A function of huntingtin in guanine nucleotide exchange on Rab11

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Received 14 August 2008; accepted 20 August 2008

DOI: 10.1097/WNR.0b013e328315cd4c

Huntingtin is ubiquitously expressed and enriched in the brain. Deletion of the huntingtin gene in mice is lethal during early embryonic development. The function of huntingtin is, however, not clear. Here, we report that huntingtin is important for the function of Rab11, a critical GTPase in regulating membrane traffic from recycling endosomes to the plasma membrane. In huntingtin-null embryonic stem cells, the levels of Rab11 on membranes and nucleotide exchange activity on Rab11 were significantly reduced compared with normal embryonic stem cells. In brain membranes, an antibody against huntingtin immunoprecipitated a nucleotide exchange activity on Rab11 and huntingtin was coprecipitated with Rab11 in the presence of guanosine diphosphate. These data suggest a role for huntingtin in a complex that activates Rab11. *NeuroReport* 19:1643–1647 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: endosomes, huntingtin, nucleotide exchange factor, Rab11

Introduction

The expansion of glutamines (>37 glutamines) in huntingtin (Htt) causes Huntingtin's disease (HD) [1], which results in widespread neurodegeneration in the brain. The mechanism of cell death caused by mutant Htt is unknown. Loss of wild-type Htt function may contribute to HD neuropathology [2]. The function of wild-type Htt, however, is unknown. Immunohistochemistry and subcellular fractionation studies reveal that Htt locates to subcellular membraneous compartments [3], including trans-Golgi network and endosomes. Palmitoylation of Htt at C214 by Htt-interacting protein 14 (HIP14) [4] and a domain in Htt (aa172–372) that directly binds acidic phospholipids [5] support Htt function on membraneous structures. Through binding partners, Htt associates with the molecular machinery for vesicular trafficking, including some Rab GTPases [6,7]. Rab5, which functions at early endosomes, and Rab8, which associates with Golgi membranes, have been shown to interact with Htt through huntingtin-associated protein-40 and optineurin, respectively [8,9]. Thus, Htt has been proposed to function in events of vesicular trafficking.

Vesicular trafficking involves several steps including formation of vesicles at the donor organelle, delivery of vesicles along cytoskeletons and fusion of vesicles with the acceptor organelle. These steps are regulated by small GTPases called Rab proteins. Rab GTPases are molecular switches and cycle between the GTP-bound active state and the GDP-bound inactive state. The GTP–GDP cycle is coupled with the cycle between membranes and the cytoplasm. These cycles are controlled by several factors including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and Rab–GDP displacement inhibitor (GDI). GDI proteins sequester GDP-bound Rab proteins in the cytoplasm as Rab–GDP/GDI complexes. A GEF activates a Rab by converting Rab–GDP into Rab–GTP after Rab–GDP is freed from the Rab–GDP/GDI complex. In contrast, a GAP inactivates a Rab by hydrolyzing GTP, thereby returning the Rab back to Rab–GDP that is extracted by GDI. To be active, Rab proteins must be associated with membranes and bound with GTP. Following their activation, Rab proteins execute functions by recruiting a cohort of effectors.

Our data suggest that the presence of Htt is important for Rab11 localization at membranes and for the activation of Rab11.

Materials and methods

Monoclonal antibodies were purchased from Aldrich-Sigma (St. Louis, Missouri, USA; tubulin, DM1A; FLAG, M2), BD Biosciences (Franklin Lakes, New Jersey, USA; Htt-N, MAB2166 and Rab11), Serotec (MorphoSys US Inc., North Carolina USA; Htt-C, MCA2051), and Covance (Princeton, New Jersey, USA; myc, 9E10). Polyclonal antibodies were

commercially available (calnexin c-terminus: Stressgen; optineurin: Cayman Chemical). Polyclonal antibody against Htt1-17 (AA1-17 of Htt) has been described elsewhere [3]. Conjugated secondary antibodies were obtained from Jackson Laboratory (Bar Harbor, Maine, USA).

Subcellular fractionation

We used a hypertonic homogenization buffer (50 mM HEPES-Na, pH 7.0, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 0.25 M sucrose containing protease inhibitors) to prevent Htt aggregation during preparation. Brain pieces in the homogenization buffer were passed through a dounce homogenizer for 12 strokes on ice. For cultured cells, lysates were prepared in a homogenization buffer by passing through a 271/2G needle for 5 strokes on ice. Crude lysates were centrifuged at 4°C 14 000 rpm for 10 min. The post nuclei supernatant (S1) was further centrifuged at 4°C 55 000 rpm for 1h in a TLA120.2 rotor (Beckman-Coulter, Fullerton, California, USA) or on a 2 M sucrose cushion in a Ti70 rotor (Beckman-Coulter). The supernatant (S2, cytosol) was collected and the pellet (P2, total membrane) was re-suspended in the homogenization buffer supplemented with 1% Triton X-100 or directly in the sample buffer. For centrifugation with the Ti70 rotor, the band (total membranes) formed between 0.25 and 2 M sucrose was collected and stored in an aliquot at −80°C until used. For Triton X-100 extraction of the postnuclear supernatant (S1) or total membranes (P2), a centrifugation at 14 000 rpm for 10 min was performed to remove Triton-insoluble proteins.

Guanine nucleotide exchange assay

[^{3}H]GDP release was used to determine the GEF activity. In brief, 0.5 μg of purified glutathione S-transferase (GST) - Rab11 was immobilized on glutathione beads and loaded with 20 picomol of [^{3}H]GDP (11.9Ci/mmol, Amersham) in 20 mM HEPES, pH 7.2, 20 mM potassium acetate, 1 mM DTT, 5 mM EDTA (total volume is 10 μl) at 30°C for 30 min. After loading, free [^{3}H]GDP was removed by two washes in a cold GDP loading buffer supplemented with 10 mM $MgCl_2$. Samples, membranes and IP complex, were diluted in 50 and 100 μl of assay buffer (20 mM HEPES, pH 7.2, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 0.75 mM GTP/GDP), respectively, and mixed with [^{3}H]GDP-GST-Rab11. The GEF reaction was initiated by incubating at 30°C. After the reaction was stopped by transferring the samples on ice, GST-Rab11 on glutathione beads (together with the GEF on protein-G agarose) was washed twice in a cold wash buffer (20 mM Tris/Cl, pH 7.4, 20 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT) and transferred into scintillation vials for scintillation counting. For all experiments, data were represented as a mean percentage of released [^{3}H]GDP. Released [^{3}H]GDP was obtained by subtracting the counting of the remaining [^{3}H]GDP on GST-Rab11 from total binding or no GEF control.

Immunoprecipitation

Immunoprecipitation was performed by standard procedures. In brief, the antibody was coupled onto preequilibrated protein G beads in 1xPBS containing 0.1% Tween-20 at 4°C for 1.5 h. After one wash in a homogenization buffer containing 1% Triton X-100, beads were incubated with samples supplemented with 1% Triton X-100 at 4°C for 2 h or overnight. Beads were washed four times each for 15 min with mixing in homogenization buffer containing 1% Triton X-100. Proteins were eluted into the sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis or silver staining. For GEF experiments, precipitated proteins on protein-G beads were further washed twice in cold assay buffer.

Results and discussion

Decreased levels of Rab11 on membranes and impairment of Rab11 activity in the absence of Htt

As Rab GTPases must have a secure attachment with membranes to execute a function, we investigated whether the absence of Htt affects the levels of Rab proteins on membranes. We prepared membrane fractions from wild-type and Htt-null ES cells and looked at the levels of Rab5 and Rab8, which indirectly associate with Htt [8,9]. Western blot analysis showed comparable levels of Rab5 and Rab8 in membrane fractions from Htt-null ES cells and wild-type ES cells (Fig. 1a). We then looked at Rab11, which functions at recycling endosomes [10]. In contrast to Rab5 and Rab8, the levels of Rab11 were reduced seven-fold in the membrane fraction of Htt-null ES cells compared with the membrane fraction of wild-type ES cells (mean ± SD, 7.4 ± 0.2 folds; Fig. 1a and b). Htt-null and wild-type ES cell lines had similar contents of Rab11 in total cell lysates (Fig. 1c). These data suggested that Htt might be needed specifically to stabilize Rab11 on membranes.

Having observed diminished Rab11 on membranes in the absence of Htt, we addressed the question whether this finding had any physiological relevance. Secure attachment of Rabs to membranes is achieved through Rab activation, which involves nucleotide exchange from GDP to GTP by a GEF [11–13]. Therefore, we determined whether Rab11 GEF activity is altered in a Htt-null background. Considering that Rab11 activation occurs on membranes, we carried out an assay using total membranes prepared from wild-type and Htt-null ES cells. After the membranes were incubated with GST–Rab11–[^{3}H]GDP in the presence of excess unlabeled GTP/GDP, we looked for the release of [^{3}H]GDP as a measure of Rab11 GEF activity. The release of GDP from GST-Rab11 catalyzed by membranes from Htt-null ES cells was reduced by 40% compared with the total membranes prepared from wild-type ES cells (mean ± SD, wild-type vs. Htt-null: Rab11 37.9 ± 2.5 vs. 22.4 ± 11.4; two-tailed Student's *t*-test: $P < 0.05$; Fig. 1d). Under the same conditions, there was no significant difference in nucleotide exchange activity on Rab5 in membranes from wild-type and Htt-null ES cells (mean ± SD, wild-type vs. Htt-null; Rab5: 29.6 ± 8.1 vs. 34.4 ± 9.6; two-tailed Student's *t*-test: $P = 0.545$; Fig. 1d). These results suggested that Htt was important for the distribution and activation of Rab11 on membranes.

Htt associates with a Rab11 GEF and prefers interaction with Rab11-GDP in brain membranes

As the presence of Htt was important for Rab11 distribution to membranes and for Rab11 activation, we next explored whether Htt associated with a Rab11 GEF at membranes and through this association supported the process of activating Rab11. As the brain is a rich source of membranes and has high levels of Htt, we prepared membrane fractions from fresh mouse brains and used anti-Htt antibody to

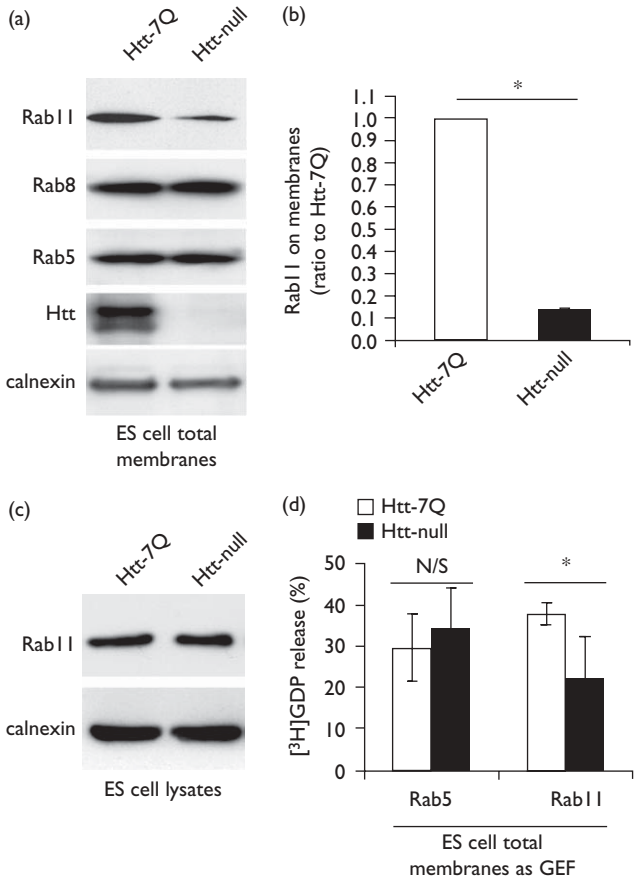

Fig. 1 Knockout of huntingtin (Htt) affects secure attachment of RabII to membranes and reduces guanine nucleotide exchange activity on RabII. (a) Absence of Htt reduces membrane association of RabII. Total membranes from wild-type and Htt-null embryonic stem (ES) cells were analyzed by SDS–PAGE and western blot with the indicated antibodies. Shown is a representative series from five experiments. (b) Quantification of western blot data in (a). Signal for RabII immunoreactivity was normalized to signal for calnexin, which was used as loading control. Results are plotted as the ratio of normalized RabII signal for Htt-null to Htt-7Q ($n=5$, Mean $\pm$ SD, 2-tailed Student *t*-test: $^*P<0.01$). (c) Contents of RabII in ES cells. 30 μg postnuclear supernatants were analyzed by western blot with indicated antibodies. Shown is one of five experiments. (d) Reduction of RabII GEF activity but not Rab5 GEF activity in Htt-null ES cells. 50 μg of total membranes from wild-type or Htt-null ES cells were extracted with 1% Triton X-100 and centrifuged. The extracted supernatants were used to carry out GDP release experiments. Bovine serum albumin (BSA) was used as no-GEF control. Data are represented as the percentage of [^{3}H]GDP released from GST-RabII or hexa histidine (6xHis)-Rab5 relative to BSA ($n=4$, Mean $\pm$ SD, 2-tailed Student *t*-test: $^*P<0.05$; N/S, not significant).

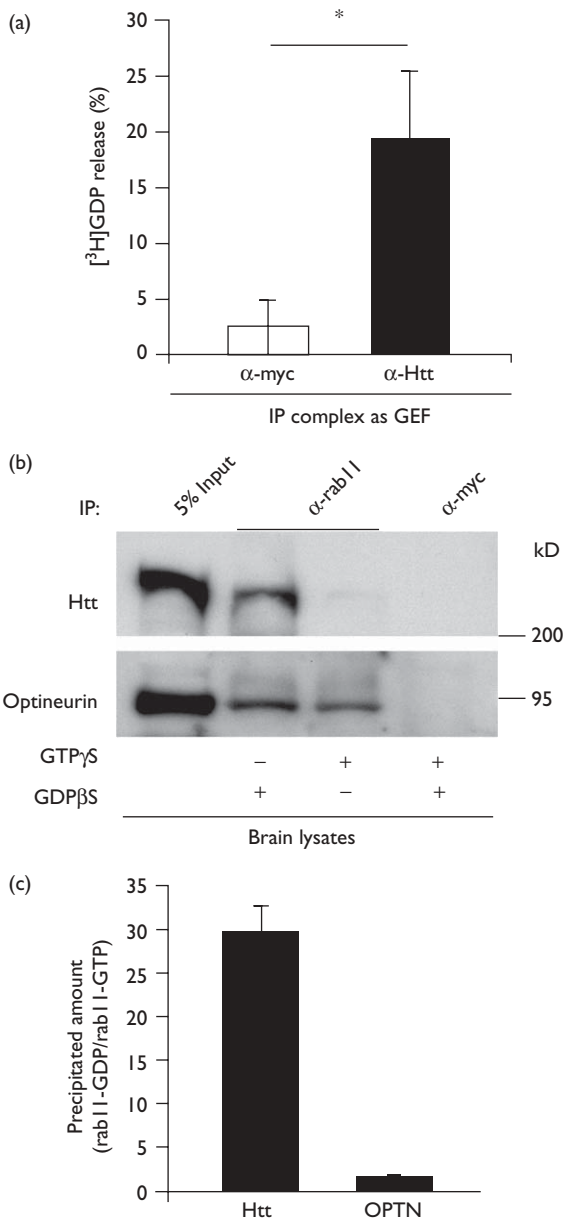

Fig. 2 Interaction of huntingtin (Htt) with a RabII GEF and RabII-GDP in mouse brain. (a) Brain membranes were treated with 1% Triton X-100 and centrifuged. The post-Triton supernatants were incubated with protein-G beads coupled to anti-Htt1-17 antibody or anti-myc antibody as control. GEF activity was examined in the precipitated complexes on beads by measuring [^{3}H]GDP release from GST-RabII for 30 min. Data are represented as the percentage of [^{3}H]GDP released from GST-RabII after incubation with the precipitates ($n=4$, Mean $\pm$ SD, 2-tailed Student *t*-test: $^*P<0.01$). (b) Coprecipitation of endogenous Htt by endogenous RabII in a GDP-favored manner ($n=5$). Postnuclear mouse brain supernatant was extracted with 1% Triton X-100 and centrifuged. The resulting supernatant was incubated with anti-RabII or anti-myc antibody-bound protein-G beads. Precipitates were analyzed by SDS–PAGE and western blot with indicated antibodies. Shown is one of five experiments. (c) Quantitative data for (b). Western blot films were scanned and each band density was measured using NIH Image]. Data are represented as the ratio of Htt or optineurin (OPTN) precipitated by RabII-GDP to that precipitated by RabII-GTP ($n=5$, Mean $\pm$ SD, 2-tailed Student *t*-test: $P<0.0001$).

precipitate Htt from the membranes. The Htt immunoprecipitates were incubated with GST–Rab11–[^{3}H]GDP in the presence of excess unlabeled GTP/GDP and the release of [^{3}H]GDP was used as a measure of Rab11 GEF activity. The precipitates obtained with anti-Htt antibody contained an activity that catalyzed nucleotide exchange on Rab11 (mean $\pm$ SD, myc vs. Htt: 2.47 $\pm$ 2.45 vs. 19.57 $\pm$ 5.89; two-tailed Student's *t*-test: $P<0.01$; Fig. 2a), suggesting that at membranes Htt associates with another protein that catalyzes nucleotide exchange on Rab11 or that Htt itself is a GEF.

To be ready for nucleotide exchange, Rab11 is bound to GDP at membranes. We reasoned that to have direct influence on Rab11 activation, Htt should associate with a complex in membranes that contains Rab11-GDP. As Rab11 and IgG light chains have a similar mobility on SDS–PAGE, we could not assess the coassociation of Htt with Rab11 by

coimmunoprecipitating Rab11 with anti-Htt antibody. Hence, we investigated interaction between Htt and Rab11 by immunoprecipitation with anti-Rab11 antibody from mouse brain followed by the detection of Htt. Endogenous Htt was coprecipitated with endogenous Rab11 significantly more in the presence of GDPβS than in the presence of GTPγS (29.6±3.1-fold increased association in the presence of GDPβS vs. GTPγS; Fig. 2b and c). These results suggested that Htt associated mainly with Rab11-GDP. In a biochemical assay, we had determined earlier that optineurin, which is known to interact with Rab8 [8], also coprecipitates with Rab11. We probed the same immunoblots with an anti-optineurin antibody to see if the interaction between optineurin and Rab11 was nucleotide dependent. Unlike the preferred association between Htt and Rab11 in the presence of GDPβS, optineurin was coprecipitated by anti-Rab11 antibody in the presence of GDPβS or GTPγS (about 1.5±0.2-fold for GTPγS vs. GDPβS; Fig. 2b and c). Thus the association between Htt and Rab11 was GDP dependent when compared with that between optineurin and Rab11.

Our biochemical analysis of Htt-null ES cells and brain membranes support a role for Htt in a process of activating Rab11, and not that of a Rab11 effector. Most Rab interactors associate with GTP-bound Rab proteins and function as Rab effectors. Htt interacts with GTP-bound Rab8 and GTP-bound Rab5 through optineurin and huntingtin-associated protein-40, respectively [8,9]; in these complexes Htt could function as a Rab effector. In this study, we found an association between optineurin and Rab11. Optineurin was originally named FIP2 but is distinct from the Rab11 effector FIP2 (Rab11-FIP2). A sequence alignment analysis reveals similarity between optineurin and Rab11-FIP2 including the

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Rab11-FIP2   13 thvqvtvlqakdlkpkgksgtndtytiiqlgkekystsvaektlepvwke  62
                :|.:::.|..|:..|...:|....:              :|...|:...|
Optineurin    2 shqplscltekedspsestgngpph-------------lahpnldtftpe  38

Rab11-FIP2   63 easfelpglliqgspekyilflivmhrslvgldkflgqvainlndifedk  112
                |..:::..||.:...|..:.|  .:::::|..:.|        ..:.:|
Optineurin   39 ellqqmkelltenhqlkeamkl--nnqamkgrfeel--------sawtek  78

Rab11-FIP2  113 qrrktewfrleskqgkriknrgeikvniqfmrnnmtasmfdlsmk-dktr  161
                |:.:.::|.::||:.|.                    .:..||.: :|.:
Optineurin   79 qkeerqffeiqskeake--------------------rlmalsheneklk  108

Rab11-FIP2  162 spfaklkdkmkgrkndgtfsdtssaiipsthmpdansefssgeiq---mk  208
                ....|||.|.:...:|.|         ..:.:|.|.:|...:::   ::
Optineurin  109 eelgklkgkserssedpt---------ddsrlpraeaeqekdqlrtqvvr  149

Rab11-FIP2  209 skpkkpfllgpqrlssahsmsdlsgshmsseklkagtigqthllghqlds  258
                .:.:|..|||        .:|:|        :||.:.:|.:.      ||
Optineurin  150 lqaekadllg--------ivsel--------qlklnssgsse------ds  177

Rab11-FIP2  259 f-----------gtvpesgslksphrrtlsfdtskmnqpdsivdegelcf  297
                |           |:|.|......| .||:|..|:.........|..
Optineurin  178 fveirmaegeaegsvkeikhspgp-trtvstgtalskyrsrsadga----  222

Rab11-FIP2  298 grqndpftnvtaslpqkfatlprkknpfeessetwdssmnlfskpieirk  347
                                  ||.||....|....:      :.:|:
Optineurin  223 ------------------------knyfeheeltvsqll------lclre  242

Rab11-FIP2  348 enk---------rekrekvslfervtgkkdsrrsd-klnnggsdspcdlk  387
                .|:         :|.:|:||.||    ||.|.||: :....||
Optineurin  243 gnqkverlevalkeakervsdfe----kktsnrseietqtegs-------  281

Rab11-FIP2  388 spnafsenrqdyfdyestnpft--akfrasnimpsssfhmsptsnedlrk  435
                     :|...|    |...|.|  ::..|.|:..:|.|       ::|::
Optineurin  282 -----tekend----eekgpetvgsevealnlqvtslf-------kelqe  315

Rab11-FIP2  436 ipdsnpfdatagyrsltyeevlqelvkhk-ellrrkdthi----------  474
                          .:..|:..|::::.::.| :.|.||::.|
Optineurin  316 -----------ahtklseaelmkkrlqekcqalerknsaipselnekqel  354

Rab11-FIP2  475 ----reledyidnllvrvmee  491
                    ::||..:::::|..:..|
Optineurin  355 vytnkklelqvesmlseikme  375
```

Fig. 3 Alignment of optineurin with Rab11-FIP2. The amino acid sequence of optineurin (NP.068815) was aligned with that of Rab11-FIP2 (NP.055719) using sequence analysis software (EMBOSS pairwise alignment) at European Bioinformatics Institute (EBI). The symbol | indicates identical amino acid and the symbol: indicates similar amino acid. The Rab11 binding domain in Rab11-FIP2 was highlighted in red.

region in Rab11-FIP2 that has a Rab11 interaction domain (Fig. 3). The sequence similarity of optineurin to Rab11–FIP2 may explain why optineurin interacts with Rab11. Unlike its reported preference for Rab8-GTP [8], we found that optineurin associated with GDP-bound and GTP-bound forms of Rab11 (Fig. 2b and c). Rab11-FIP2 was also found to interact with GDP-bound and GTP-bound Rab11 [14]. The capability to interact with both nucleotide states of a Rab protein could reflect dual roles as activator and effector. More experiments will be required to know how Htt influences Rab11 through its interaction with optineurin.

A GEF has not been identified for Rab11. If Htt were the GEF for Rab11, a direct interaction of Htt and Rab11 would be required. No direct interaction, however, was found between Htt and Rab11 in an earlier study [9]. Therefore, Htt is unlikely to be the Rab11 GEF. We speculate that Htt is in a complex with the Rab11 GEF and functions to optimize GEF activity on Rab11. Protein complexes as GEFs have been described earlier. In yeast, a ten protein complex called TRAPP II complex functions as a GEF for the yeast Rab11 homolog Ypt31/Ypt32 [15]. In mammalian cells, the complex of Elmo and Dock180 is a Rac1 GEF; Elmo stimulates the activity of Dock180 on Rac1 [16]. It will be necessary in future studies to determine the identity of the Rab11 GEF to determine the molecular mechanism by which Htt affects the catalytic activity of the Rab11 GEF.

In conclusion, our study suggests that Htt is a critical component for activating Rab11, thus clarifying a physiological function of wild-type Htt.

Acknowledgements

This study was supported by grants from NIH (to M. D. and N. A.), DANA Foundation (to M. D. and N. A.), High Q Foundation (to M. D.), and Huntingtin's Disease Society of America (to M. D.). We thank Dr Meiqian Weng for her generous gifts of fresh mouse brains. Xueyi Li is supported by a John J. Wasmuth Postdoctoral Fellowship from the Hereditary Disease Foundation. Authors declared no conflict of interests.

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