Dictyostelium huntingtin controls chemotaxis and cytokinesis through the regulation of myosin II phosphorylation

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ABSTRACT Abnormalities in the huntingtin protein (Htt) are associated with Huntington’s disease. Despite its importance, the function of Htt is largely unknown. We show that Htt is required for normal chemotaxis and cytokinesis in Dictyostelium discoideum. Cells lacking Htt showed slower migration toward the chemoattractant cAMP and contained lower levels of cortical myosin II, which is likely due to defects in dephosphorylation of myosin II mediated by protein phosphatase 2A (PP2A). htt− cells also failed to maintain myosin II in the cortex of the cleavage furrow, generating unseparated daughter cells connected through a thin cytoplasmic bridge. Furthermore, similar to Dictyostelium htt− cells, siRNA-mediated knockdown of human HTT also decreased the PP2A activity in HeLa cells. Our data indicate that Htt regulates the phosphorylation status of myosin II during chemotaxis and cytokinesis through PP2A.

INTRODUCTION

Huntingtin (Htt) is a large, evolutionarily conserved protein with a polyglutamine stretch and multiple Huntington, Elongation Factor 3, the scaffold subunit A of protein phosphatase 2A (PP2A), TOR (HEAT)-repeats. Huntington’s disease, a progressive neurodegenerative disorder, is caused by expansion of the polyglutamine stretch in Htt (Ross, 2002; Harjes and Wanker, 2003; MacDonald et al., 2003; Ross, 2004; Gil and Rego, 2008; Caviston and Holzbaur, 2009). In addition, it has been suggested that the loss of Htt function contributes to the pathogenesis of Huntington’s disease (Harjes and Wanker, 2003; Caviston and Holzbaur, 2009). Htt is present primarily in the cytosol, and a fraction of Htt associates with organelles such as membrane vesicles, nuclei, and microtubules (Harjes and Wanker, 2003; Caviston and Holzbaur, 2009). Previous studies have also shown that Htt functions as a scaffolding protein in different cellular processes, such as intracellular signaling, membrane trafficking, apoptosis, and transcription (Harjes and Wanker, 2003; Caviston and Holzbaur, 2009). However, the exact function of Htt remains largely unknown. In this report, to determine the cellular function of Htt, we deleted the Htt gene in Dictyostelium discoideum, a social amoeba that has been used to study problems relevant to many human diseases (Williams et al., 2006; Annesley and Fisher, 2009). We found that the Dictyostelium homologue of Htt is required for cytokinesis and chemotaxis through regulation of the actin-based motor protein myosin II.

In Dictyostelium and mammalian cells, both cytokinesis and chemotaxis share much underlying molecular architecture and many mechanisms, as they require myosin II for reorganization and accumulation at the equator of a dividing cell or at the rear of a chemotaxing cell, respectively (Robinson and Spudich, 2004; Vicente-Manzanares et al., 2009; Pollard, 2010). During cytokinesis, myosin II assembles into bipolar thick filaments, accumulates in the cleavage furrow, and forms a contractile meshwork with the actin cytoskeleton to help drive cytokinesis (Uyeda and Nagasaki, 2004; Reichl et al., 2008). During chemotaxis, myosin II also assembles into filaments, and then becomes concentrated in the lateral and trailing cortices of chemotaxing cells. Myosin II is involved in the posterior contraction...
of cells, suppression of lateral pseudopodia, and disassembly of the actin network (Yumura and Uyeda, 2003; Bosgraaf and van Haastert, 2006); recent evidence also points toward myosin II inhibiting leading edge signaling (Lee et al., 2010).

The localization of myosin II is regulated by at least two mechanisms in Dictyostelium. First, the ability of myosin II to form bipolar thick filaments is controlled by heavy-chain phosphorylation by myosin heavy-chain kinases (MHCKs); this phosphorylation drives the disassembly of the myosin bipolar thick filaments (De la Roche et al., 2002; Bosgraaf and van Haastert, 2006). There are at least three MHCKs (A, B, and C) that have partially overlapping functions in myosin II phosphorylation and regulate its assembly and localization (Yumura et al., 2005). In contrast, PP2A dephosphorylates myosin II, promoting its assembly. The second step in localization is recruitment of myosin II bipolar thick filaments to the cortex, which is thought to require Gbpc and Rap1 (Bosgraaf and Van Haastert, 2002; Jeon et al., 2007). Myosin II localization changes in response to the chemoattractant cAMP (Yumura and Fukui, 1985; Levi et al., 2002), a process that must be highly regulated. We found that the localization of myosin II was altered in htt− cells: myosin II failed to accumulate in the cortex of cAMP-stimulated cells or in the cleavage furrow cortex during cytokinesis due to a reduction in the activity of PP2A. Our findings suggest that Htt regulates myosin II phosphorylation/dephosphorylation during chemotaxis and cytokinesis.

RESULTS

htt− cells are defective in normal development and chemotaxis

Like human Htt, Dictyostelium Htt (DDB0238473) has a polyglutamine stretch and HEAT repeats (Supplemental Figure S1). To investigate the function of Htt, we disrupted the Htt gene by homologous recombination in Dictyostelium cells (Figure S1A) and found that Htt is important for normal development in Dictyostelium. When starved, Dictyostelium cells spontaneously aggregate and differentiate to form fruiting bodies. htt− cells were delayed in aggregation compared with wild-type cells (Figure 1A), although the morphology and density of fruiting bodies were similar in wild-type and htt− cells (Figure S2). Furthermore, individual htt− cells formed small plaques on bacterial lawns (Figure 1, B and C). Exogenous expression of Htt fused to green fluorescent protein (GFP) showed even distribution in the cytoplasm of Dictyostelium (Figure S3, B and C), similar to human Htt (Gil and Rego, 2008), and restored normal growth in htt− cells (Figure 1, B and C).

Because aggregation depends on chemotaxis toward cAMP during development (Swaney et al., 2010), we asked whether Htt was required for chemotaxis. In this assay, we developed htt− cells for 5 and 7 h and compared their chemotaxis to wild-type cells developed for 5 h. The longer time period for htt− was necessary, as the expression of developmentally regulated genes, such as the cAMP receptor cAR1 and adenylylate cyclase, is delayed in htt− cells (Figure 2A). Wild-type cells developed for 5 h and htt− cells developed for 7 h had similar amounts of cAR1 and adenylylate cyclase (Figure 2A). Consistent with these observations, the htt− cells developed for 7 h polarized normally (Figure 2, B and F) and displayed normal motility speed (Figure 2C); however, these cells showed decreases in chemotaxis speed (Figure 2D) and in chemotaxis index, which indicates the accuracy of chemotaxis (Figure 2E). These results indicate that Htt is required for normal chemotaxis.

PtdInsP3 is one of the signaling molecules that regulates actin polymerization during chemotaxis (Parent et al., 1998; Iijima and Devreotes, 2002) and is transiently generated in the plasma membrane upon cAMP stimulation. Production of PtdInsP3 is mediated by recruitment of PI3-kinase (PI3K), which phosphorylates PtdIns(4,5)P2 at the plasma membrane (Funamoto et al., 2002; Iijima and Devreotes, 2002; Iijima et al., 2004). We observed the production of PtdInsP3 in living cells by expressing GFP fused to the PH domain of CRAC, which specifically binds to PtdInsP3. GFP-PHcrac was recruited to the plasma membrane with similar kinetics in wild-type and htt− cells when stimulated with cAMP, suggesting that PtdInsP3 was produced normally in htt− cells (Figure S4A). Likewise, PI3K-GFP translocated to the plasma membrane similarly in wild-type and htt− cells (Figure S4B). These results suggested that PtdInsP3 signaling is normal in htt− cells.
FIGURE 2: Htt is required for normal chemotaxis toward cAMP and the cortical localization of myosin II. (A) Immunoblot of the cAMP receptor, cAR1, and adenylate cyclase A (ACA) in wild-type and htt− cells differentiated for 5 and 7 h. The protein-transferred membrane was stained with Coomassie Brilliant Blue (CBB stain) and shown as loading control. (B) Chemotaxis to a micropipette filled with 1 μM cAMP. The movements of developed wild-type (5 h) and htt− (7 h) cells were recorded. Images were taken at the indicated time points. (C) Motility speed was determined by measuring the position of the centroid every 30 s for a period of 10 min. Results were statistically analyzed using Student’s t test (*, p < 0.05; **, p < 0.01) in (C)–(F). (D) Chemotaxis speed was calculated as the distance toward the micropipette divided by the elapsed time (10 min). (E) Chemotaxis index was defined as the distance moved in the direction of the gradient divided by the total distance moved for 30-s intervals during a period of 10 min. (F) Roundness was determined by calculating the ratio of short axis (A_s) and long axis (A_l) of cells (A_s/A_l). Values are mean ± SEM (n = 40). Amounts of myosin II (G) and actin (H) in the Triton X-100–insoluble fraction of wild-type and htt− cells in response to 10 μM cAMP. Values are mean ± SEM (n = 3). (I) Wild-type and htt− cells carrying GFP-myosin II, which is expressed on top of native myosin II, were metabolically labeled with [32P]orthophosphate. Whole-cell lysates were subjected to immunoprecipitation using anti-GFP antibodies, and then analyzed by immunoblotting using anti-GFP antibodies and autoradiography. Band intensity was quantified relative to wild-type levels. Values are mean ± SEM (n = 3).
Myosin II in the cell cortex is decreased in htt\(^{-}\) cells

To determine whether Htt is required for myosin II assembly and localization, we determined the amount of myosin II in the Triton X-100–insoluble cytoskeletal fraction, which represents levels of assembled myosin II in the cortex (Steimle et al., 2001b). Consistent with previous observations, the amount of myosin II transiently decreased in the Triton X-100–insoluble fraction after cAMP stimulation in wild-type cells (Figure 2G). In contrast, htt\(^{-}\) cells showed levels of myosin II in this fraction to be \(\sim\)50% of the control and showed no changes after cAMP stimulation (Figure 2G), although the total amount of myosin II was not affected. As a control, the amount of actin in the same fraction, which represents the amount of filamentous cortical actin, was transiently increased by \(\sim\)2.5-fold in both wild-type and htt\(^{-}\) cells (Figure 2H). Therefore reduced myosin II levels were not simply due to reduced amounts of filamentous actin. We then metabolically labeled wild-type and htt\(^{-}\) cells expressing GFP-myosin II using \(^{32}\)Porthophosphate and performed immunoprecipitation using anti-GFP antibodies. Phosphorylation levels of myosin II were significantly increased by 2.1-fold in htt\(^{-}\) cells (Figure 2I). Thus htt\(^{-}\) cells have more phosphorylated myosin II, either due to enhanced kinase or reduced phosphatase activity.

htt\(^{-}\) cells are defective at later stages of cytokinesis

Myosin II is required for cytokinesis when Dictyostelium cells are grown on a nonadhesive substrate or in suspension culture (Robinson, 2001; Uyeda and Nagasaki, 2004). We examined the time course of cytokinesis on adhesive coverglass and less-adhesive silicon-coated coverglass. We identified cells in telophase based on cell morphology with the short-to-long axis ratio of 0.2 (Robinson and Spudich, 2004; Uyeda and Nagasaki, 2004). Wild-type and htt\(^{-}\) cells both completed cytokinesis similarly on the uncoated coverglass (Figure 3, A, C, and D). In contrast, on the silicon-coated substrate, almost three times as many htt\(^{-}\) cells as wild-type cells failed to complete cytokinesis (Figure 3, B and C). Interestingly, wild-type cells that failed to complete cytokinesis regressed the cleavage furrow, while htt\(^{-}\) cells that were unable to finish cytokinesis remained dumbbell-shaped and none regressed the cleavage furrow (Figure 3C). Of those cells that completed cytokinesis on silicon-coated coverglass, htt\(^{-}\) cells took longer to divide than wild-type cells (Figure 3D) due to a 40%
longer second phase, when the axial ratio decreased from 0.1 to zero (Figure 3E). Thus htt− cells appeared to stall cytokinesis just before completion.

**htt− cells form grape-like clusters in suspension culture**
Consistent with the cytokinesis defects on nonadhesive substrates, htt− cells were severely defective in cell growth in shaken suspension cultures (Figure 4A), but not on adhesive substrates (Figure 4B). In suspension culture, htt− cells formed clusters of cells that we named grape-like clusters (GLCs; Figure 4, C and D). Exogenous expression of Htt fused to GFP restored normal growth to htt− cells, demonstrating that GLC formation results from a lack of Htt (Figure 4A). When GLCs were placed on coverglass, htt− cells migrated away from each other and cytoplasmic connections between htt− cells were clearly observed (Figure 4E). The intercellular connections were stretched and eventually ruptured. Furthermore, when we expressed GFP or red fluorescent protein (RFP) in the cytoplasm of htt− cells and cultured them in shaking cultures for 3 d, individual GLCs contained only GFP or RFP (Figure 4F). These results suggest that htt− cells remained attached through the cytoplasmic connection in GLCs. The maintenance of GLCs appears to be independent of the rearrangement of the actin cytoskeleton, as GLCs were maintained when treated for 1 h with 5 μM latrunculin A, which blocks actin polymerization (unpublished data).

**htt− cells fail to maintain myosin II in the cleavage furrow**
To analyze the localization of myosin II, we expressed GFP-myosin II in cells and examined the ratio of GFP signals between the cleavage furrow and polar cytoplasm in dividing cells. We identified cells in telophase based on cell morphology with the short-to-long axis ratio of 0.2 and observed them until cytokinesis was completed (Robinson and Spudich, 2004; Uyeda and Nagasaki, 2004). In wild-type cells, GFP-myosin II showed ~80% stronger intensity at the cleavage furrow until the completion of cytokinesis compared with the polar cytoplasm (Figure 5, A and B), consistent with previous reports (Nagasaki et al., 2002; Robinson et al., 2002). In contrast, although myosin II initially accumulated in the cleavage furrow in cells with the short-to-long axis ratio of 0.2, it was gradually lost as cytokinesis progressed, and the short-to-long axis ratio became 0.1 and zero in htt− cells (Figure 5, A and B). These data indicate that Htt helps maintain myosin II at the cleavage furrow cortex in late stages of cytokinesis.

Dephosphorylation of myosin II stimulates assembly and recruits myosin II filaments to the cortex (Murphy and Egelhoff, 1999; Yumura et al., 2005; Bosgraaf and van Haastert, 2006). To test whether dephosphorylated myosin II could be maintained at the cleavage furrow in htt− cells, we expressed a mutant form of myosin II in which three phosphorylation sites were substituted with alanine. We found that mutant myosin II continued to accumulate at the cleavage furrow in both wild-type and htt− cells (Figure 5, C and D). Our data also suggest that dephosphorylated, assembled myosin II can be recruited to the cortex in htt− cells.

**The loss of myosin II is epistatic to the loss of Htt**

If Htt is required for completion of cytokinesis through myosin II, loss of myosin II would be epistatic to Htt loss. Indeed, hpRNA-mediated knockdown of myosin II generated multilamellated giant cells in wild-type and htt− cells (Figure 5, F and G). Therefore myosin II is required during the early stages of contractility in order to form the GLCs, and Htt is only required for maintenance of myosin II at the cleavage furrow cortex during the late stages of cytokinesis completion.

Interestingly, overexpression of MHCK-C led to the formation of large multinucleated cells in htt− cells, but not in wild-type cells, whereas overexpression of MHCK-A resulted in multinucleated cells in both wild-type and htt− cells (Figures 5F, S5, and S6). These results suggest that the loss of Htt renders cells more sensitive to excess phosphorylation of myosin II by MHCK-C.

**The activity of PP2A is reduced in htt− cells**
To understand the mechanism underlying the altered phosphorylation status of myosin II in htt− cells, we examined the amount (Figure 6A), localization (Figure 6B), CAMP-stimulated membrane recruitment (Figure S7), activity (Figure 6C), and autoprophosphorylation (Figure S8) of MHCKs. We found that MHCKs were not affected in htt− cells. In contrast, the activity of PP2A was significantly reduced in htt− cells (Figure 7B), while its amount and localization were not altered (Figure 7, A and C).

To test whether Htt also facilitates the activity of PP2A in human cells, we knocked down the expression of Htt using siRNA in HeLa cells (Figure 7D). As with Dictyostelium htt− cells, knockdown of Htt resulted in a reduction in PP2A activity without affecting total amounts of PP2A (Figure 7, D and E). We also examined whether overexpression of Htt affected PP2A activity in HeLa cells and found that overexpression of either wild-type (HTT-Q17) or mutant (HTT-Q138) Htt (Rubinsztein and Carmichael, 2003) had no effect on the phosphatase activity (Figure 7, F and G). We speculate that levels of endogenous Htt are already near saturation for the regulation of PP2A activity, therefore increasing wild-type Htt levels may not change the phosphatase activity. Concerning mutant Htt overexpression, we initially thought this could show a dominant negative effect; however, the data suggest mutant Htt does not interfere with endogenous Htt function in PP2A regulation.

**DISCUSSION**
Our study demonstrates that Htt is required for cytokinesis and chemotaxis through the regulation of myosin II phosphorylation/dephosphorylation. As cytokinesis proceeds, htt− cells gradually lose myosin II from the cortex of the cleavage furrow and fail to complete cell division, producing unseparated daughter cells connected by a thin cytoplasmic bridge. Similarly, myosin II levels in the cortex are reduced during chemotaxis in htt− cells, leading to decreased migration speed. Recruitment of myosin II to the cortex requires dephosphorylation of myosin II by the phosphatase PP2A. htt− cells displayed a reduction in PP2A activity and contained increased amounts of phosphorylated myosin II. Furthermore, our data also indicate that Htt is required for assembly of myosin II, but not for translocation to the cortex, as a phosphorylation-defective myosin II can be maintained in the cleavage furrow of htt− cells.

PP2A consists of three subunits: catalytic, scaffolding, and regulatory. The scaffolding subunit contains the HEAT domain, which binds to the catalytic subunit. Because Htt contains HEAT domains, it is tempting to speculate that Htt may directly bind to the catalytic subunit through this domain and function as a scaffold protein, modulating the phosphatase activity or its specificity toward myosin II. In addition, Htt may regulate the assembly of PP2A. Although we found no evidence of a physical interaction between Htt and PP2A in communoprecipitation studies (Y. Wang and M. Iijima, unpublished observations), it remains possible that Htt weakly and/or transiently interacts with PP2A to regulate its enzymatic activity, specificity, or assembly. Moreover, the Dictyostelium genome contains at least six PP2A regulatory subunits (Table S1), suggesting that PP2A likely has many functions and many
substrates in *Dictyostelium* in addition to myosin II. A recent study showed that a PP2A regulatory subunit, phr2αB, is required for myosin II assembly (Rai and Egelhoff, 2011). It would be interesting to test whether and how interactions of catalytic subunits with different regulatory subunits modulate the target specificity, phosphatase activity, and localization.
FIGURE 5: Htt is required for myosin II localization during cytokinesis. Wild-type and htt− cells expressing GFP fused to wild-type (A) and mutant (C) myosin II were observed during cytokinesis using time-lapse fluorescence microscopy; in (C), three phosphorylation sites (Thr₁₈₂₃, Thr₁₈₃₃, and Thr₂₀₂₉) are mutated. (B and D) Quantification of GFP-myosin II localization. The fluorescence intensity of the cleavage furrow (Iₚ) and polar cytoplasm (Iᵢ) was measured, and the ratio of Iₚ/Iᵢ is presented (E). (F) Myosin II is required for the formation of GLCs in htt− cells. Wild-type and htt− cells were subjected to shRNA-mediated knockdown of myosin II and grown in shaking culture for 3 d (mhcAhp/WT and mhcAhp/htt−). Wild-type and htt− cells were transfected with MHCK-A and -C. Cells were stained with DAPI to visualize the nuclei. (G) Immunoblotting of whole-cell lysates using antibodies for myosin II heavy chain and actin.
We found that knockdown of Htt also reduced the activity of PP2A in HeLa cells, suggesting that the role of Htt in PP2A activity is evolutionarily conserved. Although the assembly of mammalian myosin II is also regulated by heavy-chain phosphorylation (Vicente-Manzanares et al., 2009), it remains to be determined whether PP2A regulates myosin II during cytokinesis or chemotaxis in mammalian cells. Nonetheless, since the loss of normal function of Htt could contribute at least partially to the pathogenesis of Huntington’s disease (Harjes and Wanker, 2003; Caviston and Holzbaur, 2009), it would be interesting to further investigate the phosphorylation status of PP2A substrates in this disease. In addition, a recent study has shown that a PP2A-B55α complex regulates mitotic exit, most likely by dephosphorylating Cdk1 substrates in human cells (Schmitz et al. 2010). RNAi knockdown of B55α, a regulatory subunit of this PP2A complex, delays spindle disassembly, chromosome decondensation, and Golgi assembly. These findings and our current study suggest that PP2A plays a key regulatory role in the mitotic phase.

Dictyostelium cells can divide on surfaces or in suspension culture. However, suspension growth is highly restrictive for cells with defects in the cytokinetic machinery. As a result, many cytokinetic
novel role for myosin II in the completion of cytokinesis, in addition to its known role in the initiation and ingestion of the cleavage furrow. The failure of myosin II maintenance at later stages of cytokinesis likely blocks abscission in htt− cells, leading to the formation of GLCs.

MATERIALS AND METHODS

Cell culture, strains, and plasmids

All Dictyostelium discoideum cell lines were cultured in HL5 medium at 22°C. htt− cells were generated by homologous recombination. The Htt targeting vector was constructed as follows: DNA fragments containing 5′ untranslated region (UTR) of htt was amplified from genomic DNA using primers 5′-TCAGCATCACAATAGATAACAGGG-3′ and 5′-ATATACCTTCTACATTTCTGATGTC-3′. A DNA fragment containing the 3′UTR region was amplified using primers 5′-CATTGGATAGACAGAAATTCTCACG-3′ and 5′-CTTTATTGAAATAAAATCCTGATGC-3′. These two DNA fragments and the blasticidin-S-resistance cassette from pLPBLPv2 (M. Landree, Johns Hopkins University, Baltimore, MD) were cloned into pBluescript (Stratagene, Agilent, Santa Clara, CA) to generate the targeting vector. The Htt targeting vector was linearized with NotI and introduced into growing AX2 cells by electroporation (Gaudet et al., 2007). Transformants were selected in HL5 medium containing 5 μg/ml blasticidin (ICN Biomedicals, Costa Mesa, CA) on plastic dishes. After 5 d, cells were harvested and plated on SM agar plates with Klebsiella aerogenes. Randomly selected clones were screened for gene disruption by PCR and Southern blot analysis.

To clone the Htt gene, full-length Htt was amplified by PCR from three separate regions of the Htt gene of strain AX2 genomic DNA with primers based on the predicted DNA sequences of Htt in dictyBase (http://dictybase.org). To combine the three fragments, the internal restriction sites BamHI, ScaI, NciI, and XhoI were used. The full-length Htt gene was cloned into the BglII and XhoI site of GFP-PJK1 (Iijima and Devreotes, 2002). The resulting htt-GFP construct was confirmed by DNA sequencing.

Antibodies

We used antibodies against actin (C-11; Santa Cruz Biotechnology, Santa Cruz, CA), human Htt (#2773; Cell Signaling, Beverly, MA), PP2A-C subunit (HeLa cells: #2038; Cell Signaling; Dictyostelium cells: 1D6; Millipore, Billerica, MA), and PP2A-A subunit (HeLa cells: PP2A A subunit [81G5] rabbit monoclonal antibody #2041; Cell Signaling). Antibodies against Dictyostelium PP2A-A subunit mutants show quantitative defects under both growth conditions, but only fail at cytokinesis, becoming multinucleated only in suspension culture (Robinson, 2001; Uyeda and Nagasaki 2004). Therefore htt− cells, like mhcA null cells, complete cytokinesis successfully on surfaces by drawing on adhesion properties but fail in suspension culture. However, htt− cells formed GLCs, unlike mhcA null cells, which become enlarged and multinucleated. In addition, there are several Dictyostelium mutants that are defective in cytokinesis, none of which have been reported to form GLCs. Therefore Htt likely acts at an unidentified step during cytokinesis. Our study also suggests a

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were described previously (Murphy and Egelhoff, 1999), as were antibodies against MHCK-A and -C (Yumura et al., 2005).

Developmental assay
To assess developmental phenotypes, cells grown exponentially were washed twice in development buffer (DB: 10 mM phosphate buffer, 2 mM MgSO4, 0.2 mM CaCl2) and plated on 1% nonnutrient DB agar at a cell density of 5 × 10^6 cells/cm^2. To determine plaque size, cells were plated as individual clones with K. aerogenes on SM agar plates (10.0 g/l glucose, 10.0 g/l protease peptone, 10.0 g/l yeast extract, 1.9 g/l KH2PO4, 1.0 g/l K2HPO4, 0.5 g/l MgSO4, 15.0 g/l agar) and cultured at 22°C for 5 d.

Myosin II phosphorylation
Metabolic labeling with [32P]orthophosphate was performed, as previously described (Berlot et al., 1985). Approximately 8 × 10^7 aggregation-competent, caffeine-treated cells were incubated with [32P]orthophosphate at 0.5 mCi/ml for 40 min. Cell suspensions containing ~5 × 10^7 cells were mixed with an equal volume of ice-cold 2x lysis buffer (200 mM NaF, 300 mM NaCl, 2% NP-40, 2 mM EDTA, 2 mM dithiothreitol [DTT], 2 mM ATP, 10 mM phosphate buffer, pH 6.8, protease inhibitor cocktail tablet, and phosphatase inhibitor cocktail tablet [Roche Applied Science, Indianapolis, IN]) and centrifuged for 20 min at 45,000 rpm in a TLA-55 (Beckman Coulter, Brea, CA). The supernatants were incubated with GFP antibody (Santa Cruz Biotechnology) and IgG-Staphylococcus aureus (Sigma-Aldrich, St. Louis, MO) for 3 h at 4°C. The samples were centrifuged and the pellets were washed three times with 1x lysis buffer. The pellets were resuspended in SDS–PAGE sample buffer and analyzed by immunoblotting using anti-GFP antibodies and autoradiography. For immunoblotting, proteins were visualized by fluorophores conjugated to secondary antibodies (ZyMax goat anti–rabbit IgG [H + L] Cy5 conjugate; Invitrogen, Carlsbad, CA) or an enhanced chemiluminescence detection system (Thermo Scientific, Lafayette, CO) and analyzed using a PharoFX Plus molecular imager (Bio-Rad, Hercules, CA), Quantity One (Bio-Rad), and Photoshop software (Adobe, San Jose, CA).

Measuring myosin II and actin in the cortex
Cells were developed and pretreated with caffeine, as previously described (Iijima and Devreotes, 2002). To obtain the Triton X-100–insoluble fraction, 5 × 10^6 cells were harvested and lysed in Triton X-100 buffer (1% Triton X-100, 10 mM KCl, 10 mM imidazole, 10 mM EGTA, 50 μg/ml NaN3; Steimle et al., 2001b). After mixing by vortex, samples were kept on ice for 10 min, and then further incubated at room temperature for 10 min. The samples were spun at 8,000 × g for 4 min. The pellet fractions were washed once with Triton X-100 buffer and then dissolved in 2x SDS–PAGE sample buffer. Proteins were resolved by SDS–PAGE and visualized by Coomassie Brilliant Blue staining. Myosin II and actin were quantified using Image J software (http://rsweb.nih.gov/ji/download.html).

Chemotaxis assay
Cells grown in plastic dishes were washed twice with DB buffer, resuspended at 2 × 10^7 cells/ml, and shaken for 1 h before being induced to differentiate with 100 nM CAMP pulses at 6-min intervals for 4 h (Iijima and Devreotes, 2002). Chemotactic movements of cells to a micropipette containing CAMP were performed as previously described (Parent et al., 1998). Differentiated cells were plated on a chambered coverglass (Lab-Tek; Nunc, Thermo Scientific) and allowed to adhere to the surface. A micropipette filled with 1 μM cAMP was positioned, and images of moving cells were recorded. Image J software was used to collect and process data. The motility speed was determined by measuring the position of the centroid every 30 s for a period of 10 min. The chemotaxis speed was calculated as the distance toward the micropipette divided by the elapsed time (10 min). The chemotaxis index was defined as the distance moved in the direction of the gradient divided by the total distance moved for 30-s intervals averaged during a period of 10 min. Roundness was determined by calculating the ratio of short axis (A1) and long axis (A2) of cells (A1/A2) (Loovers et al., 2006).

Microscopy
Phase-contrast images of living cells in chemotaxis assays were obtained using an Olympus CKX41 inverted microscope equipped with a 10×/0.25 objective connected to a Scion CFW-1308M digital camera (Frederick, MD) that was operated with the Image J software. Differential interference contrast microscopy (DIC) and epifluorescence images of DAPI-stained cells were obtained on an Olympus IX81 inverted microscope equipped with a 40×/1.3 oil objective connected to an EM-CCD camera (C9100; Hamamatsu, Japan) and Slidebook software (3i, Denver, CO).

To observe cytokinesis on surfaces, cells were placed in Lab-Tek II chambers (Nunc, Thermo Scientific). To make the surface less adhesive, Lab-Tek II chambers were coated with Sigmacote (Sigma-Aldrich) according to the manufacturer’s instructions. Once the cells had settled, the medium was replaced with DB-2-(N-morpholino)ethane sulfonic acid (DB-MES: 20 mM MES, pH 6.5, 2 mM MgSO4, 0.2 mM CaCl2). All data were analyzed with Image J software.

To observe GFP-myosin II, an Olympus IX81 inverted microscope equipped with a 40×/1.3 oil objective connected to an EM-CCD camera (C9100; Hamamatsu, Japan) and Slidebook software (3i) was used. For quantification of GFP-myosin II distribution, the fluorescent signal intensities of the cleavage furrow (lc) and the polar cytoplasm (lp) were measured and the lc/lp ratio was calculated (Robinson and Spudich, 2000).

MHCK activity assay
Wild-type and htt+ cells were transfected with plasmids GFP-fused to MHCKs and transformants were selected in the presence of 10 µg/ml G418. The GFP fusion proteins were immunoprecipitated from 107 cells using 20 μl of packed GFP-Trap beads (Allele Biotech, San Diego, CA) following the manufacturer’s protocol, except that 0.5% Triton X-100 was used in place of 0.5% NP40. For quantification, purified kinases were subjected to SDS–PAGE with a series of known amounts of bovine serum albumin (BSA) on the same gel. After Coomassie Brilliant Blue staining, the protein bands were quantified by scanning densitometry, and the concentration of each purified fusion protein was determined by comparing the densitometry values for the purified kinases with those of the BSA standards in the same gel. Immunoprecipitated MHCK-A, -B, and -C (100 nM) were assayed for kinase activity toward MH-1 peptide substrate (50 μM) as has been described in detail previously (Steimle et al., 2001a; Egelhoff et al., 2005). Briefly, all kinase assays were performed at 25°C in 10 mM TES, pH 7.0, 2 mM MgCl2, 0.5 mM DTT, and 0.5 mM [γ-32P]ATP (250–400 Ci/mmol). The peptide sequence of the MH-1 substrate (RKKF-GESEKTKKEFL-amide) was synthesized by New England Peptide (Gärdner, MA) and contains the sequence corresponding to the mapped MHCK-A target site in the myosin II tail at residue 2029 (underlined in peptide sequence above). Kinase reactions...
were stopped by blotting a fraction of the reaction mixture onto P-81 filter paper (Whatman, Piscataway, NJ) and then processing the filter for quantification via scintillation counting.

**PP2A activity assay**

PP2A activity was measured by release of phosphates from phosphopeptides (K-R-pT-I-R-R) using a Malachite Green assay (PP2A immunoprecipitation phosphatase assay kit; Millipore) as previously described (Lee et al., 2008). The activity was normalized to cell numbers.

**Transfection of HeLa cells**

HeLa cells (2 × 10^5 cells/well) were transfected in six-well plates with 20 nM Silencer Select Pre-designed Htt siRNA (siRNA ID: S6490; Ambion, Austin, TX) using 4 μl siPORT NeoFX (Ambion) per well. The medium was replaced with fresh culture medium (DMEM supplemented with fetal bovine serum [FBS]) at 24 h after transfection. Nontargeting siRNA (AM4635; Ambion) was used as a negative control. Lipofectamine 2000 (Invitrogen) was used to transfect HeLa cells with wild-type Htt (pTre2Hyg:3xFLAG:HD138Q full length) and mutant Htt (pTre2Hyg:3xFLAG:HD138Q full length; Rubinsztein and Carmichael, 2003) plasmids according to the manufacturer’s instructions. Cells were lysed at 72 h after transfection to measure the PP2A activity.

**Statistical analysis**

All values are means ± SEM. Results were statistically analyzed using Student’s t-test (*, p < 0.05; **, p < 0.01).

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