to stress-induced apoptosis. (A) Time-course of apoptosis induced by serum-deprivation in cells transfected with HA-43CAG-GFP and HA-17CAG-GFP constructs. The fraction of cell death was determined as described in Materials and Methods. The values are represented as fold increase over the basal level in growing conditions. 500-1000 cells per samples were scored in three independent experiments. (P<0.001: uninduced versus induced cells; P<0.001: 17 CAG versus 43 CAG). The doubling time was 48 hrs for HA-43CAG-GFP and HA-17CAG-GFP cells in the absence or presence of doxycyclin. (B) JNK activation in induced cells. JNK activation was determined by immunoblot with anti-
P-JNK antibody. Uninduced and induced cells were serum-deprived for the indicated times. The blots were stripped and reprobed with the anti-JNK antibody. (C) Comparison of ROS levels in HA-43CAG-GFP transfected cells which were serum-deprived for the indicated times. The values are reported as fold increase over the basal level of uninduced cells in growing conditions. (D) Western blot analysis performed on cytosolic fractions from HA43Q-GFP cells incubated with anti-cytochrome c antibody. The same blot was probed with a-tubulin antibody to normalize the proteins loaded in each lane.

In all the experiments fusion proteins were induced for 3 days (HA-43CAG-GFP) and 6 days (HA-17CAG-GFP) in absence of doxycycline before serum-withdrawal.
Figure 3. The expression of 43 CAG fusion protein increase the ATM activity. (A) Time-course of HA-43Q-GFP induction upon doxycycline removal. A time point of HA-17Q-GFP expression is reported as control. The nuclear extracts were prepared as described in Materials and Methods and fractionated on a 12% polyacrylamide gel, blotted and incubated with phospho-Ser/Thr ATM/ATR substrates antibody. The lower part of the same gel was incubated with anti-H2A antibody to normalize the amount of loaded proteins. 3 independent clones expressing HA-43Q-GFP and 2 clones expressing HA-17Q-GFP were analyzed. Both 60 and 32 Kda phosphorylated bands were present only in HA-43Q-GFP expressing clones. (B) Densitometric analysis of the lower band. Data are representative of independent experiments. (C) Caffeine sensitivity of HA-43Q-GFP transfected cells induced for 6 days in absence of doxycycline. Western blot analysis was performed with anti-P-Ser/Thr ATM/ATR substrates antibody. Where indicated, the caffeine was added for 3 days and fresh solution was prepared every day. (D) P-ATM/ATR substrate sensitivity to treatment with a reducing agent, NAC. A stock solution of 250 mM of NAC was directly added to cells to give rise the final concentration. For each panel, one of some representative experiments is reported.
**Figure 4.** HD and SCA-2 fibroblasts have an increased rate of DNA lesions. 

(A) Nuclear extracts of fibroblasts from patients and from two different controls were analysed by western blotting with anti-P-Ser/Thr ATM/ATR substrates antibody. In the extracts from fibroblasts cells the antibody detect a band of 32 kD which is stimulated by treatment with 50 µM H$_2$O$_2$. The normalization with anti-H2A antibody is shown. Densitometric analysis of the results is reported. Each histogram corresponds to a lane on the top of panel. Data are representative of three independent experiments conducted on lines between V and IX passage. In each experiment the fibroblasts cells were harvested at the same passage number. The age of controls was comparable to that patients. 

(B) Immunofluorescence experiments performed with anti-γ-H2AX antibody. Human primary fibroblasts were seeded onto glass slides and
treated with 50 µM H₂O₂. After 15 min the cells were fixed and incubated with primary antibody as described in Materials and Methods. Upper panel shows a 40X magnification. The lower panel presents a single cell magnification to show the distinctive features of H2AX foci. The % of cells of the patients showing brighter H2AX foci (compared to control cells) was ca. 60 to 70%.
Abbreviations: GFP, green fluorescent protein; HD, Huntington’s disease; SCA-2, spinocerebellar ataxia type 2; HA, hemoagglutinin influenza human virus epitope; PC12, pheochromocytoma cell line; Dox, doxycycline hydrochloride; ROS, reactive oxygen species; ATM, ataxia telangiectasia mutated kinase; ATR, ATM and Rad3-related kinase; NAC, N-Acetyl-L-Cysteine.