Chapter III

- *in which the mechanism for wtHtts anti-apoptotic function is investigated; apoptosome assembly in different ST14A cell lines in response to an apoptotic stimulus is studied; wtHtt is shown to compromise formation of a proper apoptosome; immunoprecipitation experiments are performed to explain this effect; interaction of caspase-9 with Htt is demonstrated*

1 Introduction

The experiments in the previous chapter described and firmly established an anti-apoptotic function for the Htt protein. Moreover, a potential target for this anti-apoptotic function was identified as caspase-9. Nonetheless, activation of caspase-9 is brought about in the context of the large apoptosome complex that contains Apaf-1, caspase-9, cytochrome c, possibly heat shock proteins as well as yet unidentified proteins (Adrain et al., 1999; Cain et al., 2000; Chu et al., 2001; Srinivasula et al., 1998; Stennicke et al., 1999; Zou et al., 1999).

Htt could possibly exert its caspase-9 inhibitory effect at several points. Htt could physically disrupt formation of the apoptosome thereby preventing caspase activation. This mechanism was demonstrated to be responsible for the anti-apoptotic properties of HSP70 (Beere et al., 2000). It is also plausible that Htt acts from within the apoptosome and blocks Apaf-1 mediated caspase-9 activation. These scenarios require physical interaction of Htt with components of the apoptosome.

However, another plausible mechanism would occur after apoptosome formation. In this case, Htt would act to prevent caspase-9 activity independently of the apoptosome. This could occur through two possible pathways, which are not mutually exclusive: Firstly, Htt could interact with

the active caspase and block catalytic activity, thus acting like a cellular IAP protein; or Htt could interact with an intermediate in the caspase-9 activation process, thereby preventing caspase-9 from becoming fully active. It is also conceivable that different domains within Htt are able to perform both tasks.

However, the exact mechanisms that take place during the formation and activation of the apoptosome are not known. Neither are all of its components, which makes identification of the mechanisms of Htt function difficult.

Nonetheless, experimental verification of physical interaction with known components of the apoptosome is possible. This is most commonly done by overexpressing the target proteins in human embryonal kidney HEK293 cells followed by immunoprecipitation of one of the proteins. Interaction is then determined by Western blot with the respective antibodies. Additionally, formation of the apoptosome will be investigated using size-fractionation chromatography. Since the active apoptosome is a huge multi-protein complex of ~700kD it is possible to separate it from lower molecular weight complexes by size-exclusion Fast Protein Liquid Chromatography (FPLC) (Cain et al., 1999). Analysis will then be performed by Western blot of the individual collected fractions. By this technique, the effects of Htt on the induction of apoptosis and changes in apoptosome composition will be investigated.

Using these approaches, the molecular target of Htts caspase-9 inhibitory function will be identified. Furthermore, important clues towards the exact mechanism of Htt function will be gained by identification of the molecule targeted by Htt.

2 Results

2.1 No upregulation of heat shock proteins by Htt

Some heat shock proteins are able to inhibit caspase activities. In particular, it was demonstrated that HSP27, HSP90 and HSP70 inhibit cytochrome c-dependent caspase-9 activation, presumably by preventing recruitment of pro-caspase-9 to the apoptosome (Beere et al., 2000; Bruey et al., 2000; Pandey et al., 2000). Thus, it is a possibility that wtHtt exerts its function by transcriptional upregulation of heat shock proteins. In a collaboration with the lab of G. Cohen (University of Leicester, GB), lysates from all three cell lines were separated on SDS-PAGE and the level of heat shock protein expression was determined by Western blot. As shown (Fig. 1), no differences in the levels of HSP60, HSP70, HSC70 and HSP90 expression were observed. This indicates that upregulation of heat shock proteins is not involved in Htt protective function.

2.2 Htt is part of a large protein complex

HSP70 blocks caspase-9 activation by disruption of the apoptosome and inhibition of caspase-9 recruitment to Apaf-1. Htt might act in similarly. To test this possibility, and whether Htt is part of the apoptosome, cytoplasmic S-100 extracts from Htt expressing cells were size-fractionated using a Sephadex 200 filtration column. Cells were exposed to the non-permissive temperature to investigate the events that take place during apoptosome formation.

In the parental ST14A line at the permissive temperature (Fig. 2A), endogenous FL-Htt eluted with the high molecular weight fractions 5-9 that include molecular sizes between ~660kD-350kD. This suggests that FL-Htt forms Htt-Htt dimers or is part of a huge multi-protein complex. Possibly, an

equilibrium exists between Htt dimers and monomers. Apaf-1 eluted with fractions 6-11 (M_r~650kD-250kD) at molecular weights lower than expected for an active apoptosome. The data suggests that inactive Apaf-1 exists in various forms in a multi-protein complex, ranging from Apaf-1 dimers to higher order homomers or heteromers with unidentified molecules. Caspase-9 eluted in zymogen form with fractions 13-19 (M_r~220kD-180kD) as a higher molecular order complex, possibly with HSPs. Intriguingly, a Htt fragment was detected in fractions 14-19, co-eluting with caspase-9. As shown earlier, a short wtHtt fragment of 548 residues protected ST14A cells from apoptosis and inhibited cytochrome c-dependent caspase-9 activation. Co-elution of this fragment with caspase-9 tantalizingly hints at interaction between these two molecules. However, it is unclear how FL-Htt is processed in non-apoptotic cells.

The elution profiles looked slightly different in ST14A cells induced to undergo apoptosis by shifting to the non-permissive temperature in serum-deprived media (Fig.2B). FL-Htt eluted with fractions 5-11, indicating a change in the composition of proteins surrounding FL-Htt resulting in lower molecular weight complexes. The Htt fragment eluted in fractions 14-17. Caspase-9 elutes between fractions 9-19, indicating a different makeup of the molecules surrounding the caspase. Interestingly, in fractions 12-16 the p35 cleavage product was also observed, demonstrating activation of caspase-9. However, the lower molecular weight fractions 17-19 did not show processing of caspase-9, indicating that some of the caspase still existed in zymogen form. In marked contrast to the distribution at the permissive temperature, the Apaf-1 profile was expanded and the molecule was detected in fractions 5-12, and notably in fractions 13 and 14, where it partially co-eluted with caspase-9.

The shift of Apaf-1 to higher molecular weights suggests that an active apoptosome of ~700kD is formed. However, the significance of the shift to lower sizes is unclear. Possibly, a non-classical apoptosome is formed in ST14A cells in response to this particular apoptotic stimulus, which is smaller than the the classical apoptosome. The partially overlapping elution profiles of Apaf-1 and caspase-9 could be a sign of this different apoptosome. Nonetheless, the elution profiles alone do not clarify whether this small apoptosome represents an active or an inactive complex.

Concomitant caspase-9 activity assays showed no significant caspase-9 activity in any fraction at the permissive temperature indicating that no activated apoptosome was formed at regular growth conditions. However, two intense peaks of activity were observed at the non-permissive temperature in fractions 5-9 and 18-19, respectively, but no caspase-9 was observed in these fractions by Western blot.

This suggests that only a small fraction of available caspase-9 is recruited to the active apoptosome (fractions 5-9) or exists as free caspase-9 (18-19) that is below the detection limit of the Western blot. Most significantly, no caspase activity was observed in the fractions containg the Htt fragment and processed caspase-9. These data suggest that an active, classical apoptosome is formed, whereas the smaller Apaf-1 containing complex shows no activity.

In wtHtt expressing, cells the situation at the permissive temperature (Fig. 2C) resembled that of the parental line. Endogenous FL-Htt eluted with fractions 5-11, while the transfected fragment eluted in fractions 14-18. Caspase-9 co-eluted with the small Htt fragment in fractions 14-18, possibly suggesting association between the two molecules. Apaf-1 eluted from the column in fractions 9-12. At the non-permissive temperature (Fig. 2D) FL-

Htt was detected in fractions 7-11 and the N-terminal fragment in fractions 13-16. The elution profile of the Htt fragment again overlapped that of caspase-9, which also eluted in fractions 13-16. While the parental line showed clear processing of the caspase at the non-permissive temperature, no cleavage products were detected in wtHtt expressing cells. Most importantly, Apaf-1 remained unchanged and eluted with fractions 9-12, just as it did at the permissive temperature.

Measurement of caspase-9 activity revealed no significant catalytic activity in any fraction, paralleling the situation of the parental line at the non-permissive temperature. Thus, no changes at all were observed in wtHtt cells at the non-permissive temperature.

These data indicate that in wtHtt expressing cells recruitment of Apaf-1 to caspase-9 is impaired, both to higher and lower molecular weight complexes. This could be caused by association of the N-terminal Htt fragment with caspase-9, thus representing a possible mechanism for the observed effects of wtHtt on caspase-9 activity.

The elution profile of muHtt expressing cells at the permissive temperature (Fig. 2E) very closely resembled that of the other two cell lines. FL-Htt eluted with fractions 5-13, the N-terminal fragment eluted between fractions 14-17. Just like in wtHtt expressing cells, the profile of the Htt fragment matched that of caspase-9, which eluted in fraction 13-17. Apaf-1 was detected in fractions 7-13. At the non-permissive temperature (Fig. 2F), FL-Htt was detected in fractions 5-12, where it co-eluted with a Htt fragment (fractions 5-9). The Htt fragment was detected in fractions 13-17. The appearance of this fragment in the early fractions indicates that FL-Htt is extensively cleaved during apoptosis. Caspase-9 co-eluted with the Htt fragment in fractions 13-17. As in the parental cells, the p35 caspase-9

processing product was observed. Most interestingly, however, Apaf-1 eluted in fractions 6-12.

Thus, the Apaf-1 elution profile extends to higher molecular size, but not to lower sizes and thus does not overlap with the caspase-9 profile as observed with the parental line. This suggests that even the muHtt fragment inhibits Apaf-1/caspase-9 association and subsequent caspase activation. Thus, while a classical apoptosome is still formed, albeit at lower levels, the formation of the observed smaller apoptosome that is formed in the parental line is inhibited, just like in wtHtt expressing cells. Nonetheless, caspase-9 is processed, probably in an Apaf-1-independent manner as a result of the gain-of-function effect of the expanded poly-Q stretch.

2.3 Caspase-9 interacts with both wtHtt and muHtt

The gel filtration experiments described previously revealed partial co-elution between caspase-9 and the N-terminal Htt fragment. Thus, these two molecules could be part of the same protein complex and interact physically. To test this hypothesis, 293 cells were transiently transfected with expression constructs for a dominant-negative version of caspase-9, where the catalytic C is mutated to A, together with truncated forms of muHtt and wtHtt.

Cells that express the muHtt are susceptible to more apoptotic stimuli than cells that express wtHtt. This might be due to decreased interaction of caspase-9 with the glutamine-expanded mutant protein. After immunoprecipitation of the caspase, Western blot analysis revealed that the N-terminal Htt fragment strongly bound to caspase-9. In addition, both, muHtt and wtHtt, bound equally well to the caspase (Fig. 3). This data supports the gel filtration data and suggests a possible mechanism for the

anti-apoptotic function of the Htt protein. Furthermore, since these experiments were performed in high stringency buffer, the possibility of artifacts, often observed in 293 overexpression systems, is markedly reduced. Thus, interaction between Htt and caspase-9 represents a plausible basis for the observed effects of wtHtt.

2.4 WtHtt does not interact with Apaf-1

Because negative regulation of caspase-9 by wtHtt might also be brought about by negative regulation of Apaf-1, interaction of wtHtt with various Apaf-1 constructs was tested. The Apaf-1 constructs used were truncation mutants that contain either the CARD domain of Apaf-1 or the CARD domain plus the CED-4 homology domain, but not the WD40 repeats. Analogously to the previous experiments with caspase-9, Apaf-1 was immunoprecipitated and the immunocomplexes analyzed by Western blot. As shown, no significant interaction between Apaf-1 and wtHtt was observed (Fig. 4A). This is as expected, since the Htt constructs did not co-elute with Apaf-1 by size-exclusion chromatography. However, the elution profiles of FL-Apaf-1 and FL-Htt did overlap, therefore a possible association between the two FL molecules cannot be excluded.

2.5 WtHtt does not interact with Bcl-2 family members

In addition, analogous to the previous experiments, interaction of wtHtt with various members of the Bcl-2 family was tested in co-immunoprecipitation experiments. Since Bcl-2 family members act to regulate mitochondrial PT, interaction of Htt with these proteins was not expected. As shown, wtHtt did not significantly interact with any Bcl-2

family protein tested (Fig. 4B). This is consistent with data showing that Htt acts after mitochondrial cytochrome c release.

2.6 WtHtt preferentially interacts with the zymogen form of caspase-9

Preceding experiments identified caspase-9 as the prime target for Htt function excluded other candidates like Apaf-1 or Bcl-2 family members. These co-immunoprecipitations, however, were performed using a catalytically inactive version of caspase-9. This was done to avoid caspase-9 overexpression-induced apoptosis in 293 cells. In order to verify interaction with a fully active caspase, the experiments were repeated using the active wild type form of caspase-9. Again, the caspase was immunoprecipitated and the immunocomplexes were analyzed by Western blot. WtHtt preferentially interacted with DN-caspase-9, which retains a zymogen-like structure. No significant interaction was observed with the active, fully processed caspase (Fig. 5A).

2.7 WtHtt interacts with long pro-domain caspases

To test whether interaction was specific for caspase-9, 293 cells were transiently transfected with wtHtt and various catalyticaly inactive caspase constructs. Interestingly, Htt did not only interact with caspase-9, but also with caspase-8. However, no interaction was observed with the effector caspase-3 (Fig. 5B). The same results were obtained when muHtt was used (data not shown). Therefore, Htt only interacts with long pro-domain caspases, but not with short pro-domain caspases. This suggests not only a functional difference between these two classes of caspases, but presumably also a structural difference which Htt is able to recognize.

2.8 Htt binds the catalytic domain of caspase-9

Since Htt appeared to interact with long pro-domain caspases, like caspase-8, -9 and -10, but not with short pro-domain caspases, it was hypothesized that interaction was mediated by the CARD-domain of the long pro-domain caspases. Therefore, 293 cells were transiently co-transfected with Htt and constructs encoding either full-length caspase-9, only its CARD-domain or only its catalytic p30 subunit. Surprisingly, strong interaction was observed only when the catalytic domain construct was used, weaker interaction was seen with the full-length construct. No significant interaction was observed with the CARD-domain construct (Fig. 6A). The same results were obtained when equivalent caspase-10 constructs were used (Fig. 6B). Htt did not significantly interact with the CARD-domain, but only with the catalytic p30 domain of caspase-10. While these data identify the domain in the long pro-domain caspases responsible for interaction with Htt, it also suggests important structural differences between the catalytic domains of short and long pro-domain caspases. While these differences are hard to identify using protein sequences, apparently Htt easily distinguishes them.

2.9 Interaction between endogenous proteins

The gel filtration experiments showed that a N-terminal Htt fragment co-eluted together with caspase-9 in the same molecular weight fractions. This, however, is only indirect evidence for interaction. Direct evidence for Htt binding to caspase-9 came from co-immunoprecipitation experiments in HEK 293 cells. These experiments suffered from the massive overexpression of the two proteins that might force interaction. Although

they were performed in high stringency buffer that should disrupt any non-specific interactions, the 293 system does not represent the situation *in vivo*. In order to establish interaction between Htt and caspase-9 *in vivo*, co-immunoprecipitation experiments were performed in ST14A cells. The endogenous FL-Htt protein was immunoprecipitated and interaction determined by Western blot (Fig. 7). Immunoprecipitation with α-IgG isotype control AB did not result in co-immunoprecipitation of endogenous caspase-9. When the α-Htt AB was used, a faint band of co-immunoprecipitated caspase-9 was observed. This confirms further interaction between Htt and caspase-9.

2.10 Htt is upregulated in some cancer cell lines

The ability of Htt to influence the apoptosome and negatively regulate caspase-9 marks it as a potential oncogene. Dysregulation of Htt may be one strategy tumor cells employ to shut down apoptotic signaling pathways that would otherwise induce them to die. Accordingly, multiple tissue Northern blot analysis revealed that Htt is expressed at low levels in almost every tissue. However, robust upregulation of Htt was indeed observed in various tumor cell lines (Fig. 8) further stressing the important role Htt plays in regulating a key apoptotic process.

3 Discussion

The anti-apoptotic function of Htt takes place as far downstream as caspase-9. It leads to inhibition of caspase-9 catalytic activity and decreased caspase-9 processing. This makes the events surrounding caspase-9 activation the probable target of Htt action. It is currently clear that caspase-9 is activated through recruitment to the apoptosome (Li et al., 1997). However, the exact mechanisms remain unsolved. Cytoplasmic cytochrome c binds to Apaf-1 (Benedict et al., 2000), which leads to an ATP/dATP driven conformational change in Apaf-1. The second effect of cytochrome c binding is oligomerization of several Apaf-1 molecules (Adrain et al., 1999; Srinivasula et al., 1998; Zou et al., 1999). The exact stoichometry of this complex is unknown. The nature of any additional apoptosomal proteins has yet to be elucidated. The conformational change in Apaf-1 leads to pro-caspase-9 recruitment in a 1:1 ratio to Apaf-1. Subsequently, caspase-9 is rapidly activated (Qin et al., 1999).

The change in Apaf-1 conformation enables caspase-9 molecules, recruited to Apaf-1 via CARD-CARD domain interactions, to come in close proximity to each other. The caspase-9 zymogen has considerable intrinsic catalytic activity, which enables the juxtaposed molecules to cleave each other at D315 (Stennicke et al., 1999). Evidence points to an additional event that occurs between binding of caspase-9 to Apaf-1 and processing at D315. It appears that in order to get full activation and processing, the caspase has to undergo a conformational change into a semi-activated state. Only in this intermediate state can the intrinsic catalytic activity of the caspase become fully realized (Stennicke et al., 1999). Upon cleavage at D315 the active caspase-9 p10/p20 heterodimer is formed. The active caspase-9 dissociates

from the complex and cleaves and activates its direct downstream target pro-caspase-3.

Apparently, caspase-9 is a "special" caspase, because its activation takes place by a different mechanism than that of other (initiator) caspases. The situation is made more complicated by the enigmatic nature of the apoptosome, whose molecular members remain mostly unidentified. Additional regulation of caspase-9 activation occurs before apoptosome formation. It has been shown that HSP70 is able to inhibit apoptosome formation by binding the CARD of Apaf-1.

The gel filtration experiments only minimaly show recruitment of caspase-9 to an activated apoptosome complex, even in parental ST14A at the non-permissive temperature. This is probably a consequence of relatively low levels of caspase-9 expression in these cells so that the fraction that is recruited to the apoptosome lies below detection limits by Western blot. Furthermore, since these experiments were performed *ex vivo*, the apoptotic stimulus failed to reach all cells at the exact same time and the apoptotic program was probably not executed in a synchronized way. Nonetheless, recruitment and activation of caspase-9 must take place since, clearly, strong caspase-9 activity is observed in high molecular weight fractions (i.e. apoptosome) and lower molecular weight (i.e. free caspase). Thus, the apoptosome complex in ST14A cells is similar to the apoptosome complex of other cell types (Cain et al., 2000).

In cells protected by the N-terminal Htt fragment, no caspase-9 activity is observed, either in cytosolic extracts artificially activated by cytochrome c, or in *ex vivo* stimulated cells fractionated by FPLC. In addition, the widening of the Apaf-1 elution profile, seen in fractions of apoptotic parental cells, is not observed in wtHtt cells. This effect on Apaf-1

suggests that apoptosome formation is compromised in wtHtt expressing cells. While the lack of caspase-9 activity in the corresponding fractions supports that hypothesis, it cannot be ruled out that wtHtt directly affects the catalytic activity of caspase-9. Evidence for this possibility is seen in the matching elution profiles of caspase-9 and the N-terminal Htt fragment. Moreover, the function of Htt can possibly encompass both, the inhibition of catalytic activity as well as the blocking of apoptosome formation.

Interestingly, the muHtt fragment also inhibits the widening of the Apaf-1 elution profile towards low molecular weight demonstrating again that this fragment still possesses some wt function. Moreover, it co-eluted with caspase-9. Nonetheless, muHtt cells show some apoptosome formation and increased caspase-9 activity. However, they apparently die by a different mechanism than parental cells due to the sensitization effect, as demonstrated by caspase-3 activation. The gel filtration experiments provide a tantalizing clue toward an explanation of this observation. If muHtt still binds to caspase-9, it is possible that muHtt acquires the function to directly activate caspase-3. Therefore, muHtt acts as a "Trojan Horse", inhibiting caspase-9 while at the same time using its positioning at the center of this apoptotic pathway to directly activate downstream effector caspases. The observed cleavage of caspase-9 is then due to a retrograde cleavage/amplification process by downstream effector caspases.

Of the two known members of the apoptosome, Htt only interacts with caspase-9, but not with Apaf-1. Three independent lines of evidence show interaction. Caspase-9 co-elutes with the 548 amino acids N-terminal fragment of Htt on a size-fractionation column. In addition, this fragment co-immunoprecipitates with caspase-9 in the 293 overexpression system. Co-immunoprecipitation is also observed with the endogenous molecules.

Interaction appears to be stabilized by the presence of detergent, since immunoprecipitations of endogenous proteins that are performed in detergent-free buffer fail to support interaction (data not shown). Nonetheless, the 548 amino acids containing N-terminal wtHtt fragment still associates with caspase-9, even in the absence of detergent. It is exactly this fragment that contains anti-apoptotic function indicating interaction is crucial for execution of the anti-apoptotic activity. This has to be further confirmed by performing the experiments with a mutated Htt fragment that does not interact with caspase-9. If binding is a prerequisite for the anti-apoptotic function, this fragment would be expected not to provide protection from apoptosis.

Htt interacts with the catalytic p30 domain of caspase-9 and not with its CARD domain. This is surprising since Htt does not interact with short pro-domain caspases, suggesting that a second distinction exists between long and short pro-domain caspases that is based on different structures of the catalytic domains. The primary amino acid sequences offer no clues, since they are extremely similar. Nonetheless, these subtle sequence changes might account for a different tertiary structure that Htt is able to recognize. The solving of the crystal structure of caspase-9 will allow for comparison with the crystal structure of caspase-3 and identification of these structural differences.

Interaction of Htt with caspase-9 does not take place after the caspase has been activated since interaction is only observed with the DN form of caspase-9, which exists in zymogen form, but not with the wild type, active caspase. Interaction explains the observed complete inhibition of caspase-9 activity because wtHtt not only prevents recruitment of the caspase to the

apoptosome, and its subsequent activation, but also possibly directly inhibits the intrinsic catalytic activity of the caspase.

In this sense, the behaviour of Htt is very similar to that of XIAP. XIAP is a potent caspase inhibitor that is cleaved in the process of Fas-induced apoptosis. The resulting N-terminal fragment still inhibits caspases-3 and -7 (Deveraux et al., 1997; Deveraux et al., 1999), albeit at lower efficiency, which is probably due to further degradation. The C-terminal cleavage product, however, is a potent inhibitor of caspase-9. This seems to be reflected in the Htt mechanism, since FL-Htt is cleaved and releases the N-terminal fragment, which interacts with caspase-9. The mechanism of FL-Htt cleavage without apoptotic stimulus are unclear; however, during apoptosis caspase cleavage of Htt occurs which leads to the release of the N-terminal XIAP-like fragment.

Although the mechanism by which Htt inhibits caspase-9 is very similar to XIAP, IAPs have not been reported to affect apoptosome formation. Further, Htt lacks the characteristic BIR and RING domains of IAPs. Indeed, as mentioned earlier, Htt does not contain any domains that are recognized by BLAST or SMART database searches. Apart from the now identified anti-apoptotic N-terminal fragment and the cytotoxic effect of the expanded poly-Q stretch, no other functional data for any other part of Htt exists.

Nonetheless, this fragment comprises of 548aa and probably contains several discreet domains within. If this fragment indeed contains a dual nature, comprising of caspase-9 activity-inhibition and blockade of caspase-9 recruitment to the apoptosome, then it might be possible to separate these two functions by producing truncation mutants. However, it will be difficult to rationally design these mutants since no clues exist as to where the

domain boundaries lie. Nonetheless, these mutants will prove to be useful and indispensable tools to further understand Htt function.

Htt also interacts with the catalytic domain of other long pro-domain caspases, implying some universality in its function that is not restricted to caspase-9 and the apoptosome. It is possible that Htt acts as a general inhibitor of the catalytic function of long pro-domain caspases thus preventing initiator events. This is demonstrated by the fact that wtHtt protects from DR-induced apoptosis.

In the more specialized context of caspase-9, Htt is able to perform a second function, inhibiting formation of the apoptosome. This is important because of the central part of the apoptosome in integrating several different apoptotic signaling pathways, including the DR-pathway in type II cells. In order to perform its anti-apoptotic activity effectively, Htt has to go beyond inhibition of catalytic activity by preventing the preceding events.

4 Materials and Methods

4.1 Fast Protein Liquid Chromatography

Four 150mm confluent dishes each of ST14A, ST14A-wtHtt or ST14A-muHtt exposed to 39˚C for 12-24hrs were harvested in buffer A and cytoplasmic S-100 extracts were prepared as described. Around 800µg of extracts were loaded onto a Fast Protein Liquid Chromatography System (BioRad) equlibrated with buffer A and separated using a Sephadex 200 (10/24) column with a flow rate of 0.5ml/min. After discarding most of the void volume, fractions of 500µl were collected. Aliquots of the fractions were separated on SDS-PAGE and analyzed by Western blot using α-Htt (Chemicon), α-caspase-9 (MBL) and α-Apaf-1 (Pharmingen BD) antibodies.

4.2 Cell Culture and Transfections

HEK293 cells were grown at 37˚C in Dulbeccos Modified Eagle Medium (DMEM, BioWhittaker) supplemented with 10% FCS, 1%Pen/Strep and 1% glutamine. Transfections of 2-3x10^6 HEK293 cells were carried out using the Calciumphosphate method. Htt-N548wt and Htt-N548mt, caspase-3-AU1, myc-Apaf-1-3, myc-Apaf-1-3+4, HA-tagged Bcl-2 family member constructs and dominant-negative FLAG-tagged caspase-9, -8, -10 constructs were described previously.

4.3 Co-immunoprecipitation of overexpressed proteins

1x106 HEK 293 cells were transfected with 5µg each of the indicated plasmids. 24hrs post-transfection cells were harvested in NP-40 lysis buffer (20mM TRIS pH8, 137mM NaCl, 2mM EDTA, 10% glycerol, 1% NP-40, protease inhibitor tablets (BMB) 5mM Na_2VO_4, 0.5mM PMSF). Lysates

were adjusted to 500mM NaCl and the tagged proteins were immunoprecipitated for 4hrs at 4˚C with their respective antibodies. The immunocomplexes were precipitated with Protein-G beads and resolved by SDS-PAGE. Western blot analysis was performed using α-Htt (Chemicon), α-myc (BabCo), α-HA (BabCo), α-AU1 (BabCo) or α-FLAG M2 (SIGMA) antibody, respectively.

4.4 Co-immunoprecipitation of endogenous proteins

Eight 150mm plates of ST14A cells were grown to near confluency and shifted at 39˚C for 24hrs. Cells were harvested in NP-40 lysis buffer. Lysates were pre-cleared with BSA-blocked Protein-G Sepharose beads (SIGMA), and incubated for 4hrs at 4˚C with either α-Htt antibody or α-IgG (SIGMA). The immunocomplexes were resolved by SDS-PAGE and analyzed by Western blot with α-Htt antibody or α-caspase-9 antibody (MBL).

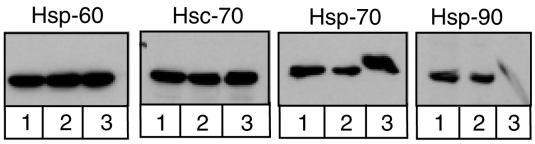
4.5 Northern blot analysis

Multiple Tissue Northern (MTN) Blots were obtained from CLONTECH. Htt-N548wt and β-Actin probes were labeled with ^{32}P-dATP using the Random Prime labeling kit (Gibco). Hybridization was carried out overnight, the blots were then washed and autoradiographed.

5 Figures and Legends

Figure 1

Equal amounts of lysates from ST14A (1), ST14A-wtHtt (2) and ST14A-muHtt (3) cells were separated by SDS-PAGE and Western blot was performed using the indicated antibodies. Lane (3) of the HSP90 blot represents an artifact caused by the end lane of the gel.

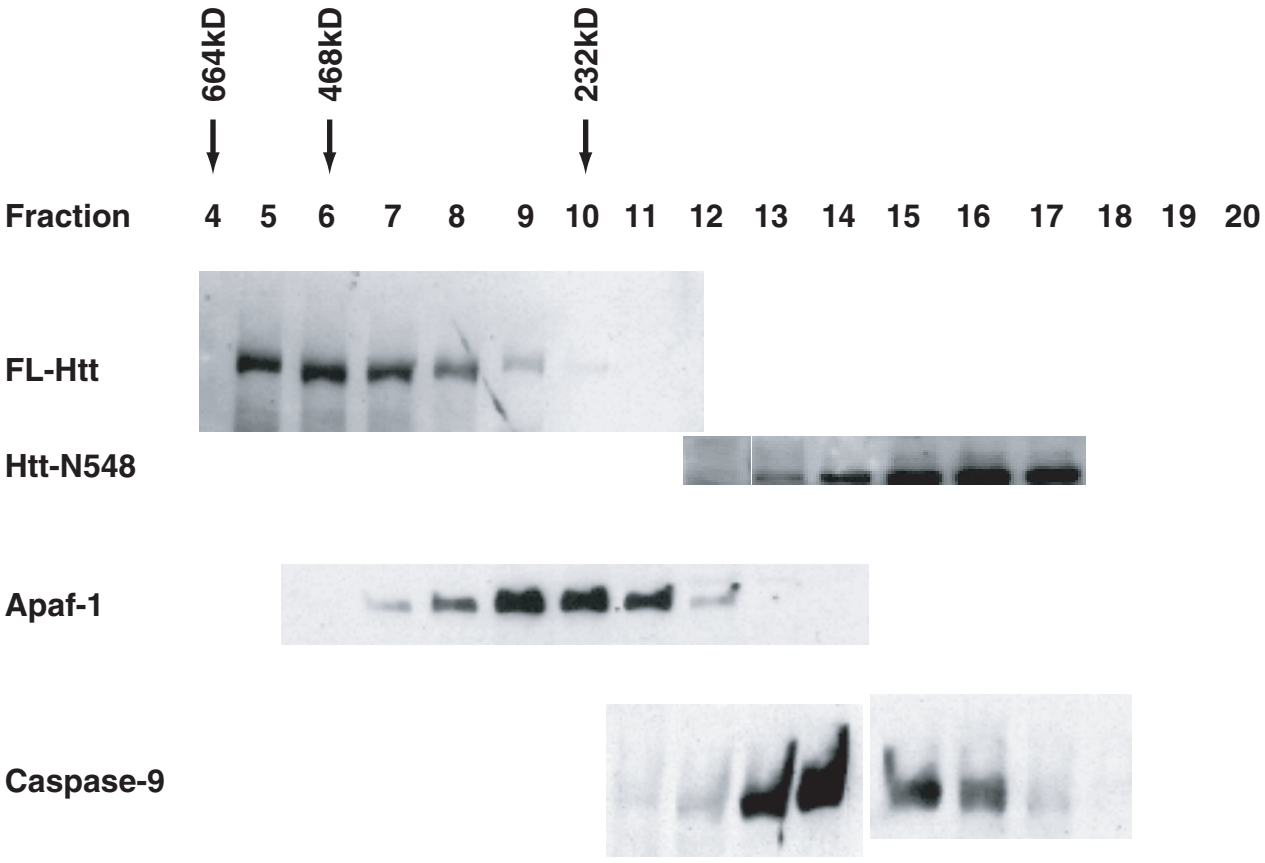


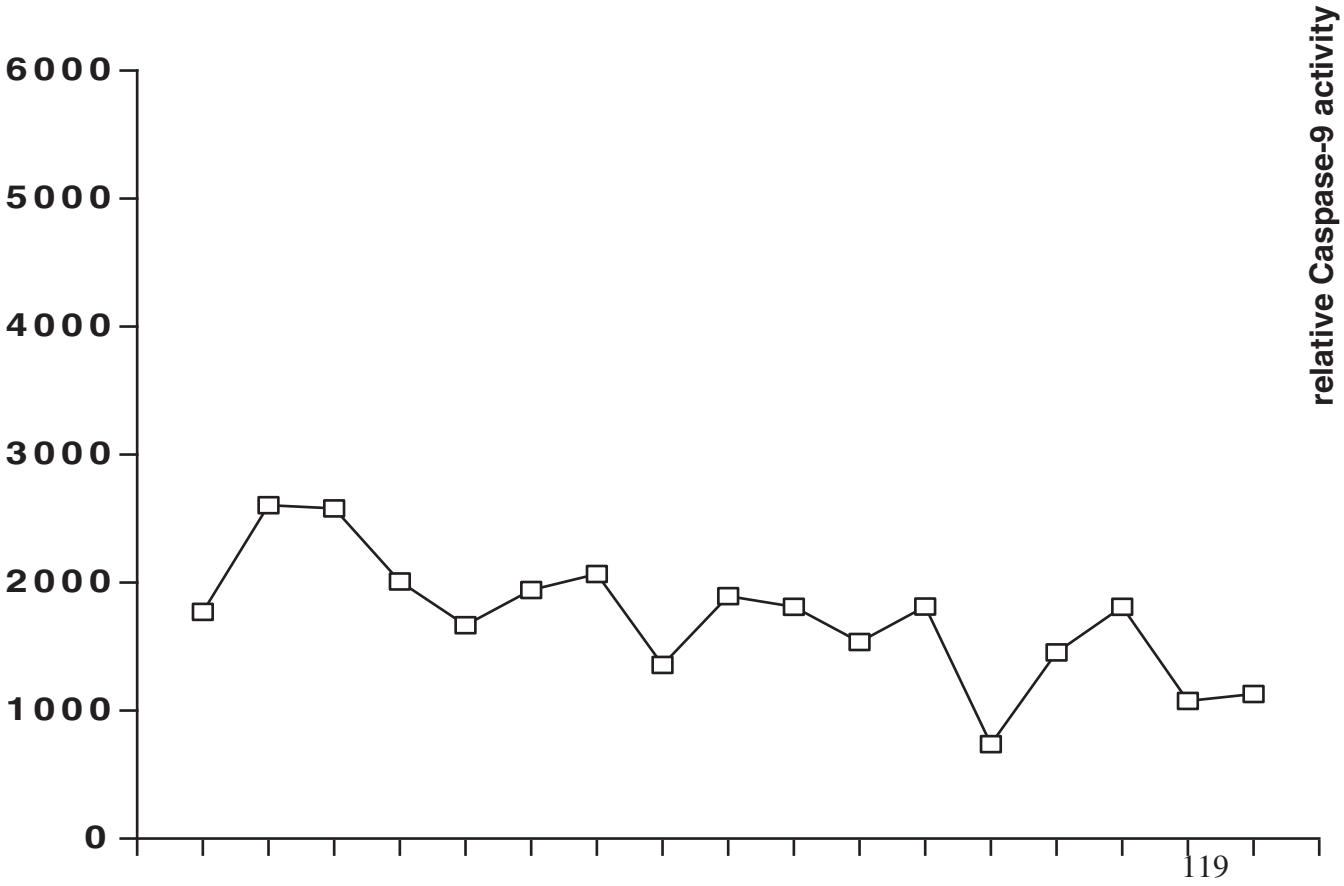
1. Parental ST14A Lysates
2. Wild-Type Htt(n548) Lysates
3. Mutant Htt(n548) Lysates

Figure 2

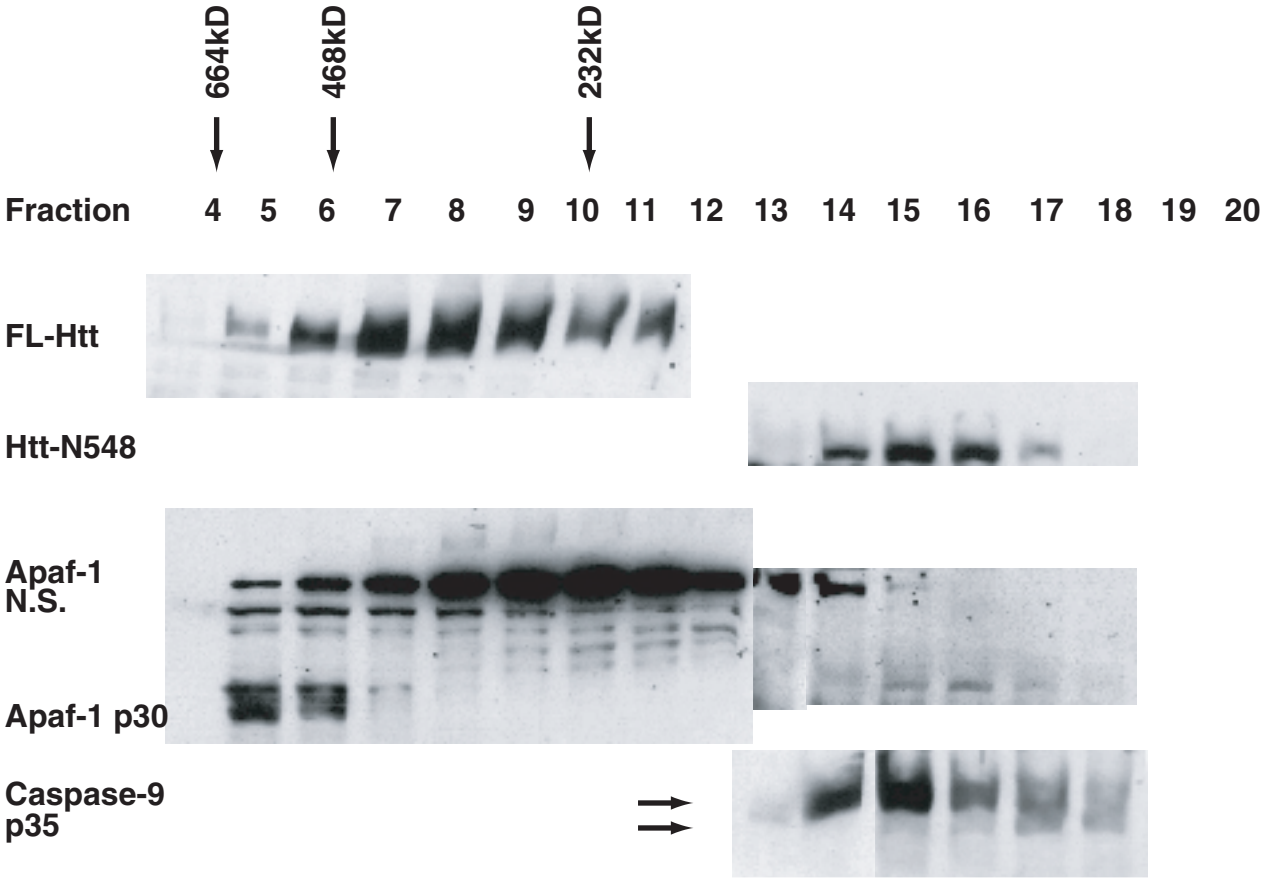
Cytosolic S-100 extracts of ST14A cell lines that were exposed to the non-permissive temperature were separated on a Sephadex 200 FPLC column. Samples of FPLC fractions were resolved on SDS-PAGE and analyzed by Western blot with the indicated antibodies. caspase activity measurements were performed with samples of equal volumes for all fractions using LECD-MAC as a substrate. Panels A, C, E show fractionated parental, wtHtt and muHtt cellular extracts, respectively, at the permissive temperature; panels B, D, F show fractionations of the same cell extracts at the non-permissive temperature.

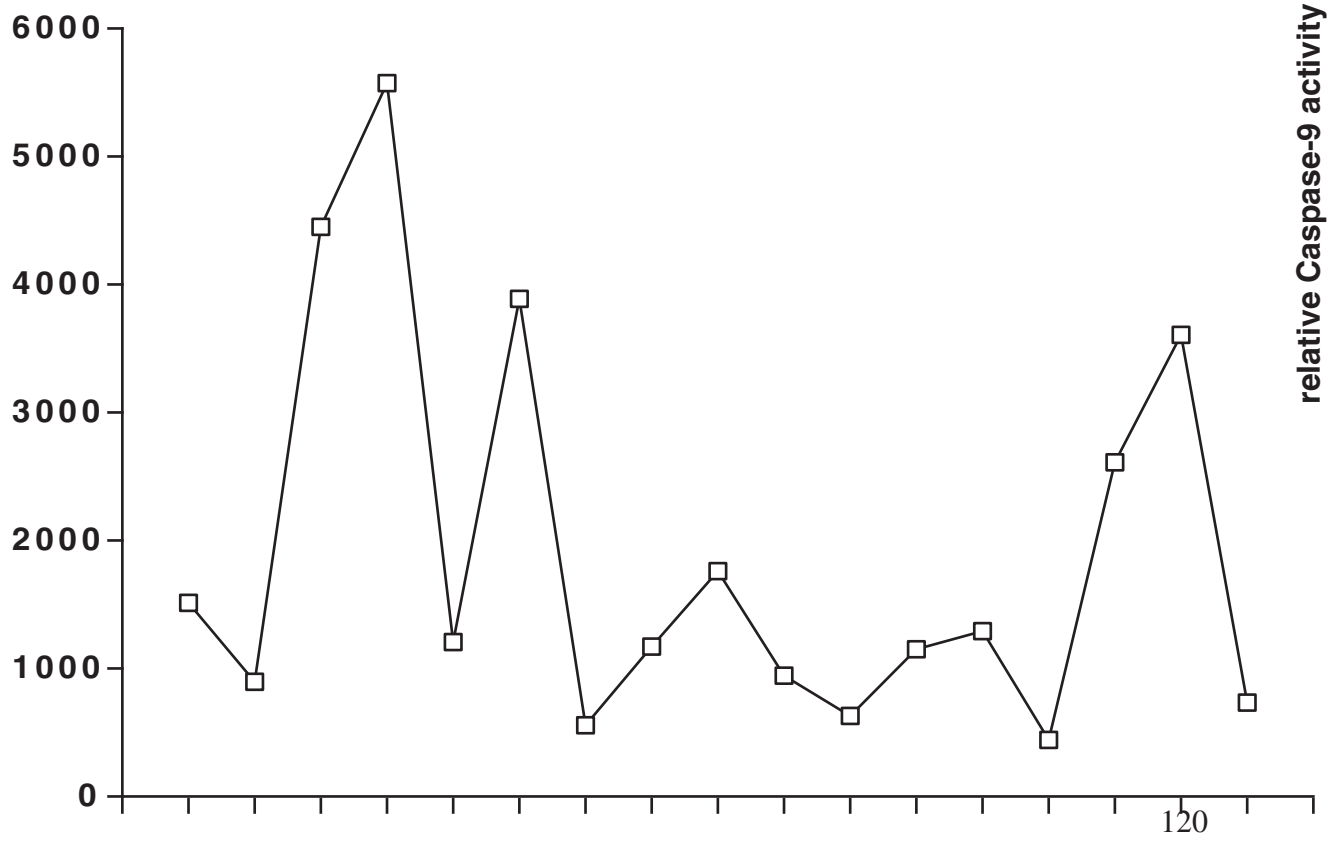
A ST14A, 33˚C


664kD
468kD
232kD
Fraction 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
FL-Htt
Htt-N548
Apaf-1
Caspase-9

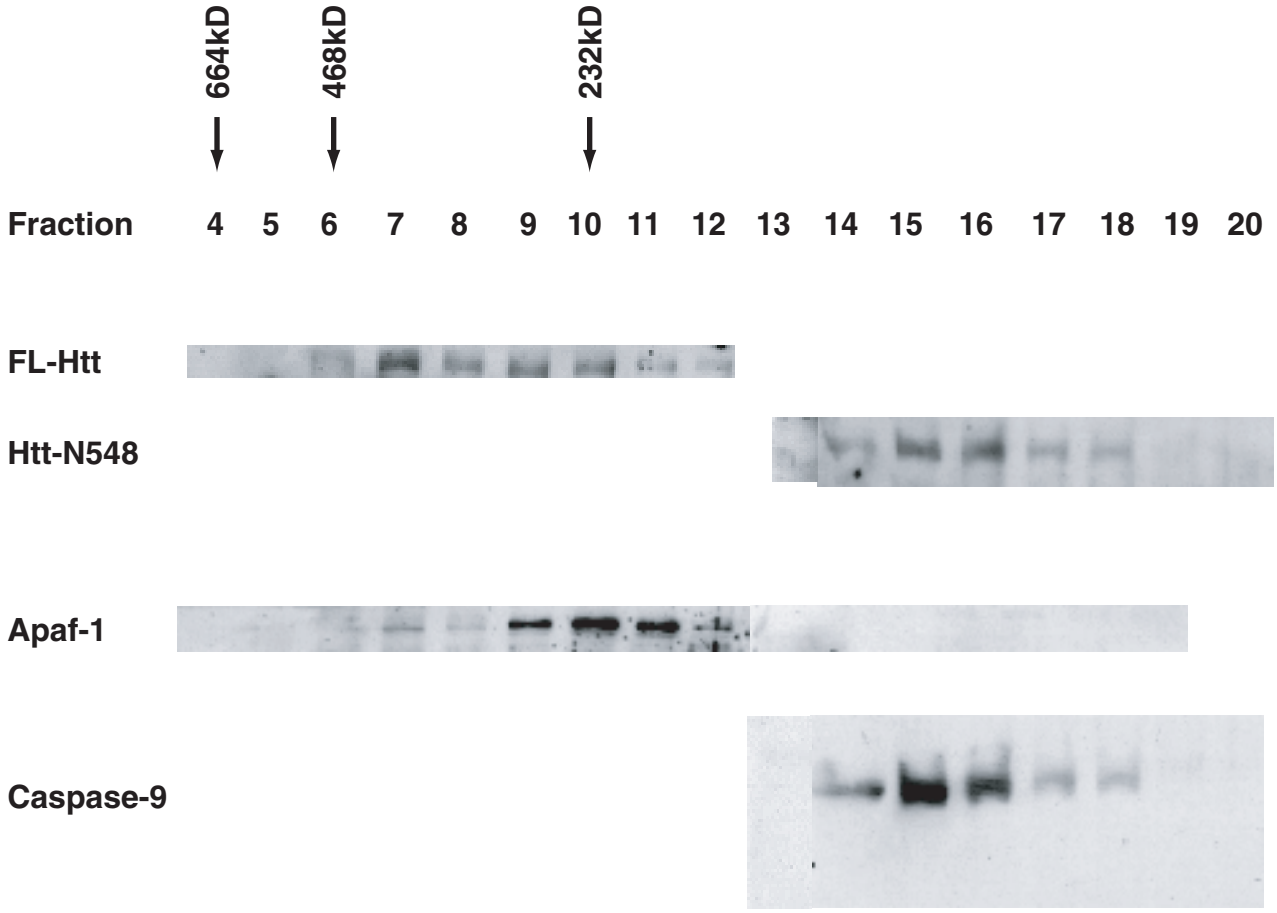

6000
5000
4000
3000
2000
1000
0
relative Caspase-9 activity

B ST14A, 39˚C

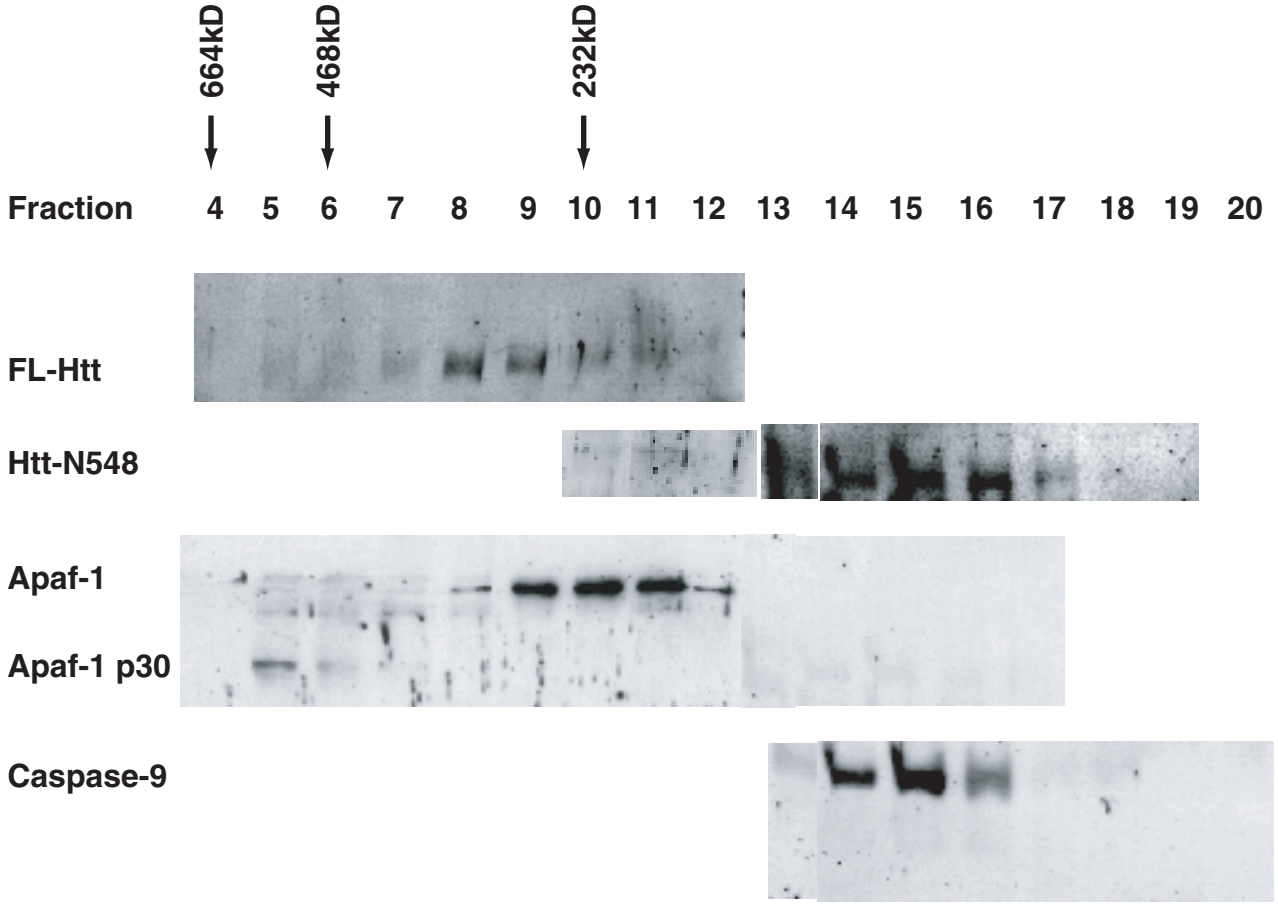

664kD
468kD
232kD
Fraction 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
FL-Htt
Htt-N548
Apaf-1 N.S.
Apaf-1 p30
Caspase-9 p35

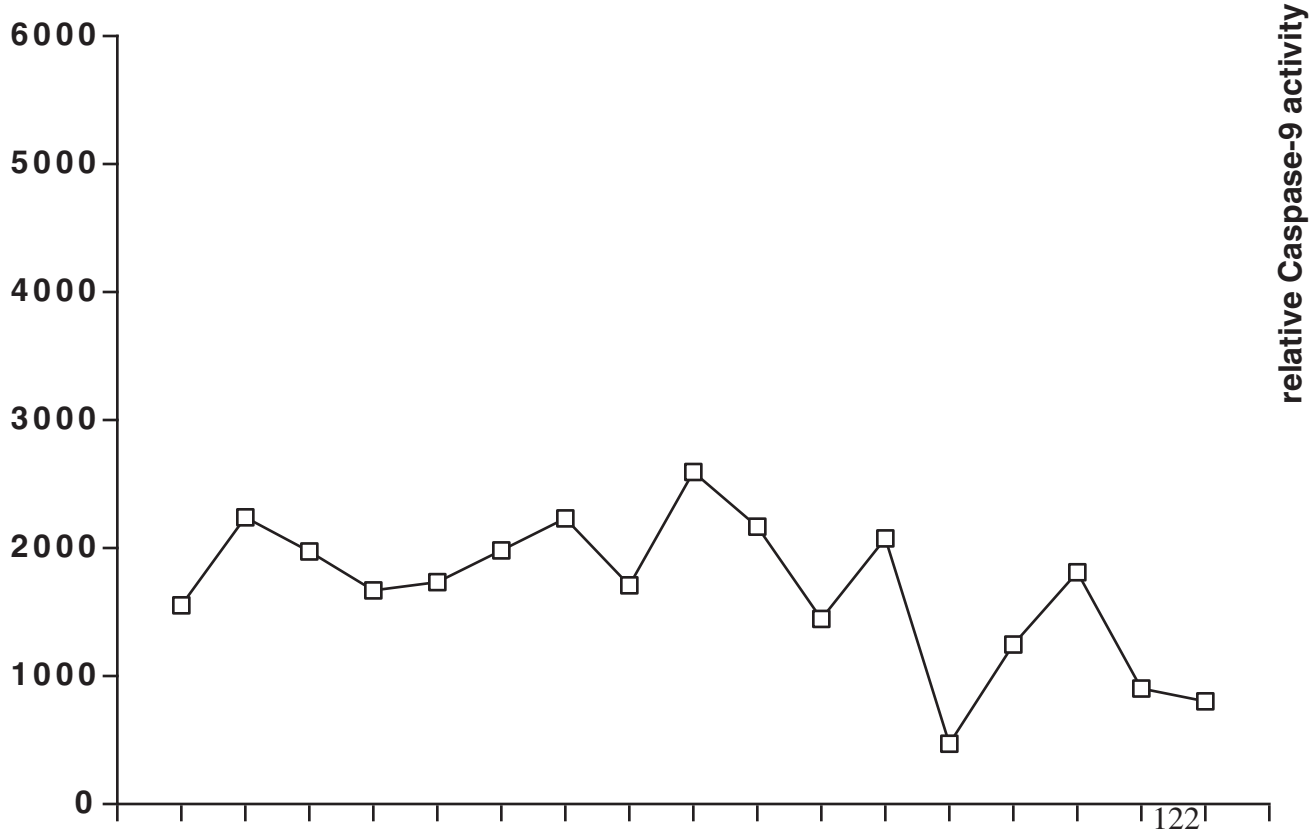

6000
5000
4000
3000
2000
1000
0
relative Caspase-9 activity

C ST14A-wtHtt, 33˚C

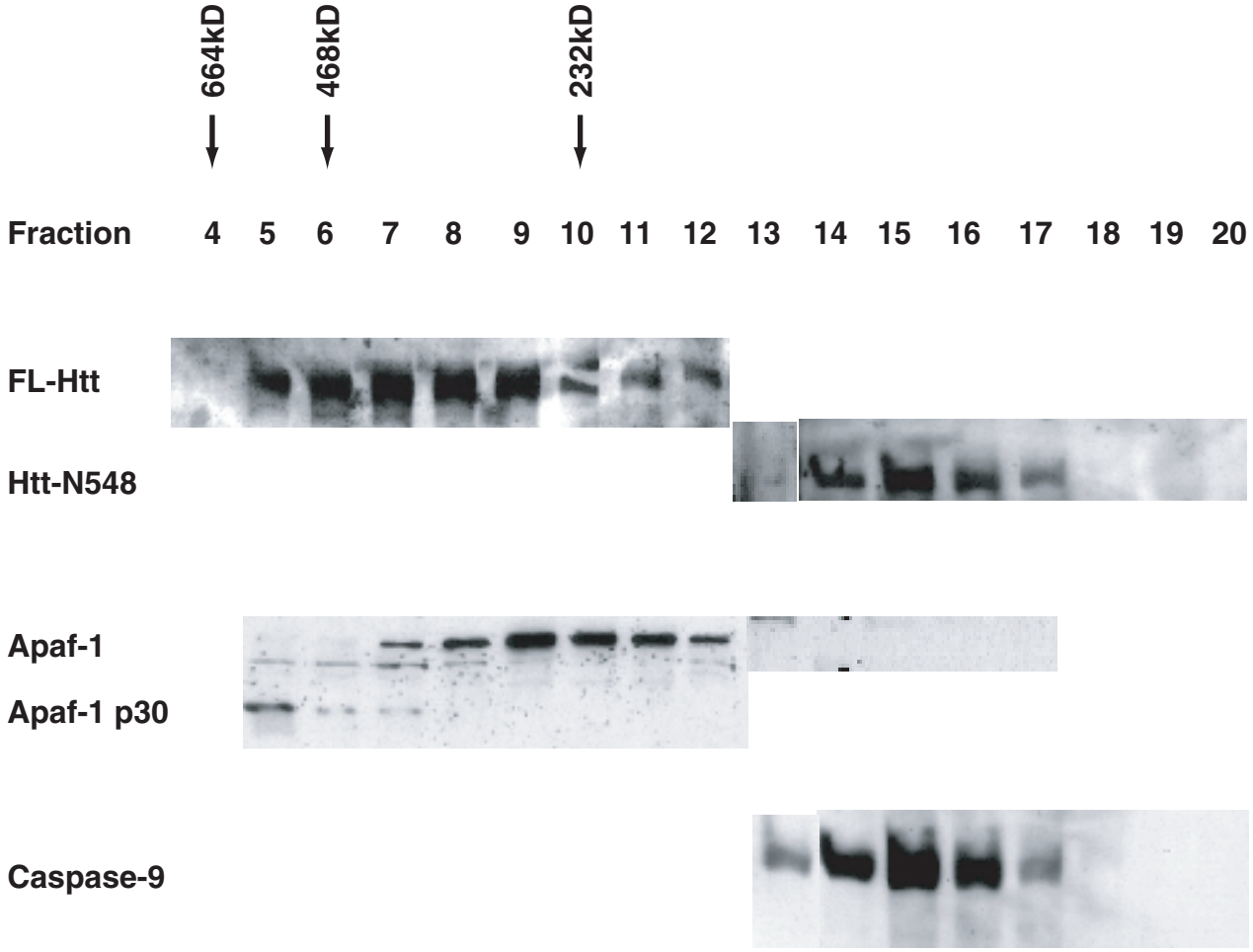


D ST14A-wtHtt, 39˚C





E ST14A-muHtt, 33˚C



F ST14A-muHtt, 39˚C

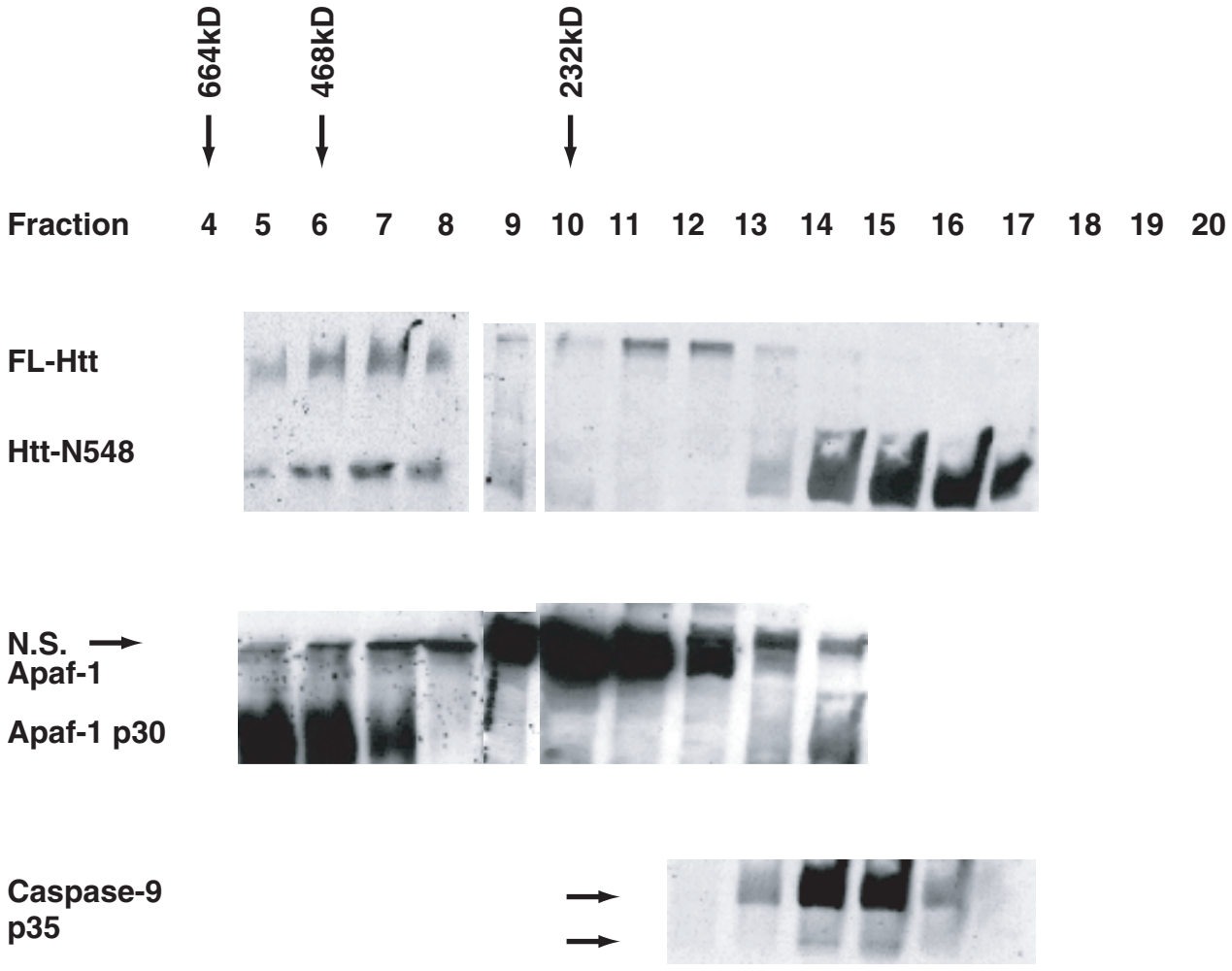


Figure 3

HEK293 cells were co-transfected with 5μg each of the indicated expression plasmids. 24hrs after transfection the cells were harvested and the lysates were immunoprecipitated with α-FLAG antibodies to immunoprecipitate the tagged caspase. After separation on SDS-PAGE and transfer, the membranes were probed with an α-Htt antibody or α-FLAG antibody, respectively. As shown, both, wtHtt and muHtt, interact strongly with caspase-9.

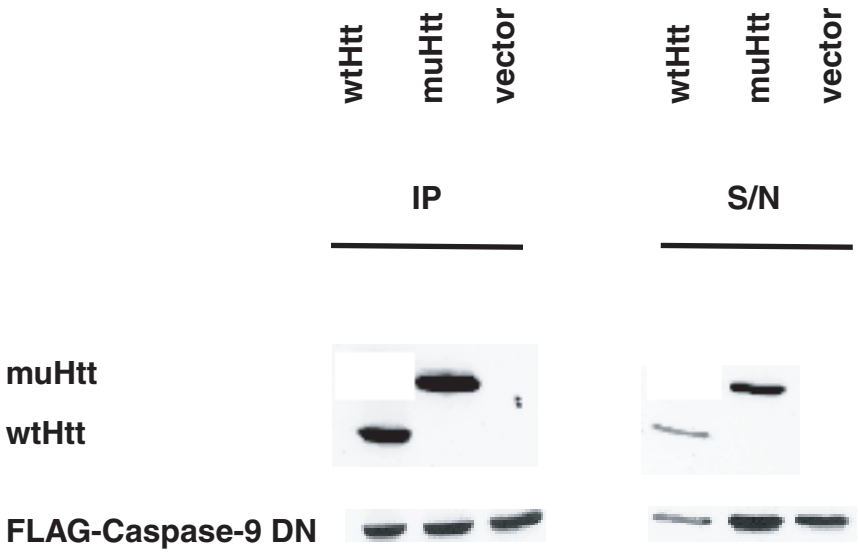

wtHtt
muHtt
vector
wtHtt
muHtt
vector
IP
S/N
muHtt
wtHtt
FLAG-Caspase-9 DN

Figure 4

HEK293 cells were transfected with the indicated expression constructs and immunoprecipitation was performed using α-HA or α-myc antibodies, respectively. After separation on SDS-PAGE, Western blot analysis was performed using α-Htt and α-HA or α-myc antibodies.

(A) No interaction of wtHtt is observed with either a short (no CARD, no WD40 domains) or a long (no WD40 domains) Apaf-1-myc construct.

(B) Likewise, no interaction between pro- or anti-apoptotic HA-tagged Bcl-2 family members and wtHtt is detected.

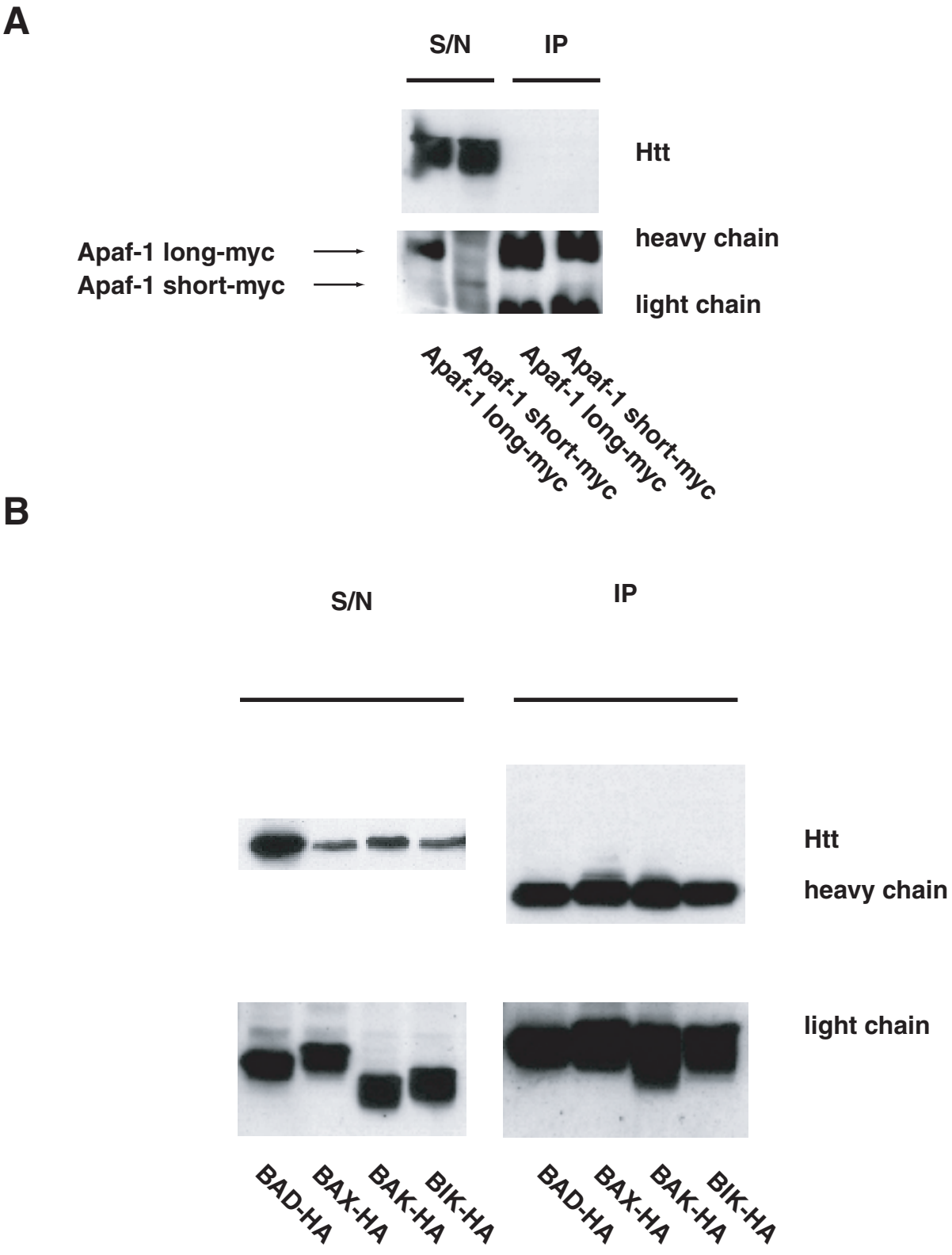

A
S/N
IP
Htt
heavy chain
Apaf-1 long-myc
Apaf-1 short-myc
light chain
Apaf-1 long-myc
Apaf-1 short-myc
Apaf-1 long-myc
Apaf-1 short-myc
B
S/N
IP
Htt
heavy chain
light chain
BAD-HA
BAX-HA
BAK-HA
BIK-HA
BAD-HA
BAX-HA
BAK-HA
BIK-HA

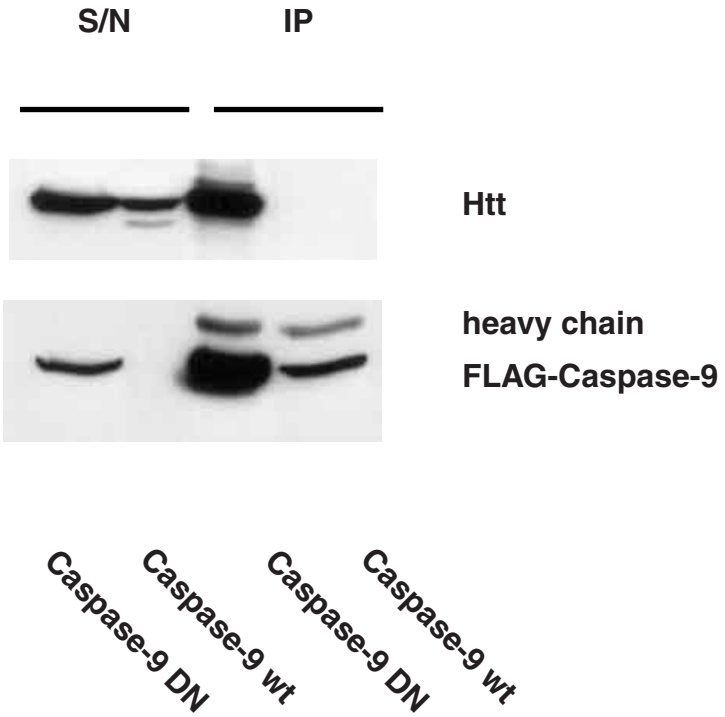
Figure 5

Expression constructs for various caspases were transfected into 293 cells together with wtHtt. After immunoprecipitation for the tagged caspase with α-FLAG or α-AU1 antibodies, respectively, Western blot analysis was performed with the α-Htt and α-FLAG or α-AU1 antibodies.

(A) WtHtt co-immunoprecipitated preferentially with a caspase-9 construct in which the catalytic C was replaced by A and which thus retains the conformation of the zymogen. No significant interaction was observed with the active caspase.

(B) Both caspase-8 and -9 are capable of interaction with Htt. The short pro-domain caspase-3, however, does not significantly bind Htt.

A


S/N
IP
Htt
heavy chain
FLAG-Caspase-9
Caspase-9 DN
Caspase-9 wt
Caspase-9 DN
Caspase-9 wt

B

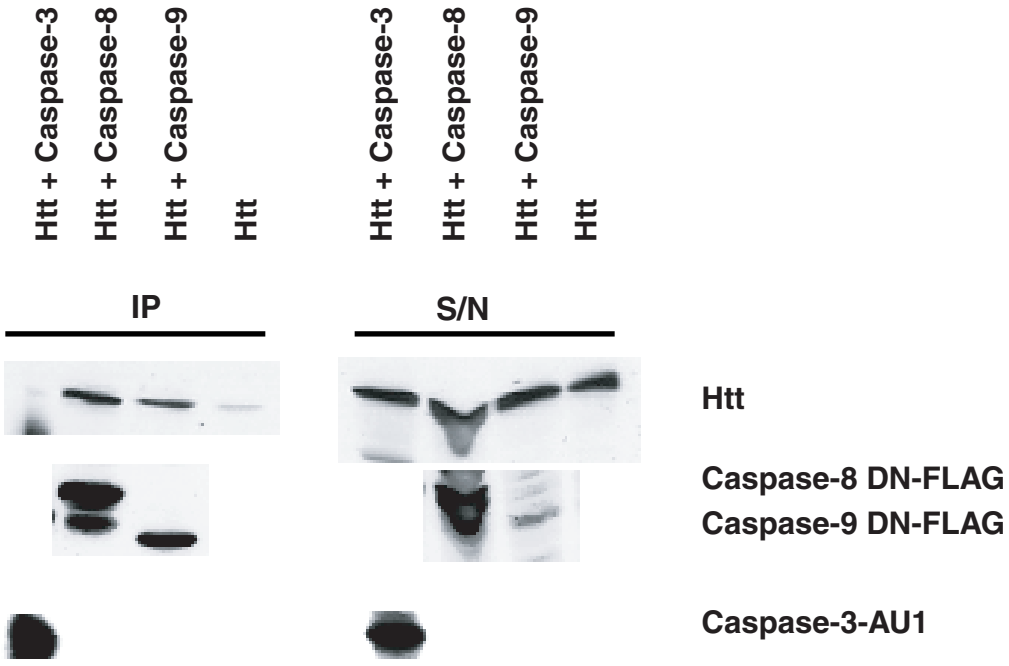

Htt + Caspase-3
Htt + Caspase-8
Htt + Caspase-9
Htt
Htt + Caspase-3
Htt + Caspase-8
Htt + Caspase-9
Htt
IP
S/N
Htt
Caspase-8 DN-FLAG
Caspase-9 DN-FLAG
Caspase-3-AU1

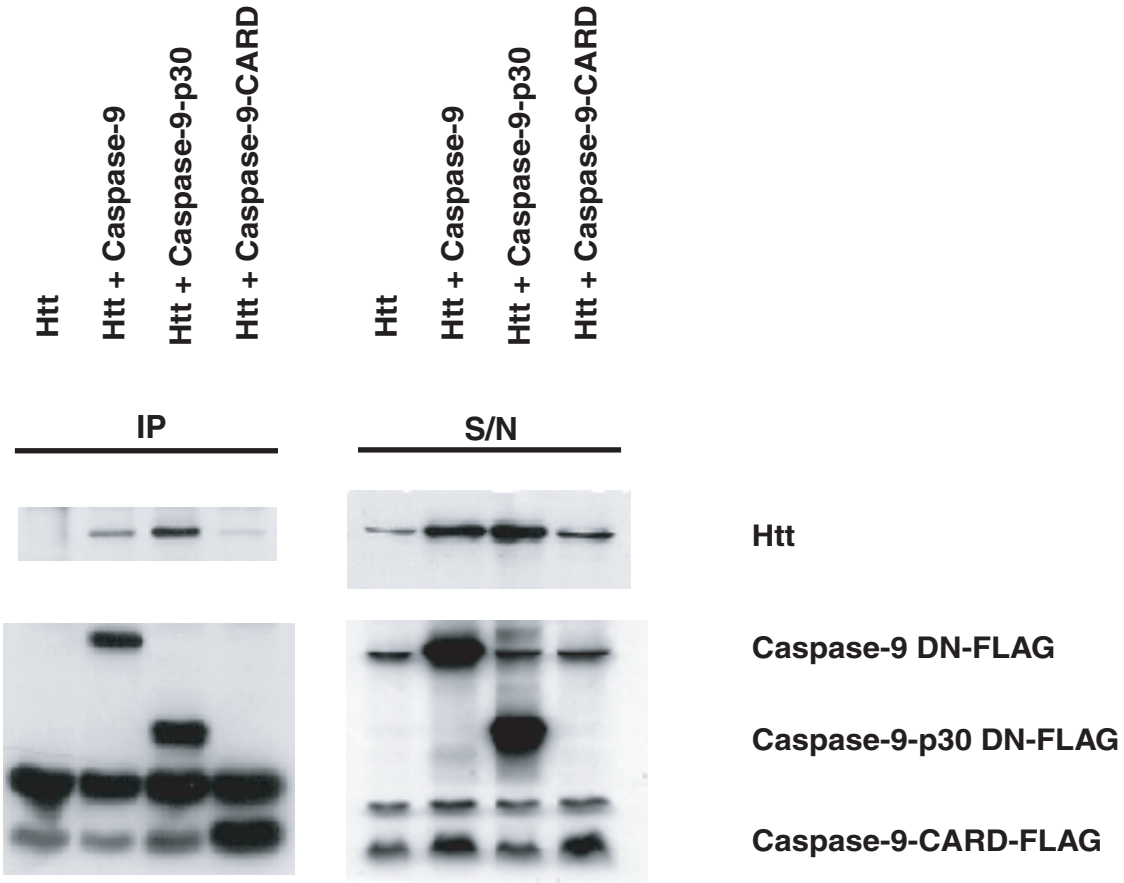
Figure 6

HEK293 cells were co-transfected with the indicated expression constructs. After cell lysis, immunoprecipitation with an α-FLAG antibody was performed to immunoprecipitate the FLAG-tagged dominant-negative caspase constructs, followed by Western blot with α-FLAG or α-Htt antibodies.

(A) Htt interacts strongly only with the catalytic domain of caspase-9 and weakly with full length caspase-9. No interaction is observed with the CARD-domain of caspase-9.

(B) Likewise, the same kind of specific interaction was observed when caspase-10 expression constructs were used.

A


Htt
Htt + Caspase-9
Htt + Caspase-9-p30
Htt + Caspase-9-CARD
Htt
Htt + Caspase-9
Htt + Caspase-9-p30
Htt + Caspase-9-CARD
IP
S/N
Htt
Caspase-9 DN-FLAG
Caspase-9-p30 DN-FLAG
Caspase-9-CARD-FLAG

B

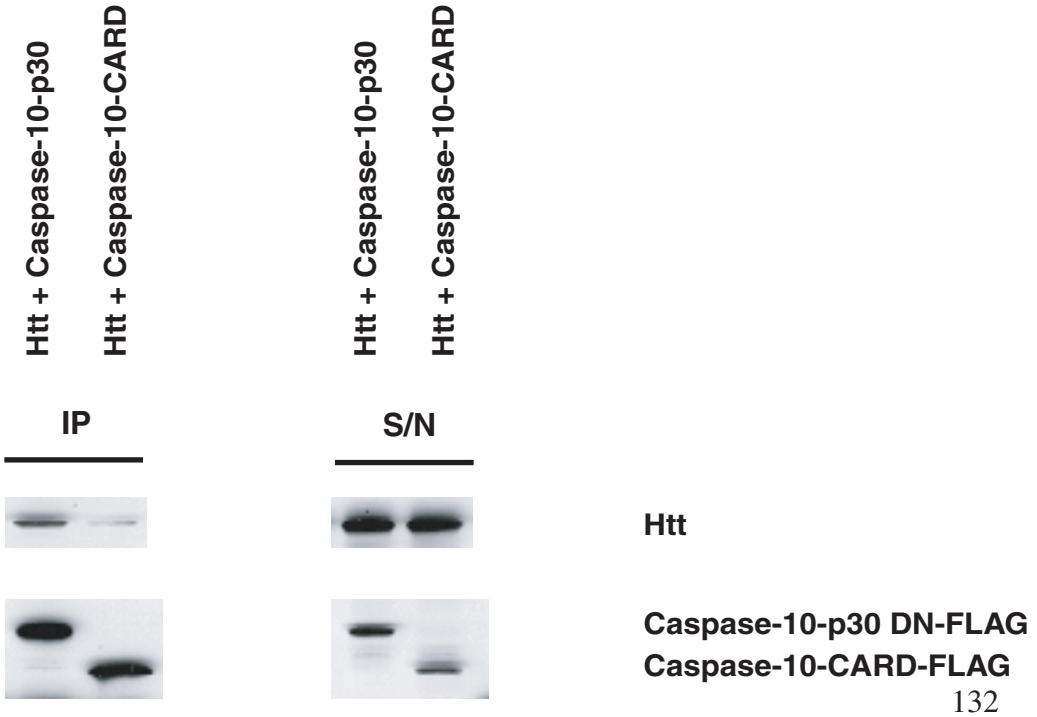

Htt + Caspase-10-p30
Htt + Caspase-10-CARD
Htt + Caspase-10-p30
Htt + Caspase-10-CARD
IP
S/N
Htt
Caspase-10-p30 DN-FLAG
Caspase-10-CARD-FLAG

Figure 7

Parental ST14A cells were harvested and low stringency co-immunoprecipitation was performed using α-Htt or α-IgG as a control, respectively. Immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot using α-Htt or α-caspase-9 antibodies. At both the permissive, as well as at the non-permissive temperature, a weak caspase-9 band was observed only when the α-Htt antibody was used, but not with the α-IgG control antibody. Insufficient separation between the heavy chain of the antibody and caspase-9 often leads to an obscuration of the caspase-9 band.

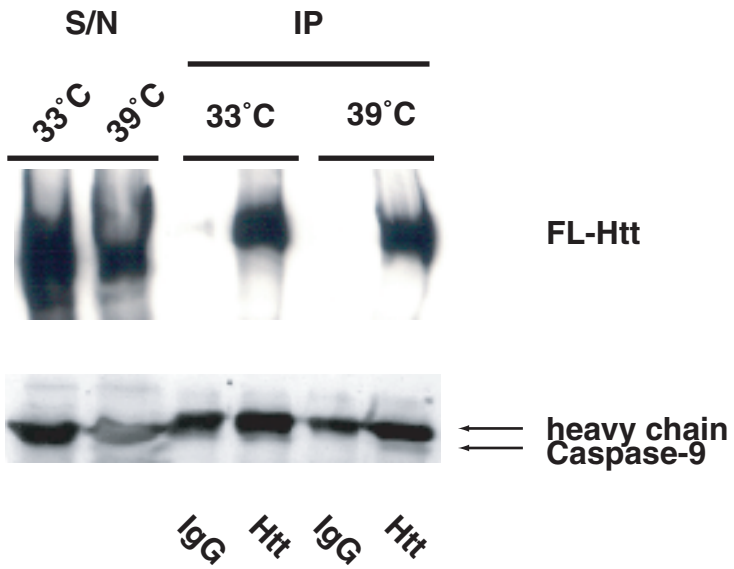
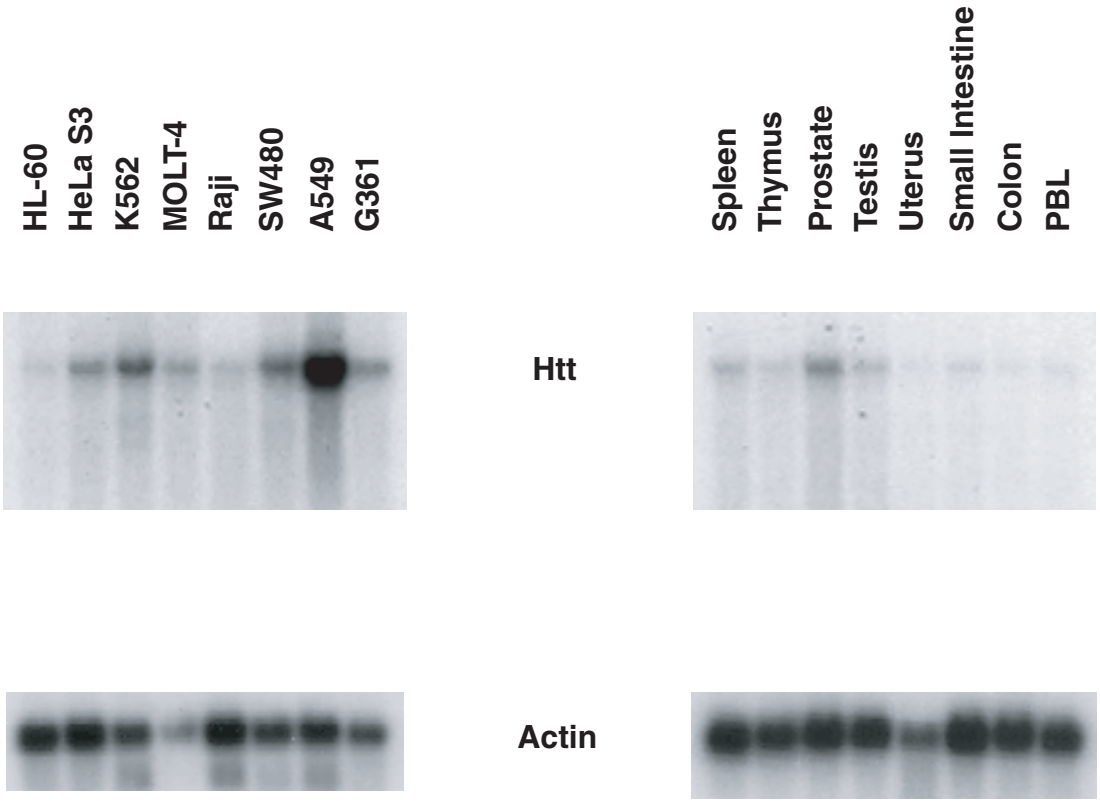

S/N
IP
33˚C
39˚C
33˚C
39˚C
FL-Htt
heavy chain
Caspase-9
IgG
Htt
IgG
Htt

Figure 8

Multiple tissue Northern blot analysis showed widespread expression of Htt. Moreover, it reveals that Htt is upregulated in various cancer cell lines. This indicates that Htt may play a role in transforming cells by blocking apoptotic pathways. Equal loading of the blots is confirmed by probing with an β-Actin probe.


HL-60
HeLa S3
K562
MOLT-4
Raji
SW480
A549
G361
Spleen
Thymus
Prostate
Testis
Uterus
Small Intestine
Colon
PBL
Htt
Actin

6 References

Adrain, C., Slee, E. A., Harte, M. T., and Martin, S. J. (1999). Regulation of apoptotic protease activating factor-1 oligomerization and apoptosis by the WD-40 repeat region. J Biol Chem *274*, 20855-60.

Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000). Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the apaf-1 apoptosome. Nat Cell Biol *2*, 469-75.

Benedict, M. A., Hu, Y., Inohara, N., and Nunez, G. (2000). Expression and functional analysis of Apaf-1 isoforms. Extra Wd-40 repeat is required for cytochrome c binding and regulated activation of procaspase-9. J Biol Chem *275*, 8461-8.

Bruey, J. M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S. A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A. P., Kroemer, G., Solary, E., and Garrido, C. (2000). Hsp27 negatively regulates cell death by interacting with cytochrome c. Nat Cell Biol *2*, 645-652.

Cain, K., Bratton, S. B., Langlais, C., Walker, G., Brown, D. G., Sun, X. M., and Cohen, G. M. (2000). Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. J Biol Chem *275*, 6067-70.

Cain, K., Brown, D. G., Langlais, C., and Cohen, G. M. (1999). caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex. J Biol Chem *274*, 22686-92.

Chu, Z. L., Pio, F., Xie, Z., Welsh, K., Krajewska, M., Krajewski, S., Godzik, A., and Reed, J. C. (2001). A novel enhancer of the Apaf1 apoptosome involved in cytochrome c-dependent caspase activation and apoptosis. J Biol Chem *276*, 9239-45.

Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., and Reed, J. C. (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. Embo J *18*, 5242-51.

Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-4.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-89.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D., and Kharbanda, S. (2000). Negative regulation of cytochrome c-mediated oligomerization of apaf-1 and activation of procaspase-9 by heat shock protein 90. Embo J *19*, 4310-22.

Qin, H., Srinivasula, S. M., Wu, G., Fernandes-Alnemri, T., Alnemri, E. S., and Shi, Y. (1999). Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1. Nature *399*, 549-57.

Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell *1*, 949-57.

Stennicke, H. R., Deveraux, Q. L., Humke, E. W., Reed, J. C., Dixit, V. M., and Salvesen, G. S. (1999). caspase-9 can be activated without proteolytic processing. J Biol Chem *274*, 8359-62.

Zou, H., Li, Y., Liu, X., and Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem *274*, 11549-56.