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ORIGINAL ARTICLE

Calretinin interacts with huntingtin and reduces mutant huntingtin-caused cytotoxicity

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Abstract

Huntington's disease (HD) is a devastating neurodegenerative disorder caused by an expansion of CAG trinucleotide repeats encoding for polyglutamine (polyQ) in the huntingtin (Htt) gene. Despite considerable effort, the mechanisms underlying the toxicity of the mutated Htt protein remains largely uncertain. To identify novel therapeutic targets, we recently employed the approach of tandem affinity purification and discovered that calretinin (Cr), a member of the EF-hand family of calcium-binding proteins, is preferentially associated with mHtt, although it also interacts with wild-type Htt. These observations were supported by coimmunoprecipitation and by colocalization of Cr with mHtt in neuronal cultures. Over-expression of Cr reduced mHtt-caused cytotoxicity in both non-neuronal and neuronal cell models of HD, whereas knockdown of Cr expression in the cells enhanced mHtt-caused neuronal cell death. In addition, over-expression of Cr was also associated with reduction of intracellular free calcium and activation of Akt. These results suggest that Cr may be a potential therapeutic target for treatment of HD.

Keywords: calcium-binding protein, calretinin, huntingtin, Huntington's disease, neuroprotection, polyglutamine.

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Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an expansion of polyglutamine (polyQ) in the N-terminal region of the huntingtin (Htt) protein (Li and Li 2004). The length of polyQ repeat is inversely correlated to the age of HD onset, such that patients with longer polyQ repeat tracts have an earlier onset age and faster progression of clinical symptoms (Slepko *et al.* 2006). The clinical symptoms of HD include chorea, psychiatric dysfunction, and cognitive decline (Bates *et al.* 2002). To date, there is no cure for the disorder.

The pathogenic mechanisms underlying HD remain largely obscure, but several lines of evidence indicate that abnormal neuronal calcium homeostasis linked to HD (Tang *et al.* 2003; Brustovetsky *et al.* 2005). First, the mutant Huntingtin (mHtt) protein is associated with mitochondria and disrupts mitochondrial calcium homeostasis (Panov *et al.* 2002). Second, mHtt also directly associates with the inositol 1,4,5-triphosphate receptor in the endoplasmic reticulum (ER) and sensitizes this intracellular calcium-release channel to facilitate its activation by IP3 (Tang *et al.* 2003). As the medium-sized spiny neurons in the striatum

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Abbreviations used: Cr, calretinin; Erk, extracellular signal-regulated kinase; GFP, green fluorescent protein; HD, Huntington's disease; Htt, huntingtin; LDH, lactate dehydrogenase; mHtt, mutant huntingtin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K/Akt, Phosphatidylinositol 3-kinase/Akt; polyQ, polyglutamine; SF-Htt, Strep/Flag-tagged huntingtin; TAP, tandem affinity purification.

of the brain express a high level of metabotropic glutamate receptors (mGluR5) acting via inositol 1,4,5-triphosphate receptor-mediated calcium release, this may explain why these neurons are the most vulnerable to mHtt (Mao and Wang 2002). Third, mHtt interacts with NMDARs and causes over-activation of the receptors, leading to an increase in calcium influx (Fan and Raymond 2007). This eventually results in the opening of the permeability transition pore in mitochondria, caspase activation, and neuronal death.

Calretinin (Cr) is a calcium-binding protein that contains six EF-hand domains, a structural motif well known for its ability to bind calcium ions (Henzi *et al.* 2009). Cr has a broad distribution in the brain and may play a protective role in neurons. Cr-positive neurons show more resistance to 6-hydroxydopamine-induced lesion than other neurons in the substantia nigra pars compacta (Tsuboi *et al.* 2000). In contrast, Cr-deficient mice reveal impaired long-term potentiation, motor coordination, and Purkinje cell activity (Schwaller *et al.* 2002). Interestingly, more recent data link Cr to HD. Cr-positive interneurons are selectively spared in Huntington's disease patients (Massouh *et al.* 2008). Moreover, Cr shows reduced expression in a HD mouse model, which is correlated with impaired neurogenesis in adult HD animals (Fedele *et al.* 2011). To date, however, direct physical and functional connections between Cr and mHtt are lacking. In this report, we demonstrate that Cr interacts with mHtt and reduces mHtt-caused cytotoxicity in both non-neuronal and neuronal cell models of HD.

Materials and methods

Plasmid construction and generation of stable cell lines expressing C-terminal SF-tagged Htt

The SF–Htt plasmids expressing Htt19Q, Htt55Q, and Htt94Q and the stable cell lines expressing these constructs have been described previously (Dong *et al.* 2012). The Cr expression plasmid construct was purchased from OriGene (Rockville, MD, USA).

Cell culture and transfection

Human embryonic kidney (HEK)293 cells stably expressing C-terminal Strep/Flag (SF)-tagged Htt containing 19Q, 55Q, or 94Q were grown in selective medium. For transfection, the different expression plasmids were introduced into HEK293 cells using Safectine RU50 DNA transfection kit according to the manufacturer's protocol (Syd Labs, Malden, MA, USA). In some experiments, the cell line stably expressing SF-tagged 94Q was cultured in the complete medium supplemented with dimethylsulfoxide (vehicle control), 20 μM LY294002 (Sigma, St. Louis, MO, USA), or 1 μM Wortmannin (Sigma) for a further 28 h before cell death or specific proteins were examined.

The methods to culture and differentiate the inducible neuronal progenitor cell lines stably expressing green fluorescent protein (GFP)-tagged Htt-exon1 containing 28Q or 74Q have been previously described (Dong *et al.* 2011). For transfection, the control or the Cr expression plasmid was introduced into the undifferentiated neurons either by electroporation using a neural stem cell Nucleofector kit and device (Lonza, Walkersville, MD, USA) or using Safectine RU50 DNA transfection kit (Syd Lab). After transfection, the cells were cultured in differentiation medium as described previously (Dong *et al.* 2011) before being used for analysis.

Primary neuronal culture derived from YAC128 HD mice

The YAC128 HD mouse that expresses a full length of mHtt containing 128Q was obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and was bred and maintained under pathogen-free conditions according to institutional guidelines under an approved protocol in the University of South Dakota Stanford School of Medicine. Hemizygous YAC128 HD mice were crossed with the wild-type mice to generate HD pups. Primary striatal neuronal culture was performed using previously described methods with modifications (Zeron *et al.* 2002). Briefly, anterior striata were isolated from pups at post-natal day 0 in ice-cold divalent-free Hank's Balanced Salt Solution (Invitrogen, Carlsbad, California, USA). During the dissection procedure, a forelimb was saved from each pup to prepare genomic DNA for PCR genotyping. The genotyping primers and protocol were according to the recommendations provided by the Jackson Laboratory. The isolated striatal tissue was dissociated in 0.25% trypsin–EDTA (Invitrogen) and plated at a density of $\sim 1 \times 10^6$ cells/mL on poly-ornithine (Sigma) -coated 24-well plates in Neurobasal medium B27 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific, HyClone, Logan, UT, USA), 100 units/mL penicillin–streptomycin (Invitrogen). Mouse cortical primary neuronal cultures were prepared from timed-pregnant HD mice at embryonic day 15 based on previously described methods (Wang *et al.* 2004). Cells were cultured at 37°C, 5% CO_2 with humidity, and were fed every 3 days. The cells were cultured for 14 days before being fixed and immunocytochemically stained and the medium was refreshed every 3 days.

Western blot analysis

The cells prepared for western blot analysis were washed twice with ice-cold phosphate-buffered saline (PBS) and were then lysed with a cell lysis buffer (50 mM Tris-HCl, pH6.8, 150 mM NaCl, 20 mM EDTA, 1 mM EGTA, 0.5% SDS, 0.5% NP-40, 0.5% Sarkosyl) supplemented with fresh protease inhibitor cocktail (Sigma) for 20 min on ice with intermittent agitation. Protein extracts were resolved on 10 or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membrane (Whatman GmbH, Piscataway, NJ, USA) under standard transfer conditions as described previously (Dong *et al.* 2011). The antibodies used were FLAG monoclonal antibody (1 : 2000, Sigma), Cr polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GFP monoclonal antibody (Santa Cruz Biotechnology), actin polyclonal antibody (Santa Cruz Biotechnology), Akt and p-Akt (Thr308) (1 : 1000, Cell Signaling, Danvers, MA, USA), extracellular signal-regulated kinase (Erk)1/2 (p44/42 MAPK, 1 : 1000, Cell Signaling), and phosphorylated Erk1/2 (Phospho-p44/42 MAPK antibody, 1 : 1000, Cell Signaling). Detections were performed using horseradish peroxidase-conjugated anti-rabbit, -mouse, or -goat antibody (Santa Cruz Biotechnology) and Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA, USA).

Immunocytochemical staining

Immunocytochemical staining of cells was performed according to our previously described method (Dong *et al.* 2011). Briefly, cells were fixed in 4% paraformaldehyde in PBS for 25 min, permeabilized with 0.15% Triton X-100 for 12 min, and incubated in blocking buffer (5% bovine serum albumin in PBS) for 45 min at 23.5°C. The cells were then incubated in rabbit anti-Cr polyclonal antibody (1 : 200, Santa Cruz Biotechnology) and mouse anti-FLAG monoclonal antibody (1 : 250, Sigma), mouse anti-polyglutamine monoclonal antibody (1 : 100, 5TF1-1C2, Millipore), or rabbit anti-NeuN polyclonal antibody (1 : 200, Millipore) for 2 h followed by incubation with Cy2-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit antibody (1 : 200; Jackson ImmunoResearch) for 45 min at (23.5°C). The nuclei were further stained with Hoechst 33342 (Invitrogen). Immunostained cells were observed using a Zeiss Axiovert fluorescence microscope and images were captured using a digital camera and Axiovision software (Zeiss, Thornwood, NY, USA).

Coimmunoprecipitation analysis

Approximately, 2×10^7 cells were lysed in 1 mL of cell lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 7.4, 1% Triton X-100, and protease inhibitor cocktail) for 20 min at 4°C with gentle rotation. The lysates were passed through a 23G3/4 needle 20 times. The insoluble debris was removed by centrifugation at 10 000 *g* for 15 min. For coimmunoprecipitation, cell lysates (about 1.2 mg protein) were incubated with 100 μL Strep Tactin matrix (IBA, Goettingen, Germany) at 4°C overnight with gentle rotation. The beads were washed three times with washing buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 0.5% Triton X100 and eluted with 80 μL of 2X SDS sample buffer at 100°C for 5 min. Alternatively, cell lysates were incubated with 2.4 μg Cr antibody (Santa Cruz Biotechnology) or 2 μg GFP antibody (Santa Cruz Biotechnology) for 2 h at 4°C with gentle rotation, then the Cr-antibody mixtures were incubated with 100 μL pre-cleared Protein G Plus-Agarose (Santa Cruz Biotechnology). The beads were then washed three times with PBS buffer containing 0.5% Triton X100 and eluted with 80 μL of 2X SDS sample buffer at 100°C for 5 min.

Cell-death assay

Cell-death analysis was performed as described previously(Dong *et al.* 2012) with either a TACS™ 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay kit (Trevigen, Gaithersburg, MD, USA) or the Trypan Blue exclusion method using a Countess™ automated cell counter (Invitrogen) according to the manufacturers' protocols. In some experiments, cells were also stained with 3 mM propidium iodide. The stained cells were observed using a Zeiss Axiovert fluorescence microscope and images were captured using a digital camera and Axiovision software (Zeiss). Alternatively, in some parallel experiments, cell death was also assessed using the lactate dehydrogenase (LDH)-release assay kit (Cayman, Ann Arbor, MI, USA) by following the manufacturers' protocols.

RNAi knockdown studies

The HC2S2 neuronal cell line stably expressing GFP–mHtt74Q was plated in 6-well plates. Twenty-four hours after plating, cells were transfected with either control or Cr TriFECTA RNAi kit (Integrated DNA Technology, Coralville, IA, USA) using Lipofectamine ™ RNAiMAX reagent (Invitrogen) according to the suggested protocol by the company. The cells were allowed to grow for 2 days before being analyzed for viability using an MTT assay kit (Trevigen).

Analysis of free intracellular calcium

Analysis of intracellular free calcium was performed by flow cytometry. Briefly, cell lines stably expressing either SF-19Q or SF-94Q were plated at a density of 1×10^6 cells/well in a 6-well plate. The cells were transfected either with an empty control vector or with a Cr expression vector. Twenty-four hours following the transfection, the cells were treated with 100 μM H_2O_2 for 7 h. After washing with PBS, the cells were trypsinized in preparation for collection of cells. The cell pellets were resuspended with control salt solution [containing (in mM): 120 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 25 Tris-Cl, 15 glucose, pH 7.4] and loaded with Fluo-3-AM (Invitrogen) for 30 min at 23.5°C. The cells were washed twice with control salt solution and then subjected to flow cytometric analysis with excitation at 488 nm using a flow cytometer equipped with argon-ion laser source (BD Accuri C6, Franklin Lakes, NJ, USA).

Statistical analysis

All numerical data represent the mean values ± SDM. One-way analysis of variance was used for statistical analysis of the experimental results and *t*-tests were used for comparisons between two different groups. $p < 0.05$ was regarded as statistically significant.

Results

Cr interacts with Htt

We recently employed the technique of tandem affinity purification (TAP) to identify novel mHtt-interacting proteins (Dong *et al.* 2012), as this approach allows efficient identification of interacting proteins under near-physiological conditions (Rigaut *et al.* 1999; Gloeckner *et al.* 2009). To obtain sufficient expression of the SF–Htt fusion proteins for the TAP assay, we generated the HC2S2 neuronal progenitor cells (Dong *et al.* 2011) and the HEK293 cell lines (Dong *et al.* 2012) stably expressing the fusion proteins. The neuronal cell line stably expressing Strep/Flag-tagged huntingtin (SF-Htt) containing 55Q was employed first for the TAP assay. Following incubation of the supernatant of the neuronal cell lysates with both Strep and FLAG superflow resin columns, the final TAP eluates containing the SF-tagged Htt and associated proteins were subjected to two-dimensional nano-liquid chromatography tandem mass spectrometry. The database search of the mass spectrometry results revealed that one of the proteins associated with the SF–mHtt protein was calretinin (Cr). The interaction of Cr with mHtt was verified by western blot analysis of the eluates from the TAP assay (Fig. 1a).

To confirm the interaction between Cr and mHtt, we transfected Cr into the HEK293 cell line stably expressing Strep/Flag (SF)-tagged Htt containing either 19Q, 55Q, or

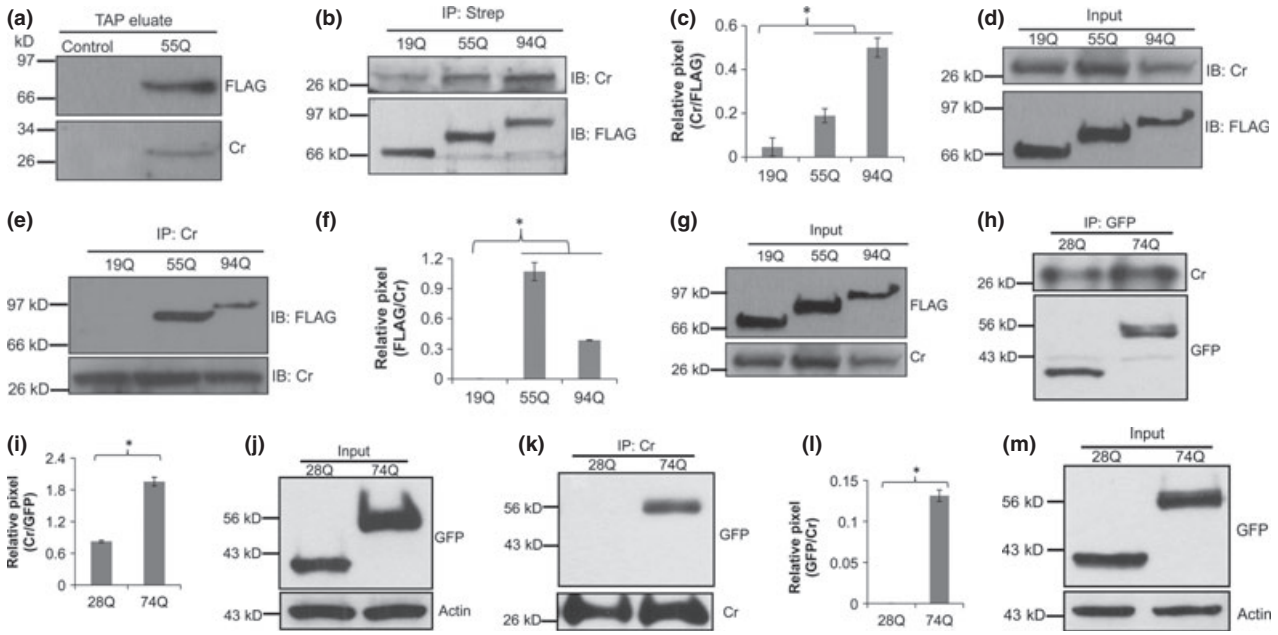

Fig. 1 Cr interacts with mHtt. (a) Western blot analysis of the proteins eluted from the tandem affinity purification (TAP) columns. Control: the proteins derived from the HC2S2 cell lysate expressing an empty vector. 55Q: the proteins derived from the HC2S2 cell lysate expressing the SF–mHtt55Q fusion protein. (b) Mutant Htt coimmunoprecipitates with calretinin (Cr) upon coexpression in HEK293 cells. The supernatants derived from HEK293 cells coexpressing Cr and SF-tagged N-terminal Htt containing either 19Q, 55Q, or 94Q were incubated with Strep-Tactin Superflow resin. After centrifugation, the bound proteins were separated by SDS–PAGE and immunoblotted for the indicated proteins. (c) Quantitation of data corresponding to (b). Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.01$. (d) Western blot analysis of input samples used in (b). (e) Cr coimmunoprecipitates with mHtt upon coexpression in HEK293 cells. The supernatants derived from HEK293 cells coexpressing Cr and SF-tagged N-terminal Htt containing either 19Q, 55Q, or 94Q were incubated with Protein G PLUS-Agarose beads that had been coupled to Cr antibody. After centrifugation, the bound proteins were separated by SDS–PAGE and immunoblotted for the indicated proteins. (f) Quantitation of data corresponding to (e). Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.01$. (g) Western blot analysis of input samples used in (e). (h) Mutant Htt coimmunoprecipitates with Cr in neuronal cells stably expressing green fluorescent protein (GFP)-tagged mHtt. The supernatants derived from the inducible neuronal cells stably expressing GFP-tagged Htt exon 1 containing either 28Q or 74Q were incubated with Protein G PLUS-Agarose beads that had been coupled to GFP antibody. After centrifugation, the bound proteins were separated by SDS–PAGE and immunoblotted for the indicated proteins. (i) Quantitation of data corresponding to (h). Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.01$. (j) Western blot analysis of input samples used in (h). (k) Cr coimmunoprecipitates with mHtt in neuronal cells stably expressing GFP-tagged mHtt. The supernatants derived from the inducible neuronal cells stably expressing GFP-tagged Htt exon 1 containing either 28Q or 74Q were incubated with Protein G PLUS-Agarose beads that had been coupled to Cr antibody. After centrifugation, the bound proteins were separated by SDS–PAGE and immunoblotted for the indicated proteins. (l) Quantitation of data corresponding to (k). (m) Western blot analysis of input samples used in (k).

94Q and immunoprecipitated SF–Htt fusion proteins with the Strep-Tactin matrix. We then examined the precipitates for coimmunoprecipitation of Cr by immunoblotting. As seen in Fig. 1b (upper panel), 1c, and 1d, more Cr was coimmunoprecipitated with SF–mHtt (55Q and 94Q) than with SF–Htt19Q protein. Similarly, when we used the approach to immunoprecipitate Cr from the cell lysates and examined the precipitates for coimmunoprecipitation of SF-tagged Htt, we detected more SF–mHtt (55Q and 94Q) coimmunoprecipitated with Cr than with the SF–Htt19 protein (Fig. 1e–g). These observations were confirmed when we repeated the experiments using the neuronal cell lines stably expressing GFP-tagged Htt exon 1 protein containing either 28Q or 74Q (Dong *et al.* 2011). Compared to the GFP–Htt28Q, Cr appeared to preferentially bind to GFP–mHtt74Q (Fig. 1h–m). These data suggest that Cr interacts with Htt, with slight but apparent preference for mHtt containing expanded polyQ repeats rather than with the Htt protein containing a normal polyQ tract.

Cr is colocalized with Htt

As mHtt interacts with Cr, we speculated that the two proteins should be colocalized in the cytoplasm. We examined this using double immunofluorescence microscopy in the HC2S2 neuronal cell line (Dong *et al.* 2011) transiently transfected with SF-tagged Htt containing either 19Q, 55Q, or 94Q. As shown in Fig. 2a, although Cr was seen in the nucleus, it was prominently present in the

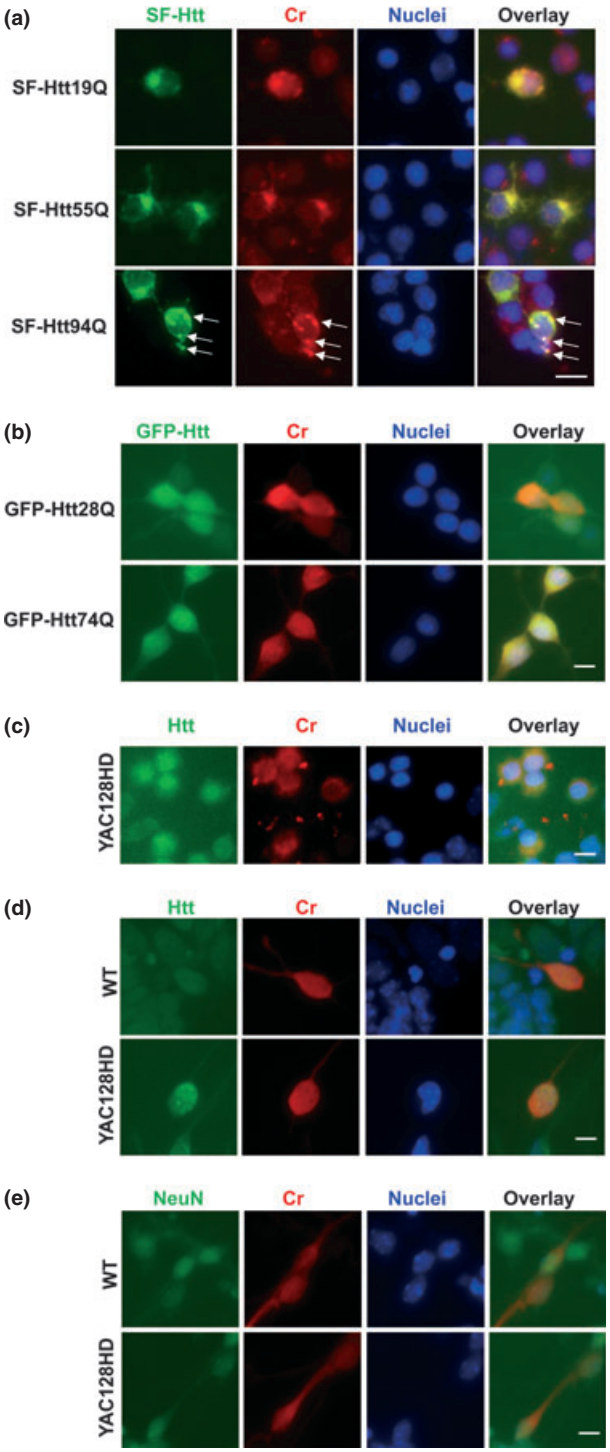

Fig. 2 Cr colocalizes with diffusible mHtt. (a) Calretinin (Cr) colocalizes with SF-tagged Htt in the inducible neurons. HC2S2 neurons were transiently transfected with SF-tagged Htt containing either 19Q, 55Q, or 94Q. The neurons were allowed to differentiate for 2 days before being immunocytochemically stained for SF–Htt (green) and endogenous Cr (red). The images were taken using a fluorescence microscope. Nuclei were stained with Hoechst 33342. Scale bar, 10 μm. (b) Cr colocalizes with green fluorescent protein (GFP)-tagged Htt in the inducible neurons. HC2S2 neurons stably expressing GFP-tagged Htt exon 1 containing either 28Q or 74Q were allowed to differentiate for 2 days before being immunocytochemically stained for endogenous Cr (red). The images were taken using a fluorescence microscope. GFP-tagged Htt was in green color. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm. (c) Cr colocalizes with endogenous mHtt in the primary striatal neuronal culture derived from a YAC128 HD mouse. Striatal neuronal cultures were performed at post-natal day 0 and fixed after 14 days *in vitro* and immocytochemically stained for the indicated proteins. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm. (d) Cr colocalizes with endogenous Htt in the primary cortical neuronal culture derived from either wild-type or YAC128 HD embryos. Cortical neuronal cultures were performed at embryonic day 15 and fixed after 5 days *in vitro* and immunocytochemically stained for the indicated proteins. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm. (e) Cr-positive cells are neurons. The primary cortical neuronal cultures corresponding to (d) were fixed and immunocytochemically stained for the indicated proteins. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm.

cytoplasm and colocalized with SF-tagged Htt in the neuronal cells as indicated by the yellow color in the overlay images. Interestingly, when aggregates were found, Cr was colocalized with SF–mHtt in the aggregates (Fig. 2a, pointed by arrows in the bottom panels). To confirm these results, we also immunostained and examined the previously established neuronal cell lines stably expressing GFP-Htt28Q or -mHtt74Q (Dong *et al.* 2011). As previously observed, without any treatment, there were no apparent GFP-tagged mHtt aggregates in the cells. Cr was colocalized with both GFP–Htt28Q and –mHtt74Q proteins (Fig. 2b). The colocalization of Cr with mHtt protein was further verified by immunochemical staining of primary striatal cell cultures (Fig. 2c) and primary cortical neuronal cultures (Fig. 2d) derived from the YAC128 transgenic mice. Using neuron-specific marker, NeuN, it was revealed that the cells positive in Cr staining were also NeuN-positive cells (Fig. 2e), suggesting that Cr is colocalized with mHtt in neurons. These results provide additional evidence that Cr interacts with Htt protein in neuronal cells.

Over-expression of Cr reduces mHtt-caused cell death in non-neuronal and neuronal cell models of HD

To examine the role of Cr in HD, we transiently transfected HEK 293 cells stably expressing SF–Htt containing an expanded polyQ (94Q) repeat with a Cr expression construct (Fig. 3a). Upon oxidative stress, the cells expressing the expanded polyQ proteins (94Q) showed a significant increase in cell death when transfected with a control vector compared to the cells transfected with a Cr plasmid (Fig. 3b and c). These results were confirmed by an MTT assay that assesses cell viability and by the LDH-release assay that examines cell death. As shown in Fig. 3d and e, over-expression of Cr

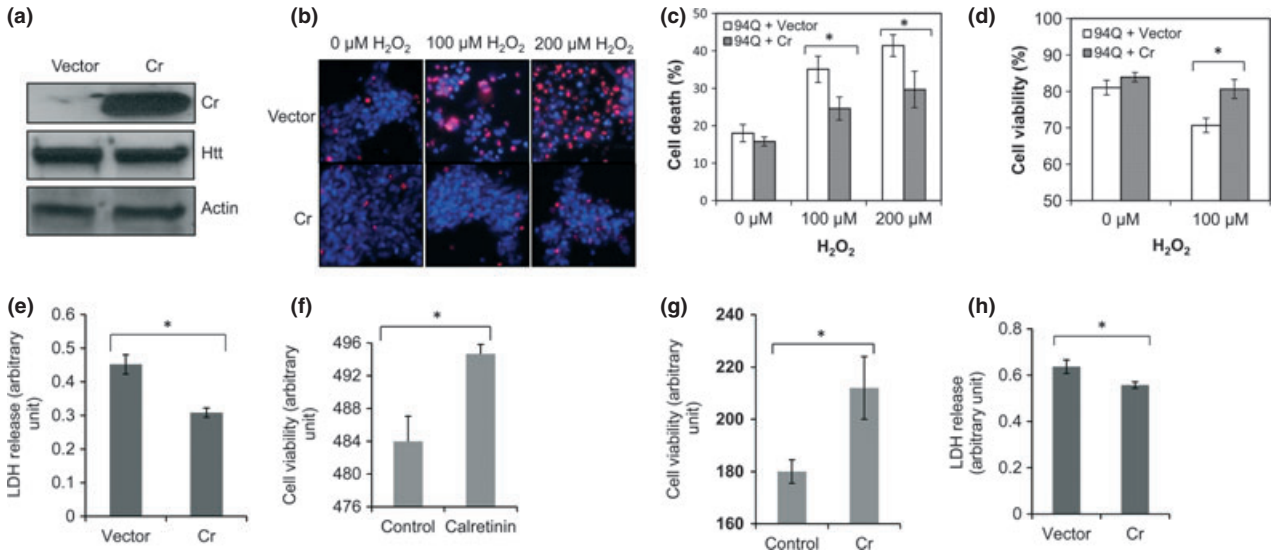

Fig. 3 Over-expression of calretinin (Cr) reduces mHtt-caused cell death in non-neuronal and neuronal cell cultures. (a) Western blot analysis showing the expression of Cr in HEK293 cells transfected with Cr or an empty control vector (Vector). An equal amount of protein from each cell line was loaded on the gel. Western blot of actin is used as a loading control. (b) Representative images of the SF–mHtt94Q stable cell line transfected with either an empty control vector (upper panels) or a Cr expression vector (lower panels). Thirty-three hours following the transfection, the cells were treated with the indicated concentration of H_2O_2 for 36 h. The cells were vitally stained with Hoechst 33342 (blue) and propidium iodide (red). Only dead cells are red in color. Scale bar, 20 μm. (c) Cell death in the HEK293 cell line stably expressing SF–mHtt94Q shown in (b) was also quantitatively analyzed by the Trypan blue method. Data are shown as mean ± SDM; $n = 6$; asterisk, $p < 0.005$. (d) Cell viability in the HEK 293 cells was quantitatively accessed by an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The HEK293 cell line stably expressing SF–Htt94Q was transiently transfected with a Cr or a control vector and then treated with the indicated concentration of H_2O_2 for 35 h. Data are shown as mean ± SDM; $n = 4$; asterisk, $p < 0.005$. (e) Cell cytotoxicity in the HEK293 cell line stably expressing SF–Htt94Q corresponding to (b) was quantitated by lactate dehydrogenase (LDH) assay. Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.05$. (f) Cell viability in the HeLa cell line stably expressing green fluorescent protein (GFP)–mHtt74Q was quantitatively accessed by an MTT assay. The stable cell line was transfected with either a Cr or a control vector for 24 h and then treated with 100 μM H_2O_2 for 8 h. Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.01$. (g) Over-expression of Cr inhibits mHtt-induced cell death in neuronal cells. The inducible neuronal cell line stably expressing GFP–mHtt74Q fusion protein was transiently transfected by electroporation with an empty control vector (Control) or a Cr expression vector (Cr) and then grown in neuronal differentiation medium for 3 days. The cells were treated with 100 μM H_2O_2 for 8 h and cell viability was quantitatively analyzed by an MTT assay. Data are shown as mean ± SDM; $n = 5$; asterisk, $p < 0.05$. (h) Cell cytotoxicity in the inducible neuronal cell line stably expressing GFP–mHtt74Q shown in (g) was quantitated by LDH assay. Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.05$.

promoted cell survival in the cells expressing SF–mHtt compared to the same cell line transfected with an empty vector. To exclude the possibility that this protection exerted by over-expression of Cr is dependent on the specific cell type, we also tested a previously generated HD HeLa cell line that expresses GFP-tagged mHtt-exon-1 containing 74Q (Wang *et al.* 2006). Over-expression of Cr in this HD cell line also showed a remarkable protective effect (Fig. 3f), suggesting that Cr suppresses mHtt-caused toxicity in different non-neuronal cell models of HD.

To further examine whether the modulation of polyQ toxicity by over-expression of Cr also occurs in neuronal cells, we transfected the Cr expression plasmid into an inducible neuronal progenitor cell model of HD that we recently established (Dong *et al.* 2011) using the Nucleofector kit and device (see Methods). Similar to the results obtained from the non-neuronal cell lines, over-expression of Cr also reduced expanded polyQ protein-induced cell death in neuronal cells (Fig. 3g and h).

Knockdown of Cr levels facilitates mHtt-caused cell death

We next examined what effect reducing the levels of endogenous Cr protein has on cell survival of the inducible neuronal cells of HD (Dong *et al.* 2011). Two days after siRNA transfection, Cr protein levels were reduced by 55% compared with the control-transfected cells (Fig. 4a and b). Knockdown of Cr expression caused a statistically significant increase in cell death in the Cr siRNA-transfected cells compared with the control siRNA-transfected cells, as detected by MTT assay (Fig. 4c). These results demonstrate that a decrease in Cr levels facilitates mHtt-caused cell death in neuronal cells.

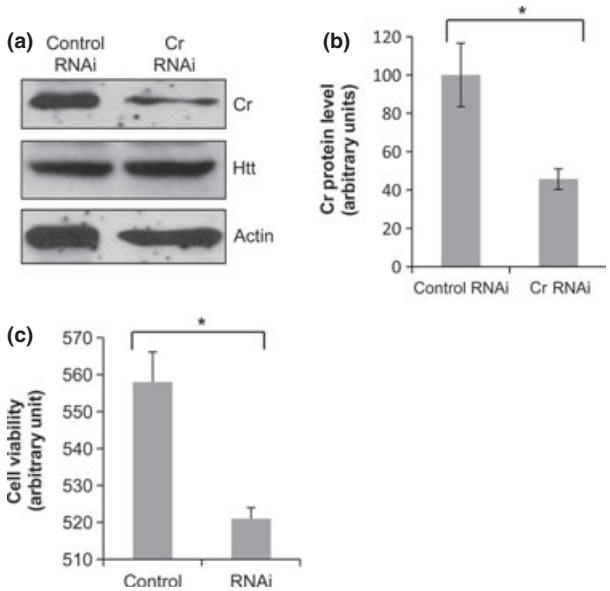

Fig. 4 Down-regulation of calretinin (Cr) in green fluorescent protein (GFP)–mHtt74Q neuronal cells enhances Htt-caused cell death. (a) Western blots showing successful knockdown of Cr protein expression by synthetic siRNAs. GFP–mHtt74Q neuronal cells were transfected either with a control siRNA or synthetic Cr siRNAs. Cells were harvested after 2 days following the transfection. An equal amount of protein was immunoblotted for Cr (upper panel) or for actin (lower panels) as a loading control. (b) Graph showing quantification of band intensity shown in (a). Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.001$. (c) Graph showing quantification of cell viability in the GFP-mHtt74Q neurons with Cr knockdown. Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.0001$.

Over-expression of Cr ameliorates intracellular free calcium rise caused by mHtt

As Cr is a calcium-binding protein, it is known to serve as a buffer against excessive intracellular calcium. To determine intracellular free calcium levels, we performed flow cytometric analysis using extensively employed fluorescent calcium indicator, fluo-3-AM (June and Rabinovitch 1994). Upon treatment with H_2O_2, the cells expressing the expanded polyQ protein (94Q) had significantly more cells positive in fluo-3 than the cells expressing the normal polyQ protein (19Q) (Fig. 5). Interestingly, over-expression of Cr dramatically reduced fluo-3-positive cells in the cells expressing mHtt containing 94Q (Fig. 5). These results indicate that over-expression of Cr reduces intracellular Ca^{2+} rise caused by mHtt.

Over-expression of Cr activates Akt pathway

In addition to functioning as calcium buffer, over-expression of Cr may activate cellular pro-survival pathways. To test this possibility, we first examined whether over-expression of Cr activates Erk pathway as activation of this pathway has been proven beneficial in HD (Bodai and Marsh 2012). However, we did not find significant alteration in phosphorylated Erk1/2 in the cells stably

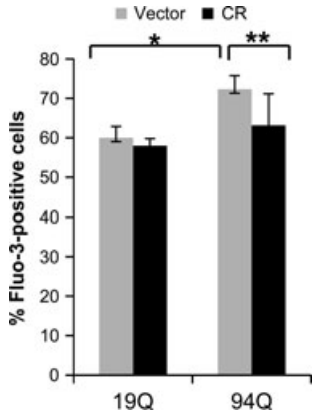

Fig. 5 Over-expression of Cr suppresses intracellular free Ca^{2+} rise induced by mHtt. HEK293 cells stably expressing either SF-Htt19Q or SF–Htt94Q were transfected with either Cr or an empty control vector. The next day, following the transfection, the cells were treated with 100 μM H_2O_2 for 7–8 h before being loaded with Fluo-3-AM. The cells were then collected for flow cytometric analysis. Data are shown as mean ± SDM; $n = 4$; *$p < 0.001$; **$p < 0.05$.

expressing SF-Htt 94Q after being transfected with Cr construct (data not shown). As some calcium-binding proteins such as calbindin and camodulin mediate cellular protection via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Coticchia *et al.* 2009; Sun *et al.* 2011), we therefore examined whether over-expression of Cr-activated Phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathway. Toward this end, we cotransfected SF–Htt94Q construct together with an empty control vector or a Cr over-expression plasmid, and after 36 h, the Akt and phosphorylated Akt (p-Akt at Thr308) proteins were detected by western blot analysis. As shown in Fig. 6a and b, over-expression of Cr was associated with a

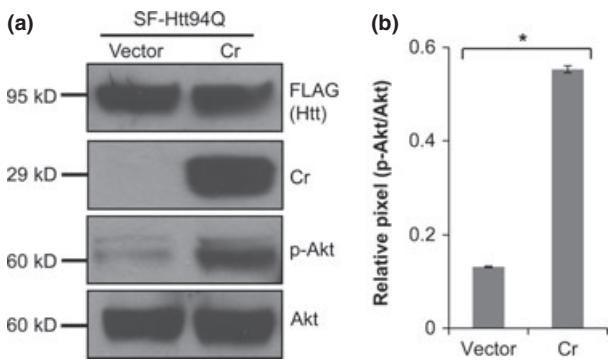

Fig. 6 Over-expression of calretinin (Cr) is associated with activation of Akt. (a) Western blot analysis of HEK293 cells cotransfected with SF–Htt94Q together with either an empty vector or a Cr over-expression plasmid. After 36 h following the transfection, the cells were collected for western blot analysis using the indicated antibodies. (b) Quantification of p-Akt band intensity shown in (a). The result has already been normalized against the total Akt. Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.001$.

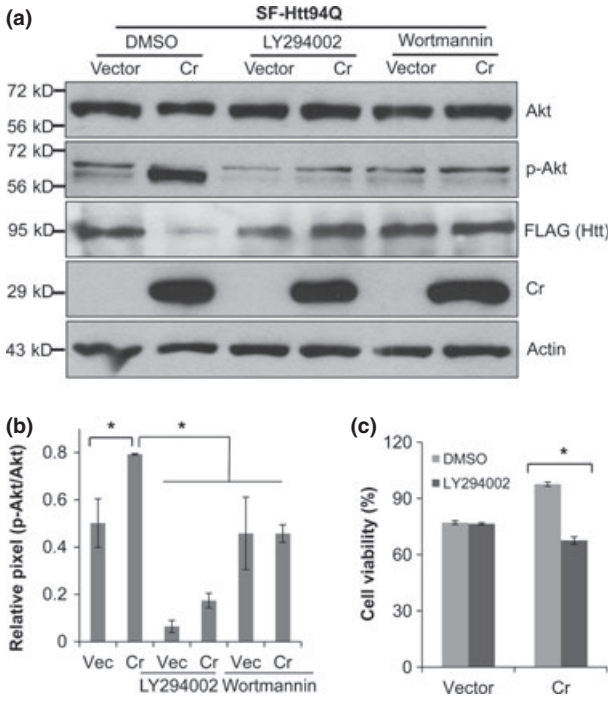

Fig. 7 PI3K inhibitors suppress calretinin (Cr)-mediated protection against mHtt-caused toxicity. (a) HEK293 cell line stably expressing SF–Htt94Q was transfected with either an empty vector or the Cr construct. Forty-eight hour following the transfection, the cells were cultured in fresh complete medium supplemented with dimethylsulfoxide (vehicle control), 20 μM LY294002, or 1 μM Wortmannin and continued cell culture for additional 28 h before being collected for western blot analysis with the indicated antibodies. (b) Quantitation of data corresponding to (a). Data are shown as mean ± SDM; $n = 3$; asterisk, $p < 0.0001$. (c) Inhibition of Phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway suppressed Cr-mediated protection against mHtt-caused toxicity. Cells corresponding to (a) was assessed for cell viability by an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

significant increase of p-Akt. In contrast, phosphorylation of Akt caused by over-expression of Cr was completely abolished in presence of PI3K inhibitors, either LY294002 or wortmannin (Holmes 2011) (Fig. 7a and b). Moreover, compared with the control treatment, inhibition of phosphorylation of Akt significantly reduced Cr-enhanced cell viability (Fig. 7c). These data suggest that activation of PI3K/Akt pathway may be, at least partially, involved in Cr-mediated protection against mHtt-caused toxicity.

Discussion

We show here that Cr protein interacts with Htt proteins, with slight, but apparent preference for the Htt proteins containing an expanded N-terminal polyQ repeat. Interaction of the two proteins is further supported by colocalization of Cr with Htt in the inducible neuronal cell lines and primary striatal neuronal cultures of HD. When over-expressed, Cr reduces the expanded polyQ protein-caused cytotoxicity both in non-neuronal and neuronal cells, whereas down-regulation of Cr enhances mHtt-caused neuronal cell death. Furthermore, over-expression of Cr also reduces intracellular free Ca^{2+} and causes activation of Akt. These data not only provide evidence showing that Cr and mHtt interact with each other but also highlight the importance of Cr, a calcium-binding protein, in suppressing expanded polyQ protein-caused cytotoxicity.

Using both neuronal and non-neuronal cell models of HD, we demonstrated that Cr not only colocalizes but also interacts with mHtt. Although Cr also interacts with wild-type Htt, it appears to preferentially bind to mHtt. It is conceivable that binding of mHtt to Cr protein may result in an alteration of Cr conformation, leading to disruption of its function and normal interactions with calcium and other proteins. In addition to calcium, Cr is also reported to interact with cytoskeletal proteins such as cytokeratin (also referred to as keratin) and α-tubulin (Marilley and Schwaller 2000). As mHtt also interacts with microtubules (Tukamoto *et al.* 1997), it is possible that Cr, mHtt, and microtubule may interact with each other and form a large complex. The association of Htt with microtubules is considered to be responsible for intraneuronal vesicular trafficking and it would be interesting to investigate whether the Cr–mHtt interaction alters this in neuronal cells.

Our data strongly support that over-expression of Cr is protective against mHtt-caused cytotoxicity in both neuronal and non-neuronal cells. This observation is in accordance with previous *in vivo* and *in vitro* studies. It has been found that neurons positive for Cr expression are selectively spared in the striatum of the HD brain (Cicchetti *et al.* 2000; Massouh *et al.* 2008). Similarly, cortical neurons expressing Cr are selectively resistant to calcium overload and excitotoxicity in cell cultures (Lukas and Jones 1994). A number of previous studies have indicated that impaired calcium homeostasis and excitotoxicity have been implicated in HD. Furthermore, Cr shows reduced expression in a HD mouse model, which is correlated with impaired neurogenesis in adult HD animals (Fedele *et al.* 2011). Therefore, enhancement of Cr expression may be a therapeutic strategy in treating HD.

The role of Cr in protecting cells from mHtt-caused toxicity may be through multiple mechanisms including its calcium-buffering capability and activation of pro-survival signaling pathways. Our data indicate that over-expression of Cr prevents cells from intracellular free Ca^{2+} rise. A number of studies have revealed that mHtt is associated with impaired calcium homeostasis partially because of the activation of NMDA receptors and subsequent influx of calcium (Fan and Raymond 2007). A high level of intracellular calcium can be harmful to cells by either triggering apoptotic cell-death cascades or activating a number of enzymes such as endonucleases, proteases, and

phospholipases that subsequently damage intracellular components and cellular structures. Like other calcium-binding proteins, one major function of Cr is to bind to the cytosolic free calcium and thus buffer intracellular free Ca^{2+}, leading to alleviation of the toxicity caused by excessive free calcium. Our results are in accordance with this speculation. In addition to functioning as a buffer against excessive intracellular free Ca^{2+}, our data also indicate activation of a pro-survival signaling pathway, the Akt pathway. This function is consistent with a previous report (Henzi *et al.* 2009), suggesting that activation of PI3K/Akt pathway may be, at least partially, involved in Cr-mediated protection against mHtt-caused toxicity. Apart from Cr, other calcium-binding proteins such as calbindin and calmodulin can also activate Akt (Coticchia *et al.* 2009; Sun *et al.* 2011). However, the significance behind this redundant function shared by different calcium-binding proteins remains unclear.

Taken together, we demonstrate that mHtt is associated with Cr. Over-expression of Cr not only suppresses mHtt-caused toxicity whereas knockdown of Cr enhances mHtt-caused cell death. In addition, over-expression of Cr reduces intracellular free Ca^{2+} rise caused by mHtt and activates Akt pathway. Thus, our results highlight the importance of maintaining intracellular calcium homeostasis via manipulation of calcium-binding protein such as Cr that might provide a therapeutic strategy for treating HD.

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Conflict of interest

The authors have declared no conflict of interest.

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