

# HAP1-huntingtin interactions do not contribute to the molecular pathology in Huntington's disease transgenic mice

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**Abstract** HAP1 (huntingtin associated protein) has previously been found to interact with huntingtin (htt) in a glutamine length dependent manner and has been proposed to play a role in the cell specific neurodegeneration observed in Huntington's disease (HD). We have isolated mouse HAP1 (hap1) and have shown that expression is not enriched in areas specifically affected in HD. We have used the yeast two hybrid system to demonstrate that htt amino acids 171–230 are necessary for the hap1-htt binding and that hap1 does not interact with the transgene exon 1 protein in a transgenic model of HD.

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**Key words:** Huntington's disease; Triplet repeat; Polyglutamine; HAP1

## 1. Introduction

Huntington's disease (HD) is one of several neurodegenerative disorders caused by a CAG/polyglutamine expansion. In addition to HD this includes spinal and bulbar muscular atrophy, dentatorubral pallidoluysian atrophy (DRPLA) and the spinocerebellar ataxias: SCA1, SCA2, SCA3 (also known as Machado Joseph disease), SCA6 (reviewed in [1]) and SCA7 [2]. The proteins harbouring the polyglutamine repeats all have wide and overlapping expression patterns. It is likely that the mutation acts by imparting a gain of function to the respective protein and that the mechanism of neuronal dysfunction and cell death is common to these diseases. Despite the overlapping expression profiles, the pattern of neuropathology differs strikingly between these diseases and the factors governing this selective vulnerability are unknown [3].

The proteins that contain the polyglutamine expansions are mostly novel and unrelated except for the presence of the polyglutamine tract. One approach to gaining insights into the normal function of these proteins, the molecular basis of the pathogenesis and the selective vulnerability of certain neurons is to identify interacting proteins. Huntingtin interactors that have been identified using the yeast two hybrid system include: htt associated protein (HAP1) [4], htt interacting protein (HIP1) [5,6] and a ubiquitin conjugating enzyme (hE2-25K) [7]. HAP1 was isolated from a rat brain cDNA library using the N-terminal 230 amino acids of htt containing 44

glutamine repeats [4]. Rat HAP1 has two isoforms, HAP1A and HAP1B, and expression was reported to be exclusively in brain [4]. HAP1 was found to bind to htt in a glutamine length dependent manner, but its failure to bind to atrophin with 21 repeats suggested that it did not bind non-selectively to the polyglutamine tract [4]. The restriction of the expression pattern to brain and the modulation of the htt-HAP1 interaction by the length of the polyglutamine repeat suggested that HAP1 might be involved in the molecular events that lead to the onset of HD.

We have recently generated lines of mice transgenic for exon 1 of the HD gene carrying highly expanded CAG repeats that express an N-terminal htt protein [8]. Three transgenic lines have been established: R6/1, (CAG)<sub>115</sub>; R6/2, (CAG)<sub>145</sub>; and R6/5, (CAG)<sub>128–156</sub> that develop a progressive neurological phenotype with similarities to HD. In order to determine whether htt-HAP1 interactions could be important to the molecular basis of the phenotype observed in these mice, we have cloned the mouse homologue of HAP1 (hap1). Expression analysis revealed high levels of expression in both brain and testis. The yeast two hybrid system was used to show that hap1 does not interact with the N-terminal exon 1 transgene protein.

## 2. Materials and methods

### 2.1. Cloning and analysis of mouse hap1 cDNA

A mouse whole brain (BALB/c P20) unidirectional cDNA library (Stratagene, 937301) was screened with a mouse hap1 probe of 306 bp (nt 889–1194 according to rat sequence [4], or nt 847–1152 according to mouse sequence) obtained by RT-PCR of mouse brain RNA. cDNAs were excised in vivo as pBluescript SK clones and sequenced (Sequenase version 2.0, USB). Further 5' sequence (nt –10 to +6) was determined by 5' RACE [9]. Sequence from the 3' UTR was obtained using an ABI 377 sequencer with primers 40093 (TGGACGAGAC-CACAAGGATG) and 40104 (GTCAGCCTCGGAAGTGCTGA). Fragments of the hap1 cDNA were cloned into the bacterial expression vector pTrcHis B (Invitrogen) to ensure that recombinant proteins of the predicted size were produced. Protein extracts, Western blots and detection of recombinant protein with the anti-Xpress antibody were performed according to the manufacturer's recommendations (Invitrogen).

### 2.2. Extraction and analysis of mouse RNA

RNA was isolated from a range of mouse tissues and Northern blots were prepared as described [8]. Mouse RNA was reverse transcribed in 75 mM KCl, 50 mM Tris-HCl pH 8.3, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM dNTPs, 7 ng/μl random hexamers with 0.8 U/μl RNasin (Promega), 13 U/μl M-MLV RTase (BRL) for 10 min at 23°C, 40 min at 37°C and then for 10 min at 94°C. The mhap1 probe used to screen the mouse brain cDNA library was amplified using primers designed from the rat sequence: 40085 (CATCTTCGAGAG-GAGGCCTC) and 40088 (ATCTGTCATGTGGGAGCCAG). PCR was performed in Promega buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs,

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**Abbreviations:** HD, Huntington's disease; HAP, huntingtin associated protein; HIP1, huntingtin interacting protein; htt, huntingtin; gln, glutamine

4 ng/μl primers with 0.02 U/μl Taq DNA polymerase (Promega). Cycling conditions were 90 s at 94°C, 34×(30 s at 94°C, 30 s at 55°C, 90 s at 72°C), 10 min at 72°C.

### 2.3. Yeast two hybrid analysis

The 1.4 kbp *Bgl*II fragment from the mouse hap1 cDNA was ligated into the *Bam*HI site of pGAD10 (Clontech). Insert orientation was determined by restriction analysis and the reading frame verified by sequencing. pBTM117C recombinant clones were generated to produce the following fusion proteins: pBTMCAG51, htt aa 1–118 (51 glns); pBTMHD0.5, htt aa 1–171 (23 glns); pBTMHD1.7, htt aa 1–588 (23 glns); pBTMHD3.6, htt aa 1–1245 (23 glns). Yeast transformations, selection systems and X-gal staining were as previously described [6].

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40
MRPKEQVQSGAGDGTGSGDPAAGTPTTQPAVGPAPEPSAE
-----D-----S-----T-----AD-----
80
PKPAPAQGTGSGQKSGSRTKTG--SFCRSMIIGDSDAPWTR
-----G-----R-R-----
120
YVFQGPYGPRAATGLGTGKAEGIWKTAAAYIGRRPGVSGPE
-I-----R-----
160
RAAFIRELQEALCPNPPTKKITEDDVKVMVLYLLEEKERD
-----R-----I-----
200
LNTAARIGQSLVKQNSVLMEEENKPKETMLGSAREEILHLR
-----L-----
240
KQVNLRDDLLQLYSDSDDDDEDEDEDEDEEEEREQR
-----EED-ED-E-----
280
DQDQQHDHPYGAPKPHPKAETAHRCPOLETLOQKRLLEE
-----P-----L-H-----A-K---K-----
320
ENDHLREEASHLDNLEDEEQMLILECQEVSFAIQQMAEL
-----K-----S-----
360
SEVLVLRLEGYERQOKEITQLQAEITKLQORCQSYGAQTE
-----
400
KLQOMLASEKGTHSESLRAGSYMODYGSRPRDAQEVGKSH
---Q---V-P---H---ER-D---
440
RQRSSMPAGSVTHYGYSVPLDALPSFPETLAEELRTSLRK
-----
480
FTTDPAYFMERRDTHCREGRKKEQRAMPPPPAQDLKPPED
-----C-R---E---GT---V-----
520
FEAPEELVPEELGAIEEVGTAEDGQAEENEQASEETEAW
-----P---T-----
560
EEVEPEVDETTRMNVVVSALASGLGPSHLDKMYVLQQLS
-----A-----
598
NWQDAHSKRQKQKQVVPKGECSRRGHPPASGTSFRSSTI HAP1A
-----Y---L---
600
DSPTPQQQTNMGGGILEQQPRV HAP1B
---A-----V---I---
628
PTQDSQRLEEDRATHSPSAREEEGPSGAT HAP1B
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Fig. 1. Comparison of the amino acid sequence of the deduced mouse hap1 and rat Hap1 proteins. The rat sequence is below the mouse sequence and identical amino acids are indicated by a dash. HAP1A and HAP1B differ at the C-terminus from residue 579 [4]. The region of rat HAP1 initially isolated by the yeast two hybrid system [4] is indicated in bold.

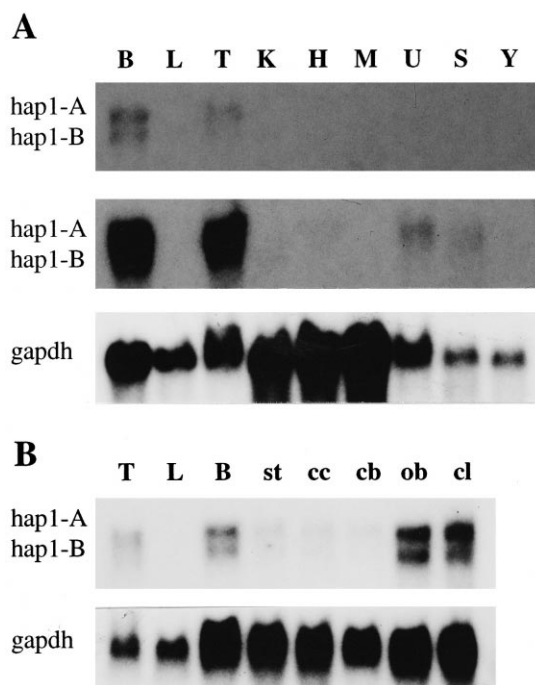


Fig. 2. Northern blot analysis of hap1 mRNA. All lanes contained 20 μg of total RNA. hap1 hybridisations were performed with a 2.0 kb 5' hap1-cDNA probe and mouse gapdh was used as a loading control. A: Expression of hap1 in a range of adult mouse tissues: the upper and lower panels show short and long exposures respectively. B: Expression of hap1 in a range of adult mouse brain regions. B = whole brain, L = liver, T = testis, K = kidney, H = heart, M = muscle, U = lung, S = spleen, Y = thymus, st = striatum, cc = cerebral cortex, cb = cerebellum, ob = olfactory bulb, cl = colliculus.

## 3. Results

### 3.1. Isolation of the mouse homologue of HAP1

A mouse total brain cDNA (BALB/c p20) library was screened with a mouse RT-PCR product amplified with primers flanking the region of hap1 that binds to htt (aa 277–445 in rat). Of 11 unique cloned cDNAs hybridising to this probe, two correspond to hap1-B (EMBL accession number AJ000262). From the remaining nine clones it was possible to deduce the mouse hap1-A sequence (accession numbers AJ002271 and AJ002272); however, the majority of these clones contained complex deletion events occurring between the 3' hap1 coding region and the distal 3' UTR. These had not arisen as a result of alternative splicing events when compared to the genomic sequence (A. Sharp, C. Ross, unpublished) and have most likely arisen as either cloning or reverse transcription artefacts. 5' RACE was used to confirm that the ATG initiation codon was at the equivalent location to that in the rat hap1 cDNA and the 5' UTR sequence was determined to nucleotide –10. From these clones, hap1-A and hap1-B were found to be 3641 and 3130 nucleotides from the initiation codon respectively. cDNA fragments were cloned into the bacterial expression vector pTrcHis to confirm that hap1 fusion proteins of the correct size were produced.

The predicted protein sequence for mouse hap1-A and hap1-B (Fig. 1) shows a high degree of homology with the rat sequence (92.5% identity for hap1-A and 92.7% identity for hap1-B). In particular, the N-terminus (aa 276–363) and C-terminus (396–450) of the region shown to bind to htt

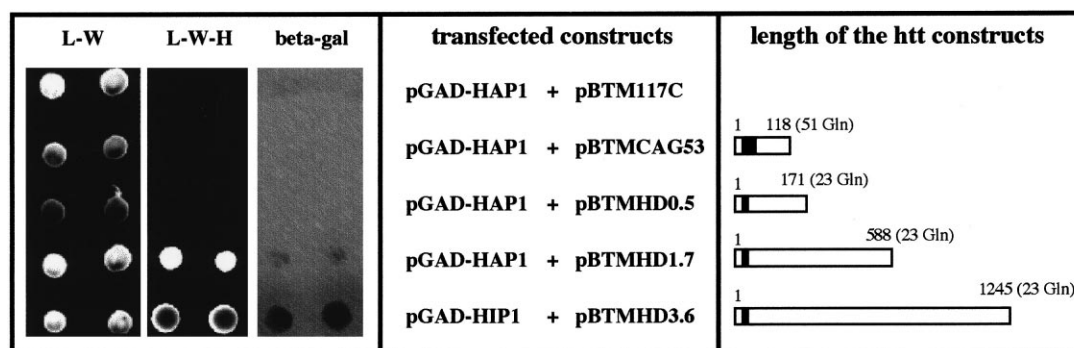


Fig. 3. Interaction of huntingtin and hap1 in the yeast two hybrid system. Activation of the *HIS3* and *lacZ* reporter genes by the interaction of hap1 or HIP1 with N-terminal fragments of htt as indicated. The *Saccharomyces cerevisiae* strain L40C harbouring pBTM117C, pBTMCAG53, pBTMHD0.5, pBTMHD1.7 and pBTMHD3.6 was transformed with pGAD-HAP1 or pGAD-HIP1. Individual Leu<sup>+</sup>Trp<sup>+</sup> transformants were selected on synthetic medium plates and replicated onto SD-Leu-Trp (L-W), SD-Leu-Trp-His (L-W-H) plates. The plates were incubated at 30°C for 3–5 days and replica filters were made for the  $\beta$ -galactosidase assay (beta-gal).

showed especially high homologies of 97.7% and 100% respectively. In the coding sequence, the only alignment discrepancy arose from the deletion of the rat codon 63 in the mouse sequence. As expected, comparison of the 3' UTRs show a higher degree of discrepancy including a deletion of the rat sequence from nt 3576 to nt 3635.

### 3.2. Expression analysis of hap1

In order to analyse the expression of hap1 in mouse tissues, we hybridised the entire coding region of hap1 to Northern blots containing a panel of mouse tissue RNAs (Fig. 2). In contrast to that observed in rat, the expression pattern of mouse hap1 was not only restricted to brain but also showed a high level of expression in testis, and a lower level in both lung and spleen. In all cases the hybridisation signal appeared as a doublet of 3.6 kb and 3.1 kb corresponding to the isoforms hap1-A and hap1-B respectively. Hap1-A appeared to be the major hap1 isoform and the hap1-A/hap1-B expression ratio did not differ between tissues. Comparison of mRNA expression between different brain regions showed a much higher level of expression in the olfactory bulb and colliculus than in striatum, cerebral cortex or cerebellum. The expression of hap1 in mouse does not mirror the major sites of HD neuropathology.

### 3.3. hap1 and htt protein interactions

As the htt-exon 1 protein has been shown to be sufficient to induce in mice a neurological phenotype with similarities to HD [8], we set out to determine whether mouse hap1 could interact with this exon 1 protein (1–90 aa with 23 gln; 1–118 aa with 51 gln). The 1.4 kb *Bgl*/II fragment coding for the first 473 amino acids of hap1 was cloned into the plasmid pGAD10 to generate a GAL4 activator domain fusion protein (pGAD-HAP1). This clone contained the entire hap1 fragment found to bind to htt, initially allowing the cloning of rat hap1 in the yeast two hybrid system [4]. pGAD-HAP1 was transformed into yeast containing different N-terminal fragments of htt fused to the binding domain of LEXA. Hap1 did not interact with htt fragments containing the first 118 aa (51 gln repeat) and 171 aa (23 gln repeat) but did interact with htt fragment 1–588 (Fig. 3). The interaction between HIP1 and htt was used as a positive control and hap1 was found to interact with these truncated htt fragments with a lower affinity than HIP1 (based on colony growth and X-gal staining).

The failure of hap1 to interact with the N-terminal 118 or 171 amino acids was not due to an absence of expression of htt in these clones which have shown positive interactions when tested with other proteins (Wanker, unpublished). The rat hap1 clone was initially isolated with a 1–230 aa htt clone and therefore, taken together, these results would indicate that the amino acids at a position between 171–230 are essential for the interaction.

## 4. Discussion

The normal function of htt is thought to be involved in vesicle trafficking and cytoskeletal functions, as within neurons htt is associated with vesicle membranes and microtubules [10,11]. This role is supported by the isolation of the htt interactors, HIP1 [5,6] and HAP1 [4]. HIP1 shows significant similarity to cytoskeleton proteins and is also enriched in subcellular compartments containing vesicle-associated proteins. HAP1 has recently been found to interact with cytoskeletal proteins, namely the p150<sup>Glued</sup> subunit of dynactin [12] and the pericentriolar protein PCM-1 (Duo) [13]. Analysis of axonal transport has shown that the rapid anterograde accumulation of both htt and HAP1 is compatible with their transport on vesicular membranes. Both proteins also show retrograde movement, possibly necessary for their degradation or for a function in retrograde membrane trafficking [14].

Insights into the molecular pathology of HD have recently been made from the analysis of the exon 1 HD transgenic mice [15]. Immunocytochemistry with antibodies raised against the N-terminus of htt (that detect the exon 1 protein) and ubiquitin identified the presence of neuronal intranuclear inclusions (NII) in symptomatic mice of all three lines prior to the onset of the phenotype [15]. NII had previously been described at the ultrastructural level in biopsy material from HD caudate [16] and more recently have been detected by immunocytochemistry with N-terminal antibodies in postmortem HD brains [17,21]. Interestingly, the inclusions are not detected with more C-terminal antibodies [17,21], and the identification of an N-terminal 40 kDa fragment on Western blots suggests that mutant htt is cleaved to release an N-terminal fragment capable of aggregation [17].

This interpretation of the neuropathological findings is supported by the production of the exon 1 protein in vitro as a glutathione *S*-transferase (GST) fusion protein, containing

CAG repeats ranging from (CAG)<sub>20</sub> to (CAG)<sub>122</sub> [18]. On removal of the GST tag, proteins containing pathogenic polyglutamine expansions form fibrillar aggregates with an amyloid-like morphology which is consistent with polar zipper interactions occurring between the polyglutamine repeats [19]. It appears that the mutant htt protein is cleaved to generate an N-terminal fragment that moves to, or is taken to, the nucleus, becomes ubiquitinated and forms insoluble amyloid-like aggregates. However, the order of these events and the underlying molecular interactions are currently unknown. It is becoming increasingly likely that the formation of fibrillar aggregates via polyglutamine interactions will be central to the molecular pathology of all CAG repeat disorders supported by the recent report of NII in post-mortem MJD [20], DRPLA [21,22] and SCA 1 brains [23].

We have shown that the htt amino acids 171–230, downstream of the polyglutamine stretch, are necessary for the interaction between htt and hap1. Therefore, hap1 is excluded from forming interactions with the exon 1 transgene protein and from playing a role in the molecular pathology of the HD transgenic mice. The demonstration that the exon 1 protein can form amyloid-like aggregates *in vitro* may indicate that further interactions are not necessary for the formation of these aggregates, and as predicted from this finding, neither the NII in transgenic mice nor in postmortem HD brains are immunoreactive for anti-HAP1 antibodies (S.W. Davies, personal communication; A. Sharp, unpublished). The selective vulnerability of specific neurons is likely to be due in part to subtle differences in expression levels [15]. However, huntingtin may be involved indirectly in cellular functions regulated by ras-related signalling pathways (via HAP1–PCIP-10 (Duo)–Rac1) [13], which may be consistent with the altered neuritic morphology seen in some surviving neurons [24].

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