Huntington’s disease (HD) is an autosomal-dominant neurodegenerative disorder with onset in midlife that is characterised by irreversible motor dysfunction, personality changes, and cognitive decline ultimately leading to death 15-20 years after disease onset. HD belongs to a family of neurodegenerative diseases caused by mutations in which the expansion of a CAG trinucleotide repeat results in an aberrantly long polyglutamine (polyQ) tract in the encoded protein. In HD, the CAG expansion occurs within exon 1 of the \( HTT \) gene and leads to an elongated polyQ tract at the N-terminus of the HD protein huntingtin (HTT), a protein of many diverse functions. Individuals with \( (CAG)_{35} \) or less remain unaffected, whereas those with \( (CAG)_{40} \) and above will develop HD within a normal lifespan. The length of the CAG expansion is inversely correlated with age of disease onset, with repeats at the higher end of the spectrum causing the juvenile form of HD.

Numerous HD mouse models have been developed and are being used to study the pathogenic pathways involved in HD. The \( HdhQ150 \) knock-in mouse carries \(~150\) CAG repeats which have been inserted into the mouse HD gene \( (Hdh) \). It has been proposed that the accumulation of a critical concentration of N-terminal HTT fragments represents a rate-limiting step in the onset and progression of HD-related phenotypes in this model. Numerous studies suggest that the smallest N-terminal HTT fragments are pivotal to the molecular pathogenesis of HD. Human post-mortem studies showed that only N-terminal antibodies detect nuclear inclusions in patient brains and formic acid solubilisation released a small N-terminal fragment. In cell models, HTT has been shown to be cleaved into smaller fragments called cp-A and cp-B or cp-1 and cp-2. Furthermore, using the \( HdhQ150 \) knock-in model we recently demonstrated that full-length HTT is cleaved into a number of N-terminal HTT fragments, the smallest of which is an exon 1 HTT protein.

The ensuing search for sites of proteolytic cleavage of human HTT raised the hypothesis that the cleavage of HTT by proteases...
contributes to the pathogenesis of HD. Studies have identified active caspase-3 (HTT-513 and HTT-552) and caspase-6 (HTT-586) sites 11, calpain sites (HTT-469 and HTT-536) 12, 13, and recently an MMP-10 site (HTT-402) 14. Additionally, a number of less well-defined HTT cleavage fragments have been isolated from HD post-mortem brains 8 and from a number of HD model systems, for which the precise cleavage sites and proteases involved remain elusive 9, 10, 15, 16, 17, 18, 19. The prevention of cleavage at the caspase-6 but not caspase-3 sites was found to be protective in the YAC128 HD mouse model 20. In that study, mutation of the aspartate at amino acid 586 ameliorated HD-phenotypes, implying that the caspase-6 mediated cleavage of HTT is critical in the pathogenesis of HD. The HTT-586 fragment could itself be pathogenic and / or cleavage at this site might initiate further proteolytic events that generate smaller N-terminal fragments with detrimental effects: the cascade hypothesis. Alternatively, the aspartate substitution may alter the conformation of HTT without altering cleavage, and it could be this structural change that improves the phenotype of these mice.

We previously mapped the location of the proteolytic cleavage sites required to generate the N-terminal HTT fragments that are present in the HdhQ150 HD model, and this suggested that Fragment 8 might be generated by caspase-6 6. To test this hypothesis, we crossed caspase-6 (Casp6)-deficient (Casp6–/–) mice with the HdhQ150 mouse model of HD and analysed the pattern of N-terminal HTT fragments. This genetic approach would test whether CASP6 actually cleaves HTT at amino acid 586 in vivo, and also assess the so called cascade hypothesis should cleavage at HTT-586 be inhibited. In concurrence with our previous findings, we were able to identify the same 14 prominent N-terminal mutant HTT fragments in the brain of the Hdh Q150 mouse. Interestingly, we showed that the ablation of CASP6 had no effect on the production of Fragment 8 or any other N-terminal HTT proteolytic fragments. In addition, the ablation of CASP6 had no effect on the levels of the full-length HTT protein. Therefore, CASP6 does not play a direct role in the molecular pathogenesis that occurs in HdhQ150 mice through the modulation of HTT proteolysis or modifying the levels of mutant HTT.

MATERIALS AND METHODS

Mouse Breeding, Maintenance, Genotyping, and CAG Repeat Sizing. All mouse experiments were performed under the Animals Scientific Procedures Act (1986) under project and personal licences approved and issued by the Home Office. The HdhQ150 knock-in mice were maintained, genotyped, and CAG repeat-sized as previously described 21. The mean repeat size was 179 ± 7.6 (±S.D.). The Casp6–/– mouse was created by Taconic Biosciences and genotyped as previously described 22. The Casp6 mice were maintained by backcrossing Casp6+/– males to C57BL/6J females (Charles River, A003). Hdh+/Q150::Casp6+/– (Dble) and Hdh+/Q150::Casp6+/+ (Hdh+/Q150) mice were generated by crossing Casp6+/– males with C57BL/6J Hdh+/– mice. Mouse brains were snap-frozen in liquid nitrogen and stored at −80°C.

Immunoprecipitation. Whole brains were homogenised in ice-cold HEPES buffer (50 mM HEPES/NaOH (pH7.0), 150 mM NaCl, 10 mM EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with 10 mM DTT, 1 mM PMSF, and complete protease inhibitor mixture (Roche). Lysates were processed for western blotting or immunoprecipitation as described 6. For immunoprecipitation, either 2 µg mlG, 2B7, S830, or 3B5H10 was used. (See Table 1 and 2 for antibody details).

Western Immunoblotting. For western blotting, 30 µg of total protein or 6 µl of immunoprecipitate in Laemmli buffer were denatured at 75°C for 10 min and separated by 8% or 10% SDS-PAGE, blotted onto nitrocellulose membranes (Whatman) and immunoprobed as described 6. Primary antibodies: MW1 (1:1,000, 23); CASP6 (1:125); ?-ACTIN (1:10,000). Secondary antibodies: anti-mouse HRP (1:3,000); anti-rabbit HRP (1:3,000). (See Table 1 and 2 for antibody details).

TR-FRET. Time-resolved Förster resonance energy transfer (TR-FRET) was performed as described previously 24. 2B7 antibody was labelled with terbium cryptate donor fluorophore (CisBio). MW1 was labelled with D2 acceptor fluorophore (CisBio). MAB2168 was labelled with Alexa-fluor (A)488 (Invitrogen).
RESULTS

*Casp6*−/−mice show no CASP6 expression in the brain. Exons 2-5 of the Casp6 gene, which encodes the catalytic domain of the caspase-6 (CASP6) protein, were removed to generate Casp6−/− mice. Casp6−/− mice are viable and fertile, and do not show any overt phenotypes. We began by performing western blots to ensure that these mice do not express CASP6 and to verify the genotypes. Protein lysates were prepared from half brains of 2 month old wild type and Casp6−/− mice and analysed by western blotting using an anti-CASP6 antibody. As previously reported, we found that the Casp6−/− mice constitutively lacked CASP6 as compared to wild type littermates (Fig. 1).

![Image](image-url)

**Fig. 1: Figure 1. CASP6 expression in Casp6−/− and wild type brains**

Western blotting of total brain lysates from 2 month old mice using an anti-CASP6 antibody shows that CASP6 was absent in Casp6−/− samples when compared to wild type (Casp6+/+) samples. Beta-ACTIN served as a loading control. ID = Immunodetection.

Genetic ablation of *Casp6* does not modify HTT proteolysis in *HdhQ150* knock-in mice. To identify N-terminal HTT fragments in the brains of *HdhQ150* knock-in mice, we previously used a combination of immunoprecipitation and western blotting that identified 14 prominent N-terminal fragments, which in addition to the full-length protein, could be readily detected in the cytoplasmic but not nuclear fractions. These fragments were present at all ages and did not arise as a consequence of the pathogenic process. Furthermore, in an attempt to determine the source of some of the N-terminal HTT fragments, proteolytic digests were performed with caspase enzymes which indicated that Fragment 8 may terminate at the caspase-6 HTT-586 cleavage site. In order to corroborate this observation, we investigated whether the presence of this proteolytic fragment, along with all other observed N-terminal HTT fragments, was modulated upon the genetic ablation of CASP6. Casp6−/− males were bred to *Hdh*+/Q150::Casp6+/− females to generate *Hdh*+/Q150::Casp6+/− (Hdh+/Q150) or Casp6 knock-out *Hdh*+/Q150::Casp6−/− (Dble) background. We obtained at least 12 mice for each genotype and all

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groups were well matched for their CAG repeat size ($Hdh^{+/Q150}$ 179 ±7.5 (±S.D); (Dble 179 ±9.5 (±S.D)).

To compare the pattern of HTT proteolytic fragments, we applied a combination of HTT immunoprecipitation and immunodetection from whole brain lysates prepared from $Hdh^{+/Q150}$ and Dble mice aged 2 months (Fig. 2). Mutant HTT was immunoprecipitated with antibodies that recognize epitopes at the N-terminus (2B7; S830; or 3B5H10) (Fig. 2B-C) and immunodetected with MW1, an antibody that recognizes the expanded polyQ tract and, therefore, does not detect wild type HTT. A mouse IgG (mIgG) antibody was used as a negative control (Fig. 2A), and Casp6 genotypes were confirmed by western blotting (Fig. 2E). In both $Hdh^{+/Q150}$ and Dble samples we were able to identify all of the N-terminal fragments identified previously, including Fragment 8 (the proposed caspase-6 HTT-586 fragment) 6. Therefore, the proteolysis of full-length HTT, to generate Fragment 8 or any other N-terminal HTT fragments was unaltered in the absence of CASP6.

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**Fig. 2: Figure 2. Analysis of N-terminal HTT fragments in Hdh+/Q150 and Dble mouse brains.**

Immunoprecipitation of mutant HTT from Hdh+/Q150 or Dble samples at 2 months of age with either: (A) mlgG (control), (B) 2B7, (C) S830, or (D) 3B5H10, and immunodetection with MW1. Fourteen prominent N-terminal fragments can be detected (Figments 1-14), in addition to 3 weaker fragments (Figments a-c), numbered and labelled in panel (B). (E) Casp6 genotypes were confirmed by western blotting, Beta-ACTIN served as a loading control. IP = Immunoprecipitation; ID = Immunodetection; In = Interface between stacking and resolving gel; FL = full-length protein. (n = 3/genotype).

Steady state wild type and mutant HTT levels are not changed in the brains of presymptomatic $Hdh^{+/Q150}$ and Dble mice. To quantify the relative levels of mutant HTT with respect to total HTT, brain lysates from 2 month-old mice were subjected to the highly sensitive TR-FRET assay (Fig. 3). This assay uses the labelled antibody pair: 2B7-Terbiu m cryptate and
Given that Casp6+/– mice do not express the CASP6 protein. We crossed the HdhQ150 mice to Casp6 mice and employed our previously described unbiased immunoprecipitation and immunodetection strategy to detect N-terminal HTT proteolytic cleavage fragments in the brains of Hdh+/Q150 mice that did or did not express CASP6. In accordance with our previous findings, we could detect the C-terminus of 14 prominent mutant HTT N-terminal fragments (Fragments 1-14) and three additional fragments (Fragments a-c) in the brain at 2 months of age. We found that the ablation of CASP6 did not modify the production of Fragment 8 (the previously proposed caspase-6 HTT-586 fragment), or any other N-terminal HTT fragment. Since the ablation of CASP6 had no effect on the proteolysis of mutant HTT we were unable to test the cascade hypothesis to determine whether the production of one fragment is essential for the generation of others.

Given that Fragment 8 is still present in the absence of CASP6, it must therefore be generated by an alternative protease that cleaves either at the HTT-586 site or at a different position nearby. If the cleavage occurs at HTT-586, an alternative protease also recognises the CASP6 recognition motif and therefore, redundancy would be expected to ensure that the site is cleaved irrespective of whether CASP6 is present or absent. This could account for a compensatory mechanism in these constitutive...
knock-out mice. It would also confound therapeutic approaches directed at CASP6 inhibition. In concurrence with our findings, it has recently been demonstrated that the ablation of CASP6 activity in an aged BACHD mouse model did not show a reduction in the proteolysis of HTT at amino acid 586 27, and our work now extends this observation to two different HD mouse models.

The ablation of CASP6 in BACHD mice aged 13 months resulted in a reduction of both wild type and mutant HTT levels, possibly due to the activation of protein clearance pathways 27. We performed TR-FRET to quantify levels of both mutant HTT and total HTT and found that the absence of CASP6 had no effect on soluble HTT levels in presymptomatic mice. Such a difference in the effect of ablating CASP6 on HTT levels in young (2 month) HdhQ150 and aged (13 month) BACHD mice is not clear. It could be that protein clearance pathways are regulated by CASP6 only at later stages of disease, particularly when toxic HTT aggregates are present.

Preventing the formation of N-terminal HTT fragments is an attractive therapeutic strategy that needs to be understood. Our data suggest that CASP6 is not necessary for the proteolysis of mutant HTT in HdhQ150 mice and by implication does not play a direct role in HD pathogenesis in this mouse model via this mechanism. Our work highlights the importance of identifying the source of HTT proteolytic fragments to allow the design of rational therapeutic strategies when considering how one should combat the many HD-related phenotypes associated with this complex neurodegenerative disease.

COMPETING INTERESTS

AW is an employee of Novartis AG.

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References


