

Metallothioneins and copper metabolism are candidate therapeutic targets in Huntington's disease

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Abstract

HD (Huntington's disease) is caused by a polyQ (polyglutamine) expansion in the huntingtin protein, which leads to protein misfolding and aggregation of this protein. Abnormal copper accumulation in the HD brain was first reported more than 15 years ago. Recent findings show that copper-regulatory genes are induced during HD and copper binds to an N-terminal fragment of huntingtin, supporting the involvement of abnormal copper metabolism in HD. We have demonstrated that *in vitro* copper accelerates the fibrillization of an N-terminal fragment of huntingtin with an expanded polyQ stretch (httExon1). As we found that copper also increases polyQ aggregation and toxicity in mammalian cells expressing httExon1, we investigated further whether overexpression of genes involved in copper metabolism, notably MTs (metallothioneins) known to bind copper, protect against httExon1 toxicity. Using a yeast model of HD, we have shown that overexpression of several genes involved in copper metabolism reduces polyQ-mediated toxicity. Overexpression of MT-3 in mammalian cells significantly reduced polyQ aggregation and toxicity. We propose that copper-binding and/or -chaperoning proteins, especially MTs, are potential therapeutic targets for HD.

Introduction

HD (Huntington's disease) is a neurodegenerative disorder caused by an abnormal polyQ (polyglutamine) expansion in the 350 kDa protein htt (huntingtin). HD is associated with intracellular aggregates (or inclusion bodies) and neuronal loss, occurring predominantly in the striatum and cerebral cortex. Post-translational modifications and cleavage of htt occur *in vivo*, and the resulting N-terminal htt fragments containing the polyQ stretch are prone to misfolding and aggregation [1]. This misfolding and/or aggregation probably leads to a toxic gain-of-function, although loss of normal htt function, which has been implicated as a participant in a variety of cellular functions, may also play a role during disease. Many cellular dysfunctions have been identified that could participate in the disease process, including impaired transcription, mitochondrial abnormalities, dysregulation of the cellular redox homeostasis and defects in protein-degradation pathways, axonal transport and synaptic function (for a recent review, see [2]).

Both iron and copper accumulate in the CNS (central nervous system) of HD mouse models and in the HD brain [3,4] and could play a role in the above-mentioned cellular

alterations that occur in HD. Indeed, several previous findings point to the importance of copper in HD. Clioquinol, a copper/iron chelator, significantly delays neuropathology in HD mice [5], and N-terminal htt binds copper both *in vitro* and *in vivo* [4]. Htt has also been shown to reduce copper(II) and increase aggregation of the full-length wild-type (non-expanded) htt protein of mice [4]. The reduction in copper and concomitant increase in oligomerization has also been demonstrated for β -amyloid and is suggested to play a role in the pathogenesis of Alzheimer's disease [6,7]. Copper is crucial for electron-transfer reactions in a number of enzymes involved in activities such as antioxidant defence and neurotransmitter biosynthesis. Despite this essential requirement for copper, an excess of this heavy metal is toxic, primarily because of its reactivity with molecular oxygen which leads to the formation of ROS (reactive oxygen species). Therefore the transport and compartmentalization of copper is highly regulated, involving copper transporters and chaperones (see Table 1). By examining published gene expression data from HD cell and mouse models and human HD brain tissue, we found that copper-binding/chaperoning proteins, including MTs (metallothioneins), were consistently up-regulated (Table 1). This could reflect a protective response against increased copper and ROS, and/or a disruption of copper homeostasis more generally. This observation led us to test whether overexpression of copper-regulating genes in yeast and mammalian cell models of HD reduce htt toxicity. Because oligomerization and the production of amyloid-like structures of N-terminal htt has been tightly linked to cellular toxicity [8–11], we have also

Key words: copper, Huntington's disease, metallothionein, polyglutamine expansion, therapeutic target.

Abbreviations used: AFM, atomic force microscopy; CNS, central nervous system; GST, glutathione transferase; HD, Huntington's disease; HRP, horseradish peroxidase; htt, huntingtin; mRFP, monomeric red fluorescent protein; MT, metallothionein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; ORF, open reading frame; polyQ, polyglutamine; ROS, reactive oxygen species.

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Table 1 | Yeast and mammalian homologues of copper-binding proteins

Altered expression in HD models and/or effects on toxicity in a yeast model of HD are indicated (data compiled from seven studies including ours). IRE, iron-responsive element.

Mammalian homologue	Expression changes in HD models	Function	Yeast homologue	Effect on httEx1 toxicity in yeast model
MT1/MT2	mRNA up-regulated (5-fold) in PC12 cells, Tet-On Q74 [21] and in human HD brain [44]	Metal donation to target apometalloproteins (particularly zinc-finger proteins and enzymes), metal detoxification, and antioxidant	Crs5 Cup1-2	Rescues Rescues
MT3				
CCS [copper chaperone for SOD (superoxide dismutase)]		Transports copper to CuZn SOD	Lys-7	Rescues
Atox1 (antioxidant protein 1)		Delivers copper to copper-binding domain of MNK/WND. Protects neurons against oxidative stress and serum starvation	Atx1	Rescues
Cox17 (cytochrome <i>c</i> oxidase 17)		Delivers copper to cytochrome <i>c</i> oxidase in mitochondria, required for functional expression of cytochrome <i>c</i> oxidase	Cox17 Cox23	Rescues Rescues (not shown)
ATP7A (MNK) (Menkes's disease protein)		Copper-translocating P-type ATPase. Delivers copper to cuproenzymes in the Golgi compartment and effluxes excess copper	Ccc2	Rescues (not shown)
ATP7B (WND) (Wilson's disease protein)	Protein up-regulated in R6/2 mice [4]	Functions within the secretory apparatus, loading cytoplasmic copper onto the plasma ferroxidase caeruloplasmin (facilitates iron export)		
Ceruloplasmin	mRNA up-regulated in striatal primary neurons (httN171Q82), R6/1, R6/2 mice and human HD brain [44-47]	Copper storage, iron metabolism	Fet3	Not tested
DMT1 (divalent metal transporter 1) [Nramp2 (natural resistance-associated macrophage protein 2)]		Non-specific bivalent cation transporter (import) for iron (contains IRE) and copper	Smf1, Smf2 and Smf3	Not tested

investigated whether copper modulates the formation of such protein assemblies using AFM (atomic force microscopy) and biochemical assays and tested whether increased expression of copper-binding proteins reduces htt aggregation.

Protein purification and AFM

pGEX-HD53/20Q plasmids were kindly provided by Paul Muchowski (Gladstone Institute of Neurological Disease, University of California, San Francisco, CA, U.S.A.)

and httEx1-GST was prepared as described previously [12,13]. The integrity of the protein was validated using SDS/PAGE. The protein was dialysed in 20 mM Tris/HCl (pH 8), 150 mM NaCl, 0.1 mM EDTA and 5% glycerol. Freshly prepared protein samples were used for each experiment performed at 4°C. CuCl₂ (10 μM) was added before the addition of PreScission protease (2 units/100 μg of protein) to 50 μM GST (glutathione transferase)-htt-Q20 or -Q53 to initiate GST cleavage. At 24 h after the addition of PreScission protease and/or CuCl₂, 5 μl (12 μg of protein) of the reaction mixture was spotted on to a freshly cleaved mica disc, incubated for 2 min and then rinsed with 200 μl of ultrapure water and dried with compressed air. The samples were imaged in air with a digital multimode Nanoscope III AFM operating in tapping mode with an uncoated silicon tip.

Cell culture, DNA transfection and adenovirus infection

HeLa cells were grown in 4500 mg/ml DMEM (Dulbecco's modified Eagle's medium) with 100 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FBS (fetal bovine serum) at 37°C under 10% CO₂. For transfection, 150 000 HeLa cells per well were plated in six-well plates/35 mm dishes and exposed to 2 μg of human MT3 DNA (SC123646; OriGene) and 4 μl of Lipofectamine per well for 5 h (24 h after seeding) in serum-free medium, after which culture medium was added. At 24 h later, cells were trypsinized and re-plated in 96-well plates. Alternatively, cells were treated with CuCl₂ or clioquinol for 24 h in six-well plates before being re-plated into 96-well plates. Before re-plating, equal numbers of cells were infected in suspension at an MOI (multiplicity of infection) of 10 with an adenovirus expressing httEx1 containing either 25 or 97 glutamine residues fused to mRFP (monomeric red fluorescent protein) at the httEx1 C-terminus. Cells were then cultured for a further 48 h in the presence or absence of CuCl₂ or clioquinol where appropriate, after which an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay was performed following standard protocols using a plate reader at a wavelength of 490 nm. Cells were fixed subsequently for immunoblot analysis. Overexpression of MT3 was verified by Western blotting.

Dot blot and Western blot analysis

Cell lysis, SDS/PAGE, preparation of samples for dot blots and Western blots were performed as described previously [14]. Dot blot samples, normalized for total protein content, were filtered on a dot blot filtration unit through a cellulose acetate membrane with a pore size of 0.2 μm that was pre-equilibrated with 2% SDS. HttEx1 was detected using a sheep anti-httEx1 antibody at a concentration of 1:6000 (S830, a gift from Gillian Bates, Kings College London, London, U.K.) followed by a secondary HRP (horseradish peroxidase)-conjugated anti-sheep antibody and enhanced

chemiluminescence detection. MT3 was detected on a Western blot using an anti-mouse MT3 antibody, followed by an HRP-conjugated secondary anti-mouse antibody and enhanced chemiluminescence detection.

Overexpression of copper-modulating genes in yeast

Yeast strains containing plasmids for the overexpression of selected genes were obtained from the Yeast ORF (open reading frame) Collection (Open Biosystems). The relevant yeast strains were grown overnight in 96-well plates containing 100 μl of selective medium supplemented with 2% glucose per well and transformed with either p425GALL-htt25Q-GFP or p425GALL-htt103Q-GFP using a high-throughput transformation method [15]. The constructs p425GALL-htt25Q-GFP and p425GALL-htt103Q-GFP were generated by amplifying the huntingtin constructs from pYES2-htt25Q-GFP and pYES2-htt103Q-GFP [16] and cloning them into the SpeI and XhoI sites of p425GALL [17]. Both htt103Q and htt25Q are GAL (galactose)-inducible, FLAG- and GFP (green fluorescent protein)-tagged constructs encoding the first 17 amino acids of htt fused to a polyQ tract. Transformants were grown to stationary phase in selective medium containing 2% glucose, serially diluted, and spotted on to selective medium supplemented with either 2% glucose or 2% galactose and 2% raffinose. Plates were incubated at 30°C for 3–5 days and yeast strains were scored for growth.

Statistical analysis

Pairwise comparisons were conducted using a two-tailed Student's *t* test and 95% (*) or 99% (**) confidence intervals were used for calculating significance. The number of experiments performed for each result is indicated in the Figure legends.

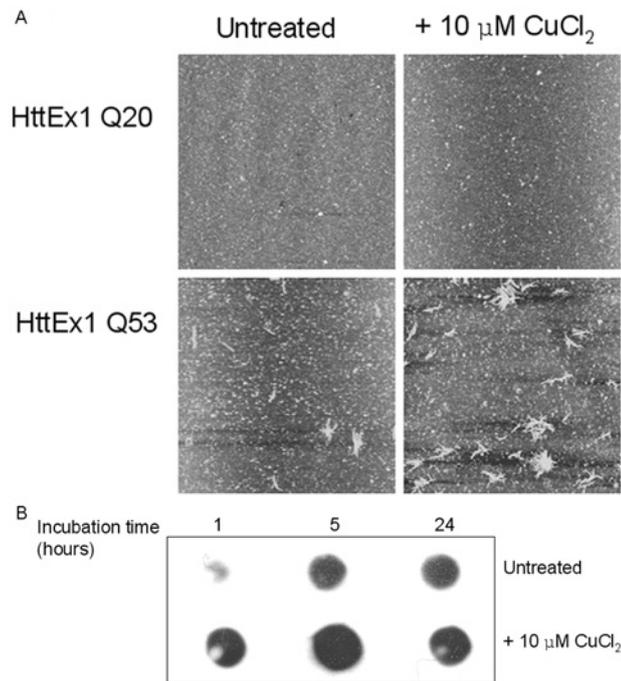
Results

We used an *in vitro* model of htt aggregation involving purification of a GST fusion protein of exon 1 of htt (httEx1) followed by cleavage of the GST tag, which induces a time-dependent aggregation of httEx1 [8,18]. Cleavage of the GST moiety from httEx1-Q20 did not lead to any detectable aggregation, but imaging of samples of httEx1-Q53 by AFM revealed amyloid-like fibrillar structures (Figure 1A). Addition of 10 μM CuCl₂ significantly accelerated the production of httEx1 fibrillar structures (Figure 1A). An increase in aggregation due to addition of CuCl₂ to purified httEx1-Q53 was also demonstrated using a filter trap assay, which detects SDS-insoluble material produced by httEx1 (Figure 1B).

Owing to the observed effect of copper on aggregation of httEx1 protein *in vitro*, we hypothesized that alterations in copper metabolism would affect aggregation and toxicity induced by expression of httEx1 *in vivo*. Therefore we tested the ability of overexpression of several yeast genes known to be involved in copper homeostasis to rescue httEx1-mediated toxicity (see Table 1). Figure 2 shows that all

Figure 1 | Copper increases aggregation of httEx1 *in vivo*

(A) Representative AFM images (scan size $10\ \mu\text{m} \times 10\ \mu\text{m}$) of recombinant httEx1-Q20 or -Q53 incubated for 24 h in the presence or absence of $10\ \mu\text{M}$ CuCl_2 . No significant aggregation was seen for httEx1-Q20, whereas fibrillar structures were evident for the httEx1-Q53 reaction and the number of fibrillar structures per $10\ \mu\text{m}^2$ area increased in the presence of CuCl_2 . (B) Dot blot of SDS-insoluble httEx1-Q53 recombinant protein, generated using a filter trap assay, probed with the S830 anti-httEx1 antibody. No insoluble material was detected for httEx1-Q20 (results not shown). Incubation of httEx1-Q53 in the presence of $10\ \mu\text{M}$ CuCl_2 markedly increases the amount of insoluble material at all time points.

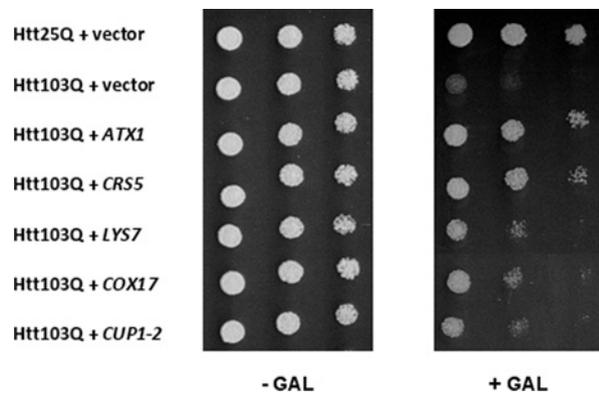


seven copper-binding genes tested provided some degree of rescue against expanded polyQ toxicity. Because one of the most effective genes in rescuing toxicity was *CRS5*, a yeast homologue of MT, we chose this protein to test its effects on polyQ aggregation and toxicity in a mammalian cell culture model of httEx1.

We first established that increased copper concentrations also increased httEx1 aggregation *in vivo* and whether clioquinol, as shown previously [5], was able to reduce aggregation. Equal expression and transgene integrity of httEx1-Q25/97 fused to mRFP (monomeric red fluorescent protein) was verified by Western blotting (results not shown). Figure 3(A) shows that, whereas additional copper enhances aggregation of HttEx1-Q97, clioquinol reduces aggregation of HttEx1-Q97 in a dose-dependent manner. Copper similarly increased aggregation in rat pheochromocytoma cells (PC12) expressing httEx1-Q97 (results not shown). Copper did not induce polyQ aggregation of httEx1-Q25 which does not normally aggregate when overexpressed at similar levels to httEx1 with a polyQ expansion [14,19–22].

Figure 2 | Overexpression of genes involved in copper homeostasis reduces httEx1 toxicity in yeast

Parental wild-type Y258 yeast containing constructs for overexpression of the indicated yeast ORFs were transformed with p425-Htt25Q or p425-Htt103Q and cellular viability determined using growth assays [16]. The expression of both the htt constructs and the indicated yeast ORFs is induced by galactose. Five-fold serial dilutions of stationary phase cultures starting with an equal number of cells of representative suppressor genes are shown. Expression of htt-Q103 induces significant toxicity compared with expression of htt-Q25. Overexpression of copper-homoeostatic genes (see Table 1 for details) shows a significant rescue effect towards htt-Q103 toxicity (*Cox23* and *Ccc2* not shown). This growth assay was performed twice with identical results.



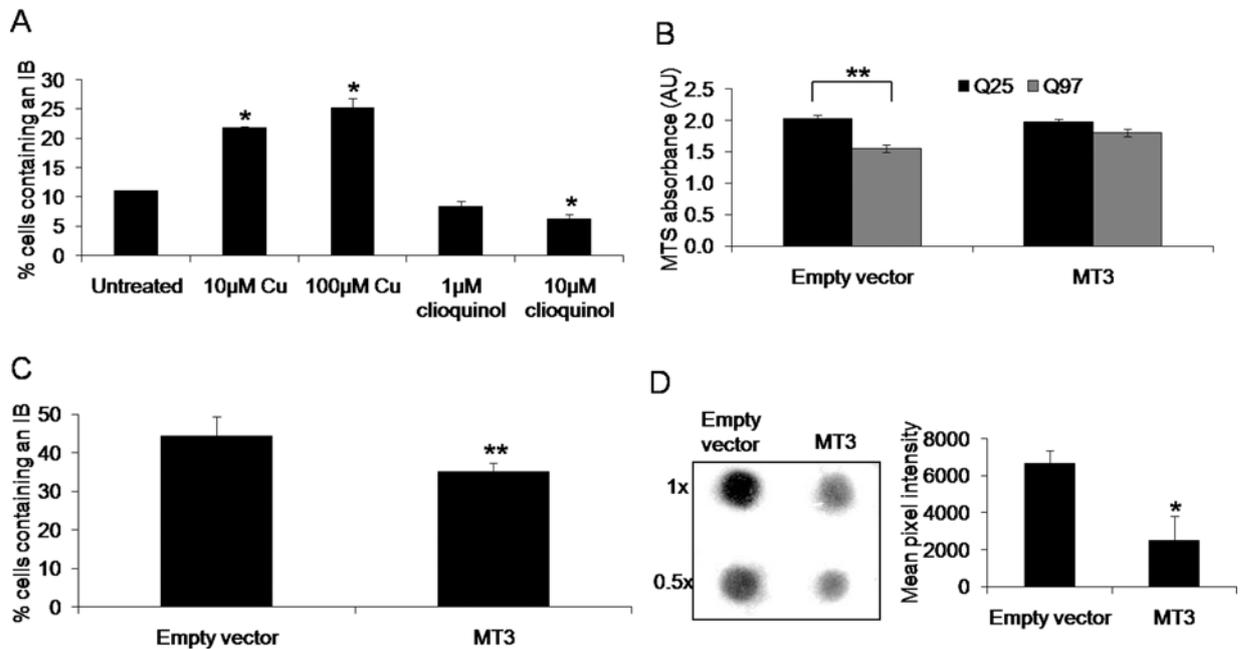
Clioquinol reduced httEx1 toxicity and copper increased toxicity in both httEx1-Q25- and -Q97-expressing cells (at the concentrations of 10 and $100\ \mu\text{M}$ tested) (results not shown). We next overexpressed human MT3 DNA for 24 h before infection with the httEx1 adenoviruses. We chose MT3 because, in contrast with other MT isoforms, it is mainly expressed in the CNS and is therefore likely to be the most relevant isoform for potentially modulating HD phenotypes. As seen in Figure 3(B), httEx1-Q97 induced significant toxicity compared with httEx1-Q25, reducing MTS activity by approx. 25% after 48 h of expression. Notably, co-expression of MT3 was protective, and restored more than 50% of the MTS activity lost due to httEx1-Q97 toxicity. In parallel with reduced levels of toxicity, MT3 overexpression significantly reduced inclusion body formation (Figure 3C) and the production of SDS-insoluble material in cells expressing httEx1-Q97 (Figure 3D).

Discussion

Our results show that altering levels of copper either by addition of exogenous copper, addition of a copper chelator or overexpression of copper-homoeostatic genes affects aggregation and/or toxicity of an aggregation-prone N-terminal fragment of htt. Copper could participate directly in HD pathogenesis by increasing aggregation of htt and/or by altering brain energy metabolism [e.g. LDH (lactose dehydrogenase) metabolism is altered in mouse HD brain]

Figure 3 | Copper increases aggregation and HT3 reduces aggregation and toxicity in a mammalian cell model of HD

(A) Percentage of HeLa cells expressing httEx1-Q97mRFP containing an inclusion body (IB) when left untreated or when treated with clioquinol or CuCl_2 (mean \pm S.E.M.; $n = 3$). The addition of CuCl_2 significantly increases the percentage of cells containing an IB, whereas the presence of clioquinol at a concentration of $10 \mu\text{M}$ causes a modest decrease in the percentage of cells containing an IB. (B) MTS absorbance measured at 490 nm for HeLa cells expressing httEx1-Q25/Q97mRFP for 48 h (mean \pm S.E.M.; $n = 3$). Expression of httEx1-Q97 induced toxicity, demonstrated by a $23 \pm 2\%$ reduction in MTS absorbance. Co-expression of MT3 ameliorated this toxicity by $60 \pm 5\%$. AU, absorbance units. (C) Percentage of HeLa cells expressing httEx1-Q97mRFP containing an IB when transfected with an empty vector or with MT3 (mean \pm S.E.M.; $n = 4$). Co-expression of MT3 significantly reduced the percentage of cells containing an IB. MT3 expression was verified by Western blotting (results not shown). (D) Dot blot of SDS-insoluble material extracted from HeLa cells expressing httEx1-Q97mRFP protein using a filter trap assay, probed with an anti-httEx1 antibody (S830). $1\times$ and $0.5\times$ indicate the relative amount of pellet loaded. No insoluble material was detected for cells expressing httEx1-Q25 as shown previously [14,22] (results not shown). Cells co-expressing MT3 produced significantly less SDS-insoluble material, compared with empty-vector-transfected control cells (mean \pm S.D.; $n = 2$).



[4]. However, it should be noted that some of the copper-homoeostatic proteins tested in yeast also regulate iron metabolism and therefore some htt toxicity-modifying genes in our analysis may be linked to iron metabolism. Copper metabolism is highly regulated and, although a chelation approach with clioquinol and tetrathiomolybdate has been partly successful in reducing pathology in an HD mouse model [5,23], a more targeted approach to restore copper homoeostasis may provide further and more consistent improvement with fewer side effects, which is particularly important for long-term treatment.

MTs could provide such a missing link. In our study, two yeast MT homologues, Crs5 and Cup1-2, suppress toxicity in a yeast model of mutant htt toxicity, with Crs5 showing the most significant rescue of the genes tested. In addition, one of the mammalian homologues of this yeast gene, MT3, was also protective against htt toxicity in HeLa cells. MTs are a family of low-molecular-mass cysteine-rich proteins which bind not only copper, but also zinc and heavy metals [24]. MTs are believed to have antioxidant properties

due to their multiple thiol groups, which interact with ROS. Previous studies have shown that MT3 overexpression protects against H_2O_2 -induced oxidative stress [25]. Under stress conditions, including oxidative stress, MTF-1 (metal-responsive transcription factor 1) activates expression of MTs via MREs (metal-response elements) present in their promoter regions. In support of a neuroprotective effect of MTs, addition of Zn(II) to cultured neurons causes an up-regulation of MTs and a correlated increase in survival of dopaminergic neurons [26]. In addition, the neuroprotective drug resveratrol (neuroprotective in HD models [27]) was also shown to up-regulate MTs [28]. How MT3 protects against polyQ toxicity is not clear, but it is likely to be linked to either the copper-binding ability of this protein or its general antioxidant actions, or both. Indeed, we have shown previously that expression of a mutant htt fragment is associated with an increase in cellular ROS production in both yeast and mammalian cells [20,29], but it is unknown how increased ROS are produced. Copper could be involved in ROS production by modulating httEx1 aggregation and/or

participate directly in Fenton-type reactions, leading to biomolecular damage due to oxidative stress. Therefore the metal-binding and antioxidant properties of MTs make these proteins good candidates for therapeutically targeting HD, especially given the importance of oxidative stress in HD (reviewed in [2,30]) and the likely importance of copper in htt aggregation/toxicity as shown in this work and in experiments performed by Fox et al. [4]. This critical study showed that copper binds to His⁸² and His⁹⁸ in a 171-amino-acid N-terminal fragment of htt. It is therefore possible that direct interactions of copper with httEx1 (which contains His⁸²) may affect the oligomerization properties of this protein and subsequent fibril formation and thereby impact on the production of toxic intermediate protein species. This hypothesis remains to be tested in the future using copper-binding-deficient htt fragments.

In summary, we have shown that copper increases polyQ aggregation *in vitro* and *in vivo* and that overexpression of MTs protects against polyQ toxicity in two cellular HD model systems. Given the importance of MTs in Alzheimer's disease [31–35] and other chronic CNS diseases such as amyotrophic lateral sclerosis [36,37] and Parkinson's disease [38–43], we propose that MTs are excellent candidate therapeutic targets for HD.

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