CHAPTER SEVENTEEN

Regulation of Endosome Dynamics by Rab5 and Huntingtin-HAP40 Effector Complex in Physiological versus Pathological Conditions

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Contents

Abstract

Vesicular transport of signaling molecules, that is, neurotrophins, in neurons is essential for their differentiation, survival, and plasticity. Neurotrophins such as neuron growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are internalized by receptor-mediated endocytosis at synaptic terminals and loaded into endosomes for microtubule-based transport along axons to the cell body where they exert their signaling function in the nucleus. The molecular mechanisms underlying this intracellular transport are not only relevant from a basic knowledge viewpoint, but have also important implications for neurodegenerative diseases. Defects in trafficking are increasingly implicated in the pathology

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of Huntington's disease (HD) and other neurodegenerative disorders. The small GTPases Rab5 and Rab7 play important roles in the endocytic trafficking of neurotrophins. We have recently identified Huntingtin (Htt) and Huntingtin associated protein of 40 kDa (HAP40) as a novel Rab5 effector complex that regulates endosome motility. In HD, we detected higher HAP40 protein levels compared with normal cells. Such increase causes an augmented recruitment of Htt onto Rab5-positive early endosomes that drastically reduces their motility by "switching" these organelles from microtubules to F-actin. These findings suggest a mechanism by which impaired Rab5-mediated trafficking of neurotrophic factors may be a key event of the pathogenetic process leading to neurodegeneration in HD. To dissect the mechanisms by which Htt, HAP40, and Rab5 function in early endosome interactions with the cytoskeleton, we developed assays to investigate endosome–cytoskeleton interactions that can be applied to normal and pathological conditions. We provide here detailed protocols for, first, an assay that measures binding of early endosomes to microtubules and F-actin. Second, we describe an improved protocol for a cell-free assay that recapitulates the motility of early endosomes along microtubules *in vitro*. These assays provide mechanistic insights into the dysfunction of endosome motility occurring in HD as well as other neurodegenerative disorders.

1. Introduction

In endocytosis, nutrients and growth factors are internalized in budding vesicles from the cell surface and targeted to a series of intracellular compartments where they are sorted and routed to the recycling and degradative pathways (Conner and Schmid, 2003; Gruenberg and Maxfield, 1995; Mellman, 1996; Pelkmans and Helenius, 2003). Endocytic transport allows signaling cargo to transduce signals to the nucleus (Lanzetti *et al.*, 2000; Miaczynska *et al.*, 2004). To accomplish these trafficking tasks, endocytic organelles such as early endosomes undergo heterotypic fusion with incoming clathrin coated vesicles and also homotypic fusion with each other, transform their geometry through membrane fission and tubulation, and move over long distances to transfer cargo to other organelles. In these dynamics, membrane interactions with the tubulin and actin cytoskeleton play pivotal roles at all the levels of trafficking events (Apodaca, 2001; Gasman *et al.*, 2003; Hoepfner *et al.*, 2005; McPherson, 2002; Nielsen *et al.*, 1999), particularly in neurons where vesicular transport of essential neurotrophins from axonal terminals over long distances to the nucleus (Deinhardt *et al.*, 2006; Hibbert *et al.*, 2006; Morfini *et al.*, 2005) calls for a coordinated interplay of vesicles with different types of motors, static anchors, and cytoskeletal fibers.

Alterations of this complex trafficking scenario are implicated in neurodegeneration in several inherited disorders such as Huntington's disease (HD), Kennedy's disease (spinal and bulbar muscular atrophy [SBMA]), spinocerebellar ataxia type 1c (SCA-1), Machado-Joseph disease (MJD) or SCA-3, and dentatorubral-pallidoluysian atrophy (DRPA) (Morfini *et al.*, 2005), to name a few. HD is a good example of a trafficking dysfunction. Its pathogenesis is triggered by an expanded, amino-terminal polyglutamine sequence (polyQ) (>35 for mutant Htt) (Gusella and MacDonald, 2000) in Htt, a large 348-kDa protein interacting with a wide panel of Htt interacting (HIPs) and associated partners (HAPs) via domains adjacent to the polyQ stretch. The expansion of the polyQ sequence in HD is thought to cause a conformational change that alters the binding affinity of mutant Htt for HIPs and HAPs (Harjes and Wanker, 2003). The wide number of HIPs and HAPs that implicate Htt in diverse cellular functions and the complexity of aberrant protein–protein interactions (Goehler *et al.*, 2004) hamper the identification of the most critical players in HD pathology. Recent work has pointed to defects in endocytosis (Trushina *et al.*, 2006) and intraneuronal transport of neurotrophins (Block-Galarza *et al.*, 1997; Engelender *et al.*, 1997; Gauthier *et al.*, 2004; Li and Li, 2005; Pal *et al.*, 2006; Rong *et al.*, 2006) as primary trigger for HD pathology. Particularly compromised microtubule-dependent vesicular transport of BDNF across cortical-striatal afferents is considered to be a key event in HD pathology (Altar *et al.*, 1997; Conner *et al.*, 1997; Gauthier *et al.*, 2004; Zuccato and Cattaneo, 2007). Clearly, a better understanding of HD pathology also requires dissecting the underlying regulation of endocytosis.

1.1. Rab Proteins are key players in the coordination of endocytic events

Several small GTPases of the Rab family are key regulators of endocytosis. They serve as membrane organizers, regulating sequential transport along endocytic transport routes (Zerial and McBride, 2001). Rab5 is among the best-characterized key players of endocytosis. Functional studies of numerous Rab effectors have broadened our view of the role of Rab5 in early endocytic transport (Bucci *et al.*, 1995; Singer-Krüger *et al.*, 1994, 1995), but also of Rab GTPases in general. By recruiting distinct subsets of downstream effectors onto the membrane, Rab5 protein is capable of assembling multiple domains for different functions (Zerial and McBride, 2001). Au1

One important aspect in the spatiotemporal regulation of endocytic events is the capability of Rab5 to coordinate the homotypic fusion of early endosomes with their active transport along microtubules through the recruitment of several effectors such as EEA1, Rabenosyn-5, and Rabankyrin-5 for fusion (Schnatwinkel *et al.*, 2004; Zerial and McBride, 2001), and the early endosome-specific kinesin motor KIF16B for motility

(Hoepfner *et al.*, 2005; Nielsen *et al.*, 1999). However, it remains unclear which parameters determine the particular effector subset that is recruited and activated at a given time and location. We gained initial clues when we functionally characterized Htt and HAP40, which we isolated by affinity chromatography on immobilized active Rab5 (Christoforidis and Zerial, 2000; Pal *et al.*, 2006). Htt/HAP40 complex is recruited by active Rab5 as novel downstream effector onto early endosomes. In HD, HAP40 protein levels are ~10-fold increased by a yet unknown mechanism, leading to augmented Htt/HAP40 amounts on Rab5-positive endosomal membranes and, in turn, to a ''switch'' of these organelles from microtubules to F-actin (Pal *et al.*, 2006). The consequence is a drastic reduction of early endosome motility along microtubules due to the preferential association with F-actin. Because we can mimic these perturbations by ectopically overexpressing HAP40, we conclude that the protein level of HAP40 is a critical key parameter in determining whether Rab5 recruits those effector subsets required for early endosome tethering and fusion (via EEA1, Rabenosyn-5, Rabankyrin-5) and motility along microtubules (via KIF16B) or for the association with F-actin (via Htt/HAP40). In line with this view, we found that KIF16B displaced from early endosomes through elevated levels of recruited Htt/HAP40 (our unpublished observation). Remarkably, ablation of HAP40 by RNAi can rescue the defects in Rab5 dynamics in HD cells, suggesting that excess of HAP40 is both necessary and sufficient to block endosome motility, independently of the polyQ motif in Htt (Pal *et al.*, 2006).

Collectively, the upregulation of HAP40 naturally occurring in HD provides an interesting model system to explore the role of Rab5, Htt, and HAP40 in the regulation of early endosome motility. A better understanding of the underlying membrane–cytoskeletal interactions will also help assessing the contribution of compromised endosome transport in HD pathology and, more in general, neurodegeneration.

1.2. Outlook: Feasible candidates for functional characterizations

How does excess of Htt/HAP40 on early endosomes effectuate the switch from microtubules to F-actin? For melanosomes, Gross and coworkers (2002) documented a coordinated activation/deactivation of microtubule- (dynein, kinesins) and F-actin–based motors (myosins) to regulate the motility of these vesicles along microtubules and F-actin as well as their switching between types of filament. Because we observe a displacement of the endosomal motor KIF16B (Hoepfner *et al.*, 2005) from early endosomes through elevated levels of HAP40 (our unpublished observation; see section 1.1.), we suggest a Rab5 effector machinery with Htt and HAP40 functioning in a similar interplay of different motors and putative anchors

(Walenta *et al.*, 2001). Some of our previous findings already hint to possible candidates in such a ''switch'': Gasman and coworkers (2003) reported that the small GTPase RhoD recruits hDia2C, a splice variant of human Diaphanous 2, onto Rab5-positive early endosomes, and activates c-Src kinase, causing an alignment of early endosomes along F-actin and a reduction of their motility. The striking resemblance of this phenotype with the perturbations caused through HAP40 upregulation in HD (Pal *et al.*, 2006) suggests a functional interaction between Rab5/Htt/HAP40 on the one hand, and RhoD/hDia2C/c-Src on the other. We thus propose to use the assays here to test the role of regulatory molecules in endosome–cytoskeleton interactions.

2. Assays for Functional Characterization of Rab5, Htt, and HAP40

Because trafficking of organelles along microtubules and F-actin and their switching between filament types represent very complex regulatory and mechanical processes, we sought to begin our dissection of the phenotypic alterations observed in HD by recapitulating basic interactions between purified early endosomes and cytoskeletal components *in vitro*. Specifically, the switch of early endosomes from microtubules to F-actin corresponds to a decreased binding of early endosomes to microtubules and increase to F-actin. This may also include an inactivation of microtubule-dependent motor proteins. Thus, we provide here detailed protocols for (1) a biochemical spin-down assay to measure binding of early endosomes to microtubules and F-actin; and (2) an *in vitro* assay that recapitulates the movement of early endosomes along microtubules. Both assays were used to explore the role of Htt and HAP40 in Rab5-dependent interactions of early endosomes with the cytoskeleton (Pal *et al.*, 2006). Candidate regulators can be added as recombinant (e.g., HAP40) or native proteins to test for their ability to alter endosome binding and motility. Alternatively, cytosol prepared from cells can be added to the reaction mixture with the candidate of interest immunoblocked (e.g., Htt) or knocked down by RNAi. The latter approach has the disadvantage of working with a less defined system due to the complexity of cytosol. These assays can help unraveling the mechanisms underlying endosome dynamics under physiological versus pathological conditions. Defects in axonal transport also occur in other polyQ-expansion diseases (Morfini *et al.*, 2005), such as in Kennedy's disease (SBMA), SCA-1, SCA-3, and DRPA, pointing to a common trigger. We thus propose to explore the protocols here for diagnostic tests, scoring for perturbed interactions of cytoskeletal filaments with vesicles prepared from such disease models.

3. Spin-Down Binding Assays

3.1. Basic principles

The protocol was adapted from Nielsen and coworkers (1999) to allow for improved accuracy in the quantification by fluorimetry. The assay exploits the differential sedimentation characteristics of cytoskeletal polymers and membrane organelles. Specifically, microtubules and F-actin pellet in 35% sucrose while early endosomes floatate under these conditions. To measure binding, Au2
purified early endosomes and freshly polymerized microtubules are mixed with candidate proteins to be tested, placed on top of a 35% sucrose layer, and centrifuged. Unbound early endosomes remain in the top layer, while microtubule-bound material is forced to co-pellet. The amount of early endosomes in the pellet is quantified and provides a measure for binding affinity. Analysis of pelleted proteins by Western blotting as described (Nielsen *et al*., 1999) provides a rather tedious and semiquantitative means of binding measurements. Our comparative analysis described in Pal *et al.* (2006) clearly required a better accuracy to reveal more subtle effects of candidate regulators on membrane-cytoskeleton binding. To this end, we pulsed HeLa cells with rhodamine-conjugated transferrin to label early endosomes prior to their isolation (Nielsen *et al.*, 2001). Following centrifugation, microtubule pellets were washed and lysed, and the rhodamine content measured in a fluorimeter to reveal the amount of bound early endosomes. Only very modest (maximum ±5% standard deviation of mean) variations were detected between triplicate samples and replica experiments (Pal *et al.*, 2006).

3.2. Materials and reagents

3.2.1. Spin-down binding assays

All buffers and stock solution should be prepared in demineralized water and sterile-filtered or autoclaved.

1. Early endosomes and cytosol prepared from HeLa cells, pulsed with rhodamine transferrin: refer to Nielsen *et al.* (2001) for detailed protocol. HeLa cell cytosol is prepared in KHMG (see following) that is identical with KEHM buffer in the original protocol except for glycerin replacing the ATP (ATP is added as part of the energy mix to the binding reaction, as discussed below). The protocol can be easily adapted to prepare cytosol with a candidate protein depleted by RNAi. Store early endosomes and cytosol aliquoted at –80°.
2. Candidate protein stocks in KHMG buffer. We prepared HAP40 as carboxyterminal-tagged GST-fusion protein from *Escherichia coli* lysates, and ditto for GDI and RN-tre (see section 3.3.3.2), but with the GST-tag

cleaved off. Preparations to obtain functional proteins must be optimized for each novel candidate. Store after aliquoting at –80°.

3. Affinity-purified candidate effector mix isolated from immobilized GTPγS-bound Rab5 (Rab5 eluate): refer to Christoforidis and Zerial (2000) for detailed protocol. Store aliquoted material at –80°.
4. Antibodies for functional blocks (e.g., against Htt from Chemicon, MAB 2166).
5. KCl stock, 1 *M* in water; autoclave and store at room temperature.
6. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) stock, 1 *M*; autoclave and store at room temperature.
7. $MgCl_2$ stock, 1 *M* in water; autoclave and store at room temperature.
8. Glycerol stock, 80% in water; autoclave and store at room temperature.
9. Creatine phosphate stock, 1 *M* in water; filter-sterilize and store aliquoted at –20°.
10. ATP stock, 1 *M* in water; filter-sterilize and store aliquoted at –20°.
11. GTP stock, 1 *M* in water; filter-sterilize and store aliquoted at –20°.
12. Imidazole, 1 *M* in water; filter-sterilize and store aliquoted at –20°.
13. KHMG buffer (must be sodium-free): 110 mM KCl from 1 *M* stock; 50 m*M* HEPES from 1 *M* stock, pH adjusted to 7.4 with KOH, 2 m*M* $MgCl_2$ from 1 *M* stock, 10% glycerol from 80% stock; store sterile at room temperature.
14. Energy mixture: 75 m*M* creatine phosphate from 1 *M* stock, 10 m*M* ATP from 1 *M* stock, 10 m*M* GTP from 1 *M* stock, and 20 m*M* $MgCl_2$ from 1 *M* stock, in KHMG buffer; store sterile and aliquoted at –20°.
15. Non-specific rabbit serum, heat-inactivated; filter-sterilize and store aliquoted at –20°.
16. Lysis buffer: KHMG buffer supplemented with 1% sodium desoxycholate; store sterile at room temperature.
17. 35% SIM buffer: 35% sucrose (v/v) solution, 40.3 g sucrose, 3 m*M* imidazole from 1 *M* stock, 1 m*M* $MgCl_2$ from 1 *M* stock in 100 ml water (prepare the night before).
18. Beckman Coulter Optima™ Max tabletop ultracentrifuge with TLA 100 rotor or similar suitable for 100,000 rcf.
19. Beckman 7 × 20-mm polycarbonate tubes #343775 for maximum 200 μl.
20. Perkin Elmer LS50B fluorimeter or similar with 100-μl sample cuvette (plastic or quartz glass).

3.2.2. Preparing taxol-stabilized tubulin

1. $MgCl_2$ stock, 1 *M*; see section 3.2.1.
2. GTP stock, 1 *M*; see section 3.2.1.
3. EGTA stock, 1 *M* in water, use acid or dipotassium salt if available, and adjust pH with KOH; filter-sterilize and store at room temperature.

4. BRB80 buffer (must be sodium-free): 80 m*M* piperazine-1,4-bis [2-ethanesulfonic acid] (PIPES), pH adjusted to 6.8 with KOH, 1 m*M* $MgCl_2$ from 1 *M* stock, 1 m*M* EGTA from 1 *M* stock; store sterile at room temperature.
5. Taxol stock, 10 m*M* in dimethylsulfoxide (DMSO); store 1-μl aliquots at –80°.
6. Purified, cycled, unlabeled tubulin (e.g., from Cytoskeleton Inc.) stock, 35 mg/ml in BRB80; store 10-μl aliquots at –80°.
7. For final ultracentrifugation, see section 3.2.1.

3.2.3. Preparing F-actin

1. Lyophilized G-actin (e.g., from Cytoskeleton Inc.).
2. Potassium iodide (KI), dry salt.
3. ATP stock, 1 *M*; see section 4.2.1.
4. DTT stock, 1 *M* in water, filter-sterilize, and store aliquoted at –20°.
5. $MgCl_2$ stock, 1 *M*; see section 4.2.1.
6. $CaCl_2$ stock, 1 *M* in water; autoclave and store at room temperature.
7. Tris-HCl stock, 1 *M* in water; filter-sterilize and store at room temperature.
8. Polymerization buffer: 2 m*M* Tris-HCl from 1 *M* stock, adjust pH to 8.0 with KOH, 0.5 m*M* ATP from 1 *M* stock, 0.5 m*M* $CaCl_2$ from 1 *M* stock, 0.2 m*M* DTT from 1 *M* stock; store sterile at –20°.
9. Stabilizing buffer: 50 m*M* Tris-HCl from 1 *M* stock, adjust pH to 8.0 with 1 *M* KOH, 1.2 *M* KI, freshly weighted dry salt, 3 m*M* $CaCl_2$ from 1 *M* stock; make fresh.
10. For final ultracentrifugation, see section 4.2.1.

3.3. Protocols for spin-down binding assays

3.3.1. Preparing microtubules

1. Prewarm TLA 100 rotor on top of a 37° water bath.
2. Prepare 1 ml of 1 m*M* GTP in BRB80 from 1 *M* stock, keep at room temperature. Immediately freeze GTP stock again.
3. Prepare 10 μ*M* taxol by diluting 1-μl aliquot of stock (10 m*M*) with 1 ml BRB80; keep at room temperature.
4. Gently thaw 10-μl stock aliquot of tubulin (35 mg/ml) at 4°; put on ice once thawed.
5. For polymerization, mix 20 μl of 1 m*M* GTP with tubulin aliquot, incubate for 20 min in 37° water bath.
6. For stabilization, add 170 μl of 10 μ*M* taxol, pipette up and down, and centrifuge for 5 min at 100,000 rcf in TLA100 rotor at 22°.

7. Carefully remove and discard the supernatant, resuspend the pellet in 50 μl of 10 μM taxol to obtain the final 7 μg/ml microtubule stock. The pellet should be 3 to 4 mm in diameter and of jelly-like appearance. Can be stored at room temperature for up to 1 week. Microtubules will depolymerize below 16°!

3.3.2. Preparing F-actin

1. Dissolve G-actin in polymerization buffer at 5 mg/ml; avoid bubbles. Proceed with 180-μl aliquot.
2. Trigger polymerization by adding KCl to final 100 mM from 1 M stock and $MgCl_2$ to final 2 mM from 1M stock. Mix and incubate for 1 h at room temperature.
3. Centrifuge in TLA 100 rotor at 100,000 rcf for 1 h at 4°.
4. Carefully remove and discard supernatant.
5. Soak pellet (F-actin) for 1 h in 100 μl stabilization buffer at 4°, and then gently resuspend by pipetting up and down.
6. Remove KI in stabilization buffer by centrifugation as described previously, and wash pellet three times in 200 μl polymerization buffer. Alternatively, dialyze against polymerization buffer overnight and centrifuge.
7. After final centrifugation, resuspend in 180 μl polymerization buffer to obtain the final 5 mg/ml F-actin stock. Can be stored at 4° for up to 1 month. Do not freeze!

3.3.3. Binding of early endosomes to microtubules or F-actin (spin-down assays)

1. Allow TLA 100 rotor to equilibrate to room temperature.
2. For each binding reaction, mix the following:
   - 15 μg prepared early endosomes (according to protein determination)
   - 5 μl energy mix
   - 1 μl nonspecific rabbit serum
   - Optional: candidate factor(s), such as HAP40-GST, 1 μM final concentration each
   - Optional: Rab5 eluate or similar at final 1 mg/ml
   - Optional: antibody against candidate, such as 1 μl undiluted Chemicon MAB 2166 against Htt
   - HeLa cell cytosol to final 1 mg/ml
3. Add KHMG buffer to final 40 μl. Incubate at room temperature for 20 min.
4. Add 16 μg microtubules or 10 μg F-actin from prepared stock, and further KHMG buffer to final 50 μl. Incubate at room temperature for 10 min.

5. Carefully pipette reaction mixture on top of a 100-μl layer of 35% SIM buffer in centrifugation tubes, and centrifuge at 22° for 20 min at 100,000 rcf.
6. Carefully remove supernatant and keep for analysis of unsedimented material (optional). Wash pellet twice in cytosol-free KHMG by centrifugation as described above.
7. Lyse pellet thoroughly in 150 μl lysis buffer.
8. Transfer to cuvette and place in fluorimeter. For rhodamine, measure at 550 nm excitation, 582 nm emission, and 10 nm slit widths.

3.3.3.1. Cytosol in binding reactions Rab5-dependent interactions of prepared early endosomes with microtubules (binding and motility) can be recapitulated *in vitro* without cytosol (Hoepfner *et al.*, 2005). However, when we tested early endosome binding to F-actin without cytosol, we observed only poor interactions with no effect of any added candidate protein. Only upon addition of 1 mg/ml cytosol, did we achieve appreciable basal binding levels under control conditions that could be modulated through the addition of candidates. Hence, the basal binding of early endosomes to F-actin required cytosolic factors. In addition, removal of active Rab5 from the prepared early endosomes with GDI or RN-tre (see section 3.3.3.2) did not reduce binding below control levels. We concluded that the basal binding was independent from Rab5, while the increase in binding through HAP40 and Htt clearly required active Rab5 on endosomal membranes (Pal *et al.*, 2006).

Collectively, to ensure consistent experimental conditions we had to assay binding of early endosomes to both types of filaments in the presence of cytosol.

3.3.3.2. Controls in binding reactions

- Autofluorescence: The assay should be performed with a reaction mixture as specified in section 3.3.3, but without early endosomes and filaments. The final fluorescence readout provides the basal autofluorescence.
- Sedimentation control: Omission of filaments in the reaction mixture must result in a final fluorescence readout indistinguishable from the autofluorescence; otherwise, the sucrose concentration is incorrect and/or the early endosome preparation used is heavily contaminated with other labeled membranous material that sediments without binding to filaments.
- Omission of early endosomes: Sedimentation in the absence of labeled early endosomes will reveal the extra contribution of sedimented filaments to the basal autofluorescence. In our hands, neither microtubules

nor F-actin led to a significant increase in basal autofluorescence (Pal *et al.*, 2006).

- ATP-dependency: The molecular nature of early endosome binding to cytoskeletal filaments is certainly complex and not completely understood. However, previous findings (Nielsen *et al.*, 1999) support our working model of functional Rab5 microdomains that recruit kinases (e.g., hVps34; see section 1.1) (Zerial and McBride, 2001) to produce the early endosome-specific lipid phosphoinositol-3-phosphate. Further downstream effectors mediating membrane–cytoskeleton interactions bind specifically to this lipid as shown for the early endosome kinesin motor KIF16B (Hoepfner *et al.*, 2005). KIF16B alone in the cytosol-free reaction was sufficient for binding of early endosomes to microtubules. Therefore, omission of ATP or its substitution by the nonhydrolysable adenylyl-imidodiphosphate (AMP-PNP) analog will block kinase activity and, thus, compromise the recruitment of downstream effectors; hence, the observed reduction in binding (Pal *et al.*, 2006). By testing whether the effect of a novel candidate on binding is ATP dependent, the investigator can gain first mechanistic insights.
- Rab5-dependency: In line with our working model portrayed above, the level of active Rab5 on early endosomal membranes should affect their binding to cytoskeletal fibers. Indeed, extracting active Rab5 from prepared early endosomes through addition of recombinant Rab-GDP-dissociation inhibitor (GDI) (Ullrich *et al.*, 1994), or RN-tre, a GTPase activating protein (GAP) for Rab5 (Lanzetti *et al.*, 2000), resulted in a decrease of binding to microtubules (Pal *et al.*, 2006). These controls have shown that the effect of Htt/HAP40 on the binding of early endosomes to F-actin is Rab5 dependent and, thus, confirmed Htt/HAP40 as downstream effector of this small GTPase. Moreover, evidencing Rab5-dependency of changes in binding raises confidence levels in ruling out nonspecific co-pelleting effects.
- Specificity of candidate proteins: To confirm that the effect of a novel candidate on binding is specific, we recommend adding the protein—for instance, as GST-tagged variant to the binding reaction, and setting up a control with an equimolar amount of GST protein that should have no effect on binding (Pal *et al.*, 2006). Au3

3.3.3.3. Data processing Since the readout of the fluorimeter is in arbitrary units, we first set up a dilution series for calibration with prepared early endosomes to express binding, such as in mass units for total endosomal proteins bound to filaments. In our hands, fluorescence emission correlated linearly with the concentration of prepared early endosomes in lysis buffer in the range from 0 to at least 35 μg/ml. The mean autofluorescence (see section 3.3.3.2.) should be used for background correction. A typical equation obtained by linear regression was $y = 4548.4x - 2653.1$, with

$R^2 = 0.9879$, where y is the emission in arbitrary units, and x the concentration of EE in protein mass units (micrograms per milliliter).

3.3.3.4. Complementary controls by western blotting

It is important to confirm that changes in fluorescence correspond specifically to changes in the amount of early endosomes bound to cytoskeletal filaments, and not of other membrane contaminants. Organelle specificity is ensured by restricting the internalization of fluorescent transferrin by HeLa cells to 5 min. Other subsequent compartments (i.e., late and recycling endosomes) are normally not entered by the label under this condition (Mellman, 1996). A more elegant way to ensure organelle specificity is to prepare early endosomes from a source expressing—for instance, GFP-tagged Rab5. We recommend confirming organelle specificity by Western blot analysis of obtained pellets. Any changes in fluorescence must correspond to changes of band intensities on blots probed for specific early endosome markers (Pal *et al.*, 2006). Conversely, probing the blots for nonearly endosome markers such as LAMP1 (for late endosomes/lysosomes) (Eskelinen *et al.*, 2003) and the Golgi marker GM130 (for Golgi) (Nakamura *et al.*, 1995) will probably reveal contaminants of early endosome preparations. By confirming that band intensities of nonearly endosome markers do not change throughout all experimental conditions, the investigator can elegantly document (1) organelle specificity and (2) rule out nonspecific co-pelleting of any membranous material in the dense cytoskeletal filament mesh (Pal *et al.*, 2006). The latter point is important to address because novel candidate proteins could lead to a bundling of filaments that will increase such nonspecific co-pelleting. In such a scenario, the investigator would misinterpret the increase of the fluorescence readout as increased binding affinity. Conversely, novel candidate factors might depolymerize filaments in the binding reaction, thus leading to less early endosomes co-pelleting. In such a scenario, the investigator would misinterpret the decrease of the fluorescence readout as reduced binding affinity. To rule out this possibility, we recommend probing blots for tubulin/actin to confirm equal amount of sedimented filaments throughout all experimental conditions.

We recommend the following antibodies for complementary Western blot analysis:

- Anti-EEA1: Cytostore, mouse monoclonal, catalog no. MAB-004; diluted 1:1000
- Anti-transferrin receptor: Invitrogen, mouse monoclonal, catalog no. 13–6800; diluted 1:1000
- Anti-LAMP1: BD Biosciences, mouse monoclonal, catalog no. 611042; diluted 1:1000

- Anti-GM130: BD Biosciences, mouse monoclonal, catalog no. 610823; diluted 1:1000
- Anti-β-tubulin: BD Biosciences, mouse monoclonal, catalog no. 556321, diluted 1:1000
- Anti-actin: Sigma Diagnostics, rabbit polyclonal, catalog no. A20660; diluted 1:200
- Anti-HAP40: Chemicon, rabbit polyclonal, catalog no. AB5872; diluted 1:200
- Anti-Htt: Chemicon, mouse monoclonal, catalog no. MAB2166; diluted 1:500

However, other validated antibodies should be equally suitable.

3.3.3.5. Critical parameters and assay optimization The ratio of early endosomes to filaments is critical in the assay: a high concentration of filaments in the binding reactions will increase nonspecific binding and co-pelleting that can obscure all specific changes in binding. Therefore, the investigator might only obtain nearly constant high basal fluorescence emission throughout all experimental conditions. Conversely, using very low concentrations of filaments will lead to a saturation of binding sites for early endosome that, again, will not allow revealing subtle changes in binding. Particularly the binding of early endosomes to F-actin requires preliminary titration series to establish the optimal ratio within a working window because these filaments tend to form fine, sticky meshes and bundles. Important parameters in this regard are the type of buffer, pH, salt concentration, and concentration of added cytosol. In our lab, 10μg F-actin and 15 μg early endosomes were optimal in the reaction mixture specified in section 3.3.3., but variations among preparations might require optimizing again.

4. *In Vitro* Motility assays

4.1. Introduction

Our original protocol (Nielsen *et al.*, 2001) allowed gaining first clues about the important role of Rab5 in early endosome motility along microtubules (Nielsen *et al.*, 1999). In brief, taxol-stabilized microtubules are perfused into a microscopy chamber and allowed to bind to the glass surface. Next, prepared early endosomes labeled with fluorescent rhodamine-conjugated transferrin are added with ATP, candidate proteins to be tested and antifade reagents. Binding to and motility events of early endosomes along microtubules are then imaged by fluorescence video microscopy.

Optimal assay conditions are on microtubule tracks evenly and loosely scattered across the field. The addition of cytosol, however, can lead to a bundling of microtubules and, hence, creation of suboptimal conditions that reduces the number of motility events and velocity, presumably by steric hindrance (our unpublished observations). Thus, we have sought to improve the assay to allow for (1) better motility, (2) a simplified setup of the microscopy perfusion chamber, and (3) omission of cytosol for a defined composition and elimination of microtubule bundling. We found that replacing the original BRB80 by KHMG buffer (see sections 3.2.1 and 3.2.2) in the assay allowed recording *in vitro* motility without cytosol, but only for plus-end motility while activity toward the minus-end of microtubules was lost. Apparently, a minimal motility machinery enriched in plus-end microtubule motors remains preserved on early endosome in the preparation. Under these experimental conditions, cytosol decreases *in vitro* motility in general, but is required to restore minus-end activity. After having confirmed that the inhibitory effect of Htt and HAP40 on early endosome motility along microtubules was similar with and without cytosol (our unpublished observations), we studied these candidates in the absence of cytosol to benefit from the improvements described above. However, we recommend testing for the requirement of cytosol for each novel candidate. Thus, cytosol is listed as an optional ingredient in the protocol provided here.

4.2. Material and reagents for *in vitro* motility

4.2.1. Preparing fluorescently labeled early endosomes

Refer to section 3.2.1.

4.2.2. Preparing microtubules

See section 3.2.2.

4.2.3. Video microscopes and data acquisition

See Nielsen *et al.* (2001).

4.2.4. Reconstituting early endosome motility along microtubules

1. Energy mix: see section 3.2.1.
2. Glucose stock, 1 *M* in water; autoclave and store sterile at room temperature.
3. $MgCl_2$ stock, 1 *M* stock in water; autoclave and store sterile at room temperature.
4. Glucose oxidase stock, 10 mg/ml in KHMG buffer; filter-sterilize and store aliquoted at –20°.
5. Catalase stock, 10 mg/ml in KHMG (see section 3.2.1) buffer; filter-sterilize and store aliquoted at –20°.

6. Antifade buffer: 10 μM taxol from 10 mM stock (one 1-μl aliquot) (see section e.2.2), 10 mM glucose from 1 M stock, 4 mM $MgCl_2$ from 1 M stock, 50 μg/ml glucose oxidase from 10 mg/ml stock, 50 μg/ml catalase from 10 mg/ml stock, 0.1% 2-mercaptoethanol in total 1 ml KHMG buffer (see section 3.2.1); make fresh.
7. Two times antifade buffer; ditto but with all ingredients at doubled concentration except of KHMG buffer (remains 1×).
8. Bovine hemoglobin, 30 mg/ml in KHMG; filter-sterilize and make fresh.
9. 10% nonspecific, heat-inactivated rabbit serum in antifade buffer; make fresh.
10. 10% nonspecific, heat-inactivated rabbit serum in 2× antifade buffer; make fresh.

4.2.5. Preparing the microscope perfusion chamber

1. Microscope slides (76 mm × 26 mm × 0.8/1 mm thick, Select Micro Slides, washed; Chance Propper Ltd., Warley, UK, catalog no. KTH 360).
2. Glass coverslips (18 mm × 18 mm, no.1, Clay Adams, Gold Seal, catalog no. 3305)
3. Standard office tape, adhesive on both sides (any manufacturer).

4.3. Protocols for *in vitro* motility assay

4.3.1. Preparing the microscope perfusion chamber

1. Place two strips of office tape in parallel about 1.5 cm apart on microscope slide. Place coverslip on top so that tape serves as spacer between glass layers. Perfusion can then be performed by capillary force. To fill the chamber, 5 μl should be sufficient; otherwise, vary space between tape strips accordingly. Au4
2. Perfuse with 5 μl of previously prepared microtubules in BRB80 buffer (see section 3.2.2) by adding the solution to one side of the chamber using an air-displacement micropipette. Allow nonspecific adherence of microtubules at room temperature over 5 min, and then proceed to section 4.3.2.

4.3.2. Reconstituting early endosome motility along microtubules

1. To set up reaction (10 μl in total), mix the following and incubate for 5 min at room temperature:
   - 1 μl of fluorescently labeled, prepared early endosomes (~5 mg/ml)
   - 1 μl energy mix
   - 1 μl candidate protein(s), optional (e.g., to final 10 μM for HAP40-GST or to final 1 mg/ml for Rab5 eluate)

- 1 μl antibody against candidate, optional (e.g., 1 μl undiluted Chemicon MAB 2166 against Htt)
- 1 μl HeLa cell cytosol to final 2 mg/ml, optional
- 1 μl bovine hemoglobin
- 4 μl of 10% nonspecific serum in 2× antifade buffer

Omission of optional ingredients should be compensated by equal volumes of water.

2. Meanwhile, perfuse chamber prepared with microtubules (see section 4.3.1) with 10 μl of 10% nonspecific serum in antifade buffer to block nonspecific binding sites and to remove BRB80 buffer. Perfuse by placing the new solution as a drop at one side of the chamber and aspirating 10 μl with a micropipette from the other side.
3. Finally, perfuse chamber with prepared reaction mix.
4. Place a drop of immersion oil on the glass coverslip, and visualize fluorescent early endosomes with appropriate filters on a time-lapse video microscope. Examples of early endosome motility on *in vitro* synthesized microtubules are shown in Pal *et al.* (2006) and Nielsen *et al.* (2001).

4.3.2.1. Controls

1. Candidate specificity: As for section 3.3.3.2, the tag part of fusion proteins should be added alone in a control reaction to test for candidate specificity.
2. Antibody specificity: Because the reaction contains an excess of nonspecific immunoglobulins (10% of nonspecific rabbit serum), it is unlikely that addition of insignificantly more antibodies against candidates can cause appreciable changes in motility through nonspecific interactions. However, we still recommend validating the use of any antibody against candidate proteins by testing other specific antibodies recognizing nonrelevant epitopes.
3. Validation of affinity-purified Rab effector mix (e.g., Rab5 eluate): Candidate proteins difficult to purify (e.g., Htt due to its large size and numerous interacting partners) can be added in mixture with other co-purified proteins. To address a particular candidate selectively, specific antibodies can be added to test for a rescue of any alteration in motility caused by, for example, the Rab5 eluate (as done for Htt with Chemicon MAB 2166) (Pal *et al.*, 2006). However, the complex nature of these purified effector mixtures calls for additional controls, that is, with eluate from Rab affinity columns preloaded with GDP instead of GTPγS (Pal *et al.*, 2006).

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REFERENCES

Altar, C. A., Cai, N., Bliven, T., Juhasz, M., Conner, J. M., Acheson, A. L., Lindsay, R. M., and Wiegand, S. J. (1997). Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* **389,** 856–860.

Apodaca, G. (2001). Endocytic traffic in polarized epithelial cells: Role of the actin and microtubule cytoskeleton. *Traffic* **2,** 149–159.

Block-Galarza, J., Chase, K. O., Sapp, E., Vaughn, K. T., Vallee, R. B., DiFiglia, M., and Aronin, N. (1997). Fast transport and retrograde movement of huntingtin and HAP 1 in axons. *Neuroreport* **8,** 2247–2251.

Bucci, C., Lütcke, A., Steele-Mortimer, O., Olkkonen, V. M., Dupree, P., Chiariello, M., Bruni, C. B., Simons, K., and Zerial, M. (1995). Co-operative regulation of endocytosis by three Rab5 isoforms. *FEBS Lett.* **366,** 65–71.

Christoforidis, S., and Zerial, M. (2000). Purification and identification of novel Rab effectors using affinity chromatography. *Methods* **20,** 403–410.

Conner, J. M., Lauterborn, J. C., Yan, Q., Gall, C. M., and Varon, S. (1997). Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. *J. Neurosci.* **17,** 2295–2313.

Conner, S. D., and Schmid, S. L. (2003). Regulated portals into the cell. *Nature* **422,** 37–44.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., Bucci, C., and Schiavo, G. (2006). Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. *Neuron* **52,** 293–305.

Delcroix, J. D., Valletta, J. S., Wu, C., Hunt, S. J., Kowal, A. S., and Mobley, W. C. (2003). NGF signaling in sensory neurons: Evidence that early endosomes carry NGF retrograde signals. *Neuron* **39,** 69–84.

Engelender, S., Sharp, A. H., Colomer, V., Tokito, M. K., Lanahan, A., Worley, P., Holzbaur, E. L., and Ross, C. A. (1997). Huntingtin-associated protein 1 (HAP1) interacts with the p150Glued subunit of dynactin. *Hum. Mol. Genet.* **6,** 2205–2212.

Eskelinen, E. L., Tanaka, Y., and Saftig, P. (2003). At the acidic edge: Emerging functions for lysosomal membrane proteins. *Trends Cell Biol.* **13,** 137–145.

Gasman, S., Kalaidzidis, Y., and Zerial, M. (2003). RhoD regulates endosome dynamics through Diaphanous-related formin and Src tyrosine kinase. *Nat. Cell Biol.* **5,** 195–204.

Gauthier, L. R., Charrin, B. C., Borrell-Pagès, M., Dompierre, J. P., Rangone, H., Cordelieres, F. P., De Mey, J., MacDonald, M. E., Lessmann, V., Humbert, S., and Saudou, F. (2004). Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell* **118,** 127–138.

Goehler, H., Lalowski, M., Stelzl, U., Waelter, S., Stroedicke, M., Worm, U., Droege, A., Lindenberg, K. S., Knoblich, M., Haenig, C., Herbst, M., Suopanki, J., *et al.* (2004). A protein interaction network links GIT1, an enhancer of huntingtin aggregation, to Huntington's disease. *Mol. Cell* **15,** 853–865.

Gross, S. P., Tuma, M. C., Deacon, S. W., Serpinskaya, A. S., Reilein, A. R., and Gelfand, V. I. (2002). Interactions and regulation of molecular motors in *Xenopus* melanophores. *J. Cell Biol.* **156,** 855–865.

Gruenberg, J., and Maxfield, F. R. (1995). Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol.* **7,** 552–563.

Gusella, J. F., and MacDonald, M. E. (2000). Molecular genetics: unmasking polyglutamine triggers in neurodegenerative disease. *Nat. Rev. Neurosci.* **1,** 109–115.

Harjes, P., and Wanker, E. E. (2003). The hunt for huntingtin function: Interaction partners tell many different stories. *Trends Biochem. Sci.* **28,** 425–433.

Hibbert, A. P., Kramer, B. M., Miller, F. D., and Kaplan, D. R. (2006). The localization, trafficking and retrograde transport of BDNF bound to p75NTR in sympathetic neurons. *Mol. Cell. Neurosci.* **32,** 387–402.

Hoepfner, S., Severin, F., Cabezas, A., Habermann, B., Runge, A., Gillooly, D., Stenmark, H., and Zerial, M. (2005). Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell* **121,** 437–450.

Lanzetti, L., Rybin, V., Malabarba, M. G., Christoforidis, S., Scita, G., Zerial, M., and DiFiore, P. P. (2000). The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5. *Nature* **408,** 374–377.

Lewin, G. R., and Barde, Y.-A. (1996). Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19,** 289–317.

Li, X. J., and Li, S. H. (2005). HAP1 and intracellular trafficking. *Trends Pharmacol. Sci.* **26,** 1–3.

Lindsay, R. M., Wiegand, S. J., Altar, C. A., and DiStefano, P. S. (1994). Neurotrophic factors: From molecule to man. *Trends Neurosci.* **17,** 182–190.

McPherson, P. S. (2002). The endocytic machinery at an interface with the actin cytoskeleton: a dynamic, hip intersection. *Trends Cell Biol.* **12,** 312–315.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* **12,** 575–625.

Miaczynska, M., Christoforidis, S., Giner, A., Shevchenko, A., Uttenweiler-Joseph, S., Habermann, B., Wilm, M., Parton, R. G., and Zerial, M. (2004). APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. *Cell* **116,** 445–456.

Morfini, G., Pigino, G., and Brady, S. T. (2005). Polyglutamine expansion diseases: Failing to deliver. *Trends Mol. Med.* **11,** 64–70.

Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E., and Warren, G. (1995). Characterization of a cis-Golgi matrix protein, GM130. *J. Cell Biol.* **131,** 1715–1726.

Nielsen, E., Severin, F., Backert, J. M., Hyman, A. A., and Zerial., M. (1999). Rab5 regulates motility of early endosomes on microtubules. *Nat. Cell Biol.* **1,** 376–382.

Nielsen, E., Severin, F., Hyman, A. A., and Zerial, M. (2001). *In vitro* reconstitution of endosome motility along microtubules. *Methods Mol. Biol.* **164,** 133–146.

Pal, A., Severin, F., Lommer, B., Shevchenko, A., and Zerial, M. (2006). Huntingtin-HAP40 complex is a novel Rab5 effector that regulates early endosome motility and is up-regulated in Huntington's disease. *J. Cell Biol.* **172,** 605–618.

Pelkmans, L., and Helenius, A. (2003). Insider information: What viruses tell us about endocytosis. *Curr. Opin. Cell Biol.* **15,** 414–422.

Peters, M. F., and Ross, C. A. (2001). Isolation of a 40-kDa huntingtin-associated protein. *J. Biol. Chem.* **276,** 3188–3194. Au5

Rong, J., McGuire, J. R., Fang, Z. H., Sheng, G., Shin, J. Y., Li, S. H., and Li, X. J. (2006). Regulation of intracellular trafficking of huntingtin-associated protein-1 is critical for TrkA protein levels and neurite outgrowth. *J. Neurosci.* **26,** 6019–6030.

Schnatwinkel, C., Christoforidis, S., Lindsay, M. R., Uttenweiler-Joseph, S., Wilm, M., Parton, R. G., and Zerial, M. (2004). The Rab5 effector Rabankyrin-5 regulates and coordinates different endocytic mechanisms. *PLoS Biol.* **2,** 1363–1380E261.

Singer-Krüger, B., Stenmark, H., Düsterhöft, A., Philippsen, P., Yoo, J.-S., Gallwitz, D., and Zerial, M. (1994). Role of three Rab5-like GTPases, Ypt51p, Ypt52p and Ypt53p, in the endocytic and vacuolar protein sorting pathway of yeast. *J. Cell Biol.* **125,** 283–298.

Slow, E. J., Graham, R. K., and Hayden, M. R. (2006). To be or not to be toxic: Aggregations in Huntington and Alzheimer disease. *Trends Genet.* **22,** 408–411.

Szebenyi, G., Morfini, G. A., Babcock, A., Gould, M., Selkoe, K., Stenoien, D. L., Young, M., Faber, P. W., MacDonald, M. E., McPhaul, M. J., and Brady, S. T. (2003). Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. *Neuron* **40,** 41–52.

Trushina, E., Singh, R. D., Dyer, R. B., Cao, S., Shah, V. H., Parton, R. G., Pagano, R. E., and McMurray, C. T. (2006). Mutant huntingtin inhibits clathrin-independent endocytosis and causes accumulation of cholesterol in vitro and in vivo. *Hum. Mol. Genet.* **15,** 3578–3591.

Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994). Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature* **368,** 157–160.

Walenta, J. H., Didier, A. J., Liu, X., and Kramer, H. (2001). The Golgi-associated hook3 protein is a member of a novel family of microtubule-binding proteins. *J. Cell Biol.* **152,** 923–934.

Zerial, M., and McBride, H. M. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* **2,** 107–117.

Zuccato, C., and Cattaneo, E. (2007). Role of brain-derived neurotrophic factor in Huntington's disease. *Prog. Neurobiol* Epub 2007 Feb 9 ahead of print.

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