Huntingtin promotes cell survival by preventing Pak2 cleavage

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Summary
Huntington’s disease is caused by a polyglutamine expansion in the huntingtin protein. Wild-type huntingtin, by contrast, appears to protect cells from pro-apoptotic insults. Here we describe a novel anti-apoptotic function for huntingtin. When cells are exposed to Fas-related signals, the ubiquitously expressed p21-activated kinase 2 (Pak2) can be activated via cleavage by caspases to release a constitutively active C-terminal fragment, which mediates cell death. Our data show that huntingtin interacts with Pak2. Overexpression of huntingtin significantly inhibits caspase-3-mediated and caspase-8-mediated cleavage of Pak2 in cells. Moreover, huntingtin prevents Pak2 cleavage by caspase-3 and caspase-8 in vitro.

Introduction
Huntingtin (Htt) is a large (348 kDa), ubiquitously expressed protein. An expanded polyglutamine (polyQ) stretch close to its N-terminus causes Huntington’s disease (HD), an autosomal-dominant, progressive, neurodegenerative disorder (Trottier et al., 1995). Normal alleles have 37 or fewer glutamines in this polymorphic tract, and 38 or more of these residues cause disease (Rubinsztein et al., 1996). In the disease range, longer polyQ stretches cause earlier symptom onset.

The exact mechanism(s) of cellular toxicity caused by mutant Htt are not completely understood, although many proteins have been identified as Htt-interacting partners and numerous different processes have been suggested. Although genetic and transgenic data argue that the primary toxicity caused by the mutation causing HD is via a gain-of-function mechanism, there have been suggestions that loss-of-function(s) resulting from the polyQ expansion might also contribute to toxicity (Cattaneo et al., 2005). A number of studies have shown that wild-type Htt can protect against a range of proapoptotic insults (Ho et al., 2001; Leavitt et al., 2001; Rigamonti et al., 2000; Rigamonti et al., 2001; Zhang et al., 2006). Htt is essential for embryonic development: its complete inactivation in mice causes embryonic death before day 8.5 (prior to gastrulation and the formation of the nervous system) (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995).

The p21-activated kinases (Paks) are serine-threonine kinases that are activated by small GTPases such as Cdc42 and Rac. Six mammalian Paks (Pak1-Pak6) have been identified to date. Paks contain catalytic C-terminal that are highly conserved. Mammalian Paks have been divided into two subfamilies according to their sequence similarity: Pak1-Pak3 and Pak4-Pak6 (Abo et al., 1998; Bagrodia et al., 1995; Cau et al., 2001; Dan et al., 2002; Jaffer and Chernoff, 2002; Manser et al., 1995; Manser et al., 1994; Pandey et al., 2002; Teo et al., 1995; Yang et al., 2001). In response to extracellular signalling, Paks are activated and regulate cell morphology and motility (Bokoch, 2003; Hofmann et al., 2004; Manser et al., 1997).

Paks also regulate cell survival and death in response to cellular stimuli. Full-length Pak1, Pak2, Pak4 and Pak5 have been reported to have anti-apoptotic roles (Cotteret et al., 2003; Jakobi et al., 2001; Qu et al., 2003; Schurmann et al., 2000). In the first Pak subfamily, Pak1 and Pak3 are tissue-specific and highly expressed in the brain, but Pak2 is ubiquitously expressed (Manser et al., 1995; Manser et al., 1994; Teo et al., 1995). Also, in contrast to Pak1 and Pak3, Pak2 can be activated via cleavage by caspase proteases to release a constitutively active C-terminal fragment (Pak2p34). Caspase-activated Pak2p34 has been observed after cells are exposed to death stimuli such as Fas ligand, TNFα, heat shock or UV radiation (Chan et al., 1998; Rudel and Bokoch, 1997; Tang et al., 1998). Studies have suggested that the formation of Pak2p34 mediates cell death in response to these stimuli and that it is an essential mediator of cell death in response to Fas-related signals (Rudel and Bokoch, 1997; Rudel et al., 1998).

Recently, we identified Pak1 as an Htt-binding protein (Luo et al., 2008). Here, we investigated the ability of Htt to physically interact with Pak2 and thus prevent Pak2 cleavage. Our data show that overexpression of Htt significantly inhibits caspase-mediated cleavage of Pak2 in cells. Also, we show that Htt reduces caspase-3- and caspase-8-mediated cleavage of Pak2 in vitro. Although Htt is cytoprotective in wild-type cells exposed to TNFα (or insults causing caspase activation, such as leukaemia-inhibitory factor or serum withdrawal from embryonic stem cells), it has no significant benefit in TNFα-treated cells with Pak2 knockdown. Thus, huntingtin exerts anti-apoptotic effects by binding to Pak2, which reduces the abilities of caspase-3 and caspase-8 to cleave Pak2 and convert it into a mediator of cell death.

Key words: Huntingtin, Caspase, Pak2, TNFα

Although huntingtin is cytoprotective in wild-type cells that are exposed to TNFα, it has no significant benefit in TNFα-treated cells with Pak2 knockdown. Thus, huntingtin exerts anti-apoptotic effects by binding to Pak2, which reduces the abilities of caspase-3 and caspase-8 to cleave Pak2 and convert it into a mediator of cell death. Supplemental material available online at http://jcs.biologists.org/cgi/content/full/122/6/875/DC1
Results

Htt binds to Pak2 and prevents Pak2 cleavage in cells

We recently identified Cdk5 as an Htt-binding protein, and showed that Cdk5 phosphorylates Htt to prevent its cleavage by caspases (Luo et al., 2005). Accordingly, we investigated the issue of whether other kinases or their substrates can bind Htt. Using immunoprecipitation, we found that Pak1 is an Htt-binding protein and that Htt interacts with the Pak1 C-terminus (Luo et al., 2008). As this region is highly conserved between Pak1 and Pak2, we investigated whether Htt also interacts with Pak2. Htt is a large and caspase-sensitive protein comprising 3144 amino-acid residues; Htt residues 1-586 (Htt586) and Htt residues 1-552 (Htt552) are two of the prominent caspase-cleavage products that include the mutant polyQ tract. Htt552 has been identified as a physiological fragment in human brain (Wellington et al., 2002). For this study, we used Htt N-terminal fragments such as Htt552 and Htt588.

We first tested and confirmed that Htt binds to Pak2 in transfected cells (Fig. 1A). Htt residues 316-488 (Htt316-488), Htt316-513 and Htt316-552 were not able to bind to Pak2, suggesting that the N-terminus of Htt is responsible for the Pak2-Htt interaction (Htt552 is wild-type with 17 glutamine repeats). We confirmed that both the first 190 residues of wild-type Htt (Htt190) and the first 315 residues (Htt315) were able to bind Pak2, although these interactions were much weaker than with Htt552 or Htt588 (Fig. 1A). These data suggest that Htt190 directly mediates Htt–Pak2 interaction, but that Htt316-552 enables much stronger binding. The ability of different Htt fragments to bind Pak2 is summarised in Fig. 1B. We also confirmed the Pak2-Htt interaction by immunoprecipitation of either Htt (Fig. 1C) or Pak2 (using different sources of Pak2 and control antibodies) in mouse brain lysate (Fig. 1D; supplementary material Fig. S1).

To test whether Htt binds to Pak2 independently of Pak2 kinase activity, we transfected wild-type Pak2, the kinase-active mutant Pak2-T402E or the kinase-dead mutant Pak2-K278R with Htt588. Supplementary material Fig. S2 shows that all three forms of Pak2 bind similarly to Htt, indicating that Htt binding to Pak2 is independent of its kinase activity.

As Pak2 is readily cleaved by active caspase-3 after various cell-death signals (Rudel and Bokoch, 1997), we tested whether Htt could protect Pak2 from cleavage under these conditions. As a death stimulus, TNFα induces two different signals that diverge downstream of the TNF-receptor-interacting protein TRADD (TNF receptor-associated death domain) (Hsu et al., 1996). One signal activates NF-kB, whereas the other leads to apoptosis. Certain anti-apoptotic genes are downstream targets of NF-kB, protecting the cell from apoptosis, and inhibition of NF-kB activation potentiates TNFα-induced apoptosis (Beg and Baltimore, 1996; Hsu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). The protein-synthesis inhibitor cycloheximide (CHX) enhances TNFα-induced apoptosis, presumably owing to inhibition of de novo synthesis of TNFα-induced gene products that protect cells from apoptosis (Hsu et al., 1996).

We transfected Pak2 with increasing levels of wild-type Htt588, then treated cells with TNFα and CHX (TNFα+CHX) to induce caspase activation and cell death. As Htt levels increased, we observed a dose-dependent reduction in N-terminal Pak2 cleavage (Fig. 2A). In supplementary material Fig. S3, data from three experiments show that Pak2 cleavage gradually decreases with increasing amounts of Htt588 in cells treated with TNFα+CHX.

Interestingly, we also observed the Pak2 cleavage fragment in Pak2-transfected cells that were not treated with death stimuli (Fig. 2B); presumably this cleavage occurs because of noninduced endogenous caspase activity. This Pak2 cleavage was also reduced by full-length Htt (Fig. 2B). As degradation of Pak2 fragments is proteasome-dependent (Jakobi et al., 2003), we confirmed that the ability of Htt to modulate levels of Pak2 cleavage product is proteasome-independent, by showing that Htt also reduced the levels of the Pak2 cleavage fragment in cells treated with the proteasome inhibitors epoxomycin and lactacystin (Fig. 2B, EPX and Lact, respectively). Both lactacystin and epoxomycin gave identical results, which confirms that the effect of Htt on Pak2 cleavage is independent of proteasome activity (Fig. 2B). We observed that epoxomycin and lactacystin can cause cell death (supplementary material Fig. S4A). Pak2 cleavage might also contribute to cell death that is induced by the proteasome inhibitors, because a greater amount of cleaved Pak2 appears in the cells treated with these drugs (Fig. 2B) than in untreated cells. Because Htt prevented the appearance of the cleavage product both in the presence and absence of these proteasome inhibitors, it is probable that Htt prevents formation of the cleavage product. Consistent with the profile of Pak2 cleavage, when cells were treated with these proteasome inhibitors, less cell death occurred in the cells transfected with Pak2 and Htt552 than in cells transfected with Pak2 alone (supplementary material Fig. S4B).

Next, we looked at which domain of Htt was necessary to prevent Pak2 cleavage. We transfected Pak2 with different variants of wild-type Htt, and found that Htt552 and Htt588 could inhibit Pak2 cleavage after TNFα+CHX stimulation, but that no protection was seen with Htt120, Htt190 or Htt315, although the expression of the Htt variants appeared to be similar (Fig. 2C). This suggests that at least part of Htt316-552 is required for this protective effect. Whereas residues 316-552 are not sufficient for Htt to bind to Pak2, Htt fragments including this region bind Pak2 more effectively than shorter N-terminal fragments that end before residue 316 (Fig. 1A). Thus, the ability of Htt to inhibit Pak2 cleavage correlates with binding efficiency.

Pak2 has been reported to be a substrate of the effector caspase, caspase-3 (Rudel and Bokoch, 1997), and also of the initiator caspase, caspase-8 (Fischer et al., 2006). We confirmed that the pan-caspase inhibitor z-VAD-fmk fully blocks Pak2 cleavage (Fig. 2D). Preferential inhibitors of either caspase-3 or caspase-8 (especially the caspase-3 inhibitor) also strongly inhibited Pak2 cleavage in HeLa Tet-on cells treated with TNFα+CHX (Fig. 2D), suggesting that caspase-3 and caspase-8 play key roles in Pak2 cleavage. Zhang et al. (Zhang et al., 2006) reported that Htt inhibits the activity of caspase-3 (but not of caspases 1, 2, 6, 8 or 9). In order to demonstrate that the effects that we observed were independent of their observation, we transfected Pak2 or Pak2-Htt552 into MCF-7 cells (which lack caspase-3 activity owing to a deletion in the caspase-3 gene) (Janicke et al., 1998; Kurokawa et al., 1999) and treated these cells with TNFα+CHX. Although much less Pak2 was cleaved in these caspase-3-null cells than in HeLa cells, Htt552 still attenuated Pak2 cleavage (Fig. 2E). Cleavage of Pak2 in these MCF-7 cells treated with TNFα+CHX was robustly attenuated by the caspase-8 preferential inhibitor-I (Fig. 2F). These data suggest that caspase-8 is a crucial protease that mediates Pak2 cleavage. Furthermore, because Htt does not appear to modulate caspase-8 activity (Zhang et al., 2006), the ability of...
Htt to prevent Pak2 cleavage in MCF-7 cells is probably due to binding of Htt to Pak2.

We also tested whether physiological levels of Htt protect against Pak2 cleavage by comparing *Htt*-null (*Htt*^−/−^) embryonic stem (ES) cells with those containing wild-type Htt. Caspase-3 activation and caspase-3-associated apoptosis occur in mouse ES cells when leukemia inhibitory factor (LIF) is withdrawn (Duval et al., 2004). Pak2 cleavage occurred in ES cells after LIF and serum were

![Image](https://example.com/image1.png)

Fig. 1. Pak2 is a Htt-binding protein. (A) Left panels: Myc-Pak2–vector (Myc-Pak2 along with empty vector), Myc-Pak2–FLAG-Htt316-488 (Myc-Pak2 along with a vector expressing residues 316-488 of wild-type Htt), Myc-Pak2–FLAG-Htt316-513 (residues 316-513 of wild-type Htt), Myc-Pak2–FLAG-Htt316-552 (residues 316-552 of wild-type Htt) or Myc-Pak2–FLAG-Htt552 (first 552 residues of Htt) were co-transfected into HeLa Tet-on cells. Doxycycline (1 μg/ml) was added to the media to induce Myc-Pak2 expression following transfection. After 24 hours, cells were lysed and anti-FLAG antibody was used for immunoprecipitation. Anti-Myc was used to detect Pak2 (top) and anti-FLAG was used to detect Htt variant proteins (bottom). Right panels: Myc-Pak2–vector, Myc-Pak2–FLAG-Htt190 (residues 1-190 of wild-type Htt), Myc-Pak2–FLAG-Htt315, Myc-Pak2–FLAG-Htt552 or Myc-Pak2–FLAG-Htt588 were co-transfected into HeLa Tet-on cells. After 24 hours, cells were lysed and anti-FLAG antibody was used for immunoprecipitation. Anti-Myc was used to detect Pak2 (top) and anti-FLAG was used to detect Htt variant proteins (bottom). (B) Schematic showing the domain of Htt that binds Pak2: –, nonbinding; +, binding; ++, stronger binding. (C) Mouse brain lysate was immunoprecipitated with anti-FLAG antibody as a control (NS; lane 2) or with anti-Htt antibody (MAB2166; lane 3), and the blot probed with anti-Pak2 antibody (left column) and anti-Htt antibody (middle and right columns). Lane 1 was loaded with 8% of total lysate. (D) Mouse brain lysate was immunoprecipitated with anti-GST antibody as a control (NS; lane 2) or with anti-Pak2 antibody (lane 3) and the blot probed with anti-Htt antibody (left column) and anti-Pak2 antibody (right column). Lane 1 was loaded with 6% of total lysate. FL-htt, full-length Htt.
withdrawn from the culture media (Fig. 3A). An increased amount of truncated Pak2 was observed in *Htt*+/− ES cells than in *Htt*+/+ ES cells after serum and LIF withdrawal (Fig. 3A). Indeed, we detected active caspase-3 in serum-starved *Htt*+/− and *Htt*+/+ ES cells. There was an increased amount of cleaved caspase and more cell death in *Htt*+/− cells than in *Htt*+/+ cells under serum starvation conditions (Fig. 3B,C), corresponding to more Pak2 cleavage in *Htt*+/− cells under serum starvation. These data suggest that Pak2 cleavage might accelerate caspase cleavage via positive feedback.

Mutant Htt partially protects against Pak2 cleavage

We confirmed that the pathogenic polyQ-expanded Htt (muHtt) could also bind to Pak2 (Fig. 4A). We co-transfected either FLAG-tagged residues 1-588 of wild-type Htt (wild-type Htt588, with 17 glutamine repeats) or FLAG-tagged residues 1-588 of muHtt (muHtt588, with 138 glutamine repeats) along with Myc-Pak2 into HeLa Tet-on cells. After immunoprecipitating with anti-FLAG, we detected Pak2 (Fig. 4A). The input versus immunoprecipitate ratio for wild-type Htt588-Pak2 compared with muHtt588-Pak2 suggested that wild-type Htt588 interacted more strongly with Pak2 than did muHtt588. The quantified data suggested that the strength of muHtt588-Pak2 interaction is 30-50% of the wild-type Htt588-Pak2 interaction (Fig. 4B). These data are consistent with those for the wild-type Htt-Pak1 or muHtt-Pak1 interactions (Luo et al., 2008).

We then tested whether muHtt (with 138 glutamine repeats) could also reduce Pak2 cleavage after death stimulation. A substantial...
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The effects of Htt on the levels of the Pak2 C-terminal cleavage product were reduced in the presence of Htt552 (Fig. 6D-F). We also confirmed that the levels of the Pak2 C-terminal cleavage product were saturating levels of caspase-3 relative to Htt in our system. However, the protective effect of recombinant caspase-3 to about one third of that in the absence of Htt (lane 2, Fig. 6A) remained intact in the absence of recombinant active caspase-3. However, Htt reduced the levels of the Pak2 N-terminal cleavage product in the presence of recombinant caspase-3 to about one third of that in the absence of Htt552 (Fig. 6A,B). These effects were not due to any abolition of caspase-3 activity by Htt in this in vitro system (Fig. 6C) (as there is stronger inhibition of caspase-3 activity by Htt in this in vitro system). To further confirm that the Htt-Pak2 interaction is important in reducing Pak2 cleavage, we performed cleavage assays of Pak2 by itself (lane 1, Fig. 6A) or with Htt (lane 2, Fig. 6A) remained intact in the absence of recombinant active caspase-3. However, Htt reduced the levels of the Pak2 N-terminal cleavage product in the presence of recombinant caspase-3 to about one third of that in the absence of Htt552 (Fig. 6A,B). These effects were not due to any abolition of caspase-3 activity by Htt in this in vitro system (Fig. 6C) (as there might be saturating levels of caspase-3 relative to Htt in our system). We also confirmed that the levels of the Pak2 C-terminal cleavage product were reduced in the presence of Htt552 (Fig. 6D-F). The effects of Htt on the levels of the Pak2 C-terminal cleavage product are similar to those for the N-terminal cleavage product. Pak2 has also been reported to be a substrate for caspase-8, although caspase-8 has much weaker effects than caspase-3 (Fischer et al., 2006). Also, caspase-8 activity is not affected by wild-type Htt (Zhang et al., 2006). Consistent with our data above, we observed that Htt552 inhibited cleavage of Pak2 by caspase-8 in vitro (Fig. 7A,B).

The anti-apoptotic effects of Htt involve interactions with Pak2

Next, we tested whether the anti-apoptotic effects of Htt were related to its ability to attenuate Pak2 cleavage. We transfected Pak2 into HeLa cells with empty vector or with Htt588, Htt552, Htt315 or Htt190, then induced cell death with TNFα+CHX. Htt315 and Htt190, which do not influence Pak2 cleavage (Fig. 2C), did not inhibit cell death (Fig. 8A), but Htt552 and Htt588, which do prevent Pak2 cleavage, protected against cell death (Fig. 8A). The similar expression levels of the different Htt variants are confirmed in Fig. 2C and in supplementary material Fig. S5.

To confirm the hypothesis that the anti-apoptotic effects of Htt involve interactions with Pak2, we tested whether Htt could protect against the cell death caused by a truncated Pak2 (Pak2p34). Consistent with our data showing that Htt protects against cell death by reducing Pak2 cleavage, rather than by acting downstream of the production of the toxic Pak2 cleavage product, we observed no effects of Htt588 on the cell death caused by the cleavage product Pak2p34 (Fig. 8B).

We then knocked down Pak2 in HeLa cells with siRNA (Fig. 8C) and tested whether Htt still had protective activity in Pak2-knockdown cells. The Pak2 siRNA-knockdown cells were not as sensitive to TNFα+CHX toxicity as were control siRNA-knockdown cells, confirming the previous conclusion that Pak2 is a key factor mediating certain forms of apoptosis (Rudel and...

![Fig. 3. Pak2 cleavage, caspase-3 activation and cell death in Htt+/+ or Htt−/− ES cells under serum-withdrawal conditions. (A) Htt+/+ and Htt−/− mouse ES cells were cultured in media containing serum and LIF (lanes 1, 2). For assay of Pak2 cleavage, cells were starved in media containing neither serum nor LIF for 24 hours (lanes 3, 4). Cells were harvested and subjected to SDS-PAGE and blotting with anti-Pak2 antibody (C-terminal). The blot is representative of data from three independent experiments. (B,C) Htt+/+ or Htt−/− ES cells were each divided into two sets and cultured in complete ES cell medium for 2 days. One set of Htt+/+ or Htt−/− ES cells were starved in serum-free DMEM for 18 hours (lanes 3, 4). The other set of ES cells was continuously cultured in complete ES cell medium (lanes 1, 2). (B) The cell lysates were subjected to SDS-PAGE and western blotting with anti-caspase-3 antibody. FL, full length. (C) Cell death was evaluated by staining with Trypan blue. Samples are in triplicate. Comparisons were performed using the Student's t-test; error bars are s.d.; *P<0.0039.
Importantly, the effect of Htt overexpression on protection against cell death from TNFα+CHX was largely abolished in cells in which Pak2 had been knocked down (Fig. 8D).

Htt protection against TNFα-induced cell death is dependent on Pak2 cleavage

Full-length Pak2 has anti-apoptotic effects in cells. To preserve its anti-apoptotic activity, we generated a Pak2 mutant that was resistant to caspase cleavage and that could be expressed in cells in which we had knocked down the endogenous Pak2 with siRNA. We first selected a series of Pak2 siRNAs that were potentially able to target human Pak2 but would not affect mouse Pak2 (mPak2), because the selected siRNAs had four to seven mismatches to the mRNA sequence of mPak2. All of these siRNAs knocked down Pak2 in HeLa cells (Fig. 9A). However, Fig. 9B shows that, whereas Pak2 Smartpool siRNA and one of the oligonucleotides (siRNA-2) knocked down mPak2, siRNA-1 and siRNA-3 did not affect mPak2 levels. Consequently, we used siRNA-3 for further study. Note that mPak2 is ~98% identical to its human counterpart. Supplementary material Fig. S6 confirms that mPak2 is capable of binding to Htt (supplementary material Fig. S6A), and that Htt588 prevents mPak2 cleavage (supplementary material Fig. S6B).

To test whether the ability of Htt to protect against cell death induced by TNFα is dependent on Pak2 cleavage, we generated a noncleavable mPak2 mutant (mPak2-D212E) and confirmed that this mutant was resistant to cleavage in cells treated with pro-apoptotic insults (Fig. 9C). Htt588 protected against cell death induced by TNFα+CHX when wild-type mPak2 was introduced into cells in which endogenous human Pak2 was knocked down (Fig. 9D). However, in Pak2-knockdown cells in which the

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**Fig. 4.** Wild-type Htt interacts more strongly with Pak2 than does muHtt. (A) Myc-Pak2–vector, Myc-Pak2–FLAG-Htt588 (wild type, 17 glutamine repeats) or Myc-Pak2–FLAG-muHtt588 (138 glutamine repeats) were transfected into HeLa Tet-on cells. After 20 hours, the transfected cells were harvested and lysed. The cellular lysates were subjected to anti-FLAG (M2) agarose immunoprecipitation. The immunoprecipitates (lanes 4-6) and total lysates (lanes 1-3) were used for SDS-PAGE. The blot was probed with anti-Myc (top panel) and anti-FLAG (bottom panel) antibodies. (B) Quantification of the interaction strength between Pak2 and either wild-type Htt588 or muHtt588 was made using data from Fig. 2A and two other similar experiments: y-axis shows ratio of immunoprecipitated (IP) Pak2 to total Pak2. The ratio of IP Pak2 to total Pak2 when wild-type Htt588 was used for Pak2 immunoprecipitation was set as 1. Data were from three independent experiments. Comparison was performed using the Student’s t-test; error bar is s.d.; *P=0.00855.

**Fig. 5.** Mutant Htt reduces Pak2 cleavage in cells. (A) Pak2 was transfected with empty vector (lanes 1-3), wild-type (wt) Htt588 (lanes 4-6) or muHtt588 (lanes 7-9) into HeLa cells. Transfected cells were treated with TNFα+CHX for 0, 3 or 6 hours. The lysates were subjected to SDS-PAGE and blotting with anti-Pak2 antibody (C-terminal) (top panel) and anti-FLAG antibody for Htt (bottom panel). The molecular mass of the Pak2 C-terminal fragment was ~34 kDa. Blot is representative of data from four independent experiments. [We believe that 50 kDa is likely to be a nonspecific band as it was only present with certain batches of the antibody under certain conditions (incubation time and washing).] (B) Myc-Pak2 was transfected with empty vector (lanes 1-3), wild-type Htt588 (lanes 4-6) or muHtt588 (lanes 7-9) into HeLa Tet-on cells. Transfected cells were treated with TNFα+CHX for 0, 3 or 6 hours. The lysates were subjected to SDS-PAGE and blotting with anti-Myc antibody to detect an N-terminal Pak2 fragment (top panel), and with anti-FLAG antibody for Htt (bottom panel).
After caspase-mediated cleavage, Pak2 releases a functionally unknown N-terminal fragment and a toxic C-terminal fragment. Thus, one can assay Pak2 cleavage by analysing the levels of either the C-terminal or N-terminal fragments (or both, as we have done). Our data provide a novel mechanism for the general cytoprotective role of Htt in the context of Fas-mediated apoptosis. By binding to Pak2 and thus preventing its activation via cleavage, both wild-type Htt and muHtt abolish a pathway that is crucial for caspase-associated cell death. These data have been confirmed both in cell lines and in vitro, suggesting that this is a specific and direct property of the Htt-Pak2 interaction. Our data on Pak2 cleavage mediated by both caspase-3 and caspase-8 in MCF-7 cells and in vitro show that these effects are independent of any effects of Htt on caspase-3 activity (although one could easily envisage additive effects of the caspase-3 inhibition by Htt and our observed effects on Pak2 cleavage). Indeed, our data suggest that inhibition of Pak2 cleavage is a major component of the protective effect of Htt, at least in the context of Fas-related cell-death pathways, as Htt is cytoprotective in wild-type cells exposed to TNFα, but has no significant benefit in cells with Pak2 knockdown that are treated with TNFα.

The ability of both muHtt and wild-type Htt to inhibit Pak2 cleavage is compatible with data in the literature that suggest that noncleavable mutant mPak2-D212E was overexpressed, there were lower levels of cell death after the pro-apoptotic insult, and Htt no longer exerted a protective effect. These data confirm that the prevention of Pak2 cleavage by Htt is a major factor in the protective effects of Htt against TNFα-induced cell toxicity.

These data support the hypothesis that the ability of Htt to prevent Pak2 cleavage is a key contributor to its protective effects in this cell death paradigm (supplementary material Fig. S7).

**Discussion**

Htt, like Pak2, is a ubiquitously expressed protein (Teo et al., 1995; Trottier et al., 1995). However, much of the research on the normal functions of Htt has been restricted to neuronal systems, even though many of its normal functions might be generic. We previously reported that Pak1 binding to Htt promotes Htt-Htt interaction, and thus enhances muHtt oligomerisation and toxicity (Luo et al., 2008). Pak1, Pak2 and Pak3 have highly conserved protein sequences and similar functions (Bokoch, 2003). However, Pak2 is cleaved and releases a toxic p34 species in response to cell-death signals (Rudel and Bokoch, 1997). The widespread expression pattern of Pak2 prompted us to consider the possibility that Htt might modulate Pak2 cleavage.

![Fig. 6. Htt prevents Pak2 cleavage by caspase-3 in vitro. (A) Myc-Pak2-vector or Myc-Pak2–Htt552 was transfected into HeLa Tet-on cells. Cells were harvested and lysed. Cell lysates were cleaved by caspase-3 in vitro (37°C, 1 hour) (lanes 3, 4) or mock-cleaved in vitro (buffer, 37°C, 1 hour) (lanes 1, 2). The cleaved lysates were subjected to western blot and probed with an anti-Myc antibody. The molecular mass of the Pak2 N-terminal fragment was ~29 kDa; * indicates a nonspecific band. (B) The plots for experiments performed as in A were quantified with a ChemiImager. The ratio of caspase-3-cleaved Pak2 to full-length (FL) Pak2 in the Pak2-alone lysate was set to 1. The relative level of caspase-3-cleaved Pak2 to full-length Pak2 in Pak2-Htt552 cells is shown. Five independent experiments were performed. Comparison was performed using the Student’s t-test; error bar is s.d.; ***P=0.00004. (C) Assays of caspase-3 activity were performed for the samples in A; n=3. (D) Pak2-vector or Pak2-Htt552 was transfected into HeLa cells. Cells were harvested and lysed. Cell lysates were cleaved by caspase-3 in vitro (37°C, 1 hour) (lanes 3, 4) or mock-cleaved in vitro (buffer, 37°C, 1 hour) (lanes 1, 2). The cleaved lysates were subjected to western blot and detected with anti-Pak2 antibody that recognizes the C-terminus of Pak2. (E) The plots for the experiments performed as in D were quantified with a ChemiImager. The ratio of caspase-3-cleaved Pak2 to full-length Pak2 in the Pak2-alone lysate was set to 1. The relative value of caspase-3-cleaved Pak2 to full-length Pak2 in Pak2-Htt552 cells is shown. Three independent experiments were performed. Comparison was performed using the Student’s t-test; error bar is s.d.; *P=0.00483. (F) Assays for caspase-3 activity were performed for the samples in D; n=3.
muHtt retains many of the functions of its wild-type counterpart, although there might be some partial loss of certain activities. Indeed, our data suggest that muHtt binds more weakly to Pak2 than does wild-type Htt. This might account for the weaker protection against Pak2 cleavage afforded by muHtt than by wild-type Htt. However, it is interesting to note that the embryonic lethality associated with complete loss of Htt is rescued by YAC (yeast artificial chromosome) overexpression of either muHtt or wild-type Htt (Hodgson et al., 1999). Thus, the anti-apoptotic effects of muHtt might be sufficient if they are relevant in this context. As muHtt is itself toxic owing to its gain-of-function properties (Rubinsztein, 2003), one cannot always make simple inferences regarding its potential anti-apoptotic properties in cell culture or in vivo, especially if one uses conditions that might activate pathways that potentiate muHtt toxicity. For example, although both muHtt and wild-type Htt are reported to inhibit caspase-3 activation (Zhang et al., 2006), elevated caspase-3, caspase-8 and caspase-9 activities were observed in lymphoblasts from patients with heterozygous or homozygous mutant Htt alleles (Maglione et al., 2006). In other words, if muHtt expression enhances the toxicity of a specific toxin, then this might be due to the gain-of-function toxicity of the mutant protein, which could be unrelated to, or might indeed dominate, its anti-apoptotic properties (in other contexts). Interestingly, Maglione et al. suggested that the increased caspase activities in lymphoblasts with muHtt might enhance Htt cleavage (Maglione et al., 2006). Smaller Htt fragments lose the ability to protect against Pak2 cleavage.

In contrast to the effects of Pak1 on muHtt oligomerisation and toxicity, these new data provide a novel mechanism for the protective role of Htt by showing that it affects Pak2 cleavage. The current data, along with our previous data showing that Pak1 enhances muHtt toxicity, suggest that the effects of Pak-family members on muHtt toxicity might be either deleterious or protective, depending on the family member involved. It would also be interesting to investigate whether Htt is involved in other normal functions of Pak-family proteins.
The functional links between Htt and Pak2 might provide leads for further understanding of cell death and cell survival. Prevention of fragmentation of muHtt or wild-type Htt allows Htt to maintain its ability to inhibit Pak2 cleavage, thus decreasing the toxicity resulting from Pak2p34 (the cleaved C-terminal fragment). By mimicking the relevant Pak2-binding domain structures of Htt, one might also be able to design (or screen for) small molecules that inhibit caspase-mediated cleavage of Pak2 artificially in order to control cell survival.

Materials and Methods

Vectors and DNA construction

FLAG-tagged N-terminal Htt constructs Htt552 (aa1-552), Htt315 (aa1-315), Htt190 (aa1-190) and Htt120 (aa1-120) were generated by PCR and sub-cloned into pCI vector flanked by MluI and NotI. All the constructs were confirmed by DNA sequencing. The other Htt constructs were generated as described previously (Luo et al., 2005). Human Pak2 (Pak2) expression vector was from Origene. Tet-on expression plasmids pRevTRE-Myc-Pak2, GFP-Pak2 and GFP-Pak2p34 (all N-terminal-tagged) were provided by Rolf Jakobi (Kansas City University of Medicine and Biosciences, Kansas City, MO) (Jakobi et al., 2001). Mouse Pak2 (mPak2) cDNA was provided by Geneservice (Cambridge, UK), clone ID 6849008. mPak2 was sub-cloned into pcDNA3 and pCMV-6M (a Myc-tagged protein-expression vector). Noncleavable mPak2 (mutant mPak2-D212E), the kinase-dead mutant Pak2-K278R and the kinase-activated mutant Pak2-T402E were generated with Stratagene Quikchange Mutagenesis kit. All sequences were confirmed by DNA sequencing.

Antibodies and reagents

Anti-rabbit polyclonal antibodies used in this study were anti-Pak2 (1:1000; Cell Signaling), anti-Myc (1:1000; Sigma) and anti-GST (Sigma). The goat polyclonal antibody used was anti-Pak2 (1:1500; Santa Cruz). Anti-mouse monoclonal antibodies used were anti-Htt (MAB2166; 1:1000), anti-FLAG (M2; 1:1000), anti-tubulin (1:1000; Sigma) and anti-Myc (9E10; 1:1000; Sigma). Anti-FLAG M2-agarose affinity gel, or anti-mouse or anti-rabbit IgG agarose (Sigma) were used. TNFα, recombinant caspase-3 and recombinant caspase-8 were purchased from Chemicon International. TNFα was a product of Calbiochem. CHX, exopomycin and lactacystin were from Sigma. Capase-3 inhibitor (Ac-DEVD-CHO), caspase-8 inhibitor-I (Ac-
the observer blinded to the identity of the slides. The figures show data from counted in multiple random visual fields per slide. All coverslips were scored with.

Estimation of cell death
To measure cell death, approximately 200 transfected GFP-positive cells were counted in multiple random visual fields per slide. All cover slips were scored with the observer blinded to the identity of the slides. The figures show data from representative experiments in triplicate. Cell death was monitored by scoring the transfected cells that had an apoptotic nuclear morphotype: either fragmented or pyknotic nuclei. P values were determined by unconditional logistical regression analysis by using the general loglinear option of SPSS 9.1 software (SPSS, Chicago, IL).

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