Chapter II

- *in which the question of the function of wtHtt is addressed; a novel cellular system is introduced in which wtHtt exhibits an anti-apoptotic function; experiments are performed to place wtHtt in the context of the known apoptotic pathways; the anti-apoptotic function of wtHtt is located downstream of cytochrome c-release at the level of caspase-9*

1 Introduction

A recurrent motif in working with neurodegenerative diseases is the lack of an appropriate, easily manipulatable experimental system. Mouse models allow assessing general disease pathology but do not directly address events on a molecular level; questions of interaction networks or substrate specificity can only be perfunctorily answered. Mechanistic questions are conveniently examined in cellular systems that can be manipulated by introduction of exogenous gene products and change of culture conditions.

Primary cell culture is routinely employed to study neurodegenerative diseases, partly because established cell lines do not faithfully reproduce neuronal phenotypes. Additionally, often neurodegenerative diseases are very cell specific. Therefore, cells are needed that not only behave like neurons, but like a specific subset of neurons.

Primary neuronal cell culture faces its own difficulties. For HD, striatal neurons need to be isolated and kept in culture. Obtaining large quantities of striatal neurons is a very difficult and time-consuming procedure, given that neurons are postmitotic and therefore do not expand in culture. Also, the striatum is a very small structure and hard to isolate, which makes it challenging to routinely gather sufficiently pure striatal neurons. Manipulation of freshly isolated neurons is mostly done by microinjection, which is very stressful to the cells and limits the analysis to the handful of

neurons that can be conveniently injected and are still alive after the procedure. A striatal neuronal cell line that behaves like primary cells is therefore of great benefit.

This study employs the ST14A cell line that was developed by our collaborators in the laboratory of E. Cattaneo at the University of Milano (Cattaneo and Conti, 1998). Striatal neurons were isolated from embryonic rat brains at day E14 and immortalized with a temperature sensitive allele of the large T-antigen from SV40 virus. At the permissive temperature of 33˚C ST14A cells behave like an immortalized cell line (Ehrlich et al., 2001): They are easy to culture as adherent cells in standard media and proliferate with a doubling time of ~36hrs. In addition, ST14A cells are highly transfectable (transfection efficiencies of up to 40% were routinely achieved). However, ST14A cells still retain markers of CNS progenitor cells, markedly nestin expression.

At the non-permissive temperature of 39˚C the large T-antigen gets rapidly inactivated and the immortalized phenotype reverses back to a neuronal phenotype. The cells stop proliferating and the doubling time increases to about 120hrs. They also begin a differentiation process during which the cells acquire dendritic trees and even axonal outgrowths. Nestin expression is downregulated. Indeed, when implanted back into rat brains, ST14A cells migrate to the striatum and perform neuronal functions (Cattaneo et al., 1994; Lundberg et al., 1997). When serum is withdrawn from cells maintained at 39˚C, death occurs within 2-3 days.

2 Preliminary Studies

ST14A cells were stably transfected with truncated wtHtt and muHtt constructs of the IT15 gene. These fragments contain the N-terminal 548aa, including the poly-Q stretch, the poly-P region, the caspase cleavage site and the conserved Akt phosphorylation site (Fig. 1). Expression of the Htt constructs was confirmed by western blotting (data not shown).

2.1 Effects of Htt on viability at the non-permissive temperature

The stable Htt cell lines were exposed to the non-permissive temperature in serum deprived media to elucidate the function of Htt in a neuronal context (Fig. 2).

While all three cell lines were indistinguishable at the permissive temperature, at the non-permissive temperature differences became apparent. The parental ST14A stopped proliferating and began the differentiation process. The cells started growing dendritic trees and axonal structures; eventually, apparently for lack of trophic support, the cells rounded up, detached from the substratum and died. The muHtt cell line rounded up earlier and to a greater extent than the parental line, indicating a toxic function of muHtt. In contrast, cells expressing wtHtt rounded up much slower and seemed protected from death.

These findings are in agreement with the phenotype of the Htt -/- mouse. Even though the Htt -/- mouse shows increased apoptosis in the epiblast, it does not follow conclusively from this that the function of Htt is to prevent this cell loss. The data presented here is the first clear indication that wtHtt exhibits a pro-survival function since it directly links expression of wtHtt to inhibition of cell loss.

2.2 WtHtt prevents the appearance of apoptotic markers

To test whether the cells die by apoptosis when shifted to the non-permissive temperature, chromosomal DNA was isolated and separated on an agarose gel (Fig. 3).

All three cell lines cell lines, showed no DNA fragmentation at the permissive temperature. However, exposure to the non-permissive temperature yielded the typical DNA-ladder pattern of nucleosomal fragments indicative of apoptosis in the parental line. This pattern became more pronounced with prolonged exposure to the non-permissive temperature. The same DNA-ladder pattern was observed in the muHtt cell line, but began to occur at earlier time points and was more pronounced. In marked contrast, the wtHtt cell line showed delayed, if not completely blocked, occurrence of the DNA-ladder.

Thus, while muHtt acts as expected and accelerates apoptotic death of a striatal cell line, wtHtt protects striatal neurons from apoptotic cell death, quite neatly confirming the underlying assumption of wtHtt as an anti-apoptotic protein.

3 Results

3.1 The anti-apoptotic function of wtHtt

Exposure of ST14A cells to the non-permissive temperature in serum deprived conditions leads to apoptosis. However, it is unclear which pathways are employed and how cell death is executed. In order to establish the anti-apoptotic function of wtHtt more clearly and to shed light on a possible mechanism for this effect, a more defined apoptotic stimulus is needed.

To this end, an assay to quantitate apoptosis was developed. Terminal Transferase dUTP Nick End Labeling (TUNEL) labels nicks in double stranded DNA with a fluorescent dye (Gorczyca et al., 1993). TUNEL labeled cells are quantitated on a Fluorescence Activated Cell Sorter (FACS). Since CAD cleaves DNA during apoptosis, stronger incorporation of the dye is indicative of increased apoptosis.

ST14A cell lines were transiently transfected with Green Fluorescent Protein (GFP) as a transfection marker and gating was subsequently performed on the population of green cells (Fig. 4A). Several pro-apoptotic molecules were co-transfected together with GFP to evaluate the ability of wtHtt to counter their apoptotic activity.

When overexpressed, DR trimerize in a ligand-independent manner. As a consequence, the DISC forms and apoptosis is induced. Upon overexpression in ST14A cells, the prototype DR p55/TNFR induced robust apoptosis in the parental cell line, as did its close relative DR3. Surprisingly, Fas, the other prototype DR, induced no recognizable apoptosis and neither did its close relative DR4 (Fig. 4B). This indicates that ST14A cells lack Fas/DR4-specific upstream signaling molecules and therefore cannot efficiently execute apoptosis by this class of DR.

Overexpression of pro-apoptotic Bcl-2 family proteins induces apoptosis by shifting the ratio of pro- to anti-apoptotic Bcl-2 proteins in favour of the former. Anti-apoptotic Bcl-2 members are no longer able to inactivate all pro-apoptotic Bcl-2 proteins, which leads to their aggregation in the mitochondrial membrane and subsequent PT transition and apoptosis.

The parental ST14A cell line was susceptible to massive apoptosis induced by the pro-apoptotic Bcl-2 family members BIK and BAK (Fig. 5A). In contrast, BAD was not able to induce apoptosis in these cells. Pro-apoptotic BAD does not usually induce apoptosis because it is retained in the cytosol in an inactive state through phosphorylation by the Akt protein kinase (Datta et al., 1997; del Peso et al., 1997).

Overexpression of caspases leads to their aggregation by means of their pro-domain. Due to the intrinsic catalytic activity of the zymogen, this leads to auto-activation of the enzyme. However, caspase-3 does not induce apoptosis when overexpressed due to a safety-catch mechanism that keeps this caspase in a dormant state (Roy et al., 2001). The safety-catch consists of an Asp-tripeptide in the p10 subunit of the enzyme that blocks access to the cleavage site that is utilized in activation. Not surprisingly, therefore, caspase-3 was not able to induce apoptosis in ST14A cells.

In contrast, the wtHtt expressing cells were completely protected from death induced by any molecule tested. DR-induced apoptosis was reduced to background levels; massive apoptosis induced by pro-apoptotic Bcl-2 family members was strongly inhibited. Most importantly, wtHtt expressing cells were also protected from events that take place after the mitochondrial step, such as overexpression of caspase-9. This indicates that wtHtt exerts an anti-apoptotic function against a wide variety of stimuli and probably acts as a very general death inhibitor. Furthermore, since wtHtt protects from

caspase-9-induced apoptosis, the point of protection in the apoptotic pathway lies at least as far downstream as caspase-9.

The experiment was also performed using muHtt expressing cells to assess the effect of poly-Q expansion on apoptosis induced by various stimuli. The results (Fig. 5B) were indicative of the dual nature of muHtt. Some protection against DR- and pro-apoptotic Bcl-2 family-induced apoptosis was still observed. This suggests that even the poly-Q expanded Htt retained protective activity. It was not as complete, however, as observed in wtHtt cells. Apparently, the gain-of-function effect induces apoptosis by a mechanism that cannot be protected against by Htt. In an alternative scenario, the expanded poly-Q weakened the protective function of muHtt. This loss-of-function effect could be verified by using muHtt constructs that differ in their poly-Q lenghts.

No protection against caspase-9 was detected. Surprisingly, strong apoptosis was observed with caspase-3 and BAD, circumventing/disabling the safety mechanisms of these molecules. Therefore, while muHtt still retained some of the protective function of wtHtt, it also gained a new function that fed into a pathway, which activated BAD, and caspase-3, or both.

3.2 WtHtt acts downstream of cytochrome c-release

If the anti-apoptotic activity of wtHtt lies indeed as far downstream as caspase-9, cytochrome c-release from mitochondria should still take place in wtHtt expressing cells. Thus, the localization of cytochrome c in response to an apoptotic stimulus was investigated. The extent to which cytochrome c was released from mitochondria was determined by immunocytochemistry

with an anti-cytochrome c antibody after exposure of the ST14A cell lines to 39˚C in serum deprived media.

At the permissive temperature, cytochrome c staining was grainy throughout the cytoplasm (Fig.6A, panels a-f) and correlated with staining by MitoTracker™, a mitochondria specific stain (data not shown). Staining of chromosomal DNA with 4',6-Diamidino-2-phenylindoledihydrochloride (DAPI) revealed faint diffuse staining in the nucleus. In some instances, bright, filamental nuclear staining was observed in cells that were in the process of undergoing mitosis.

At the non-permissive temperature, diffuse cytochrome c staining was observed, indicative of release of cytochrome c into the cytosol. In the parental cell line, nuclear staining took on a dotted pattern, demonstrating condensed and fragmented chromatin during apoptosis. When apototic nuclei were matched to their corresponding cytochrome c staining, it became apparent that cytochrome c release correlated well with chromatin condensation and fragmentation (Fig. 6A, panels g-f). This was also true for muHtt cells, but the effect was stronger, since the cells underwent a stronger apoptotic response. As expected, wtHtt expressing cells still showed cytochrome c-release, which, however, was associated with much less chromatin condensation than in the parental line.

For quantification, cells with released cytochrome c were counted and their chromatin assessed (Fig. 6B). The ratio of cells with released cytochrome c that showed chromatin condensation was ~80% in the parental line, while the correlation was almost 100% in the muHtt line. This effect was significantly lower in wtHtt than in parental or muHtt cells, at about 55%. These data indicate that cytochrome c is still released in protected

cells, but that in non-protected cells chromatin condensation follows. Thus, Htts protective function takes place after cytochrome c release.

3.3 WtHtt inhibits cytochrome c-dependent caspase-9 activity

In the next step after cytochrome c-release, Apaf-1 mediates caspase-9 activation. Recent studies showed that detergent free cytosolic extracts can be activated to undergo "apoptosis" by addition of dATP and cytochrome c, resulting in the formation of the apoptosome complex and subsequent cleavage and activation of caspase-9 and -3 (Li et al., 1997).

Therefore, the effect of wtHtt expression on caspase-9 was investigated. Cytoplasmic S-100 extracts from ST14A cell lines grown at 33°C were prepared and cytochrome c-dependent caspase-9 activity was assayed by addition of dATP, cytochrome c and the fluorogenic caspase-9 substrate LEHD-MCA.

Parental ST14A cells showed increasing release of the fluorogenic moiety over time, which was dependent on the addition of exogenous cytochrome c (Fig. 7A). The release of the fluorogenic moiety was completely blocked when the inhibitory peptide LEHD-Ac was added to the lysates (data not shown). In contrast, wtHtt expressing cells showed drastically reduced cytochrome c-dependent caspase-9 activity (Fig. 7B), which was even lower than the baseline (without cytochrome c) of parental cells. MuHtt expressing cells exhibited greatly elevated levels of caspase-9 activity, as expected (Fig. 7C).

3.4 WtHtt prevents cytochrome c-dependent cleavage of pro-caspase-9

To determine whether the decreased activity of caspase-9 was due to inhibition of its catalytic activity by Htt or due to decreased processing of pro-caspase-9, *in vitro* caspase-9 cleavage assays were performed.

Extracts of ST14A cell lines were incubated with cytochrome c and ^{35}S -labeled *in vitro* transcribed/translated pro-caspase-9 (Fig. 8A). Increasing amounts of cytochrome c lead to increased processing of pro-caspase-9 and the emergence of the p35/p37 cleavage products in parental ST14A cells. Recent studies showed that the p35 fragment is a direct consequence of cleavage at D315 by caspase-9 itself, whereas the p37 fragment is subsequently generated by cleavage at D330 by caspase-9-activated caspase-3 (Srinivasula et al., 1998; Stennicke et al., 1999). In wtHtt expressing cells, caspase-9 processing only occurred at higher cytochrome c concentrations. Furthermore, the appearance of the p37 fragment was blocked.

The muHtt cell line extracts processed pro-caspase-9 at lower cytochrome c concentrations than the parental cell line extracts. The emergence of the p37 fragment was not inhibited, but rather accelerated.

These findings are in accordance with the TUNEL results that showed that muHtt activates caspase-3 activity. To confirm this, cleavage site mutants of caspase-9 were used (Stennicke et al., 1999). Mutation of D315 to A lead to disappearance of the p35 band, whereas the p37 band was still observed (Fig. 8B). Mutation of both cleavage sites completely abolished caspase-9 processing. This indicates that muHtt circumvents the caspase-9 activation step and triggers a signaling pathway which directly results in the activation of caspase-3.

To test further the possibility that wtHtt directly or indirectly affects the catalytic activity of caspase-9, the caspase cleavage assay was performed with ^{35}S -labeled caspase-3 (Fig. 8C), the downstream target of caspase-9 activity. Without cytochrome c, no processing of caspase-3 could be detected. Addition of cytochrome c to parental ST14A and muHtt extracts generated the two p18 and p20 bands of cleaved caspase-3. In wtHtt expressing cells, the occurrence of both bands was inhibited. These data show that wtHtt acts directly on caspase-9 to prevent its complete activation.

4 Discussion

The ST14A cell system is a valuable tool in examining striatal neurodegeneration. At the permissive temperature, ST14A cells are immortalized and show characteristics of neural precursors; at the non-permissive temperature, they can be induced to differentiate and behave like mature neurons. This dual phenotype makes them interesting to study both in itself and in the context of HD.

The best cellular system to study HD is one in which Htt with an expanded poly-Q specifically kills neurons and affects only those neurons that are suceptible *in vivo*. In addition, such a system would use FL-Htt, or a long truncation mutant, instead of sa hort, Exon 1-like mutant. Ideally, such a system would also allow for easy manipulation. The ST14A system presented here fulfills these criteria. Other cellular models have been employed to study the function of Htt. The best model to date employs primary striatal neurons isolated between embryonic days E16-18 into which Htt constructs of comparable length to those used in this work were transfected (Saudou et al., 1998). Data obtained with this system resemble very closely the results reported here, since the N-terminal caspase cleavage fragment of poly-Q expanded Htt induces cell death by an apototic mechanism that is independent of inclusion formation. In the ST14A system, muHtt requires an additional stimulus like serum-withdrawal to effectively induce cell death of the differentiated neurons. In this respect, the ST14A model is closer to the *in vivo* situation than the primary cell culture models, since neurodegeneration in HD is a slow process that is probably not executed as soon as muHtt is expressed in striatal neurons.

Furthermore, the study mentioned above reveals that the N-terminal fragment is only capable of inducing apoptosis when it is localized to the

nucleus. This finding is in stark contrast to data that shows that an expanded poly-Q stretch is capable of oligomerization of cytoplasmic caspase-8 in primary striatal neurons, thereby activating it (Sanchez et al., 1999). However, this report only used a poly-Q stretch without the context of the large protein, which could explain the different mechanisms these two groups propose for muHtt induced neurodegeneration. Neither of these studies, however, addresses the question of wtHtt function.

To address the question of the exact physiological role of wtHtt in CNS cells, and whether and how it relates to muHtt action, ST14A cells stably expressing a wt or mu poly-Q stretch in the context of the N-terminal 548aa truncation were obtained. In this cellular system, wtHtt increases cellular survival of cells shifted to the non-permissive temperature. Furthermore, closer analysis of dying cells shows that their demise occurs by an apoptotic mechanism. WtHtt expressing cells block the occurrence of apoptosis. Thus, this pro-survival function of wtHtt is more accurately defined as an anti-apoptotic function.

In order to delineate the mechanism of wtHtt anti-apoptotic function, several pro-apoptotic genes were transiently expressed in the different ST14A lines. Cells expressing wtHtt are protected from cell death induced by death receptors, by pro-apoptotic Bcl-2 family members (BIK and BAK) as well as by caspase-9. This protection from multiple apoptotic inducers further confirms the hypothesis that wtHtt is an anti-apoptotic molecule and implies that wtHtt is a general death inhibitor which acts on components of the common death effector pathway. Furthermore, the protection from pro-apoptotic Bcl-2 homologues implies that wtHtt is acting on mitochondrial or post-mitochondrial apoptotic events.

To test further this possibility the effect of Htt expression on cytochrome c release was assessed. When exposed to serum-deprived conditions at the non-permissive temperature all three cell lines show release of cytochrome c from mitochondria into the cytosol. While cell viability is not compromised and the nuclei appear intact, the wtHtt expressing cells still exhibit cytochrome c release. These data are consistent with the TUNEL data that show protection by wtHtt as far downstream as caspase-9. Thus, Htt does not affect mitochondrial events, but rather inhibits targets downstream of these.

Following cytochrome c-release, Apaf-1-dependent activation of caspase-9 occurs. In order to test whether Htt affects this caspase-9 step, cytochrome c-dependent caspase-9 activity was measured in cellular extracts. In the wtHtt cell line no significant caspase-9 activity is detected. Therefore, the function of wtHtt takes place at the level of caspase-9. Since caspase-3 does not induce apoptosis in ST14A cells, the caspase activity data provide the most direct evidence for Htt function upstream of the first effector caspase. This reduces the window for Htt function significantly, because only events around caspase-9 activation need to be considered.

Thus, the level of cytochrome c-dependent caspase-9 processing was measured. It is found that caspase-9 processing is delayed in extracts from wtHtt cells. Furthermore, higher concentrations of cytochrome c are needed to achieve similar processing as observed in the parental line. However, caspase-9 is still processed to some extent at 1.3nM cytochrome c, the amount used for the activity assays, whereas catalytic activity is practically non-existent. These data indicate that while some processing can occur, catalytic activity of the caspase is severely compromised.

Taken together, these data strongly suggest that the anti-apoptotic function of wtHtt takes place at the level of caspase-9. The zymogen form of caspase-9 has been shown to contain residual catalytic activity (Stennicke et al., 1999), which is needed to perform the cross-auto activation that takes place when Apaf-1 oligomerizes upon cytochrome c binding (Srinivasula et al., 1998). Therefore, Htts function is possibly exerted through inhibition of the intrinsic proteolytic activity that resides within the zymogen form of caspase-9.

Whereas wtHtt exhibits strong anti-apoptotic function, muHtt only moderately protects from DR and BIK/BAK induced apoptosis. It is generally believed that HD is a gain-of-function disease and that therefore muHtt retains some of the functions of the wt protein. The observed moderate protection is consistent with this hypothesis.

The gained function of muHtt accelerates apoptotic cell death induced by serum deprivation. Furthermore, it also turns on pathways that result in activation of caspase-3 and/or BAD. However, the gain-of-function effect is more a sensitization, rather than a pro-apoptotic effect because muHtt cells survive at 33˚C just fine. However, if the cells are induced to die by serum deprivation this sensitization takes effect by accelerating cell death. The sensitization pathway is observed in the TUNEL experiments and acts via caspase-3 activation.

The sensitization/death acceleration is also observed in the caspase-9 assays. This demonstrates that the cellular components of this pathway are already present in ST14A cells that were never exposed to the non-permissive temperature. While these observations do not rule out transcriptional effects of muHtt, they do not support them either.

A recent study describes increased caspase-9 and -3 activity in lymphoblasts of HD patients (Sawa et al., 1999). This is the first report that describes effects of muHtt outside the nervous system. Therefore, expression of muHtt sensitizes all tissues to apoptotic cell death. But since different anti-apoptotic mechanisms are employed in different cells of an organism, some cells prove more sensitive to apoptosis than others. Also, certain cell types might be sensitized to very specific forms of apoptosis. Because muHtt might potentially only activate very specific apoptotic pathways, this represents a possible explanation for tissue specificity and long incubation times in HD.

5 Materials and Methods

5.1 Plasmids and constructs

The Htt expressing vectors pcDNA3-N548wt (nt1955-Q15) and -N548mu (nt1955-Q128) were a kind gift of M. Hayden. The pCMV-EGFP plasmid was constructed by cloning of the BglII/HindIII fragment of pcDNA3 into the MCS of pEGFP-1 (CLONTECH). The pet23-caspase-9, -caspase-9D315A and –caspase-9D315A/D330A constructs were a kind gift of G. Salvesen.

5.2 Cell Culture

ST14A cells, ST14A-N548wt and ST14A-N548mu cells were grown in Dulbeccos Modified Eagle Medium (DMEM, BioWhittaker) supplemented with 10% FCS, 1%Pen/Strep and 1% glutamine at 33˚C. For induction of apoptosis, cells were shifted at the non-permissive temperature of 39˚C in serum-free DMEM with 1% BSA. For establishment of stable Htt cell lines, 10 µg of cDNA were transfected using Lipofectamine (Gibco-BRL). Resistant clones were selected with 3µg/ml puromycine. Transfection with the empty plasmid was also performed. For each construct, approximately 20 colonies were isolated with cloning rings and analyzed.

5.3 DNA fragmentation assay

Cells were harvested in 10mM Tris pH8, 20mM EDTA, 2% TritonX-100. Low molecular weight DNA was extracted by phenol/chloroform extractions and DNA precipitated with isopropanol. DNA fragmentation was detected on a 1% agarose gel.

5.4 TUNEL assay

2-3x10^5 cells were transfected with 250ng pCMV-EGFP and 1µg of the indicated plasmids (0.4µg for Bik and Bak). Expression from the transiently transfected plasmids was confirmed by western blot and found to range between 10 and 15 times above background. After 24 hrs the cells and their supernatant were harvested and terminal transferase dUTP nick-end labeling (TUNEL) was essentially performed as described previously using PE-conjugated Avidin (BMB) (Gorczyca et al., 1993). Stained cells were analyzed by dual colour FACS, collecting at least 10,000 events. The number of TUNEL-positive cells was estimated by gating on the EGFP expressing cells which represented ~30% of the total. Background death as estimated by transfection of control protein 14-3-3 was subsequently subtracted from the data.

5.5 Cytochrome c release

2-3x10^5 ST14A, ST14A-N548wt and ST14A-N548mu cells were seeded on coverslips and shifted to 39˚C for 24hrs. The cells were fixed in 4% Formaldehyde (Electron Microscopy Sciences) containing 0.05% Saponin (SIGMA). Coverslips were then incubated with α-cytochrome c antibody (1:500 dilution, clone 6H2.B4, PharMingen), followed by incubation with α-mouse FITC-conjugated secondary antibody (1:5000, SIGMA) and 100ng/ml DAPI (SIGMA). Cells were scored by fluorescence microscopy for diffuse cytochrome c staining and condensed nuclei.

5.6 Caspase-9 processing and activity assays

S-100 extracts were prepared from six confluent 125mm dishes of ST14A, ST14A-N548wt and –N548mu expressing cells. Cells were harvested in buffer A (20mM HEPES-KOH pH7.5, 10mM KCl, 1.5mM MgCl$_2$, 1mM EDTA, 1mM EGTA, 1mM DTT, protease inhibitors, 0.5mM PMSF) and homogenized by passing through a 26 gauge syringe needle. The supernatant was centrifuged at 100,000xg to obtain the cytosolic S-100 fraction. ^{35}S-labeled caspase-9 and -3 were prepared using the TNT-QUICK *in vitro*-transcription/translation kit (Promega) with the above described pet23 vectors expressing the caspases as a template.

For caspase-9 processing assays, 20µg of extract was incubated with ^{35}S-caspase-9, 1mM dATP and increasing amounts of cytochrome c at 37˚C for 30min. Reactions were separated on SDS-PAGE and the dried gel was autoradiographed. caspase-3 processing assays were performed using 1mM dATP and 19.5nM cytochrome c at 30˚C for 1hr.

For measurements of caspase-9 activity, 40µg S-100 extracts were incubated with 10mM DTT, 1mM dATP, 1.3nM cytochrome c and 200nM of the fluorogenic caspase-9 substrate LEHD-MCA (Peptide Institute). Reactions were incubated at 37˚C and release of the free MCA moiety over time was measured using a CytoFluor II fluorometer (Perseptive Biosystems).

6 Figures and Legends

Figure 1

The Htt constructs used throughout this work (as described in Materials and Methods) carry 15 Q-residues in case of wtHtt and 128 Q for muHtt. Both constructs span the N-terminal 548aa, which contain the Akt phosphorylation site and extend 35aa beyond the caspase-3 cleavage site at D513. Therefore, the constructs roughly resemble the fragment that is found in IIN.

Htt constructs used in this work

510 DSVD 513

548

1

416 RSRSGS 421

41 PPPPP[...]P 78

41

wtHtt: N548-15Q

QQQQQQQ[...15...]QQQQQQQ

18

41

muHtt: N548-128Q

QQQQQQQQQQ[......128....]QQQQQQQQQQQQQQ

18

Figure 2

ST14A, ST14A-wtHtt and ST14A-muHtt cells were exposed for 24hrs to the non-permissive temperature in serum-deprived medium (SFM). At the permissive temperature, all three cell lines looked similar by light microscopy and showed no signs of cell death (A, C, E for parental, wtHtt and muHtt, respectively). At the elevated temperature, the parental line showed signs of a differentiation process, as the cells developed axonal and dendritic outgrowths, that eventually resulted in rounding up of the cells and subsequent cell death, probably due to lack of trophic support (B). MuHtt cells were sensitized to cell death, as demonstrated by the increased amount of detached, floating cells (F). However, wtHtt expressing cells showed almost no detached cells and remained confluent (D), indicating little, or no cell loss and thus were protected from cell death induced by the experimental conditions.

33°C FCS   39°C SFM

A   B
ST14A

C   D
wtHtt

E   F
muHtt
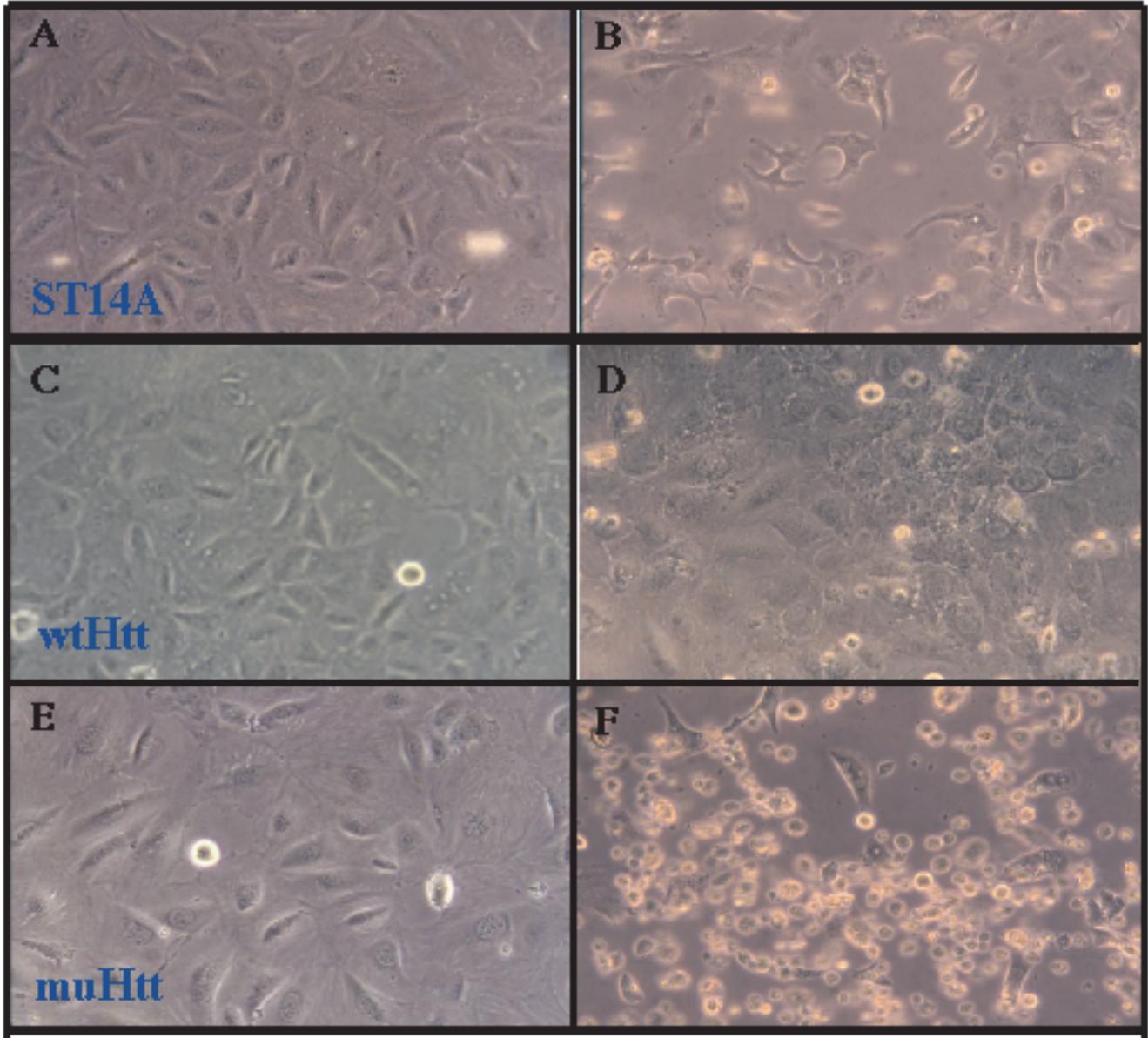
Figure 3

To determine, whether cell death observed at the non-permissive temperature followed an apoptotic mechanism, cell lines were shifted to 39˚C for various amounts of time. After harvest, DNA was extracted and analyzed by agarose gel electrophoresis.

No DNA degradation was observed in all three cell lines at regular growth conditions. The parental cells developed a regular DNA ladder pattern with roughly 200bp periodicity after 20hrs of exposure that is a hallmark sign of apoptosis. This pattern already faintly showed as early as four hours. The occurrence of the DNA ladder was greatly increased and showed very strongly already at four hours exposure in muHtt cells. WtHtt cells did not develop a DNA ladder.
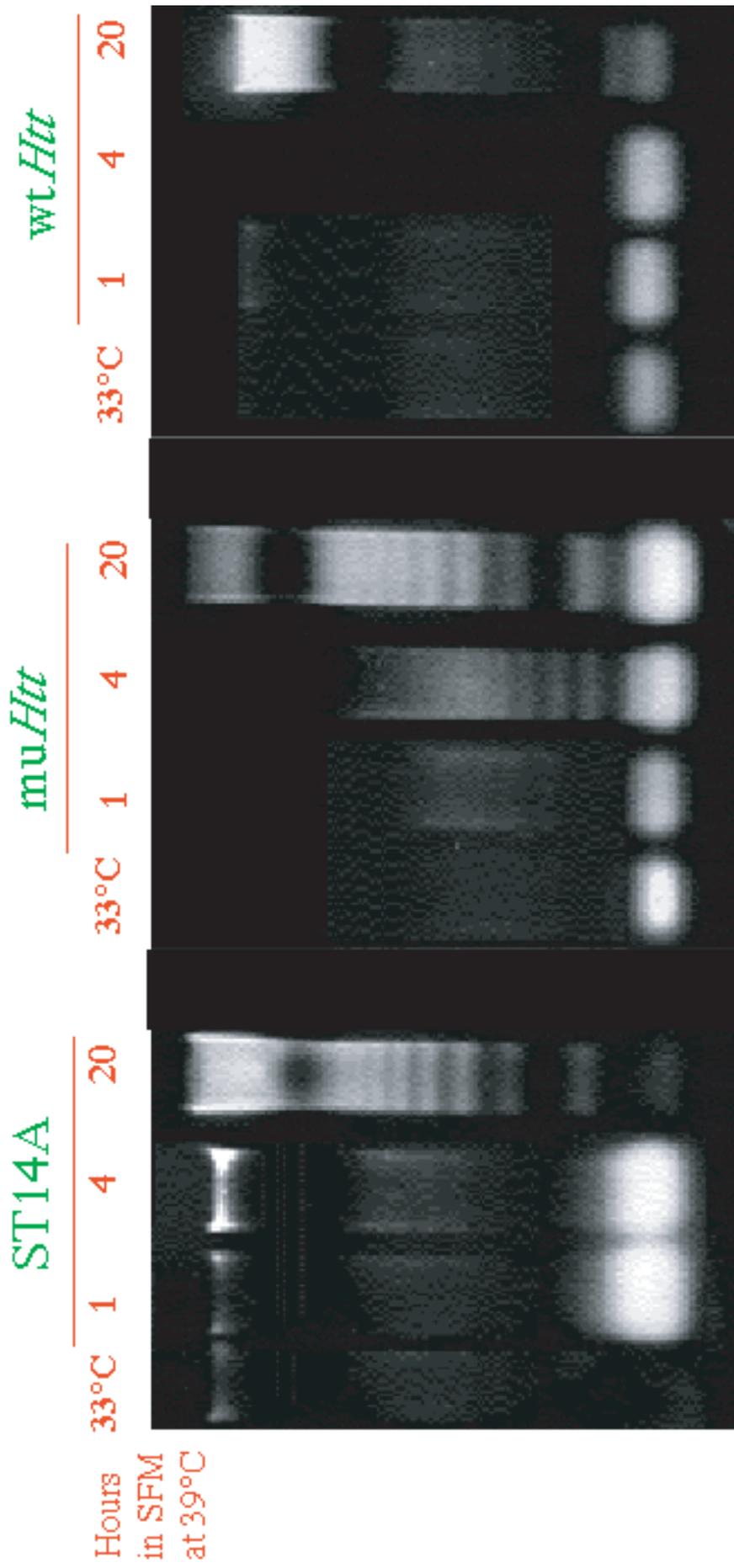
Figure 4

(A) ST14A cell lines were transfected with a GFP-expressing plasmid as a transfection marker and analyzed for presence of green fluorescence. A typical FACS readout is shown with empty vector as a control. In a typical experiment, between 30-40% of all cells were positive for GFP and thus regarded transfected. For all subsequent experiments, GFP was co-transfected with the indicated plasmids and only the GFP-positive cells were analyzed for incorporation of PE-label.

(B) Typical FACS readouts are shown for DR transfected parental ST14A cells. Gating was performed on GFP-positive cells which were analyzed for PE-staining. The number of PE/TUNEL-positive cells above that of control protein 14-3-3 is shown for a typical experiment. While Fas and its relative DR4 had no appreciable TUNEL-staining above background, p55 and its relative DR3 stained showed dramatic increase in staining, indicating a strong apoptotic response to these DR.

A

B

-12.4   22.6

% TUNEL positive above control

- 6.5   36.1
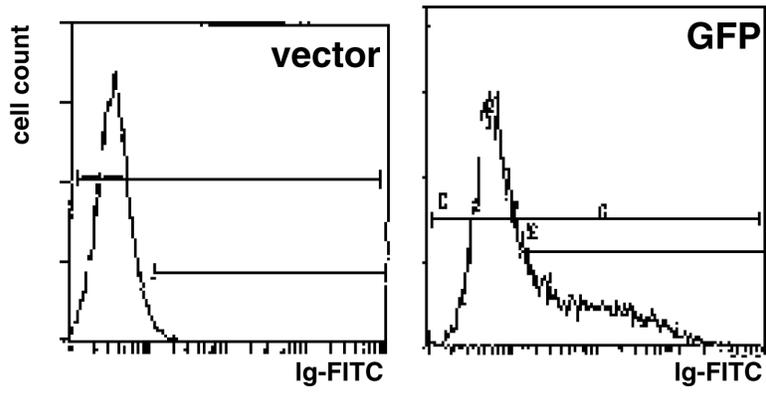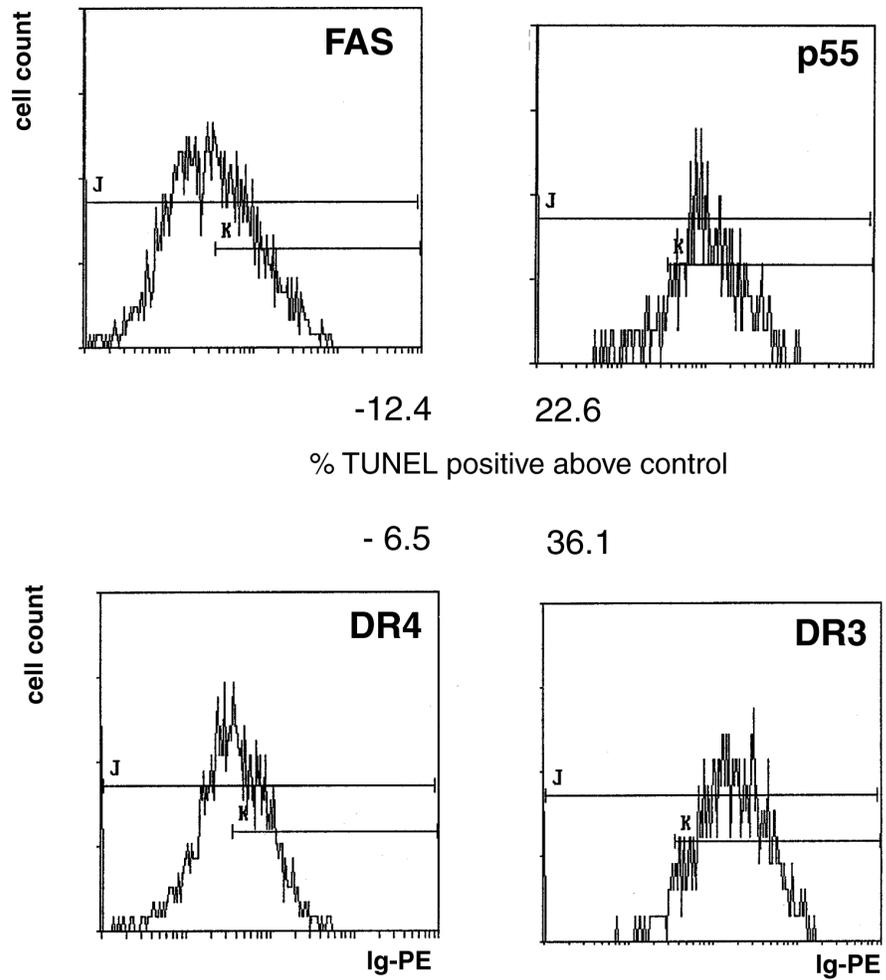
Figure 5

(A) ST14A and ST14A-wtHtt cells were transfected with the indicated death-inducers and cell death determined by TUNEL. Quantification of the TUNEL experiments was performed by subtracting background death observed by transfection of control protein 14-3-3. White bars represent parental cells, black bars ST14A-wtHtt cells. Except for caspase-3 and BAD, all transfected pro-apoptotic molecules induced robust apoptosis in parental ST14A cell. In contrast, no cell death was observed in cell expressing the wtHtt construct.

(B) Similarly, muHtt cells were analyzed by TUNEL staining following transfection with the indicated plasmids. White bars represent parental cells, gray bars ST14A-muHtt cells. While some protection was still observed against pro-apoptotic BIK and BAK, only very weak protection occured against DR-induced apoptosis. No protection was observed when caspase-9 was transfected. Remarkably, caspase-3 and BAD induced significant TUNEL staining in ST14A-muHtt cells.

A

B

% TUNEL-positive above control

p55   BIK   BAK   BAD   Caspase-3   Caspase-9

40   30   20   10   0   -10
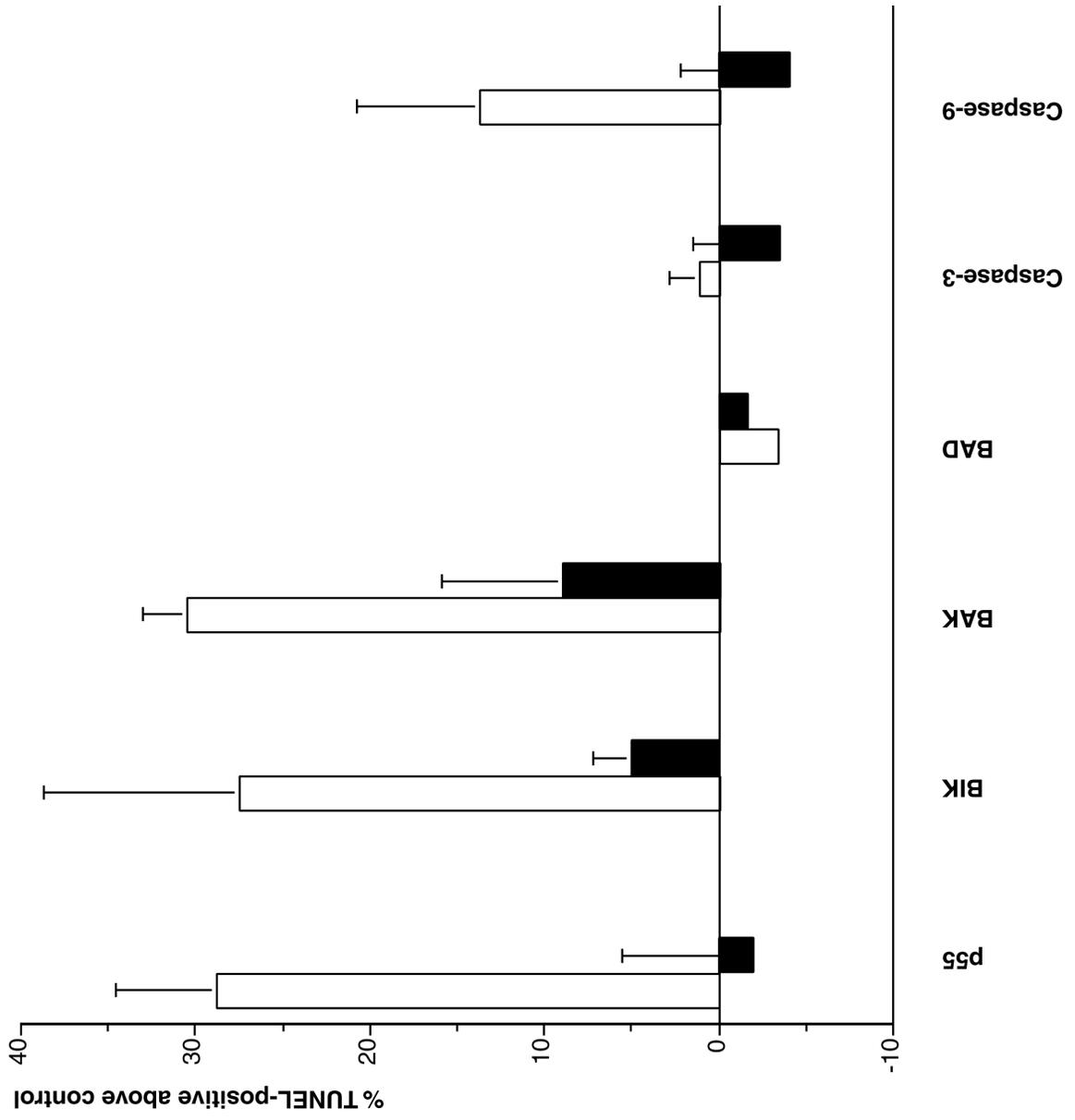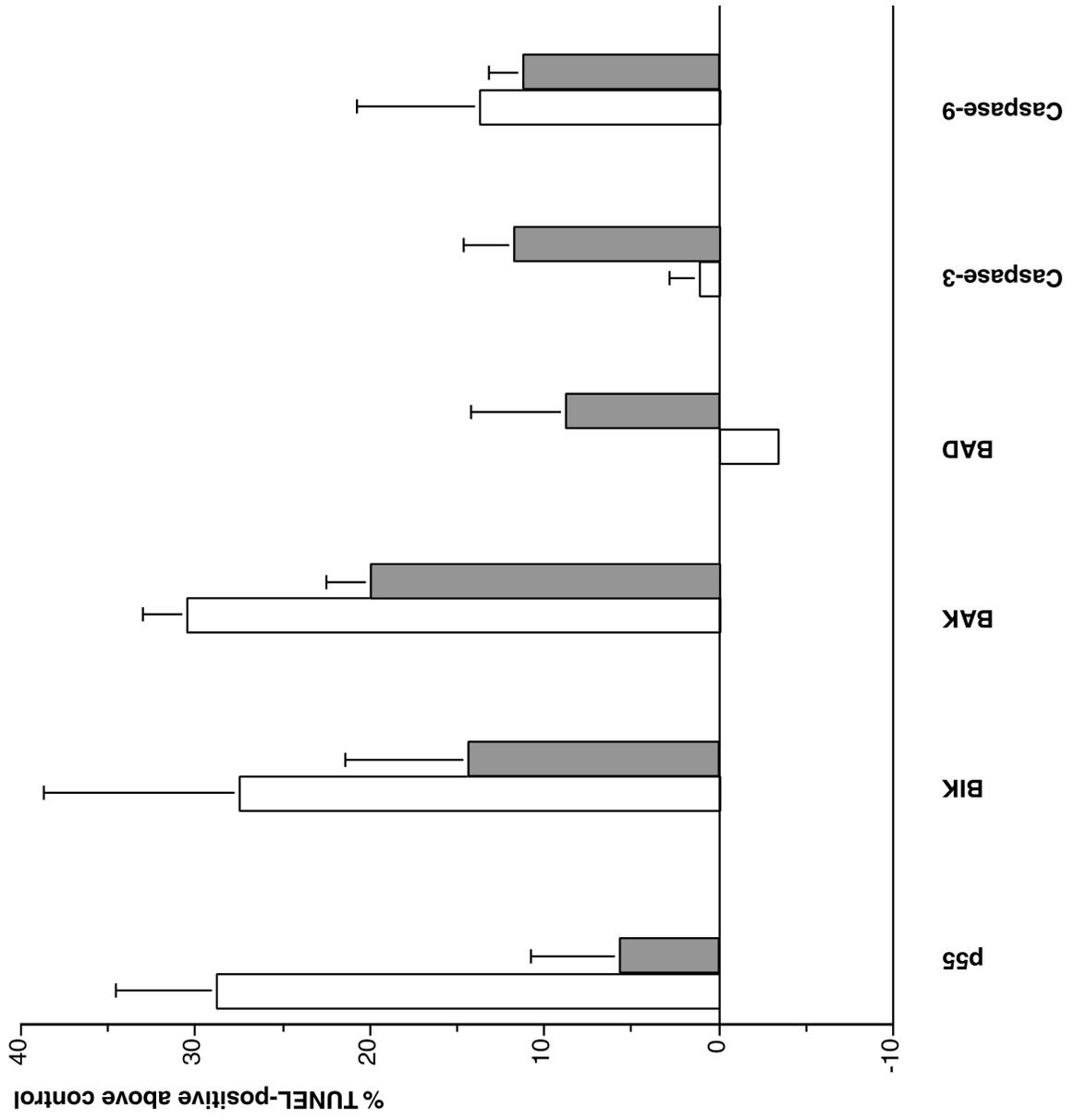
Figure 6

(A) Stably transfected ST14A cells were incubated at 33˚C or shifted to 39˚C for 24hrs. At the regular growth temperature all three cell lines were healthy and showed cytochrome c localized to mitochondria as verified by MitoTracker staining (α-cytochrome c antibody: panels A, C, E; DAPI: panels B, D, F; white arrows depict cells undergoing mitosis). In parental ST14A and muHtt cells cytochrome c-release at the elevated temperature was accompanied by chromatin condensation and fragmentation of the nuclei. In contrast, while wtHtt cells still showed cytochrome c-release from mitochondria, changes in nuclear morphology were not observed. (α-cytochrome c antibody: panels G, I, K; DAPI: panels H, J, L; white arrows depict cells with released cytochrome c and condensed chromatin, red arrows depict cells with released cytochrome c without nuclear condensation)

(B) For quantification, cells with released cytochrome c were counted and assessed for nuclear morphology. The percentage of cells with released cytochrome c and condensed chromatin is shown. Error bars represent the standard deviation of three independent experiments.

ST14A-mtHtt
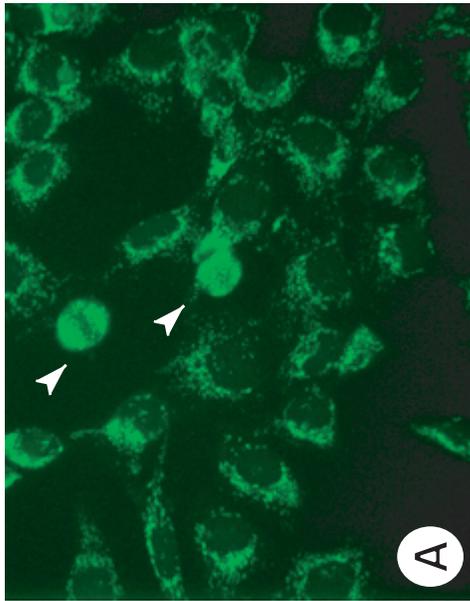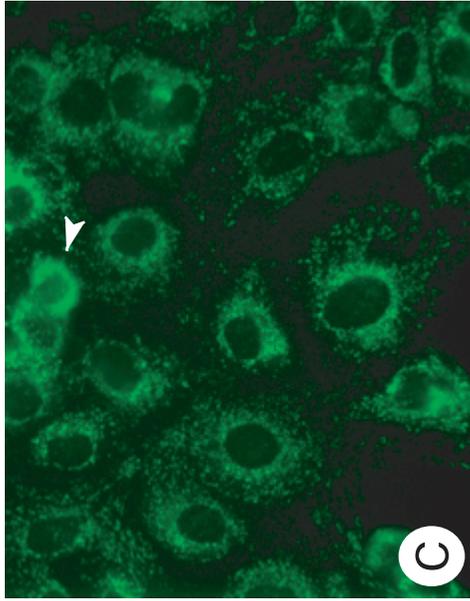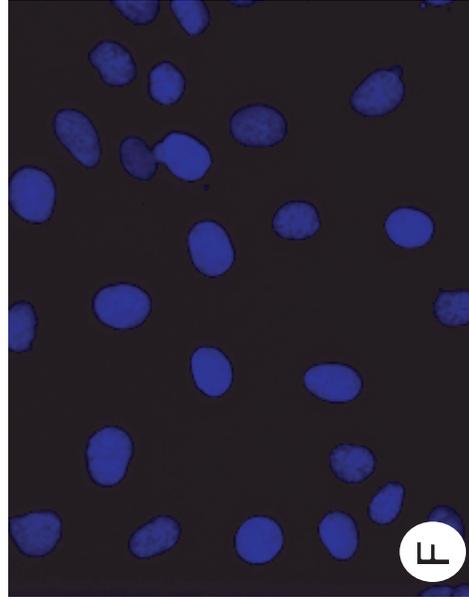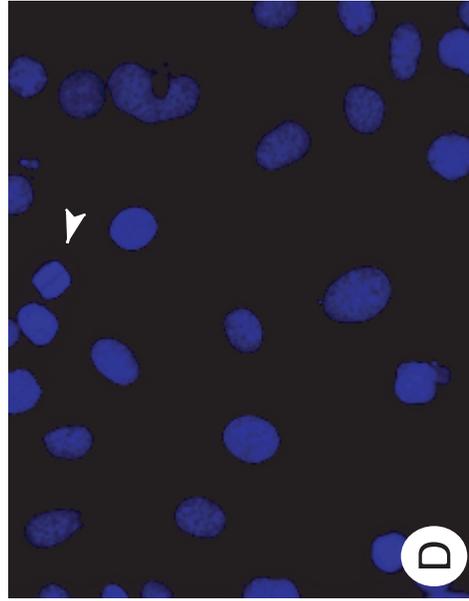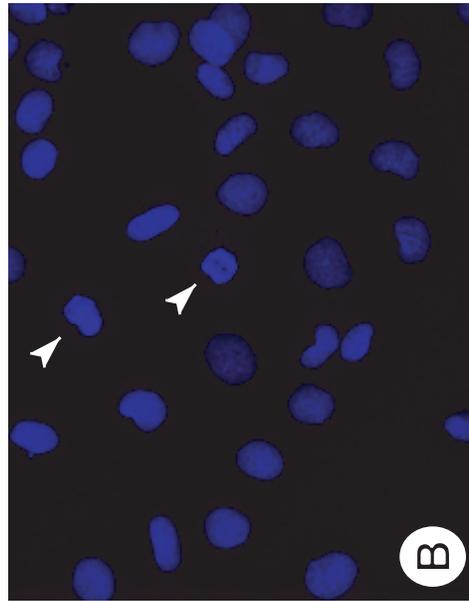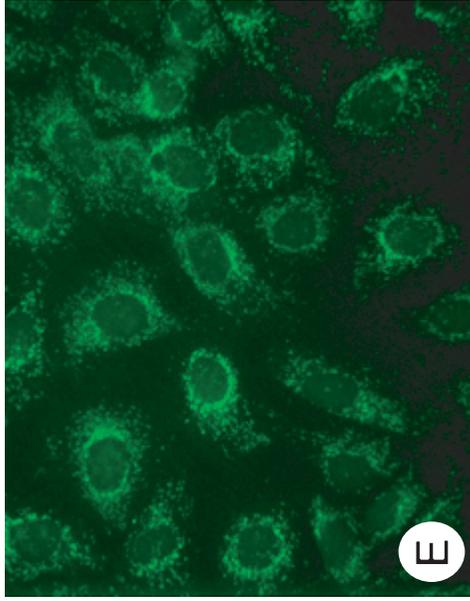
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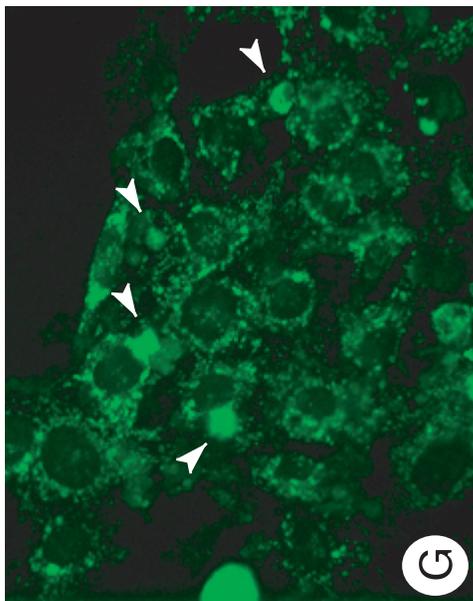
ST14A

ST14A-mtHtt

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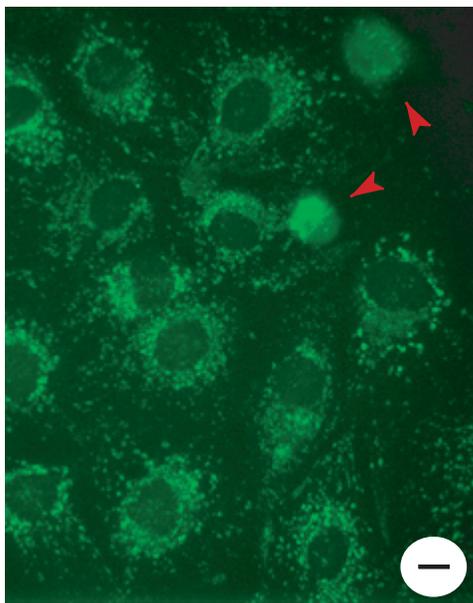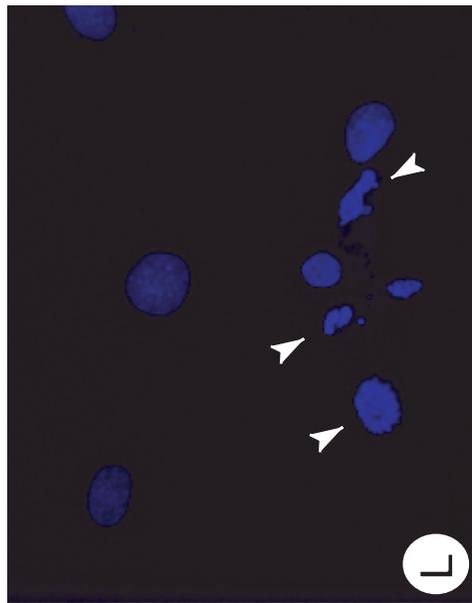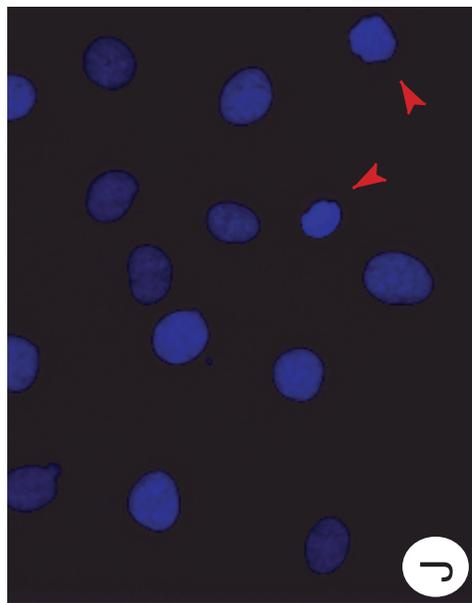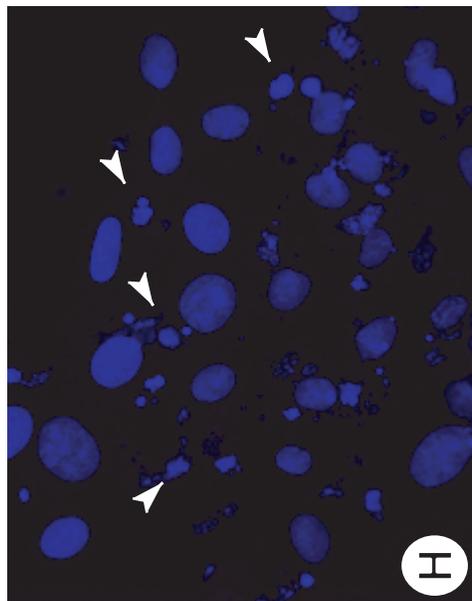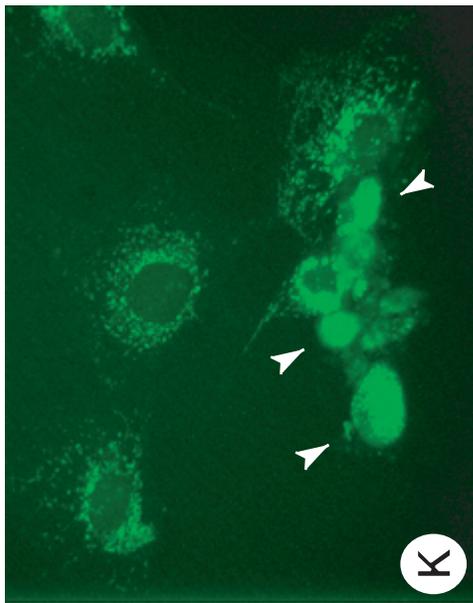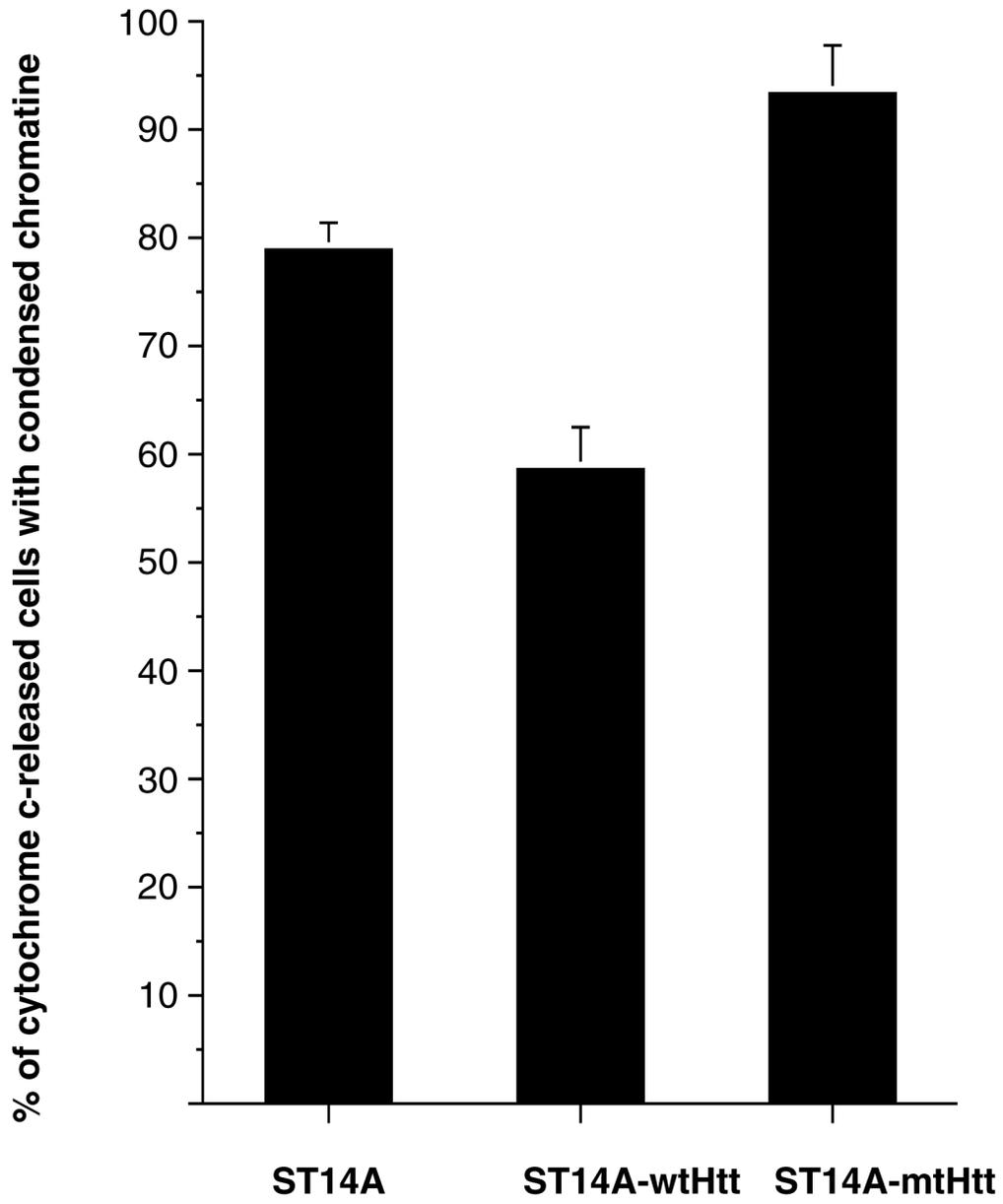
ST14A

Figure 7

Cytoplasmic S-100 extracts of ST14A cells that were stably transfected with wtHtt or muHtt were prepared and incubated with dATP, cytochrome c and the caspase-9 specific fluorogenic substrate LEHD-MAC as described in Materials and Methods. Release of the fluorogenic moiety was monitored over time.

(A) Parental ST14A cells showed vigorous caspase-9 activity, that was dependent on the presence of cytochrome c (parental ST14A: open triangles; no cytochrome c: black triangles).

(B) WtHtt expressing cells showed almost no caspase-9 activity (two independent clones of ST14A-wtHtt: open and black squares).

(C) In contrast, muHtt expressing cells showed elevated caspase-9 activity that even superseded that of the parental line (two independent clones of ST14A-muHtt: open and black circles; no cytochrome c: open diamond). Error bars shown are the average deviation of at least two independent experiments.
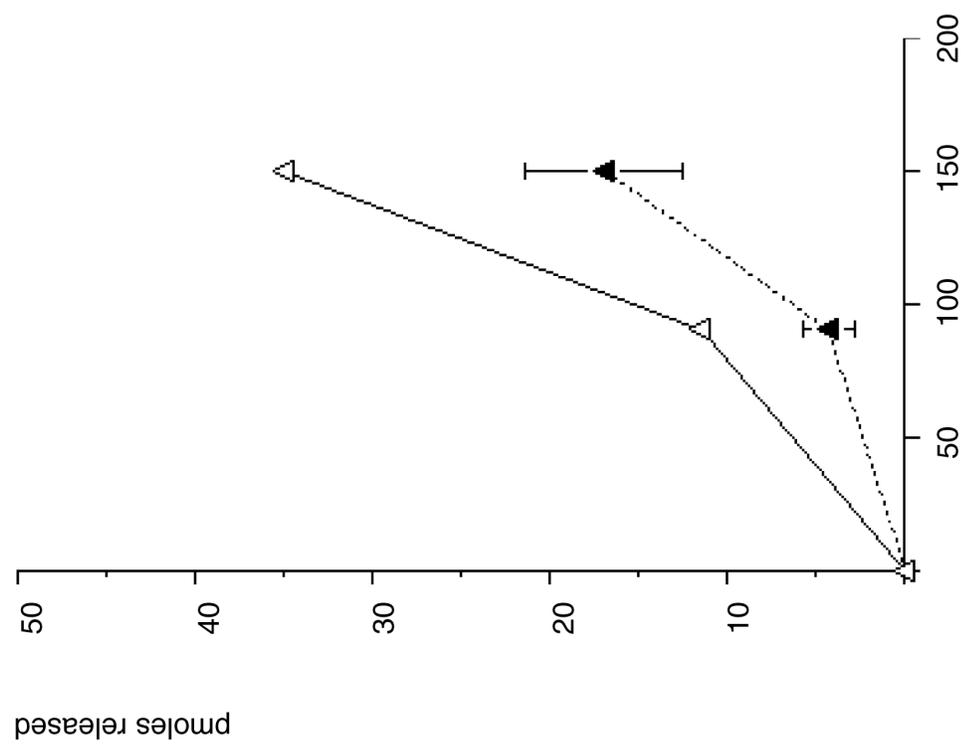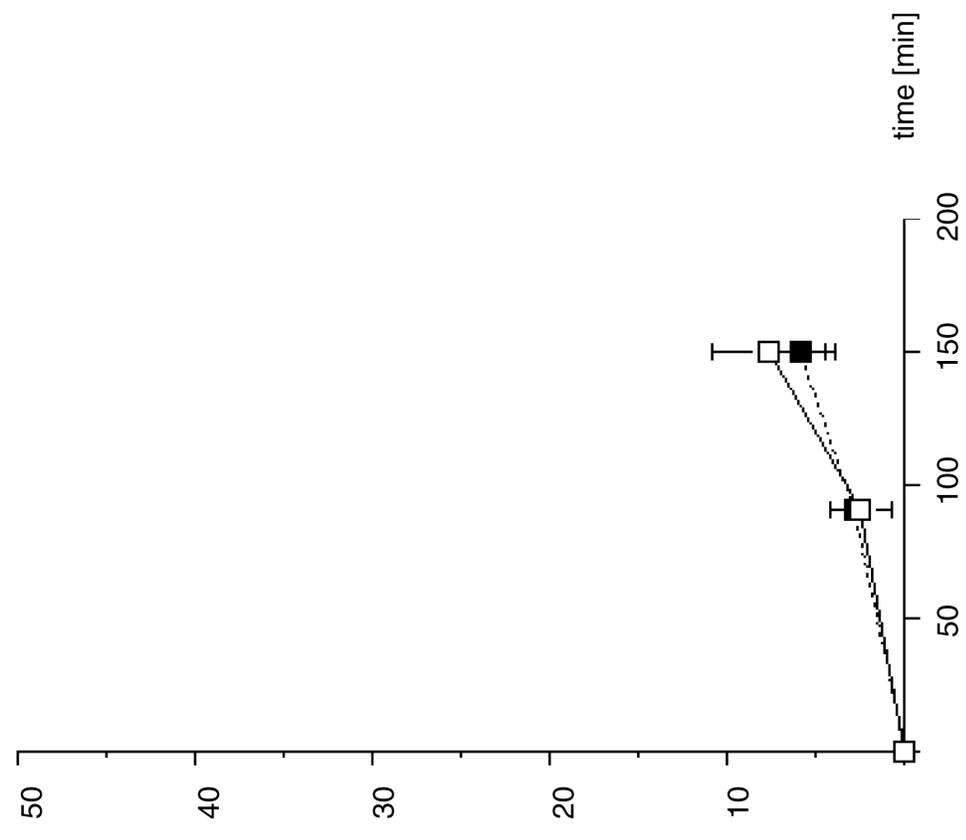
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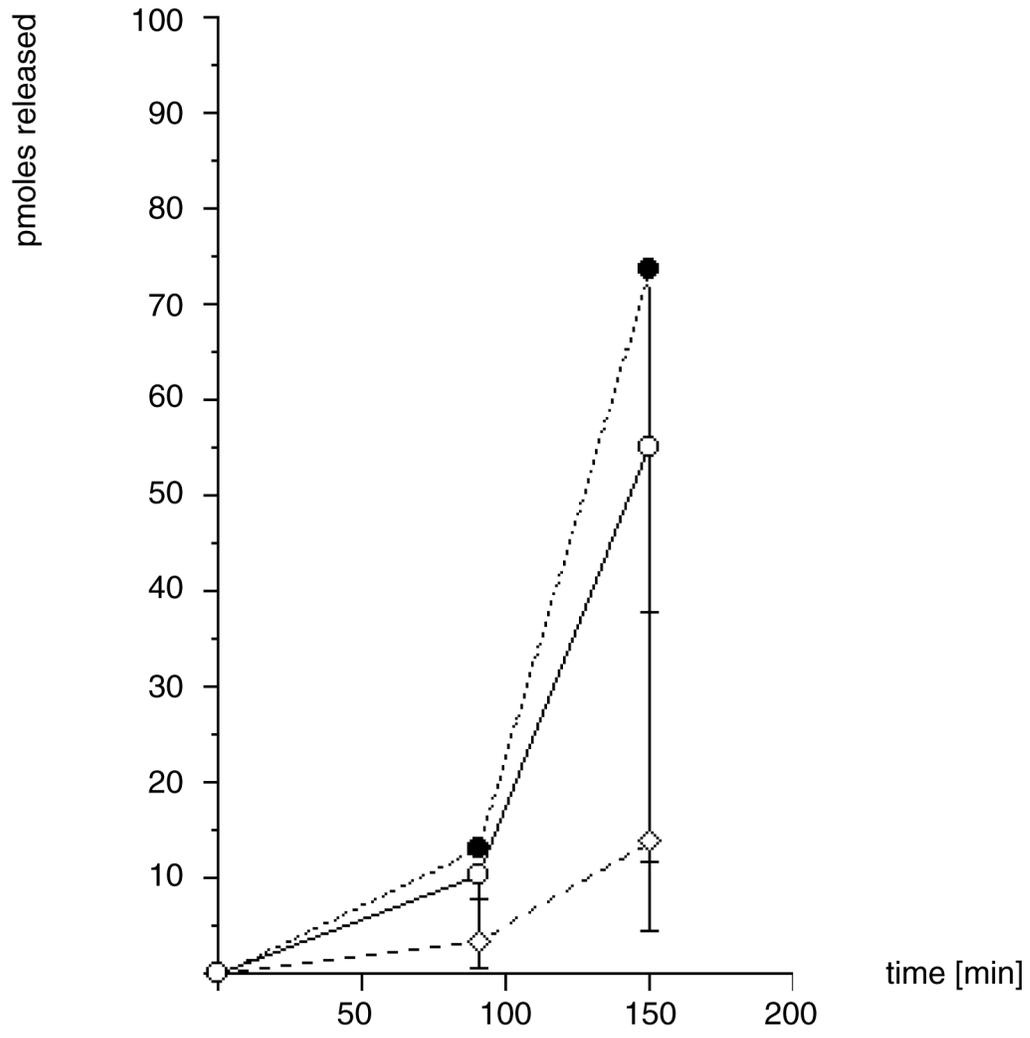
Figure 8

Cytosolic extracts from ST14A, ST14A-wtHtt and ST14A-mtHtt were incubated with cytochrome c, dATP and ^{35}S -labeled caspases.

(A) Increasing amounts of cytochrome c (from left: 0, 0.25, 1.3, 10nM) lead to increased processing of caspase-9 and the occurrence of two p35 and p37 cleavage products. MuHtt cells processed caspase-9 at the lower cytochrome c concentration.

(B) Extracts from muHtt cells were incubated at 1.3nM cytochrome c with ^{35}S -labeled cleavage site mutants of caspase-9. Mutation of the autoprocessing site lead to the disappearance of the p35 fragment, while the p37 fragment was still generated. Mutation of both cleavage sites abolishes caspase-9 processing.

(C) Extracts from all three cell lines were incubated at 19.5nM cytochrome c with ^{35}S -labeled caspase-3. While no processing of caspase-3 was observed when cytochrome c was omitted, addition of cytochrome c generated two cleavage products in parental and muHtt cells. In contrast, the appearance of the cleavage products was inhibited in wtHtt cells.

A

ST14A-mtHtt   ST14A-wtHtt   ST14A

Cytochrome C
Caspase-9
p37
p35

B

Caspase-9
p37
p35

Caspase-9 D315A   Caspase-9 D315/330A   Caspase-9

ST14A-mtHtt

C

Cytochrome C
Caspase-3
cleavage products

ST14A-mtHtt +   ST14A-wtHtt +   ST14A + −
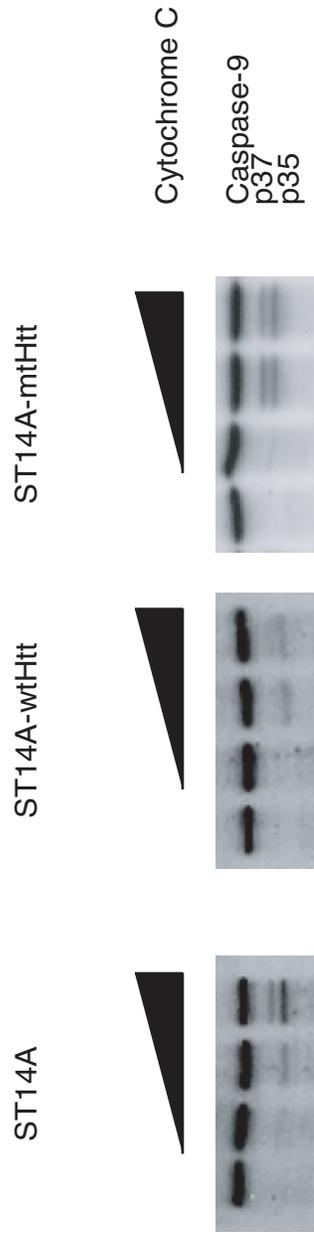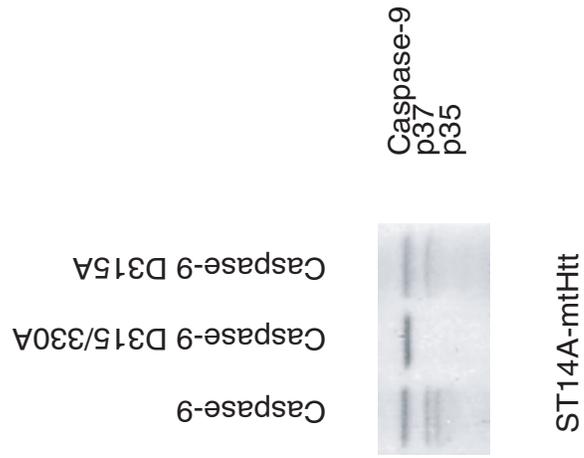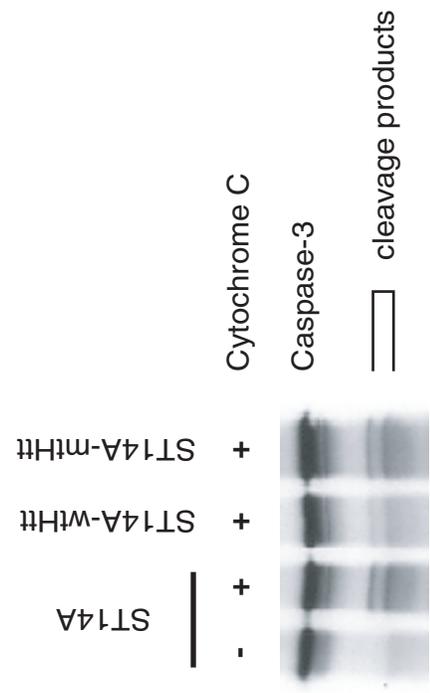
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