A Series of N-terminal Epitope Tagged Hdh Knock-In Alleles Expressing Normal and Mutant Huntingtin: Their Application to Understanding the Effect of Increasing the Length of Normal Huntingtin’s Polyglutamine Stretch on CAG140 Mouse Model Pathogenesis

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A series of N-terminal epitope tagged \textit{Hdh} knock-in alleles expressing normal and mutant huntingtin: their application to understanding the effect of increasing the length of normal huntingtin’s polyglutamine stretch on CAG140 mouse model pathogenesis

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Abstract

\textbf{Background:} Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease that is caused by the expansion of a polyglutamine (polyQ) stretch within Huntingtin (htt), the protein product of the HD gene. Although studies in vitro have suggested that the mutant htt can act in a potentially dominant negative fashion by sequestering wild-type htt into insoluble protein aggregates, the role of the length of the normal htt polyQ stretch, and the adjacent proline-rich region (PRR) in modulating HD mouse model pathogenesis is currently unknown.

\textbf{Results:} We describe the generation and characterization of a series of knock-in HD mouse models that express versions of the mouse HD gene (\textit{Hdh}) encoding N-terminal hemaglutinin (HA) or 3xFlag epitope tagged full-length htt with different polyQ lengths (HA7Q-, 3xFlag7Q-, 3xFlag20Q-, and 3xFlag140Q-htt) and substitution of the adjacent mouse PRR with the human PRR (3xFlag20Q- and 3xFlag140Q-htt). Using co-immunoprecipitation and immunohistochemistry analyses, we detect no significant interaction between soluble full-length normal 7Q- htt and mutant (140Q) htt, but we do observe N-terminal fragments of epitope-tagged normal htt in mutant htt aggregates. When the sequences encoding normal mouse htt’s polyQ stretch and PRR are replaced with non-pathogenic human sequence in mice also expressing 140Q-htt, aggregation foci within the striatum, and the mean size of htt inclusions are increased, along with an increase in striatal lipofuscin and gliosis.

\textbf{Conclusion:} In mice, soluble full-length normal and mutant htt are predominantly monomeric. In heterozygous knock-in HD mouse models, substituting the normal mouse polyQ and PRR with normal human sequence can exacerbate some neuropathological phenotypes.

\textbf{Keywords:} Huntington, Epitope tag, Knock-in, Polyglutamine, Proline-rich region, Sequestration, Huntington’s disease
Background

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder that is caused by the expansion of a CAG triplet repeat encoding polyQ (>36Q) within the first exon of the HTT gene [1,2]. The length of the expanded polyQ stretch correlates inversely with age at onset, and moderate polyQ expansions (40-50Q) in htt are usually associated with disease onset at middle age. Unfortunately, despite a number of therapies targeted at individual symptoms, there is currently no way to delay or halt progression of the disease and death results ~10-20 years after diagnosis. Neurons in the striatum and deeper layers of the cortex are affected predominantly, although neuronal cell death and white matter loss are also detected in many other areas of the brain [3]. A neuropathological hallmark of HD is the appearance of nuclear and cytoplasmic (neuropil) inclusions of aggregated N-terminal fragments of mutant htt [4-6]. Despite a correlation between the appearance of htt aggregates and behavioral deficits in the majority of HD mouse models, the role of these inclusions in the mechanism of HD pathogenesis is still uncertain, as the results from in vitro experiments and some HD mouse models have suggested that large visible mutant htt inclusions are neuroprotective [7-11]. However, such aggregates not only have the ability to recruit toxic soluble fragments or oligomers of mutant Htt, but they can also sequester other polyQ-containing proteins, including wild-type htt [12-15]. In vitro studies, for example, have demonstrated that aggregates containing both mutant and wild-type htt N-terminal fragments are formed when mutant and wild-type truncated htt expression constructs are co-expressed. A Q20 polypeptide can augment Q7 aggregation in vitro by enhancing nucleation kinetics, and co-expression of a Q30 version of htt exon 1 with Q93-htt exon 1 accelerated aggregation and increased toxicity in a Drosophila model [16]. Wild-type htt is an essential protein during early embryogenesis, neurogenesis, and in adult neuronal homeostasis. Loss of murine huntingtin (htt) expression results in progressive neurodegeneration [17], increased apoptosis [18], axonal transport deficits in neurons [19,20], altered mitotic spindle orientation in dividing neuronal progenitor cells [21], and hypomorphic primary cilia [22]. Thus, potential sequestration of wild-type htt by mutant htt in mouse models is lacking.

Immediately adjacent to the htt polyQ stretch is a proline-rich region (PRR) that is thought to have evolved in vertebrates with the polyQ stretch [25]. Data from in vitro and cell culture experiments suggest that aggregation of mutant htt N-terminal fragments and potentially sequestration of wild-type htt can also be modulated by the adjacent PRR [26-30]. A normal htt exon 1 construct containing the PRR can ameliorate the toxic effects of an N-terminal 103Q construct, while a construct expressing normal htt exon 1 without the PRR does not [31]. The mouse htt PRR is a 32 amino acid domain consisting of P9, P10, P2, and P3 stretches interrupted by short Q-rich stretches 1-3 amino acids in length. The human PRR is slightly longer (38 amino acids) and consists of P11 and P10 sequences interrupted by a proline-rich 17 amino acid stretch. It is not yet known if expression of a humanized version of normal htt with a non-pathogenic polyQ stretch and the human PRR can influence HD mouse model phenotypes.

To determine the extent of potential dominant-negative interactions in vivo, and to begin to explore the effect of expressing a non-pathogenic humanized allele of Hdh encoding htt with a 20Q stretch and human PRR on HD mouse model pathogenesis, we have generated a series of knock-in HD mouse models expressing (1) the mouse HD gene (Hdh) encoding full-length normal htt (7Q and mouse PRR) with hemaglutinin (HA) or triple Flag N-terminal epitope tags (HA7Q- and 3xFlag7Q-htt), (2) a humanized normal Hdh allele encoding a 3xFlag-tagged version of htt with a 20Q stretch adjacent to the human PRR (3xFlag20Q-htt), and (3) a humanized Hdh allele encoding a 3xFlag-tagged version of mutant htt with a 140Q stretch adjacent to the human PRR (3xFlag140Q-htt). By co-immunoprecipitation, we find that soluble full-length murine 7Q-htt does not associate stably with itself or with 140Q-htt. However, we can detect a very low level of interaction between full-length 3xFlag20Q-htt and 140Q-htt. In addition, we observe a significant increase in the number of nuclear inclusions, and in the mean size of aggregates detected in Hdh140Q/3xFlag20Q mice compared with Hdh140Q/3xFlag7Q mice. These observations, together with an increase in gliosis and lipofuscin accumulation in the Hdh140Q/3xFlag20Q brain in comparison to the Hdh140Q/3xFlag7Q brain, suggest that replacing the mouse polyQ and PRR stretches with normal human sequence can exacerbate some aspects of the CAG140 HD mouse model phenotype.

Results

To insert N-terminal HA and 3xFlag epitope tags into the Hdh locus, we assembled gene targeting constructs by replacing an endogenous Hdh exon 1 AlwNI – XmnI
restriction fragment with a PCR-generated synthetic fragment containing either the HA or 3xFlag epitope tag inserted between htt amino acids 1 and 2. The 140Q stretch was derived from our CAG140 targeting construct [32], and contains the human proline-rich region (PRR) that is adjacent to the polyQ stretch. The 20Q stretch was obtained by PCR amplification of a human wild-type allele using the same procedure employed to isolate the CAG140 stretch, and it also contains a human PRR (Figure 1). Germline transmission was obtained from three independent ES cell clones (Hdh<sup>HA7Q/+</sup> and Hdh<sup>3xFlag7Q/+</sup>) or two independent clones (Hdh<sup>3xFlag20Q/+</sup> and Hdh<sup>3xFlag140Q/+</sup>). All mice have been backcrossed to the C57BL/6J strain for at least six generations.

To establish that the addition of the HA or 3xFlag N-terminal epitope tags did not affect htt’s essential functions during embryonic development, the genotypes of progeny from heterozygous intercrosses were evaluated for any deviation from the expected Mendelian frequency (Table 1). Homozygous Hdh<sup>HA7Q/HA7Q</sup>, Hdh<sup>3xFlag7Q/3xFlag7Q</sup>, and Hdh<sup>3xFlag20Q/3xFlag20Q</sup> mice were obtained with the expected frequency, and mice hemizygous for the 3xFlag epitope-tagged 7Q allele (Hdh<sup>3xFlag7Q/-</sup>) were obtained from crosses with Hdh<sup>+/−</sup> mice. Hdh<sup>3xFlag140Q/3xFlag140Q</sup> progeny were also obtained.

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**Figure 1** Diagram of the epitope tag-modified Hdh exon 1. The wild-type Hdh exon 1 contains a short polyQ stretch (7Q) and an adjacent proline-rich region (mouse PRR, gray). An HA N-terminal epitope tag (green) was inserted between amino acids 1 and 2 of the Hdh exon 1 containing the wild-type mouse polyQ stretch (Hdh<sup>HA7Q</sup>), while a 3xFlag N-terminal epitope tag (yellow) was inserted in the wild-type Hdh exon 1 (Hdh<sup>3xFlag7Q</sup>), a chimeric mouse/human exon 1 containing a normal human polyQ stretch (Hdh<sup>3xFlag20Q</sup>), and a chimeric mouse/human exon 1 with an expanded polyQ stretch (Hdh<sup>3xFlag140Q</sup>). The human portion of the chimeric exon 1 is indicated in light blue and darker blue (the human PRR), while the polyQ stretches are displayed in shades of red. The sequences of the epitope tags are indicated along with key restriction sites used in the construction of the gene targeting vectors. A, AlwNI; X, XmnI; K, KpnI.
from heterozygous intercrosses, but the number of homozygous progeny trended less than that predicted by the Mendelian frequency, (this difference, however, did not reach significance in the χ² test; Table 1), suggesting the possibility for either increased toxicity or that 3xFlag140Q-htt may not function as efficiently as expected, there is extensive overlap in the expression of HA7Q- and 3xFlag7Q-htt expression could be detected using the appropriate anti-epitope tag antibodies (Figure 2B).

To visualize simultaneously both normal (7Q) and mutant (140Q) htt expression using anti-epitope tag antibodies, fresh frozen brain sections from 12 month old Hdh3xFlag140Q/HA7Q mice were analyzed by confocal microscopy following incubation with FLAG M2 and HA antibodies to detect 3xFlag140Q- and HA7Q-htt, respectively. Htt inclusions were visualized with the aggregation-specific mEM48 (MAB5374) antibody, and nuclei were stained with the fluorescent DNA dye, Topro-3 (Figure 3A). In cortex, diffuse and bright punctate neuropil staining , in addition to nuclear staining in a portion of the cells, was observed with the FLAG antibody. In contrast, diffuse HA7Q-htt staining was observed in the neuropil, and in nuclei that also stained with the 3xFlag epitope. HA staining was also detected in several punctae that co-stained with the Flag antibody. Flag staining was present in the neuropil, in a subset of nuclei (Figure 3A), and was detected in neurite, perinuclear, and nuclear punctae that co-stained with the mEM48 antibody (Figure 3B). Thus, the 3xFlag epitope can be detected in presumptive soluble mutant htt, nuclear inclusions, and neuropil inclusions, while the HA epitope can be detected predominantly in the neuropil, in a subset of nuclei that also stained with mutant htt, and in some htt inclusions.

Soluble full-length 7Q-htt does not interact with itself or with full-length 140Q-htt

Prior in vitro observations, cell culture studies, and experiments in Drosophila have demonstrated that mutant htt can sequester normal htt in protein aggregates, but recent analyses of human postmortem brain extracts and protein extracts derived from HD mouse models using blue native polyacrylamide gel electrophoresis (BNP) have shown that full-length normal and mutant htt are predominantly monomeric [33]. To confirm that epitope-tagged normal htt exists predominantly as a monomer in our mouse models, we immunoprecipitated 3xFlag7Q-htt from cerebellar and striatal protein extracts prepared from 1 month-old Hdh3xFlag7Q/HA7Q mice, and forebrain extracts prepared from 12 month-old Hdh3xFlag7Q/HA7Q mice (Figure 4). Flag antibody-bound and non-bound fractions were then analyzed by western blotting using FLAG and HA antibodies. In both 1 month and 12 month-old mice, Flag3x7Q-htt was efficiently immunoprecipitated. HA7Q-htt, in contrast, was detected only the antibody non-bound fractions. Thus, in both young and older mice, epitope-tagged htt exists primarily as a monomer in our protein extracts using our co-immunoprecipitation conditions.
We next prepared striatal and cerebellar protein extracts from 6 month-old \textit{Hdh}^{140Q/3xFlag7Q} mice to determine if soluble full-length mutant \textsc{htt} can interact with normal \textsc{htt}. Following immunoprecipitation of 3xFlag7Q-\textsc{htt} with anti-FLAG agarose beads, antibody-bound and non-bound fractions were analyzed by western blotting using Flag and expanded polyQ (1C2) antibodies (Figure 5A). While 3xFlag7Q-\textsc{htt} was enriched in the antibody bound fractions, 140Q-\textsc{htt} was detected only in the antibody non-bound fraction. We interpret this result to suggest that little, if any, stable interaction between normal and mutant \textsc{htt} occurs in the 6 month-old \textit{Hdh}^{140Q/3xFlag7Q} brain. To determine if an association between normal and mutant \textsc{htt} can be

![Figure 2 Expression of HA7Q-\textsc{htt} and 3xFlag7Q-\textsc{htt} in selected brain regions. (A) Confocal microscopy of HA epitope (HA), 3xFlag7Q epitope (FLAG), and \textsc{htt} (Htt) expression in the 24 month-old \textit{Hdh}^{3xFlag7Q/HA7Q} striatum and cortex. Nuclei were visualized with the DNA dye To-Pro-3 (blue). Arrows indicate examples of where co-localization of the HA and 3xFlag epitopes, or of the epitopes with \textsc{htt} occurs. Scale bar = 25 μm. (B) Western blot analysis of HA7Q-\textsc{htt} (left panel) and 3xFlag7Q-\textsc{htt} (right panel) in 50 μg total protein isolated from \textit{Hdh}^{HA7Q+/+} and \textit{Hdh}^{3xFlag7Q+/+} testis and various brain regions (brain stem: br stem, cerebellum: cb, thalamus: thal, striatum: str, and cortex: ctx). The antibodies used (monoclonal antibodies HA.11 and MAb FLAG M2) and the position of a 200 kD protein marker are indicated.](image-url)
detected in older Hdh\textsuperscript{140Q/3xFlag7Q} mice, co-immunoprecipitation experiments were performed using whole brain extracts prepared from 13 month-old Hdh\textsuperscript{3xFlag140Q/HA7Q}, and 27 month-old Hdh\textsuperscript{140Q/3xFlag7Q} mice. Cytoplasmic extract from the Hdh\textsuperscript{3xFlag140Q/HA7Q} and Hdh\textsuperscript{140Q/3xFlag7Q} brains was immunoprecipitated with anti-expanded polyQ (1C2) or Flag antibodies, using Protein G-agarose beads, and western blots of antibody-bound and non-bound fractions were probed with 1C2 (recognizing 3xFlag140Q-htt and 140Q-htt), and MAB2166 (recognizing both normal and mutant htt) antibodies (Figure 5B, C). HA7Q-htt did not co-immunoprecipitate with 3xFlag140Q-htt, and similarly 140Q-htt did not co-immunoprecipitate with 3xFlag7Q-htt, suggesting that in older mice, a stable interaction between full-length 7Q-htt and 140Q-htt does not occur. To control for non-specific association of htt with the Protein G agarose-beads, whole extract was incubated with Protein G-agarose beads in the absence of 1C2 or Flag antibodies, and the antibody-bound and non-bound fractions were probed with 1C2 and 2166 antibodies (Figure 5B, C). Htt was not detected in the control bound fractions, indicating that little, if any, non-specific binding of htt to the agarose beads occurred.

A low level of interaction between full-length 20Q-htt and 140Q-htt can be detected by co-immunoprecipitation

To determine if normal htt with a 20Q stretch and human PRR can interact with full-length mutant htt, we performed FLAG immunoprecipitations with Protein-G agarose beads using whole brain extracts prepared from 16 month-old Hdh\textsuperscript{140Q/3xFlag20Q} mice, and probed the Flag antibody-bound and non-bound fractions with 1C2 and 2166 antibodies (Figure 5C). A very low level of interaction between 140Q- and 3xFlag20Q-htt was detected in the whole brain protein extracts. We estimate that under our co-immunoprecipitation conditions, < 2.5% of soluble full-length mutant htt was associated with 3xFlag20Q-htt (see Methods).

\textbf{Sequestration of normal htt in Hdh\textsuperscript{140Q/3xFlag7Q} and Hdh\textsuperscript{140Q/3xFlag20Q} striatal inclusions}

To explore the effect of expressing a version of htt with a normal human polyQ stretch and human PRR on HD mouse model phenotypes, we first compared

\begin{figure}
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\includegraphics[width=\textwidth]{image.png}
\caption{Expression of 3xFlag140Q-htt and HA7Q-htt in 12 month-old Hdh\textsuperscript{3xFlag140Q/HA7Q} cortex and striatum. (A) Confocal microscopy of fresh frozen brain sections containing the striatum and piriform cortex were immunostained with rabbit anti-Flag (green) and mouse anti-HA antibodies (red), while nuclei were visualized with the DNA dye To-Pro-3 (blue). In both the cortex and striatum, the Flag antibody stains a fraction of the nuclei, punctae, and to a lesser extent, the neuropil. HA staining is found diffusely in both the neuropil and in some nuclei. A few punctae co-stain with both Flag and HA antibodies in the cortex and striatum (open arrowheads), and filled arrowheads mark examples of nuclei co-staining with both antibodies. (B) In striatal sections co-immunostained with both Flag and an aggregation-specific antibody (mEM48; MAB5374), the majority of htt inclusions in the neuropil are stained with both antibodies (open arrowheads). Scale bar = 25 μm.}
\end{figure}
and HDh140Q/3xFlag20Q striatum. At 24 months of age, however, we observed a significant increase in the mean aggregate size in the HDh140Q/3xFlag20Q striatum in comparison to the HDh140Q/3xFlag7Q striatum.

To compare the extent of the co-localization of 3xFlag20Q-htt with 140Q-htt vs. 3xFlag7Q-htt with 140Q-htt in aggregates, we quantified the percentage of htt aggregates that co-stained with both Flag and MW8 antibodies in the HDh140Q/3xFlag7Q and HDh140Q/3xFlag20Q striatum using confocal microscopy (Figure 8A). At 6 months of age, we observed no significant difference in the percentage of htt aggregates that contained the 3xFlag epitope in the HDh140Q/3xFlag7Q and HDh140Q/3xFlag20Q striatum (Figure 8B). At 24 months of age, there was a trend towards increased co-localization of epitope-tagged normal htt with mutant htt in the HDh140Q/3xFlag20Q brain in comparison to the HDh140Q/3xFlag7Q brain (P=0.06) (Figure 8B). This result suggests that 3xFlag20Q-htt may be recruited into inclusions more efficiently than 3xFlag7Q-htt, and this could contribute to the increased average size of aggregates we observed in the HDh140Q/3xFlag20Q striatum.

To confirm that normal htt was sequestered in mutant htt inclusions, we first performed cellulose acetate filter trap assays [34] with the initial 800xg pellet fraction from 24 month-old HDh140Q/3xFlag7Q whole brain extracts (containing crude nuclei and insoluble htt aggregates) (Figure 9A). 1C2, mEM48, and Flag-positive htt inclusions trapped on the cellulose acetate were detected by western blotting, while an underlying PVDF membrane was positive for SDS-soluble 140Q- and 3xFlag7Q-htt species, but negative for mEM48-positive aggregates.

In both full-length and truncated mutant htt HD mouse models, the predominant species of mutant htt found in macroscopic inclusions consists of N-terminal fragments [35-37]. To determine if epitope-tagged N-terminal fragments of normal htt were present in the inclusions, we extracted the SDS-insoluble 800xg pellet fraction obtained from 24 month-old HDh140Q/3xFlag7Q brains (containing crude nuclei and insoluble htt aggregates) with formic acid to solubilize the htt aggregates [38-40], and performed western blotting using 140Q- and 3xFlag7Q-htt antibodies to visualize full-length and N-terminal fragments of normal and mutant htt (Figure 9B). In the formic acid-solubilized SDS-insoluble nuclear fractions, we could detect 140Q- and 3xFlag140Q-htt fragments with the 1C2 antibody that ranged in size from ~100 kD to ~250 kD, and apparently full-length mutant htt. The Flag antibody detected an increased level of 3xFlag20Q-htt fragments with the 1C2 antibody that ranged in size from ~100 kD to ~250 kD, and apparently full-length mutant htt. The Flag antibody detected an increased level of 3xFlag20Q-htt fragments in comparison to 3xFlag7Q-htt fragments.
Increased formation of small neuropil htt aggregates occurs when mouse htt’s 7Q stretch and PRR are replaced by a 20Q stretch and the human PRR in Hdh\textsuperscript{140Q/3xFlag20Q} mice

The large neuropil and nuclear inclusions that we visualized by standard immunohistochemical methods using the MW8 and mEM48 (MAB5374) antibodies may not represent all the sites where aggregates are forming. To visualize these sites, treatment of tissue sections with formic acid to expose the expanded polyQ epitope within the htt aggregates prior to antibody staining is required [41]. Using this method, small neuropil aggregates and aggregation foci located in striatal neuronal processes resembling chains of immuno-positive punctae were observed [41]. Such 1C2\textsuperscript{+} punctae were detected in both Hdh\textsuperscript{140Q/3xFlag7Q} and Hdh\textsuperscript{140Q/3xFlag20Q} striata.
For quantification, we measured the total pixel area of the 1C2+ punctae, as they were difficult to resolve clearly. We observed a significant increase in 1C2 staining following formic acid treatment in the Hdh\(^{140Q/3xFlag20Q}\) striatum in comparison to the Hdh\(^{140Q/3xFlag7Q}\) striatum at both 6 months and 24 months of age (Figure 10B). Unfortunately, we could not detect co-localization of the 3xFlag epitope with the expanded polyQ epitope, as we were unable to recover FLAG immunoreactivity following formic acid treatment (data not shown). Nevertheless, our observations suggest that increasing the length of the normal htt polyQ stretch and/or replacing the mouse PRR with the human PRR can enhance the formation of small cytoplasmic htt aggregates.

**Increased astrocytosis and lipofuscin accumulation is observed in Hdh\(^{140Q/3xFlag20Q}\) mice compared to Hdh\(^{140Q/3xFlag7Q}\) mice**

In HD mouse models and in the HD brain, reactive astrocytosis occurs as a consequence of mutant htt expression [42-44]. To determine if astrocytosis in Hdh\(^{140Q/3xFlag20Q}\) mice is altered in comparison to that observed in Hdh\(^{140Q/3xFlag7Q}\) mice, we examined glial fibrillary acidic protein (GFAP) expression by immunohistochemistry in the striatum of 24 month-old wild-type,
As expected, GFAP immunostaining was relatively low in the wild type striatum (measured as total pixel area of GFAP signal/field), likely reflecting the basal expression of GFAP in resident astrocytes. In all three HD mouse models, however, GFAP immunostaining was significantly higher in the mutant striatum in comparison to the wild type striatum (Figure 11B).

A significant increase in astrocytosis was observed in the Hdh<sup>140Q/3xFlag7Q</sup> striatum compared to wild-type striatum, and in the Hdh<sup>140Q/3xFlag20Q</sup> striatum compared to the Hdh<sup>140Q/3xFlag7Q</sup> striatum.

In addition to the gliosis observed in HD, elevated levels of lipofuscin are detected in HD mouse models, and in the HD brain [43,45,46]. Lipofuscin is an autofluorescent aging pigment that accumulates over time.
in neurons and other postmitotic cells. It is composed primarily of cross-linked lipid, and is generated by the autophagic catabolism of membrane and organelles [47]. In HD, oxidative stress can result in the increased accumulation of perinuclear lipofuscin deposits in neurons. We assessed lipofuscin accumulation in the striatum of 24 month-old wild-type, \( Hdh^{140Q/3xFlag7Q} \), and \( Hdh^{140Q/3xFlag20Q} \), to \( Hdh^{140Q/3xFlag140Q} \) mice, and found that there was a trend towards increased lipofuscin accumulation in the striatum: wild-type<\( Hdh^{140Q/3xFlag7Q} \)<\( Hdh^{140Q/3xFlag20Q} \)<\( Hdh^{140Q/3xFlag140Q} \). Both \( Hdh^{140Q/3xFlag20Q} \) and \( Hdh^{140Q/3xFlag140Q} \) mice had significantly more striatal lipofuscin in comparison to wild-type mice, but there was no significant difference in the amount of striatal lipofuscin observed between wild-type and \( Hdh^{140Q/3xFlag7Q} \) mice (\( P=0.056 \)), between \( Hdh^{140Q/3xFlag20Q} \) and \( Hdh^{140Q/3xFlag7Q} \) mice (\( P=0.6 \)), or between \( Hdh^{3xFlag140Q/140Q} \) and \( Hdh^{140Q/3xFlag20Q} \) mice (\( P=0.46 \)) (Figure 11C, D).

**Discussion**

Our results are compatible with the hypothesis that the expanded polyQ stretch within mutant htt can interact in vivo with non-pathogenic lengths of polyQ in wild-
type htt and potentially other cellular proteins containing a polyQ stretch. Sequestration between expanded and normal-length polyQ stretches occurs predominantly between proteolytically processed N-terminal fragments of htt, as we detect little interaction between soluble full-length 140Q-htt and 3xFlag20Q-htt. Moreover, this level of interaction is so low (<2.5% of mutant htt is associated with normal htt) that it is unlikely that sequestration is contributing to potential loss-of-function phenotypes in vivo by reducing the steady-state levels of normal htt. Our co-immunoprecipitation data also suggest that full-length wild-type mouse htt (7Q-htt) does not interact stably with itself, and supports prior work suggesting that htt is predominantly a monomeric protein in vivo [33,48,49], with the caveat that our conditions of co-immunoprecipitation select for stable interactions, and a weak or transient association between htt monomers would likely escape detection.

The number of htt inclusions detected in older mice using the aggregation-specific MW8 antibody was not altered significantly when the polyQ stretch in normal htt was increased from 7Q to 20Q, and the mouse PRR was substituted with a human PRR, despite a significant increase in the number of Hdh<sup>140Q/3xFlag20Q</sup> nuclear inclusions that was observed at 6 months of age. However, the mean size of htt aggregates was significantly larger in the Hdh<sup>140Q/3xFlag20Q</sup> striatum in comparison to Hdh<sup>140Q/3xFlag7Q</sup> striatum at 12 and 24 months of age. A potential explanation for these observations is that an interaction between N-terminal fragments of...
3xFlag20Q-htt and 140Q-htt could enhance nucleation kinetics, facilitating small htt inclusion formation in \( Hdh^{140Q/3xFlag20Q} \) mice. Larger numbers of small htt inclusions could then increase the probability that they can coalesce into larger inclusions that are recognized by the MW8 antibody in the \( Hdh^{140Q/3xFlag20Q} \) brain.

Our data obtained using formic acid to both expose the expanded polyQ epitope in tissue sections and to solubilize nuclear-associated htt aggregates, provide evidence supporting this scenario. Small cytoplasmic htt aggregates were increased at both 6 months and 24 months of age in the \( Hdh^{140Q/3xFlag20Q} \) striatum in

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**Figure 10** Formic acid treatment reveals an increase in small 1C2\(^+\) inclusions in the \( Hdh^{140Q/3xFlag20Q} \) striatum in comparison to \( Hdh^{140Q/3xFlag7Q} \) striatum. (A) Images of the striatum from 6 month-old and 2 year-old \( Hdh^{140Q/3xFlag7Q} \) and \( Hdh^{140Q/3xFlag20Q} \) mice probed with 1C2 antibody (red) following formic acid treatment. Nuclei were visualized with To-Pro-3. Examples of "aggregate chains" are indicated with white arrows. Scale bar = 25 \( \mu \)m. (B) Quantification of the total 1C2\(^+\) pixel area per microscopic field in the striatum of \( Hdh^{140Q/3xFlag7Q} \) and \( Hdh^{140Q/3xFlag20Q} \) mice at 6 months and 24 months of age (n=3 mice for each age and genotype, mean ± SEM). *P<0.05.
comparison to Hdh\textsuperscript{140Q/3xFlag7Q} striatum, and higher levels of 3xFlag20Q-htt N-terminal fragments were detected in nuclear-associated formic acid-solubilized aggregates in comparison to 3xFlag7Q-htt fragments in age-matched controls. These data are compatible with prior in vitro observations suggesting that normal length polyQ polypeptides enhance the nucleation kinetics of an expanded polyQ polypeptide in a process that is both concentration- and polyQ length-dependent [16].

An alternative to the polyQ-dependent enhanced nucleation hypothesis that cannot be excluded by our data is that substitution of the mouse PRR with the human PRR in 3xFlag20Q-htt also contributed to the phenotypic differences between the Hdh\textsuperscript{140Q/3xFlag7Q} and
Hdh140Q/3xFlag20Q mice that we observed. Mammalian cell culture experiments with truncated N-terminal wild-type and mutant htt expression constructs have provided data suggesting that htt amino acids located C-terminal to the polyQ stretch can influence co-aggregation of mutant and wild-type htt fragments [30]. Moreover, data from both yeast and mammalian cell culture studies suggest that the htt PRR may be involved in the formation of the aggresome [28,29]. A future test of this hypothesis would require the generation of Hdh alleles encoding full-length 7Q-htt with the human PRR and 20Q-htt with the mouse PRR.

The htt aggregation phenotype we detect in the Hdh140Q/3xFlag20Q mouse was accompanied by enhanced gliosis and lipofuscin accumulation. In HD, however, it is unclear whether or not increasing the length of the CAG repeat in the normal HTT allele can affect pathogenesis. In a study combining data from 533 individuals from the Huntington’s Disease Modifiers of Age at Onset in Pairs of Siblings (HD-MAPS) study and 221 individuals from the Huntington Disease Center with Walls (NEHD) study, evidence was found supporting a genetic interaction between the normal and mutant HTT alleles – age at onset decreases when the normal CAG repeat length increases from 6–17 to 18–19 repeats, and increases when the CAG repeats range from 20–34 [50]. This effect on age-at-onset from the normal allele is small, however (1–2 year difference in age-at-onset), and may reflect the difficulty associated with determining a precise time for symptomatic onset. In a more recent study involving 921 subjects, increasing the size of the normal htt polyQ stretch correlated with a lower age-at-onset and increased clinical severity if the mutant htt polyQ stretch length was between 36Q and 44Q, while the opposite effect was observed if the mutant Htt polyQ stretch was >44Q [51]. However, two more recent studies could find no evidence supporting the hypothesis that the size of the polyQ stretch in the normal HTT allele influences age-at-onset [52,53]. Our data suggest that some neuropathological phenotypes are enhanced by expressing 3xFlag20Q htt in the CAG140 mouse model. However, additional studies will be needed to determine if the age-at-onset, severity, and progression of all knock-in HD mouse model phenotypes are similarly affected. In addition, we will need to examine if substitution of the mouse PRR with the human PRR is also contributing to the differences observed between the Hdh140Q/3xFlag20Q and Hdh140Q/3xFlag2Q mice.

The generation of Hdh knock-in alleles expressing N-terminal epitope-tagged 7Q-, 20Q-, and 140Q-htt provide additional tools for understanding normal and mutant htt function in the mouse. However, we caution that the incorporation of the N-terminal epitope tag may perturb htt function. In yeast, a Flag epitope tag at the N- or C-terminus of constructs expressing truncated N-terminal fragments of mutant htt enhanced cytotoxicity [54]. Similarly, our 3xFlag or HA tags may influence the structure and function of both normal and mutant htt in our knock-in mouse models. Nevertheless, we obtained the predicted numbers of Hdh+3xFlag7Q/3xFlag7Q homozygotes, and Hdh+3xFlag7Q/+ hemizygotes, suggesting that normal htt’s essential developmental functions were not affected significantly by the addition of the 3xFlag epitope tag. We also were able to obtain Hdh+3xFlag140Q/3xFlag140Q homozygotes from heterozygous intercrosses, but future studies comparing both the behavior and neuropathology in Hdh140Q/140Q and Hdh140Q/140Q mice are needed to determine to what extent an N-terminal tag on mutant htt could affect HD mouse model phenotypes.

Conclusion

The generation of new Hdh knock-in alleles expressing N-terminal epitope-tagged htt with various polyQ lengths has allowed us to begin to explore the interaction between normal and mutant htt, and to investigate the consequence of expressing a normal htt with a 20Q stretch and human PRR in the CAG140 knock-in model for HD. Substitution of the normal mouse exon 1 sequence in the wild-type Hdh allele with normal human sequence can exacerbate some phenotypes in a heterozygous CAG140 knock-in mouse model for HD, suggesting that the length of the normal polyQ stretch and/or the presence of a human PRR in normal huntingtin can modulate HD mouse model pathogenesis. We hope that these new Hdh knock-in alleles can be of use to the research community.

Methods

All experiments with mice were carried out in accordance with the ethical guidelines described in the “Guide for the Care and Use of Laboratory Animals”, Institute of Laboratory Animal Resources, National Research Council, 1996 edition. All procedures were reviewed and approved by the Animal Care and Use Committee of the University of Virginia.

Generation of mice

Partially complementary oligonucleotides containing sequence encoding an HA or 3xFlag N-terminal epitope tag, an AlwNI restriction site at the 5’ end, and an Xmnl restriction site at the 3’ end, and an XmnI restriction site at the 3’ end were annealed, repaired with the Klenow fragment of DNA polymerase I, and then digested with AlwNI and Xmnl restriction enzymes to generate a synthetic DNA fragment that was used to replace an endogenous Hdh exon 1 AlwNI–Xmnl fragment encoding the N-terminal amino acids of htt. Oligonucleotides used were, HA-f: 5’-GTCTTTCAGGGTCTGTCCCATCGGGCAGGA AGCCG TCAITGTACCCATCGACTACGCTACGG CT-3’, HA-r: 5’-CGACTCAGGAAGCCTTCATCAGCTT

http://www.molecularbrain.com/content/5/1/28
TTCCAGGGTTCGACGATGTCTGGGACGTCGTATG
GTTA-3', Flag3x-f: 5'-GTCTTCCAGGGTCTGCCCATC
GGGAGAAGCCTGTCATGACTCAAGACAGACGATGACAA-3', Flag3x-r: 5'-CGACTCTGA
AAAGCTTCTACACGTCCTTCTCCAGGGTTCGCTCAG
TCGTCGTCCTTTTGGC-TAGTC-3'. For assembly of the Hdlh3xFlag20Q gene targeting vector, An XmnI–KpnI fragment containing human wild-type HTT sequence was obtained by PCR from genomic DNA according to the method described in [32]. This fragment contains the human XmnI restriction site, a normal 20Q stretch, the human proline-rich region (PRR), human sequence extending to the end of exon 1, a 100 bp intron 1 deletion near the 5'-splice site of intron 1, and a KpnI restriction site. For assembly of the Hdlh3xFlag140Q gene targeting vector, the 3xFlag epitope tag AlwNI–XmnI restriction fragment was used to replace the corresponding AlwNI–XmnI fragment in our CAG140 targeting vector. Thus, this vector also contains both the human PRR and intron 1 deletion. In contrast, insertion of the epitope tag sequence was the only modification made to the vector also contains both the human PRR and intron 1 deletion. In contrast, insertion of the epitope tag sequence was the only modification made to the XmnI restriction site. A wild-type Hdh allele will generate a 112 bp PCR product, while the wild-type Hdh, Hdlh3xFlag20Q, and Hdlh140Q alleles lacking the intron 1 deletion generate a 235 bp product. The CAG repeat lengths in the Hdlh3xFlag20Q (23Q), Hdlh3xFlag140Q (136Q) alleles were determined by PCR sequencing (Laragen, Inc.) of genomic DNA isolated from tail biopsies.

Co-immunoprecipitation

Whole brain tissue was dounce homogenized in extraction buffer [50 mM Tris–HCl pH 8.8, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA pH 8.0, 0.5% (v/v) NP40, protease inhibitor cocktail (Thermo Scientific), with 5 mM NaF and 1 mM sodium vanadate] on ice, and then centrifuged at 16,100×g for 10′ at 4°C to obtain a crude cytoplasmic supernatant fraction. Co-immunoprecipitation was performed by incubating protein extracts with either FLAG M2 antibody-agarose beads or by incubating antibody (FLAG M2 or 1C2) with the extract, followed by capture of the antigen-antibody complex with Protein G-agarose beads. In the first method, 0.5–1.0 mg of the protein lysate was incubated by rotation o/n at 4°C with 12–20 μl FLAG M2 antibody-agarose beads (Sigma) that were pre-washed three times with 0.5 ml extraction buffer according to the manufacturer’s instructions. Following incubation of the antibody-agarose beads with the extract, the beads were pelleted by centrifugation at 8,200×g for 30 sec at 4°C, and the supernatant was carefully removed and saved as the antibody non-bound fraction (NB). The beads were then washed three times with 0.5 ml ice-cold extraction buffer. Antibody-bound protein was eluted with two sequential incubations in 30 μl 0.4 mg/ml 3xFlag peptide (Sigma) dissolved in extraction buffer. Both elution fractions were then combined as the antibody-bound (B) fraction. NB- and B-samples were fractionated on 5% SDS-PAGE and then transferred electrothermally o/n at 30V in 25 mM Tris base, 192 mM glycine, 10% methanol, 0.025% SDS to 0.45 μm PVDF membranes (Millipore). In the second method, 500 μg cytoplasmic extract was incubated o/n at 4°C with 5–6 μg FLAG M2 or 1C2 antibody. 20 μl Protein-G agarose beads (Upstate Biotechnology; washed once in extraction buffer, blocked for 1 h at 4°C in 1% blocking powder {Boehringer} dissolved in TBS, and then washed 3X in extraction buffer prior to resuspending in the original volume of the same buffer), was then added to the mixture of protein extract and antibody. This mixture was then rotated for 3 hr at 4°C to bind the antibody-antigen complexes to the beads. The beads were

Alternatively, the Hdlh3xFlag20Q and Hdlh3xFlag140Q alleles can be genotyped by using 140for: 5′-CTGCAACGCAGGCTGAGTC-3′ and 140rev: 5′-GAAGGACTGGAGTCTGAGTC-3′. These primers flank the 100 bp intron 1 deletion that is present in the Hdlh3xFlag20Q, Hdlh3xFlag140Q, and Hdlh140Q alleles, and will generate a 150 bp PCR product, while the wild-type Hdh, Hdlh3xFlag20Q, and Hdlh140Q alleles, and will generate a 150 bp PCR product, while the wild-type Hdh, Hdlh3xFlag20Q, and Hdlh140Q alleles, and will generate a 150 bp PCR product, while the wild-type Hdh, Hdlh3xFlag20Q, and Hdlh140Q alleles, and will generate a 150 bp PCR product, while the wild-type Hdh, Hdlh3xFlag20Q, and Hdlh140Q alleles, and will generate a 150 bp PCR product, while the wild-type Hdh, Hdlh3xFlag20Q, and Hdlh140Q alleles, and will generate a 150 bp PCR product.
then pelleted by centrifugation, the supernatant was saved as the NB-fraction, and then the beads were washed as described previously for the anti-FLAG M2-agarose beads. The B-fraction was eluted from the beads by adding 50 μl 3x SDS-PAGE sample loading buffer to the beads, and incubating for 5 min at 99°C in a heating block. The beads were then pelleted, and the supernatant transferred to a fresh tube. 5% of the NB-fraction and 50% of the B-fraction were analyzed by SDS-PAGE and western blotting as described above. Western blot membranes were blocked in 5% milk in TBS-0.05% Tween 20 (TBST) prior to incubation o/n at 4°C with either FLAG M2, (Sigma), HA.11 (Covance), the expanded polyQ-specific monoclonal antibody 1C2 (MAB1574, Millipore) or MAB2166 (Millipore). Membranes were washed five times with TBST before incubation with secondary goat anti-mouse IgG-HRP conjugated antibodies (Pierce) for 1 h at room temperature. Following an additional five washes, the membranes were incubated with West-Dura chemiluminescence substrate (Pierce) and then exposed to film. For quantification of the blots, exposures within the linear range of the film were scanned, and then analyzed by Image J software (http://image.nih.gov/ij/download.html). The percentage of 140Q-htt co-immunoprecipitating with 3xFlag20Q-htt was determined by dividing the pixel intensity of the B-fraction 140Q-htt signal by the NB-fraction signal, and then adjusting for the fraction of each sample loaded, and also for the efficiency of 3xFlag20Q-htt immunoprecipitation as determined from the 2166 western blots (e.g. ~20% of 3xFlag20Q-htt was immunoprecipitated in the experiment shown in Figure 5C, and the % of 140Q-htt associating with 3xFlag20Q-htt would then be corrected by a factor of 100/20).

Western blot analysis of formic acid-solubilized htt aggregates

Brains were cut in half (sagittally), and each half brain was dounce-homogenized in 2 ml extraction buffer on ice, before centrifugation at 800×g for 15’ at 4°C to obtain a crude nuclear pellet fraction. Formic acid-solubilized nuclear htt aggregates were obtained following the procedures of [39] and [40] with modifications. The crude nuclear fraction was first resuspended in extraction buffer, and then boiled 15 min in the presence of SDS prior to sonication with a Fisher Scientific 550 probe sonicator (power level 5 for 20 sec with pulses of 0.6 sec on/0.4 sec off). Following sonication, the sample was centrifuged at 16,100xg for 15’ at RT, and the SDS-insoluble pellet was resuspended in formic acid and incubated for 1 h at 37°C at 350 rpm. The sample was then dried in a speed-vac and resuspended in 1M Tris base to neutralize residual formic acid. An equal volume of 2X SDS-PAGE loading buffer was added, and then one third of the sample was incubated 5 min at 97°C prior to fractionation on 4%–15% gradient SDS-PAGE (BioRad) and blotting onto 0.45 μm PVDF membrane. Blots were reprobed with Flag (FLAG M2, Sigma) and expanded polyQ-specific (1C2, Millipore) antibodies, and imaged using a ChemiDoc XRS+ (BioRad) with West-Dura chemiluminescence reagents (Pierce).

Filter trap assay for htt aggregation

Whole brains were dounce homogenized in extraction buffer and centrifuged at 800×g for 15’ at 4°C to obtain a crude nuclear pellet fraction. Aliquots of this fraction were treated for 60 minutes on ice with 0.1 mg/ml DNase I, SDS was then added to 2% final concentration, and incubated 5 minutes at 99°C prior to spotting onto a cellulose acetate/PVDF membrane sandwich. SDS-resistant htt inclusions are trapped on the cellulose acetate membrane while soluble 3xFlag7Q-htt and 140Q-htt species are retained on the underlying PVDF membrane. Htt inclusions were detected by western blotting with the 1C2 and MAB5374 antibodies, while sequestered 3xFlag7Q-htt was detected with the FLAG M2 antibody.

Immunohistochemistry

Dissected brains were flash-frozen in isopentane on dry ice, and then serially sectioned at 14 μm on a cryostat (Bright Instrument Co.). Sections were either used immediately or stored at ~80°C until use. Sections were washed briefly in PBS, fixed for 10 min in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4, and washed in PBS three times before blocking with a 1:100 dilution of monovalent Fab fragments (donkey anti-mouse IgG (H+L), Jackson ImmunoResearch) in blocking buffer (5% donkey serum, 0.1% Triton X100 in PBS) for 1 h at RT, followed by three washes in PBS, and then incubated o/n at 4°C with primary antibody diluted in blocking buffer. Primary antibodies used were mouse monoclonal MW8 (1:70, Developmental Studies Hybridoma Bank), mouse monoclonal MAB5375 (1:100, mEM48, Millipore), mouse monoclonal FLAG M2 (1:100, Sigma), mouse monoclonal HA.11 (1:100, Covance), and rabbit anti-GFAP (1:1000, AB5804, Millipore). Following incubation with the primary antibody, sections were washed three times in PBS, and then incubated with secondary antibody (Cy3- or FITC-conjugated donkey anti-mouse or donkey anti-rabbit-IgG, Jackson ImmunoResearch) together with the fluorescent DNA stain To-Pro-3 iodide (Invitrogen) in blocking buffer for 1 h at RT. Sections were then washed with PBS before treating with Auto fluorescence Eliminator Reagent (Millipore) following the manufacturer’s instructions. Sections were then mounted with Vectashield (Vector Laboratory) and examined using either an Olympus BX51 microscope equipped with a MagnaFire CCD camera or a Nikon CI confocal system.
Immunohistochemical detection of htt aggregates following formic acid treatment
The method described in [41] was adapted for fresh frozen sections. Sections were washed briefly in PBS, and fixed in 4% paraformaldehyde in 0.1M PB for 15 min at RT. Sections were then washed twice in PBS, once with water (10 min each), and then treated with formic acid (Sigma) three times for 10 min each. The sections were rinsed three times in water and washed twice for 10 min each in PBS, followed by 30 min incubation in 1% sodium borohydride (w/v) in PBS, and then washed three times for 10 min each in PBS prior to immunohistochemical staining using the monoclonal 1C2 antibody (1:100, Millipore).

Quantification of htt aggregates using the MW8 antibody
Tissue sections were imaged with a 60X objective using a Nikon C1 confocal microscope, and the number of MW8\(^{\text{\textregistered}}\) huntingtin aggregates was counted blind to genotype from sagittal sections through the striatum (8 images from sections separated by 280 μm through the striatum, n=4 mice of each genotype) using ImagePro 4.5 software (Media Cybernetics). Neuropil and nuclear inclusions were counted separately. To determine the mean size of inclusions, the total pixel area was divided by the number of inclusions in each imaging field.

Lipofuscin analysis. Imaging and quantification of lipofuscin was performed as described in [46].

Statistical analyses
χ\(^2\) analyses of the genotypes were performed using SigmaStat (Systat software). Student’s t-test was used to evaluate significance in the quantification of aggregate numbers, aggregate mean size, and co-localization of MW8 and 3xFlag epitopes in htt aggregates.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SZ characterized the phenotypes of the epitope-tagged Hdh knock-in mice, NG participated in the immunohistochemical analyses and generated the epitope-tagged Hdh knock-in mice, J-PL carried out the formic acid western analyses and participated in drafting the manuscript, and SOZ conceived of the study, participated in its design and coordination, performed some of the co-immunoprecipitation experiments, and helped to draft the manuscript. All authors read and approved the final manuscript.

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