VCP recruitment to mitochondria causes mitophagy impairment and neurodegeneration in models of Huntington’s disease

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Mutant Huntingtin (mtHtt) causes neurodegeneration in Huntington’s disease (HD) by evoking defects in the mitochondria, but the underlying mechanisms remains elusive. Our proteomic analysis identifies valosin-containing protein (VCP) as an mtHtt-binding protein on the mitochondria. Here we show that VCP is selectively translocated to the mitochondria, where it is bound to mtHtt in various HD models. Mitochondria-accumulated VCP elicits excessive mitophagy, causing neuronal cell death. Blocking mtHtt/VCP mitochondrial interaction with a peptide, HV-3, abolishes VCP translocation to the mitochondria, corrects excessive mitophagy and reduces cell death in HD mouse- and patient-derived cells and HD transgenic mouse brains. Treatment with HV-3 reduces behavioural and neuropathological phenotypes of HD in both fragment- and full-length mtHtt transgenic mice. Our findings demonstrate a causal role of mtHtt-induced VCP mitochondrial accumulation in HD pathogenesis and suggest that the peptide HV-3 might be a useful tool for developing new therapeutics to treat HD.
Huntington’s disease (HD) is a fatal and inherited neurodegenerative disorder. The disease is caused by an abnormal expansion of a CAG repeat located in exon 1 of the gene encoding the huntingtin protein (Htt), which confers a toxic gain of function to the protein. The most striking neuropathology in HD is the preferential loss of medium spiny neurons in the striatum. Although the genetic defect that causes HD has been identified as mutant huntingtin (mtHtt), a causative pathway from the disease mutation gene to neuronal death remains elusive. Neither a cure nor disease-modifying treatment is currently available.

Evidence suggests that mtHtt causes neurotoxicity by evoking defects in mitochondria, which in turn leads to a bio-energetic treatment is currently available. death remains elusive. Neither a cure nor disease-modifying treatment is currently available.

In this study, we report for the first time that VCP is aberrantly translocated to the mitochondria and bound to mtHtt in a variety of HD models. This accumulation of VCP on mitochondria results in excessive mitophagy and subsequent neuronal degeneration. Blocking VCP translocation to mitochondria by a novel peptide HV-3 that interferes with VCP and mtHtt interaction, inhibits VCP-mediated mitophagy impairment, and reduces HD-associated neuropathology and motor deficits in HD transgenic mouse models. Our results suggest that VCP recruitment to mitochondria by mtHtt is a crucial step in the initiation of neuropathology in HD.

**Results**

VCP is recruited to mitochondria by mtHtt in HD. We used HD mouse striatal HdhQ111 (mutant) and HdhQ7 (wild-type, wt) cells to profile the interactors of mtHtt on the mitochondria (Fig. 1a, Supplementary Fig. 1). HdhQ111 and Q7 cells were immortalized from knock-in mice carrying 111 and 7 CAG repeats, respectively, in the mouse htt gene. We isolated mitochondria from these cells, and conducted immunoprecipitation (IP) of mitochondrial fractions with anti-MAB2166 antibody that recognizes both wt and mutant (mt) Htt (Supplementary Fig. 1a). Tandem mass spectrometry analysis following affinity purification identified 9 proteins that putatively bound to mitochondria-associated mtHtt in HdhQ111 but not wt Htt in HdhQ7 cells (Fig. 1a, Supplementary Fig. 1b). Among these proteins, VCP was the leading candidate that bound to mtHtt on the mitochondria of HdhQ111 cells (Fig. 1a, Supplementary Fig. 1c).

Before validating the interaction between VCP and mtHtt, we examined whether VCP is localized on mitochondria in models of HD. Western blot analysis of cellular fractionations revealed that VCP was markedly enriched in the mitochondria of HdhQ111 cells relative to those in HdhQ7 cells (Fig. 1b), while there was no increase in the recruitment of VCP to the ER in HdhQ111 cells compared with that of HdhQ7 cells (Fig. 1b). Reduction of mtHtt levels by treatment of HdhQ111 cells with Htt silencing RNA (siRNA) abolished VCP translocation to mitochondria (Fig. 1c), indicating that mtHtt is required for VCP recruitment to mitochondria. Confocal imaging analysis consistently showed increased localization of VCP on the mitochondria, but not on the ER and endosome of HdhQ111 cells, relative to that in HdhQ7 cells (Fig. 1d, Supplementary Fig. 2a). Immunogold electron microscopy (EM) found more particles of immuno-labelled VCP localized on the surface of mitochondria in HdhQ111 cells than that in HdhQ7 cells (Fig. 1e). A similar enrichment of VCP on mitochondria was observed in mitochondrial fractions isolated from the striata of both R6/2 mice at the age of 9 weeks and YAC128 mice at the age of 6 months (Fig. 1f). To test whether VCP accumulation on mitochondria also exists in human HD, we analysed VCP localization on mitochondria by confocal microscopy in the caudate nucleus of postmortem brains from three HD patients and three normal subjects. We observed greater localization of VCP on mitochondria in HD patient brains than in normal subjects (Fig. 1g). These data collectively demonstrate that VCP is recruited to and accumulated on mitochondria in HD.
Because there was no evidence of increased VCP recruited to mitochondria in response to Parkinson’s disease-associated stress, it is likely that this recruitment is disease or stress dependent.

VCP binds to mtHtt on mitochondria in HD. Next, we isolated mitochondrial, ER, and cytosolic fractions from HdhQ7 and HdhQ111 cells, and conducted IP with anti-VCP antibody followed by immunoblotting (WB) with anti-1C2 antibody. Surprisingly, we observed mtHtt proteins in VCP immunoprecipitates of mitochondrial fractions, not in those of ER and cytosolic fractions in HdhQ111 cells (Fig. 2a, left panel). Although VCP interacted with wt Htt on the mitochondria in HdhQ7 cells, the extent is smaller than in HdhQ111 cells only expressing mtHtt (Fig. 2a, left panel). To further validate the interaction between VCP and mtHtt, we performed IP analysis with anti-VCP antibody followed by IB with anti-1C2 antibody that recognizes only expanded polyQ proteins. As shown in Fig. 2a, right panel, VCP bound only to mtHtt in mitochondrial fractions, not in ER or cytosolic fractions of HdhQ111 cells, even though mtHtt was expressed in the ER and cytosolic fractions. Consistently, mtHtt recognized either by anti-1C2 antibody or by anti-EM48 antibody was observed in VCP immunoprecipitates of mitochondrial fractions isolated from the striata of YAC128 mice at the age of 6 months (Fig. 2b). Again, there was no obvious
binding of VCP and mtHtt observed in the ER or cytosolic fractions of YAC128 mouse striata (Fig. 2b). We confirmed the interaction of VCP with mtHtt on the mitochondria but not in the ER or cytosolic fractions of HD patient fibroblasts (Fig. 2c). Furthermore, IP analysis of cortical brain lysates from postmortem brain tissues of HD patients showed that VCP bound to mtHtt in HD patients who exhibited moderate to severe neuronal loss and brain atrophy, but not in the patient with subtle neuropathology (Fig. 2d, Supplementary Fig. 2c, d). Altogether, these findings not only support our observation that VCP/mtHtt binding is implicated in HD pathogenesis, but also suggest a relevance of this binding to the severity of HD pathology. A recent proteomic analysis of the Htt interactome in total brain lysates of BACHD transgenic mice supported our finding that VCP is a binding protein of Htt and that increased interaction between VCP and mtHtt is relevant to HD. Now we are able to locate this interaction with mitochondria in models of HD in culture and in animals, as well as in patient cells. However, the mechanism underlying such a specific interaction between mtHtt and VCP on mitochondria requires further investigation.

VCP plays a central role in protein degradation via the ubiquitin–proteasome system by binding to its substrates and mtHtt can be degraded by the ubiquitin–proteasome system. We found that treatment with either MG132 (a proteasome
inhibitor that prevents protein degradation) or Eeyarestatin I (Eer1, an inhibitor that blocks VCP substrate degradation), did not affect Htt or mtHtt protein levels in HdhQ or HdhQ111 cells, respectively (Supplementary Fig. 3). These data exclude the possibility that Htt or mtHtt is a substrate of VCP on the mitochondria.

HV-3 peptide interferes with Htt/VCP interaction. We next ask what specific function VCP mediates on mitochondria from HD models. VCP knock-out is lethal in mice. Compounds that inhibit VCP function, such as N2,4-dibenzyquinazolone-2,4-diamine and Eeyarestatin I, are non-specific and rapidly lead to cell death. We previously demonstrated that short peptides interfering with specific protein–protein interactions, such as Drp1 peptide inhibitor P110 (or peptide inhibitors for protein kinase C (PKC, ref. 37), can be used as pharmacological tools in cell, animal, and human models to identify the role of interacting proteins in the pathogenesis of human diseases. Given that mtHtt is required for VCP translocation to mitochondria (Fig. 1c) and that VCP and mtHtt selectively interact on the mitochondria (Fig. 2), we sought to develop a peptide that blocks VCP association with mitochondria by interfering with VCP/Htt interaction.

Similar to the peptide designs for Drp1 peptide P110 or PKC peptide inhibitors, we used L-ALIGN sequence alignment software and identified two different regions of homology between VCP (human, AAI21795) and Htt (human, NP_002102; Fig. 3a). The four regions are marked as regions HV-1 to HV-4. We synthesized peptides corresponding to the four homologous regions between VCP and Htt (Fig. 3a), and conjugated them to the cell permeating TAT protein-derived peptide, TAT47-57, to enable in vivo delivery. These peptides are referred to as HV-1, HV-2, HV-3 and HV-4. By incubating these peptides with a mixture of GST–VCP and total lysates of mouse brain (expressing full-length Htt) followed by GST pull-down analysis, we found that only the addition of peptide HV-3 blocks the interaction of VCP/Htt in this in vitro binding assay (Supplementary Fig. 4a). In HEK293 cells co-expressing Myc-tagged full-length Htt with 23 or 73 CAG repeats (Myc-23Q FL or Myc-73Q FL, respectively) and green fluorescent protein (GFP)-VCP, consistent with our observation (Fig. 2), VCP was preferentially bound to Myc-73Q FL (mtHtt) over Myc-23Q FL (Fig. 3c). Of the four peptides tested, only HV-3 peptide significantly blocked the VCP/mtHtt interaction in Myc-73Q FL expressing cells (Fig. 3c, Supplementary Fig. 4b). The IC50 of HV-3 in blocking VCP/mtHtt interaction in Myc-73Q FL expressing cells was 2.11 μM (Supplementary Fig. 4c). Peptide HV-3 is derived from the Htt c-terminal and corresponds to a sequence in the D1 domain of VCP (Fig. 3a,b). The sequence of HV-3 in Htt is highly conserved among species (Supplementary Fig. 4d). With the exceptions of Htt and VCP, there is no sequence identity or similarity found between HV-3 and other proteins. Notably, treatment with HV-3 did not influence the interaction of mtHtt with Tim23, an event previously reported, nor did it influence the interaction between VCP and its known binding protein UBBD1 (ref. 39; Fig. 3d), suggesting a selectivity of HV-3.

Molecular docking analysis indicates that HV-3 is bound to the surface of the VCP structure (Supplementary Fig. 4e). Deletion of the sequence corresponding to HV-3 in VCP abolished the interaction between Htt and VCP (Supplementary Fig. 4f). Thus, HV-3 may represent an important interaction region for VCP in Htt. To test whether HV-3 exerts its effect through direct interaction with VCP and to determine the affinity of this interaction, we carried out isothermal titration calorimetry (ITC) with recombinantly expressed and purified full-length VCP (Fig. 3e). The heat exchanged as a result of the interaction between VCP and HV-3 peptide was used to calculate the binding affinity (Kd). Our analysis of the binding isotherms clearly showed that HV-3 binds to VCP with a Kd of 17.9 μM (Fig. 3e). To further examine the specificity of HV-3, we incubated biotin-conjugated HV-3 or TAT with total protein lysates of HD mouse striatal cells and HD YAC128 mouse brain followed by IP analysis. We found that biotin–HV-3, but not biotin–TAT, pulled down VCP and that biotin–HV-3 bound to VCP more strongly in HdhQ111 cells and YAC128 mouse brains relative to that in wt counterparts (Fig. 3f). No detectable bindings were observed between biotin–HV-3 and the cytosolic protein Enolase or between biotin–HV-3 and the mitochondrial protein Clpp (Fig. 3g). Therefore, HV-3 is most likely targeting VCP to interfere with the interaction of VCP/mtHtt.

Next, we test whether the peptide HV-3 affects the VCP association with mitochondria in HD models. In HdhQ111 mouse striatal cells, treatment with HV-3 abolished VCP translocation to the mitochondria relative to cells treated with TAT (Fig. 3g). In YAC128 mice, which express a full-length human mtHtt, the treatment blocked VCP translocation to mitochondria in the striatum at the age of 6 months relative to YAC128 mice treated with control peptide TAT (Fig. 3h, treatment timeline in Supplementary Fig. 5a). Similarly, HV-3 treatment suppressed the VCP translocation to mitochondria that occurred in striatum of HD R6/2 mice expressing an N-terminal mtHtt fragment (Fig. 3i, treatment timeline in Supplementary Fig. 5a). HV-3 treatment had no effects on VCP total protein levels in the above HD cell cultures and HD animal brains (Supplementary Fig. 5b). Thus, we selected HV-3 as a peptide candidate to inhibit VCP mitochondrial accumulation and to further determine its activity in HD models.

HV-3 treatment reduces mitochondrial damage and cell death. Mitochondrial depolarization and mitochondrial fragmentation are featured in experimental models of HD and human HD. Treatment with HV-3 markedly improved the mitochondrial membrane potential (MMP) in HdhQ111 cells, compared with the cells treated with control peptide TAT (Fig. 4a). Downregulation of VCP by VCP siRNA in HdhQ111 cells similarly promoted the MMP. However, HV-3 had no additional protection on the MMP in the presence of VCP siRNA (Fig. 4a), suggesting that VCP is required for HV-3 on improvement of mitochondrial function. Treatment with HV-3 also reduced the number of fragmented mitochondria (Fig. 4b) and increased mitochondrial length (Fig. 4c) in HdhQ111 cells. Using EM analysis, we further observed an increase in the number of mitophagosomes in HdhQ111 cells, whereas treatment with HV-3 reduced this accumulation (Fig. 4c). In HdhQ111 striatal cells subjected to 24 h of serum withdrawal, HV-3 treatment reduced the release of high mobility group box 1 (HMGB1) and lactate dehydrogenase (LDH), two indicators of cell death (Fig. 4d,e). We found that HV-3 treatment had no effect on the ER stress response (Supplementary Fig. 6a), excluding the possibility that the protection provided by HV-3 to mitochondria is a secondary consequence of the inhibition of ER stress.

Neurons derived from HD patient-induced pluripotent stem cells (HD-iPS cells) exhibited mitochondrial damage and increased cell death. In neurons immunopositive for both anti-DARPP-32 (a marker of medium spiny neurons) and anti-Tubulin β-III (a marker of neurons), treatment with HV-3 reduced neurite shortening compared with patient neurons treated with control peptide TAT (Fig. 4f,g). The neuroprotective effects of HV-3 were consistently observed in all cell lines tested. The neuroprotective effects of HV-3 were consistently observed in all cell lines tested.
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**Figure 3 | HV-3 peptide blocks Htt/VCP binding.** (a) Sequence of homology between VCP (human, AA121795) and Htt (human, NP_002102). Amino acids are represented by the one-letter code; stars (*) indicate identical amino acids; Columns (:) indicate high similarity between amino acids. (b) Stick drawings of VCP and Htt main domains. Highlighted in the same colours are the two regions of homology between the two proteins, regions HV-1 and HV-3 in Htt and the corresponding regions HV-2 and HV-4 in VCP. (c) HEK293 cells were transfected with Myc-full-length Htt with 23 Q or 73Q (Myc-23Q FL or Myc-73Q FL) for 48 h following treatment with HV-3 or TAT (3 µM per day, each). The total lysates of cells were subjected to IP followed by WB with the indicated antibodies. (d) The total cell lysates were subjected to IP followed by WB in the indicated groups. (e) Gel filtration chromatogram and SDS-PAGE gel of recombinantly expressed and purified full-length mouse VCP/p97 (upper). Equilibrium binding isotherm for VCP titrated against HV-3 peptide at 15 °C (lower). Each downward spike is from a single injection of HV-3 into the sample cell. The heat exchanged during each injection is calculated from the heat exchanged during each injection is calculated from the area under the spike and fit to a binding isotherm. The Kd and n for HV-3 binding are 17.9 ± 0.65 Kcal mol⁻¹ and 2.02 ± 0.23, respectively. The values of ΔH and ΔS are −2.145 ± 0.65 Kcal mol⁻¹ and 13.97 ± 3.4 cal mol⁻¹ deg⁻¹, respectively. (f) Biotin-conjugated HV-3 or TAT (10 µM, each) was incubated with total lysates of HD cells or YAC128 mouse brains. Immunoprecipitates were analysed by WB with the indicated antibodies. All Blots shown above are representative of three independent experiments. (g) HD cells were treated with TAT or HV-3 (3 µM per day for 3 days), n = 3. (h) YAC128 or wild-type mice from 3–6 months of age and (i) R6/2 or wild-type mice from 5 to 9 weeks of age were received either TAT or HV-3 (3 mg kg⁻¹ per day), n = 6 mice/group. VCP mitochondrial levels were determined by WB. Loading control: VDAC. Data are mean ± s.e.m. (g-i) ANOVA with Holm-Sidak post hoc test.
associated with improved MMP and mitochondrial length along neurites (Fig. 4h). Further, HV-3 treatment suppressed neuronal cell death in neurons subjected to growth factor withdrawal (Fig. 4i). Taken together, these results demonstrate that treatment with HV-3 protects against mitochondrial damage and cell death under HD-associated conditions.

We found that the peptide HV-3 had only minor effects on VCP mitochondrial levels, MMP, and mitochondrial morphology, as well as cell survival rate in wt counterparts of the above HD models (Figs 3 and 4). This is likely the result of less binding between VCP and wt Htt under basal conditions (Figs 2a and 3c). Normal and mutant polyglutamine proteins...
interact with VCP, but only mutant proteins specifically affect the activity of VCP and impair its function,26, thus it is also possible that disruption of wt Htt/VCP interaction by HV-3 results in only minor physiological impacts.

**VCP translocation to mitochondria impairs mitophagy in HD.** Apoptosis and autophagic cell death are manifested in HD neuropathology.24,32. Blocking VCP recruitment to mitochondria by treatment with HV-3 did not affect apoptosis, as evaluated by the activity of caspase-3 (Supplementary Fig. 6b). In contrast, HV-3 treatment greatly reduced the accumulation of mitophagosomes in HdhQ111 cells (Fig. 4c). Down-regulation of VCP by VCP siRNA in HdhQ111 cells reduced the levels of mitochondria-associated LC3 II, which is a marker of mitophagy44 (Fig. 5a). Expression of Flag-VCP in wt mouse striatal cells induced GFP-LC3B association with mitochondria, which could be inhibited by treatment with HV-3 (Fig. 5b). Note that HV-3 treatment had no effect on the total protein level of GFP-LC3B (Supplementary Fig. 6c). Thus, we speculated that mtHtt-induced VCP accumulation on mitochondria triggers mitophagy-associated autophagy.

In HdhQ111 cells, we observed an increased number of GFP-LC3B puncta, a specific marker for autophagosomes, and hyperactivity of lysosome enzyme Cathepsin B, both of which were reduced by treatment with HV-3 (Fig. 5c,d). Similarly, neurons derived from HD-iPS cells exhibited lower mitochondrial mass and lysosome hyperactivity, whereas treatment with HV-3 corrected these aberrant events (Fig. 5e,f). Further, we examined the ultrastructure of striatal mitochondria in YAC128 mice. Consistent with the findings in cells (Fig. 4c), we observed an increase in the number of mitophagosomes in 9-month-old YAC128 mice treated with the control peptide TAT, which was reduced by HV-3 treatment (Fig. 5g). These findings suggest that inhibition of VCP mitochondrial accumulation in HD by HV-3 suppress excessive mitophagy and improve mitochondrial quality.

LC3 in mammals or Atg8 in yeast plays a key role in both autophagosome membrane biogenesis and cargo recognition.45. In yeast, Atg32 functions as a receptor on mitochondria to initiate mitophagy through interaction with Atg8 (refs 46,47). Similarly, mammalian mitophagic adaptors, such as FUNDCL1 (ref. 48), p62 (ref. 49), BNIP3 (ref. 50) and AMBRA1 (ref. 51), all bind to LC3 via a typical linear motif with a core consensus sequence of W/Y/F X L/I/V, also called LC3-interacting region (LIR)52. Given the above findings, we hypothesized that VCP might bind to LC3 on mitochondria to enhance mitophagosome production. Using an iLIR server53, we found that VCP contains two segments of sequence (LEAYRPR and AVEFKVVE) located in the β strands of the N-terminal (Fig. 6a) that fulfill the characteristics of the LIR52. To determine whether VCP binds to LC3 via putative LIR motifs, we generated two mutants (VCP-YIAA and VCP-FVAA) in which Y/I and F/V were all replaced by alanine, respectively. In HeLa cells co-expressing Myc-VCP and GFP-LC3B, we found that Myc-VCP was bound to GFP-LC3B in the mitochondrial fractions of cells (Fig. 6b). Expression of either VCP-YIAA or VCP-FVAA abolished the VCP/LC3 interaction (Fig. 6b). Moreover, expression of VCP-YIAA or VCP-FVAA reduced LC3 association with the mitochondria (Fig. 6c) and increased mitochondrial mass (Fig. 6d) compared to cells expressing VCP wt. These data demonstrate that mitochondria-accumulated VCP accelerates mitophagy by interacting with LC3 through the LIRs.

To determine direct consequences of VCP mitochondrial accumulation on mitophagy and cell survival, we generated a construct encoding VCP fused to a flag-vector containing a mitochondrial targeting sequence (MTS) (Flag-mtVCP). In HeLa cells expressing Flag-mtVCP, we confirmed the enrichment of Flag-mtVCP on mitochondria (Supplementary Fig. 7a). We further observed that expression of Flag-mtVCP induced a transcriptional upregulation of the mitochondria network, forming mitochondrial aggregates around the perinuclear envelope (Supplementary Fig. 7a), which is an intermediate step of mitophagy.51,54. The occurrence of mitochondrial aggregates in cells expressing Flag-mtVCP increased approximately sevenfold relative to cells not expressing Flag-mtVCP (Supplementary Fig. 7b). Moreover, the presence of Flag-mtVCP in cells decreased MMP and mitochondrial mass (Supplementary Fig. 7c,d), but induced an increase in the percentage of GFP-LC3B colocalizing with Tom20-labelled mitochondria (Supplementary Fig. 7e). On treatment with bafloimycin A to prevent autophagosome-lysosome fusion, Flag-mtVCP expression elevated the autophagic flux of the mitochondria (Supplementary Fig. 7f), indicating an increased rate of mitochondrial degradation.

In rat primary striatal neurons, expression of Flag-mtVCP-WT elicited mitochondrial aggregates and caused neurite shortening in medium spiny neurons that were labelled by anti-DARPP-32 antibody (Fig. 6e,f-top panel, g,h). In contrast, neurons expressing either Flag-mtVCP-FVAA or Flag-mtVCP-YIAA exhibited fewer mitochondrial aggregates and longer neurites of medium spiny neurons (Fig. 6e,f-middle and bottom panels, g,h). Thus, mitochondria-accumulated VCP contributed to mitochondrial and neuronal damage in primary striatal neurons via impairment of the mitophagic process.

**Figure 4 | HV-3 treatment reduces mitochondrial damage and cell death in HD cell cultures.** Mouse HdhQ7 and HdhQ111 striatal cells were treated with control peptide TAT or peptide HV-3 (3 μM/day for 3 days). (a) Left panel: Mitochondrial membrane potential (MMP) was determined by TMRM fluorescent dye. Right panel: HdhQ111 cells were transfected with control siRNA (siCon) and VCP siRNA (siVCP) for three days. The MMP was determined by TMRM fluorescent dye. Scale bar: 10 μm. The percentage of cells with fragmented mitochondria relative to the total number of cells was quantitated. At least 100 cells per group were counted. (c) Mitochondrial morphology was determined by EM. The length of mitochondria and the number of mitophagosomes were quantitated. At least 90 mitochondria per group were counted. (d) HD striatal cells were subjected to serum starvation for 24 h. HMGBl release into culture medium was determined by ELISA analysis with anti-HMGBl antibody. (e) HD striatal cells were subjected to serum starvation for 24 h. Cell death was determined by the release of LDH. Control and HD patient-iPS cell derived neurons were treated with HV-3 or TAT at 1 μM per day for 5 days starting 30 days after initiation of neuronal differentiation. (f) Left: Neurons were stained with anti-DARPP-32 and anti-Tuj-1 antibodies to indicate medium spiny neurons. Upper: a cluster of small neurites. Scale bar: 10 μm. (g) Quantification of neurite length of medium spiny neurons. At least 50 neurons per group were counted by an observer blind to experimental conditions. (h) Left: the MMP was determined by TMRM fluorescent dye. Right: Mitochondria were stained by anti-Tom20 antibody. Mitochondrial length along neurites of DARPP32/Tuj1-positive neurons was quantitated. (i) Neuronal cell death induced by the withdrawal of the growth factor BDNF for 24 h was determined by the release of LDH. All data are mean ± s.e.m. from at least three independent studies. ANOVA with Holm-Sidak post hoc test.
HD R6/2 mice treated with the control peptide TAT exhibited decreased horizontal and vertical activities as well as less total distance travelled in the test of spontaneous locomotion when evaluated at the age of 13 weeks. Treatment with HV-3 markedly corrected these motor deficits (Fig. 7a). The severity of clasping behaviour in R6/2 mice treated with HV-3 was significantly lower over the 4-week observation period than it was in those treated with the control peptide TAT (Fig. 7a). HV-3 treatment also resulted in increased body weight and survival rate of R6/2 mice (Fig. 7b,c). The treatment had no effects on motor ability, body weight, or life span in wt mice (Fig. 7a-c), suggesting a lack of toxicity of HV-3 treatment.

YAC128 mice exhibited progressively deficits in motor activities; they showed gradually decreasing motor coordination activity on the rotarod and defects in general motility measured by locomotor activity chambers. Sustained treatment with HV-3 improved general movement activity and rotarod performance of YAC128 mice starting at the age of 6 months, and the protection lasted until the age of 12 months (Fig. 7d,e). Again, the treatment did not affect motor activity in wt mice from 3 to 12 months of age. We found that HV-3 at 3 mg kg\(^{-1}\) per day was not toxic to naive mice (Supplementary Fig. 8). Treatment with HV-3 had no significant effects on the immunodensity of CD3, a marker of T-cell for adaptive immune response, in brain and spleen samples.
Figure 6 | VCP causes excessive mitophagy by binding to LC3 via LIRs. (a) Putative LIR sequences in VCP were aligned manually for comparison with the classical LIR motifs of ATG32, FUNDC1 and p62. The amino acids in blue indicate the conserved core residues of LIR. (b) GFP-LC3B was co-expressed with the indicated plasmids in HeLa cells. Mitochondrial lysates were subjected to IP with anti-GFP antibody, and immunoprecipitates were analysed by WB with anti-Myc and anti-GFP antibodies. Representative blots are from three independent experiments. (c) GFP-LC3B was co-transfected with the indicated plasmids in HeLa cells. Mitochondria were isolated and GFP-LC3B mitochondrial protein levels were determined by WB. Data are mean ± s.e.m. from four independent studies. (d) HeLa cells were transfected with the indicated plasmids. Mitochondria were stained with anti-Tom20 antibody. Mitochondrial mass was determined by quantitating fluorescent density of Tom20 immunostaining. At least 100 cells per group were counted. Data are mean ± s.e.m. from three independent studies. Primary rat striatal neurons (DIV 7) were transfected with either Flag-mtVCP-WT, or Flag-mtVCP-FVAA or Flag-mtVCP-YIAA plasmids for 3 days. (e) Neurons were stained with anti-Tom20 (green) and anti-Flag (red) antibodies. Mitochondrial morphology was examined by microscopy. (f) Medium spiny neurons were labelled with anti-DARPP-32 (green). Arrows indicate the cells that were not transfected with Flag-VCP. Arrowheads show the cells with transfected Flag-VCP. (g) Mitochondrial aggregates in neurons expressing Flag-mtVCP or Flag-mtVCP-FVAA or Flag-mtVCP-YIAA were quantitated. (h) Neuronal morphology was imaged and the neurite length of medium spiny neurons expressing Flag-mtVCP or Flag-mtVCP-FVAA or Flag-mtVCP-YIAA was quantitated. At least 50 neurons per group were counted by an observer blind to experimental conditions. Scale bars: 10 μm. All the data are mean ± s.e.m. from three independent experiments. ANOVA with Holm-Sidak post hoc test.
HV-3 treatment reduces neuropathology of HD mice. The levels of dopamine signalling protein, DARPP-32, enriched in medium spiny neurons are decreased in the striatum of HD patients and mouse models\(^5\). Thus, DARPP-32 has been used as a marker to assess neuronal degeneration in HD mouse models. Western blot analysis of striatal extracts revealed a significant reduction of DARPP-32 protein levels in both R6/2 and YAC128 mice. HV-3 treatment significantly increased DARPP-32 levels in the two mouse models (Fig. 8a). In HD R6/2 mice, we consistently observed a decrease in the area occupied by DARPP-32-immunostained cells in the striatum, which was increased by HV-3 treatment (Fig. 8b,c). To further assess whether HV-3 treatment can suppress neurodegenerative pathology in HD, we conducted unbiased stereology analyses to measure the number of striatal neurons in YAC128 mice at the age of 12 months. We found that treatment with HV-3 significantly increased the number of neurons positive for anti-NeuN immunostaining in the dorsolateral striatum (Fig. 8d).

**Discussion**

In this study, we reported that mtHtt-induced recruitment of VCP to mitochondria caused HD-associated neurodegeneration,
as evidenced by the fact that blocking VCP mitochondrial accumulation with the peptide HV-3 corrected excessive mitophagy and mitochondrial dysfunction, and reduced HD neuropathology in vitro and in vivo (Fig. 8e). Thus, the mitochondria-accumulated VCP might represent a new therapeutic target for combatting neurodegeneration in HD. VCP has been shown to bind to Htt in HD mouse brains and in postmortem patient brains. Here, we further showed that the binding of VCP and mtHtt mainly occurred on the mitochondria of HD cell cultures and animal brains. It is possible that VCP was aberrantly recruited to mitochondria via mitochondria-bound mtHtt through protein-protein interactions. Indeed, our in vitro and in vivo data showed that blocking VCP/mtHtt binding with HV-3 abolished VCP translocation to mitochondria and reduced mitochondrial damage, further emphasizing that the binding of VCP/mtHtt is required for VCP relocation to the mitochondria. Significantly, inhibition of VCP/mtHtt binding reduced HD-related behavioural and pathological phenotypes in two HD transgenic mouse models. Thus, the formation of the aberrant VCP/mtHtt complex on the mitochondria may be a key step in initiating mitochondrial injury, which in turn results in the neuronal pathology of HD.

The peptide HV-3 is derived from Htt and represents a sequence homologous to VCP. We further showed that HV-3 binds to VCP; HV-3 has a relatively high affinity for VCP (Fig. 3). These findings suggest that HV-3 might compete with Htt binding to VCP or that it targets VCP and prevents the
exposure of the VCP-binding site on Htt. Fujita et al showed that mtHtt weakly affects VCP enzyme activity25, suggesting that the enzyme activity of VCP might not be a key in mediating the binding of VCP to mtHtt. Currently we are determining whether HV-3 affects VCP conformational changes using VCP crystal structures, which may alter VCP activity.

We found that HV-3 can block VCP accumulation on mitochondria and provide neuroprotection in HD R6/2 mice in which an N-terminal mtHtt fragment is expressed. VCP has been shown to bind to Htt exon 1 fragment with expanded polyQ via the polyQ tract sequence and co-localize with mtHtt in perinuclear cytoplasmic region of neurons in R6/2 mice28. Because N-terminal mtHtt fragments can co-localize and co-aggregate with normal Htt fragments28, it is possible that VCP, mtHtt fragment and endogenous Htt form a protein complex in HD R6/2 mice. HV-3 may block VCP accumulation on the mitochondria in R6/2 mice by disrupting the interaction of the complex, thus reducing subsequent mitochondrial damage. However, other mechanisms may exist, which remain to be further investigated.

VCP translocation to mitochondria mediates turnover of the mitochondrial fusion protein Mitofusin 1 and subsequent Parkin-related mitophagy in HeLa cells.15 In our study, we found that targeting VCP to mitochondria is sufficient to induce massive mitochondrial clearance in HeLa cells and HD striatal cells when Parkin is not present (Supplementary Fig. 7g). Thus, VCP-mediated excessive mitophagy in HD may be independent of the Parkin-related pathway, although the detailed mechanism requires further investigation. While a basal level of mitophagy is essential for neuronal health, excessive mitophagy may cause harm by removing too many mitochondria, which leads to rapid neuronal death44,57 and is centrally implicated in the pathogenesis of neurodegenerative diseases58. Because mtHtt is ubiquitously expressed and is required for VCP translocation to mitochondria (Fig. 1c), it is conceivable that mtHtt causes excessive accumulation of VCP on the mitochondria, which disrupts the balance of mitophagy, leading to excessive mitochondrial degradation and subsequent neuronal death.

Htt can function as a scaffold protein for autophagy, and C-terminal Htt has been suggested to be essential for mitophagy induction under physiological conditions59,60. Expression of the C-terminal Htt fragment in rat primary cortical neurons or striatal cells caused cell death59,61. Similarly, we found that targeting VCP to mitochondria is required for mitophagy and leads to death of medium spiny neurons, again supporting the idea that well-balanced mitophagy is critical for maintaining neuronal survival. Although there are recent findings that suggest that the peptide HV-3 is well tolerated in normal mice. Thus, development of inhibitors, such as HV-3-like reagents, may have the potential to open up a new therapeutic route for HD and multiple polyglutamine diseases in which VCP translocation to mitochondria is characterized.

Methods

Antibodies and reagents. Protein phosphatase inhibitor and protease inhibitor cocktails were purchased from Sigma-Aldrich. VCP inhibitor Erz 1 and proteasome inhibitor MG132 were from Tocris Bioscience. Antibodies for Tom20 (sc-11415, 1:1,000), c-Myc (sc-40, 1:1,000), GFP (sc-9996, 1:1000), GST (sc-138, 1:500), CD3 (sc-20047, 1:500), Enolase (sc-15343, 1:1,000), Tim23 (sc-51463, 1:500) and Parkin (sc-32382, 1:1,000) were from Santa Cruz Biotechnology. Full-length Htt (NM_172374, 1:1000), polyQ (MAB1574, 1:1000), EM48 (MAB5377, 1:1000) and NeuN (MAB377, 1:500) antibodies were from Millipore. Pan-actin (A1978, 1:10,000) and Flag (F3165, 1:5,000) antibodies were from Sigma-Aldrich. Antibodies for VDAC (ab14734, 1:2,000), Clpk (ab124822, 1:1,000), UBXD1 (ab103651, 1:500) and VCP (ab109240, 1:10,000) antibodies were from Cell Signalling. WFS1 (NB100-1918, 1:1,000) antibody was from Novus, HMBG1 (10829-AP-1, 1:1,000) antibody was from ProteinTech, and GRP78 (ADI-SPA-826, 1:1,000) and Calnexin (AD1-SPA-860, 1:1,000) antibodies were from Enzo Life Sciences. Anti-mouse IgG and anti-rabbit IgG, peroxidase-linked, species-specific antibodies were from Thermo Scientific.

Constructs and transfection. Myc-tagged full-length Htt with 23Q or 73Q plasmid was obtained from the CHDI Foundation. The full-length VCP wt and GFP-LC3B plasmids were obtained from Addgene. To construct the mitochondria-targeting VCP plasmid, CMV-mito-GERMEDIC1 was digested with BamHI and HindIII, and VCP was PCR-amplified and inserted into the plasmid backbone. Site-directed mutagenesis of VCP plasmid was performed using a site-mutagenesis kit (Agilent Technologies, Inc.). Cells were transfected with TransIT-2020 (Mirus Bio, LLC) following the manufacturer’s protocol.

Cell culture. Immortalized striatal cell lines HdhQ111 mutant and HdhQ7 wt cells were obtained from the CHDI Foundation. Cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418. Cells were grown at 33 °C in a 5% CO2 incubator. Cells within 1-4 passages were used in all experiments.

Huh7 hepatocarcinoma cells (HeLa cells) and HEK293 cells were maintained in DMEM supplemented with 10% FBS and 1 (v/v) penicillin/streptomycin. HD patient fibroblasts (HD1: GM21756, HD2: GM03621; purchased from Coriell Institute, USA) and normal fibroblasts (Con 1, fibroblasts from adult, HDFa; Con 2, fibroblasts from juvenile, HDFj; purchased from Invitrogen) were maintained in MEM supplemented with 15% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. Primary striatal neurons from E18 rat midbrain tissue (BrainBits, Springfield, IL, USA) were seeded on cover slides that were coated with poly-D-Lysine/laminine and grown in neurobasal medium supplemented with 2% B27 and 0.5 mM glutamate. At 7 DIV, cells were transfected with the control vector or flag-VCPmt using TransIT-2020 Transfection Reagent combined with formulated TransIT-2020 transfection media for primary neurons (BrainBits, USA).

iPS cells from a normal subject and a HD patient (carrying 41 CAG repeats) were differentiated into neurons using the protocol from our previous studies5. Briefly, iPS cells were plated onto 6-well plates precoated with 2.5% Matrigel and allowed to reach 90% confluence in feeder-free medium. For the first 10 days, cells were treated with SB431542 (10 μM; Tocris Bioscience) and Noggin (100 ng/ml) in Neural Media (NM) containing Neurobasal and Dexam (1:1), B27 supplement minus vitamin A (50 ×, Invitrogen), N2 supplement (100 ×, Invitrogen), GluMax (Invitrogen, 100 ×), FGF2 (20 ng/ml) and EGF (20 ng/ml), 100 units per ml penicillin and 100 μg/ml streptomycin. For the next 10 days, cells were treated with human recombinant Sonic hedgehog (SHH, 200 ng/ml), DKK1 (100 ng/ml) and BDNF (20 ng/ml) and 10μM Y27632 (Sigma, 1:1,000) and Y27632 (Sigma, 1:1,000) and Y27632 (Sigma, 1:1,000). Normal fibroblasts from Coriell Institute, USA) and normal fibroblasts (Con 1, fibroblasts from adult, HDFa; Con 2, fibroblasts from juvenile, HDFj; purchased from Invitrogen) were maintained in MEM supplemented with 15% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin.

All of the above cells were maintained at 37 °C in 5% CO2–95% air.

RNA interference. For silencing Htt and VCP in HD striatal cells, control siRNA, mouse Htt and mouse VCP siRNA were purchased from Thermo Fisher Scientific. HdhQ111 cells and HdhQ7 cells were transfected either with control siRNA or Htt or VCP siRNA using TransIT-TKO Transfection Reagent (Mirus Bio, LLC), according to the manufacturer’s instructions. The sequences for the siRNAs used in
this study are as follows: mouse Ht, 5′-GGUUUAUGACACUUUGUUUGT-3′; Moose VCP, 5′-GAAGCAGCAGACCGUUAG-3′; control non-targeting siRNA, 5′-TTCTGCCAGAAGTTCAGCGTF-3′.

**Isolation of subcellular fractions.** Cells were washed with cold PBS and incubated on ice for 30 min in a lysis buffer (250 mM sucrose, 20 mM HEPES-NaOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Mouse brains were minced and homogenized in the lysis buffer and then placed on ice for 30 min. Collected cells or tissue were disrupted by repeated aspiration through a 25-gauge needle, followed by a 30-gauge needle. The homogenates were spun at 800 g for 10 min at 4 °C, and the resulting supernatants were spun at 10,000 g for 20 min at 4 °C. The pellets were washed with lysis buffer and spun at 10,000 g again for 20 min at 4 °C. The final pellets were suspended in lysis buffer containing 1% Triton X-100 and were mitochondrial-rich lysate fractions. The supernatant was centrifuged at 10,000 g, 4 °C, for 1 h. The pellets were suspended in lysis buffer containing 1% Triton X-100 as ER fractions. The final supernatant was cytosolic fractions. The mitochondrial proteins VDAC, the ER protein WFS1 and the cytosolic protein Enolase were used as loading controls for mitochondrial, ER and cytosolic fractions, respectively.

**Immunoprecipitation.** Cells were lysed in a total cell lysate buffer (300 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitor) or in a mitochondrial isolation buffer above. Total or mitochondrial lysates or the mixture of ER and cytosolic fractions were incubated with the indicated antibodies overnight at 4 °C followed by the addition of protein A/G beads for 1 h. Biotin-HV-3 and Biotin-TAT (10 μM each) were incubated with total lysates of cell cultures or mouse brains for overnight at 4 °C followed by the incubation with streptavidin beads for 1 h. Immunoprecipitates were washed four times with cell lysate buffer and were analysed by SDS–PAGE and IB.

**Rational design of peptide inhibitor.** Two nonrelated peptides that interact in an inducible manner have often shared short sequences of homology that represent sites of both inter- and intra-molecular interactions63,65. Similar to the peptide design for PKC peptide deltaV1-1 (ref. 37) and Drp1 peptide P110 (ref. 36), we targeted the conserved regions of human, mouse, rat and fish. We synthesized the four peptides at American Peptide Company (Sunnyvale, CA) corresponding to regions HV-1-4 and HV-5-6. The purity was assessed as >99% by SDS–PAGE analysis, were pooled and concentrated to a final concentration of 5 mM and loaded into the syringe. The peptide concentration of 0.5 μM tetramethyl rhodamine (Invitrogen Life Science) for 4 h. The fluorescence of Tom20 (1:500) or mitotracker green was quantitated using NIH Image J software.

**Electron microscopy.** The HdhiQ7 and HdhiQ111 cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Small pieces of the striata tissue of mice were fixed by immersion in triple aldehyde-DMSO. After rinsing in 0.1 M phosphate buffer (pH 7.3), the samples were post-fixed in ferrocyanide-reduced osmium tetroxide.

**Animal model of HD.** All experiments in animals were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and were performed based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sufficient procedures were employed for reducing pain or discomfort of subjects during the experiments.

Male R6/2 mice and their wt littermates (4 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME; B6CBA-TgN (HD exon 1)^62; JAX stock 004984). These mice (C57BL/6 and CBA genetic background) are transgenic for the 5′ end of the human HD gene carrying 100–150 glutamine (GQ) repeats; an observation time of 15–18 months.

YAC128 (FVB-Tg[YAC128]B5H/N), JAX stock number: 004938) breeders (FVB/N genetic background) were purchased from Jackson Laboratories. The sample in the cell was replaced by Buffer C and all other conditions were kept identical.

**Measurement of cell viability.** HdhiQ7 and Q111 mouse striatal cells were treated with the HV-3 peptide or the control peptide TAT (3 μM each) in an FBS-free DMEM medium or in DMEM containing 10% serum for 24 h. Medium from the cultured cells was harvested. Proteins from the medium were purified using Amicon Ultra 0.5 ml centrifugal filters (Millipore). HMGB1 release into the medium was then analysed by Western blotting with anti-HMGB1 antibody. In parallel, cell death was determined by measuring LDH release into the culture medium, using LDH-Cytotoxicity Assay Kit II (Roche, USA) by following the manufacturer’s instruction.

**Immunocytochemistry.** Cells cultured on coverslips were washed with PBS and fixed in 4% formaldehyde, and then permeabilized with 0.1% Triton X-100. After incubation with 2% normal goat serum, fixed cells were incubated overnight at 4 °C with indicated primary antibodies. Cells were washed with PBS and incubated with Alexa Fluor 568, 488 or 405 secondary antibody, followed by incubation with Hoechst dye (1:10,000; Invitrogen). Coverslips were washed, and slides were imaged by confocal microscopy (Fluoview FV100; Olympus).

To determine mitochondrial mass in cultures, cells were stained with antibodies against Tom20 or stained with MitoTracker green. The fluorescent density of Tom20 (1:500) or mitotracker green was quantitated using NIH Image J software. To measure the membrane potential of mitochondria in cultures, cells were incubated with 0.25 μM tetrataethyl rhodamine (Invitrogen Life Science) for 20 min at 37 °C. To determine lysosomal activity, cells were incubated with 1 μM of Red dye (Enzo Life Science) for 30 min at 37 °C. The images were visualized by microscope and quantitation of the density of red fluorescence was carried out using NIH ImageJ software. For immunocytochemistry study, at least 100 cells per group were counted and quantitated by an observer blind to experimental conditions.

**Patient-iPS cell-derived neurons.** To ensure the observation of mitochondria in the medium spiny neurons, the cells were stained with a mitochondrial marker (anti-TOM20, 1:500) and markers for medium spiny neurons (DARP-32 and Tu1 (neuron-specific class III beta-tubulin, 1:5,000, Covance). At least 50 neurons/group were counted.

**VCP expression and purification.** The full-length mouse VCP/p97 (residues 2-806) was expressed with an N-terminal 6His-tag (Addgene: plasmid # 12373) in the Rosetta (DE3) strain of Escherichia coli (Novagen) as previously described64. Briefly, the cells were grown in LB media supplemented with Kanamycin and induced at OD~1.0 for 16 h at 18 °C. Cells were homogenized in Buffer A (500 mM KCl, 10 mM HEPES pH 8.0, and 100 mM imidazole) and were purified by passing through Superdex 200 increase 10/300 GL (GE Healthcare) size exclusion column equilibrated with Buffer B (250 mM KCl, 25 mM HEPES pH 8.0, 2 mM β-mercaptoethanol and 1 mM MgCl2). The fractions containing the pure sample, as determined by SDS–PAGE analysis, were pooled and concentrated to a final concentration of ~3–5 mg ml⁻¹.

**Isothermal titration calorimetry.** Binding of HV-3 peptide to VCP was measured by isothermal titration calorimetry on a Microcal ITC200 (GE Healthcare). Before the calorimetric titration, the protein was exchanged into Buffer C (100 mM NaCl, 10 mM Hepes, pH 8.0) on a PD-10 column (Amersham Biosciences) and concentrated to ~5–6 mg ml⁻¹. The final concentration of protein in the cell (~60–70 μM) was determined on a NanoDrop Spectrophotometer (Thermo Scientific NanoDrop 2000) using a molecular weight of 89,32 kDa and a molar extinction coefficient of 36.62 M⁻¹ cm⁻¹. The HV-3 peptide was dissolved in Buffer C to a final concentration of 2 mM and loaded into the syringe. The measurements were made at a constant cell temperature of 15 °C and repeated at least three times. Thirty successive injections of 1.2 μl each were titrated into the cell with 40 μl at a constant rate of 1,001.0 μl/min. 15 injections were set between consecutive injections. The binding isotherms were analysed with the Microcal Origin software. For measurements of heat of dilution, the protein sample in the cell was replaced by Buffer C and all other conditions were kept identical.
YAC128 mice contain a full-length human huntingtin gene modified with a 128 CAG repeat expansion in exon 1. The mice were mated, bred and genotyped in the animal facility of Case Western Reserve University. Male mice at the ages of 2, 3, 6, 9 and 12 months were used in the study. All of the mice were maintained with a 12-h light/dark cycle (on 06:00 hours, off 18:00 hours).

**Systemic peptide treatment in HD mice.** All randomization and peptide treatments were prepared by an experimenter not associated with behavioural and neuropathology analysis.

Male hemizygous R6/2 mice (Tg) and their age-matched wt littermates (5-week-old) were implanted with a 28-day osmotic pump (Alzet, Cupertino CA) containing TAT control peptide or HV-3 peptide, which delivered peptides to the mice at a rate of 3 mg/kg per day. The first pump was implanted subcutaneously in the back of 5-week-old mice between the shoulders and replaced once, after 4 weeks.

YAC128 mice (Tg) and their age-matched wt littermates were implanted with an osmotic pump containing TAT control peptide or HV-3 peptide (3 mg/kg/day, each) starting from the age of 3 months. The pump was replaced once every month. By the age of 12 months, the treatments were terminated and the mouse samples were harvested for analysis.

**Behavioural analysis in HD mice.** All behavioural analyses were conducted by an experimenter who was blind to genotypes and treatment groups.

Gross locomotor activity was assessed in R6/2 mice and age-matched wt littermates at the ages of 13 weeks and in YAC128 mice and age-matched wt littermates at the ages of 2, 3, 6, 9, and 12 months. In an activity chamber (Omnitech Electronics, Inc), mice were placed in the center of the chamber and allowed to explore while being tracked by an automated beam system (Vertax, Omnitech Electronics Inc). Distance moved, horizontal, vertical, and rearing activities were recorded. Because R6/2 mice were sensitive to changes in environment and handling, we only conducted one-hour locomotor activity analysis for R6/2 mice and wt littermates. We performed 24 h of locomotor activity analysis for YAC128 mice and their wt littermates.

Hindlimb claspings was assessed with the tail suspension test once a week from the ages 8 to 11 weeks in R6/2 mice. Mice were suspended by the tail for 60 s and the latency for the first hindlimb or all four paws to clasping was recorded as the score system[67]. Clasping over 10 s, score 3; 5–10 s, score 2; 0–5 s, score 1; 0 s, score 0.

The motor coordination and balance of YAC128 mice were tested on an accelerating Rotarod (IITC Life Sciences, Serials 8) at the ages of 2, 3, 6, 9 and 12 months. Training and baseline testing for motor function tasks were conducted at 2 months of age. For training, mice were given three 120-s trials per day at a fixed-speed of 15 r.p.m. for three consecutive days. During the testing phase, the Rotarod accelerated from 5 to 40 r.p.m. over 3 min; the maximum score was 300 s.

The body weight and survival rate of HD mice and wt littermates were recorded throughout the study period.

**Immunohistochemistry and stereological measurements.** Mice were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde in PBS. Brains were processed for paraffin embedment. Brain sections (5 μm, coronal) were used for immunohistochemical localization of DARPP-32 (1:500, Epitomics) using the HAC Select HRP/AB kit (Millipore). Quantification of DARPP-32 immunostaining was conducted using NIH image J software. The same image exposure times and threshold settings were used for sections from all treatment groups.

To measure the number of NeuN-positive cells, a series of 25 μm thick coronal sections spaced 200 μm apart spanning the striatum were stained with NeuN antibody (Millipore, 1:500) and visualized by diaminobenzidine. For neuropathological analyses, brain sections were analysed stereologically. Briefly, unbiased stereological counts of NeuN-positive neurons within the striatum were performed using unbiased stereological principles and analysed with Stereo Investigator and the neuronal density is presented as NeuN positive cell number per mm3.

Quantitation was conducted by an experimenter blind to the experimental groups.

**Western blot analysis.** Protein concentrations were determined by Bradford assay. Protein was resuspended in Laemmli buffer, loaded on SDS-PAGE, and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibodies, followed by visualization with ECL. Representative blots have been cropped for presentation. Images of full-size blots are presented in Supplementary Fig. 9.

**GST pull-down assay.** Bacteria-expressed GST or GST–VCP, or GST–VCP truncated mutants were immobilized on glutathione-Sepharose 4B beads (GE Healthcare) for three hours and then washed three times. Beads were incubated with total lysates of mouse brains overnight at 4 °C. Beads were then washed with a GST binding buffer (100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% Triton-X-100 and protease inhibitor cocktail) and were analysed by SDS–PAGE and IB.

**Molecular docking.** We constructed models of VCP with the homology modelling software MODELLER9.9 using the crystal structure of p97 (PDB ID 3CF1) as the template. The structure alignment with VCP with 3CF1A (p97) showed two gaps in the 1–21 and 707–806 amino acid regions of VCP. Thus, a homologous model of the 22–706 amino acids of VCP could be readily generated. Therefore, the 1–21 and 707–806 amino acids of VCP were not taken into account in the present work. The model of HV-3 peptide was built with Amber12 in our laboratory.

All simulations were performed using the Amber12 software package together with the s99SB parameters for proteins, and the PTRAJ module of Amber12 was used to analyse the computational results. The starting models were solvated in a rectangular box of TIP3P (explicit water model) water molecules with a minimum distance of 1 Å between any protein atom and the box boundaries. To neutralize the models, three chloride ions were added. Before MD simulation, a series of minimizations were performed. All water molecules were first minimized while restraining the positions of the atoms of the protein with a harmonic potential. The whole system was then energy minimized without restraint for 2,000 steps using a combination of the steepest descent and conjugated gradient methods. After gradually heating the system from 10 to 310 K over 100 ps using the NVT ensemble, a 1 ns simulation was performed at 1 at and 300 K with the NPT ensemble to equilibrate the whole system. For production runs, MD simulations were performed in the NPT ensemble for 40 ns for VCP and for 400 ns for HV-3.

For all simulations, all bonds involving hydrogen atoms were constrained using the SHAKE algorithm. A time period of 2.6 and a non-bonded interaction cutoff radius of 10 Å were used. The particle-mesh Ewald method was employed to calculate long-range electrostatic interactions. During the sampling process, the coordinates were saved every 5 ps for further analysis.

We analysed the HV-3 and VCP with the rigid-body docking programme in Discovery Studio 2.5. The angular step size sets as 15, RMSD cutoff 0.5, interface cutoff 9.0, and 2000 configurations are generated. Supplementary Fig. 4e showed the most plausible configuration according to the comprehensive results of maximum score in ZDOCK, Density maximum and Cluster minimum.

**Statistical analysis.** Sample sizes are determined by power analysis based on pilot data collected by our labs or published studies. In animal studies, we used n = 15–20 mice/group for behavioural tests, n = 6 mice/group for biochemical analysis and n = 6 mice/group for pathology studies. In cell culture studies, we performed each study with at least three independent replications. For all of the animal studies, we have ensured randomization and blinded conduct of experiments. For all imaging analysis, the quantitation was conducted by an observer who was blind to the experimental groups. No samples or animals were excluded from the analysis.

Data were analysed by Student’s t-test or analysis of variance (ANOVA) with post hoc Holm-Sidak test for comparison between two groups. Survival, behavioural test and body weight were analysed by repeated-measures two-way ANOVA. Data are expressed as mean ± s.e.m. Statistical significance was considered achieved when the value of P was <0.05.

**Data availability.** The data that support the findings of this study are available from the corresponding author on request.

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Author contributions

X.G. performed all experiments in cell cultures and biochemical analyses of animal models and patient samples; X.Y.S. maintained HD mice and conducted animal behavioural analysis; D.H. examined VCP in Parkinson’s disease models; Y.-J.W. performed proteomic analysis of mHtt interactors; H.F. conducted electron microscopy analysis; R.V. and S.C. purified VCP protein and performed the ITC analysis of HV-3; A.U.J. and D.M.-R. conducted the toxicity analysis of HV-3 in mice; Y.L. conducted stereology analyses of the striatal neuronal number in mice; X.Q. conceived, designed and supervised all the studies and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: A patent on the design and applications of the HV-3 peptide inhibitor has been filed. The authors declare no conflict of interest.

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Supplementary Figure 1: Proteomic analysis of Htt interactors on mitochondria in HD knock-in mouse striatal cells. (a) Mitochondria were isolated from either HdhQ7 or HdhQ111 mouse striatal cells and subjected to immunoprecipitation (IP) with anti-Htt antibodies (MAB2166, recognizing full-length wtHtt or mtHtt). The immunoprecipitates were eluted from beads followed by tandem mass spectrometry analysis. (b) Venn diagram showing the protein numbers and overlaps of Htt binding proteins on the mitochondria in HdhQ7 and HdhQ111 cells. Four proteins were identified in both HdhQ7 and Q111 mitochondria, nine proteins only in mitochondria of HdhQ111 cells, and four proteins only in mitochondria of HdhQ7 cells. (c) Putative protein candidates that bind to mtHtt in HD mouse striatal cells. Spectral counts were used here as semi-quantitation (1).
Supplementary Figure 2: VCP localization on subcellular organelles and the information of HD patient samples. (a) Localization of VCP (red) in the ER (green, marked with anti-calnexin antibody) or endosome (green, marked with anti-EEA1 antibody) was determined by confocal microscopy. Pearson’s coefficiency was used to quantitate the extent of colocalization of VCP/calnexin or VCP/EEA1. Nuclei were stained with DAPI. The data are mean ± SEM of three independent experiments. Scale bar is 10 µm. (b) HEK293 cells were transfected with Parkinson’s disease-associated mutants LRRK2 G2019S or alpha-synuclein A53T for 24 hours. The control vector, wildtype LRRK2 or wildtype alpha-synuclein, was also expressed for comparison. Mitochondria were isolated at the indicated groups and VCP mitochondrial levels were examined by Western blot analysis. There was no evidence of VCP translocation to mitochondria observed in these mutants-expressing cells relative to those in cells expressing wild-type counterparts. The representative blots were from two to three independent experiments. (c) Data of normal subjects and HD patients shown in Fig. 2D. The evaluation of neuropathology of HD patients was summarized. (d) Total cortical protein lysates from postmortem brain tissues of a normal subject (5248) and a HD patient (5374) were subjected to IP with anti-VCP antibody followed by Western blot analysis with anti-1C2 antibody. Arrows indicate mtHtt recognized by 1C2 antibody which does not detect wt Htt. There was no binding between VCP and mtHtt observed in this patient brain tissue. The HD patient (5374) had very subtle loss of neurons based on the neuropathology evaluation shown in (c).
Supplementary Figure 3: Htt and mtHtt are not substrates of VCP. Left: HdhQ7 and HdhQ111 cells were treated with MG132 (10 µM for 16 hours). Protein levels of Htt were determined in the total lysates of the cells. Actin was used as a loading control. The representative blots were from three independent experiments. Right: HdhQ7 and HdhQ111 were treated with Eer I (Eeyarestatin I, 5µM for 16 hours). Protein levels of Htt were determined. Mcl-1, a mitochondrial Bcl-2 family protein, was used as a positive control. Mcl-1 has been reported to be a substrate of VCP on mitochondria and it is degraded via the ubiquitin-proteasome system. The representative blots were from three independent experiments.
Supplementary Figure 4: Rational design of a peptide inhibitor to block VCP/Htt interaction. (a) GST or GST-VCP was incubated with total lysates of mouse brain and the indicated peptides (3 µM) for 16 hours, followed by immunoblotting with anti-Htt antibodies. Representative blots are from three independent experiments. Quantitation of VCP/Htt binding was shown below the blot as the mean of three independent experiments. Data are mean ± SEM. *, p<0.05 vs. TAT-treated group (Paired Student’s t test). (b) HEK293 cells were co-expressed with GFP-VCP and Myc-73Q FL plasmids as indicated. After 48 hours incubation with the indicated peptides (3 µM/day, each), immunoprecipitation analysis was performed. The shown blots are from three independent experiments. (c) HEK293 cells were co-expressed with GFP-VCP and Myc-73Q FL plasmids as indicated. After 48 hours incubation with HV-3 at various dosages arranging from 0 to 4 µM, immunoprecipitation analysis was performed followed by western blot analysis. The blots of the bindings of mtHtt/VCP were quantitated with NIH Image J software. The IC50 was calculated. (d) Upper: sequence of the HV-3 peptide and the control peptide TAT. Lower: HV-3 peptide sequence is highly conserved among species. (e) The HV-3 peptide was docked to the VCP. Upper: Cartoon representing the predicted VCP structure using the mouse crystal structure of p97 (PDB ID 3CF1); sticks represent the structure of HV-3 (see supplementary method). The blue is the N-terminal and the red is the C-terminal of VCP. The HV-3 binds to the hydrophilic cavity of the VCP surface (marked in...
green). Lower: the enlarged area is labeled in green. (f) The sequence in VCP corresponding to HV-3 in Htt was deleted (ΔVCP). GST, GST-VCP, or GST-ΔVCP was incubated with total lysates of mouse brain for 16 hours followed by Western blot with anti-Htt antibodies. The representative blots are from three independent experiments.

Supplementary Figure 5: Treatment of peptide inhibitor HV-3 in HD animal models. (a) HD R6/2 mice and wildtype littermates were treated with either a control peptide or peptide HV-3 (3 mg/kg/day, subcutaneous administration with an Alzet osmotic pump) from 5 to 13 weeks of age. The pump was replaced every four weeks. YAC128 mice and wildtype littermates were treated with control peptide TAT or HV-3 peptides (3 mg/kg/day, each, subcutaneous administration with an Alzet osmotic pump) from the age of 3 months to the age of 12 months. Mouse behaviors and HD-associated pathology were determined every three months after beginning treatment. The pump was replaced every four weeks. (b) Total protein lysates were extracted from HdhQ111 striatal cells and the striata of HD R6/2 and YAC128 mice. VCP protein levels were determined by Western blot analysis. Actin was used as a loading control. Representative blots from three independent experiments are shown.
Supplementary Figure 6: Peptide HV-3 does not affect ER stress response and apoptosis. (a) HD mouse striatal cells were treated with thapsigargin (1μM) or tunicamycin (10μg/ml) for 16 hours in the presence or absence of HV-3 (3 μM). GRP78 total protein levels were determined by western blot analysis. Representative blots of three independent experiments are shown. Actin was used here as a loading control. (b) Twenty-four hours of serum starvation was performed in HD mouse striatal cells. Caspase-3 activity analysis was performed in the indicated groups. Data are mean ± SEM of three independent experiments. (c) Flag-VCP and GFP-LC3B were co-transfected into HdhQ7 striatal cells. Total lysates were isolated after 36 hours of transfection. The GFP-LC3B levels were examined by western blot analysis. Actin was used as a loading control. Histogram: quantitation of GFP-LC3B total protein level. Data are mean ± SEM of three independent experiments.
**Supplementary Figure 7: VCP accumulation on mitochondria elicits excessive mitophagy.** (a) HeLa cells were transfected with either the control vector or flag-mtVCP for 36 hours. Cells were stained with flag (red) and Tom20 (green) antibodies. Mitochondrial morphology was analyzed. Scale bar: 10 μm. The appearance of mitochondrial aggregates (b), mitochondrial mass (c), and mitochondrial membrane potential (d) in cells expressing flag-mtVCP were quantitated as described in the Method section. Data are mean ± SEM of three independent experiments. Paired Student’s t test. (e) HeLa cells were transfected with flag-mtVCP and GFP-LC3B for 36 hours. Cells were stained with flag (blue) and Tom20 (red) antibodies. Scale bar: 10 μm. GFP-LC3B/Tom20 colocalization was determined using confocal microscopy. At least 100 cells per group were counted by an observer blind to experimental conditions. The data are mean ± SEM from three independent experiments. Paired Student’s t test. (f) Wild-type striatal cells were transfected with either the control vector or flag-mtVCP for 36 hours. Mitochondria were isolated. The autophagic flux of mitochondria was determined by quantitation of LC3 II mitochondrial levels in the presence or absence of BFA (20 nM). The data are mean ± SEM from three independent experiments. Paired Student’s t test. (g) Parkin total protein levels were determined in HdhQ7 and HdhQ111 cells. Parkin in HEK293 cells was used as a positive control. Actin was a loading control. There was no Parkin expression in HdhQ7 and Q111 cells. The representative data are from two independent experiments.
Supplementary Figure 8: Toxicity analysis of HV-3 in naïve mice.
We performed a toxicity analysis of HV-3 in mice. Six-week-old C57Black mice were treated with saline or peptide HV-3 at 3mg/kg/day by Alzet pump implantation. After 4-week treatment, the plasma, blood and whole mouse bodies of mice were collected. Examination on blood chemistry (Supplementary Table 1), hematology (Supplementary Table 2) and general necropsy of mice were performed at the Animal Diagnostic Laboratory at Stanford University. The experimenter who conducted the analysis was blind to the treatment groups. Automated hematology was performed on the Sysmex XT-2000iV analyzer system. Blood smears were made for all full CBC samples and reviewed by a medical technologist. Manual differentials were performed as indicated by species and automated analysis. Chemistry analysis was performed on the Siemens Dimension Xpand analyzer.
(a) Frozen tissue sections of brain and spleen were stained with anti-CD3 antibody by immunohistochemistry. There is no observed difference in the immuno-density of CD3 between saline- and HV-3-treated groups. Scale bar: 100 µm. (b) The size and weight of spleens in mice treated with saline or peptide HV-3 were compared. Left: the ratio of spleen weight to body weight was calculated and expressed as mean ± SEM of five mice in each group. Right: the images of spleens of mice treated with saline or peptide HV-3. There is no observed difference in the size of the spleens. Paired Student’s t test. Four-week sustained treatment with HV-3 by pump implantation had no effects on blood chemistry (Supplementary Table 1), hematology (Supplementary Table 2), and gross necropsy of naïve mice when compared to mice treated with saline. A four-week treatment with HV-3 had minor effects on immuno-density of CD3, a marker of T-cell for adaptive immune response, in the brains and spleens of naïve mice (a). Treatment with HV-3 had no effects on the size and weight of the spleens (b).

Reference:
Table 1: Effects of 4-week treatment with HV-3 on blood biochemistry of naïve mice

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Table 2: Effects of 4-week treatment with HV-3 on hematology of naïve mice

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