A Huntingtin-Mediated Fast Stress Response Halting Endosomal Trafficking is Defective in Huntington’s Disease

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Abstract

Cellular stress is a normal part of the aging process and is especially relevant in neurodegenerative disease. Canonical stress responses, such as the heat shock response, activate following exposure to stress, and restore proteostasis through the action of isomerases and chaperones within the cytosol. Through live cell imaging, we demonstrate involvement of the Huntington's disease (HD) protein, huntingtin, in a rapid cell stress response that lies temporally upstream of canonical stress responses. This response is characterized by formation of distinct cytosolic puncta, and reversible localization of huntingtin to early endosomes. Formation of these puncta, which we have termed huntingtin stress bodies (HSBs), is associated with arrest of early-to-recycling and early-to-late endosomal trafficking. The critical domains for this response have been mapped to two regions of huntingtin flanking the polyglutamine tract, and we observe polyglutamine-expanded huntingtin expressing cells to be defective in their ability to recover from this stress response. We propose that HSB formation rapidly diverts high ATP use from vesicular trafficking during stress, thus mobilizing canonical stress responses without relying on increased energy metabolism, and that restoration from this response is defective in HD.
Huntingtin is a 350 kDa, 3144-residue protein involved in a variety of cellular functions including transcriptional regulation, mitotic spindle orientation, and vesicular trafficking through energy-dependent molecular machinery(1-8). Initially identified as the protein product of the \textit{HTT} gene implicated in Huntington’s disease (HD)(9, 10), huntingtin is essential for development(4), and lowered huntingtin protein levels have been shown to sensitize cells to stress through specific restructuring of the endoplasmic reticulum (ER)(11). Structurally, huntingtin is composed largely of helix-turn-helix HEAT repeat motifs, characteristic of scaffolding proteins(12). Additionally, huntingtin has an amino-terminal alpha-helical domain, termed N17, that has been shown to modulate the protein’s intracellular localization, toxicity and function(13, 14), as well as tethering huntingtin directly to the ER outer membrane and ER-derived vesicles. In HD, a CAG triplet repeat expansion in excess of 37 in the first exon of the coding region of the \textit{HTT} gene leads to a polyglutamine tract expansion in huntingtin, and consequently, pathology.

We have previously established that huntingtin is involved in the cell stress response, as it translocates from the ER to the nucleus in a stress-dependent manner(14). More recently, we have also demonstrated that this localization can be modulated by a family of small molecules called kinase inhibitors, which may hold therapeutic benefit in HD(15). Upon induction of cell stress, huntingtin can also localize to nuclear cofilin-actin rods, similar to those seen in the cell cytosol in Alzheimer’s disease(16, 17). Cofilin-actin rods function to transiently halt actin remodeling and thus increase available ATP during stress(18). Mutant huntingtin is defective in its ability to participate in this response, forming persistent rods that are unable to recapitulate actin dynamics(16). Moreover, an aberrant ATP/ADP ratio has been found in HD mouse and cell culture models, as well as HD brains(19). HD models have also been noted to have chronic ER stress via the unfolded protein response (UPR)(20).

Here, we build on our previous work and further investigate the temporal role of huntingtin in the cell stress response. By visualizing huntingtin in live cells under heat shock stress, we observe a very rapid, dynamic cell stress response involving reversible accumulation of the protein at early endosomes. This localization is
characterized by the formation of distinct cytosolic puncta, which we have termed huntingtin stress bodies (HSBs), and is associated with an arrest in early-to-late and early-to-recycling endosome fusion. In the context of HD, we demonstrate that cells expressing mutant huntingtin display a persistent HSB phenotype, requiring a longer time period to recover from this stress response when compared to cells expressing wild-type huntingtin.

We hypothesize that huntingtin has a critical function as a very early stress response protein, acting to arrest the energy-intensive process of endosomal trafficking, in addition to actin remodeling, to free pools of ATP for use within the cell during stress. HSB formation may therefore provide the critical energy required immediately for canonical stress responses, without relying on increased metabolism to produce ATP.

Results

Huntingtin is involved in a rapid cell stress response

To observe huntingtin biology under stress conditions, we performed immunofluorescence on wild-type (STHdhQ7/Q7) mouse striatal-derived cells and cells derived from a knock-in mouse model of HD (STHdhQ111/Q111). Cells were either kept at 33°C (steady state), or challenged with heat shock stress for 10 minutes at 42.5°C. Cells kept at steady state showed diffuse staining of huntingtin (Figure 1A, images A and C), while stressed cells formed distinct cytosolic puncta (Figure 1A images B and D), which we have termed huntingtin stress bodies (HSBs).

The dynamics of HSB formation were analyzed in STHdhQ7/Q7 cells transfected with a construct encoding the first 586 residues of huntingtin with a wild-type polyglutamine tract length of 17 repeats, fused at its carboxyl-terminus to eYFP (1-586 Q17-eYFP). Live cell imaging revealed that HSB formation was rapid, occurring within 30 seconds of induction of stress, and that HSBs are initially dynamic, but stationary following formation (Figure 1C, Supplementary Video 1). We challenged STHdhQ7/Q7 cells transfected with huntingtin 1-586 Q17-eYFP with cold shock, ATP depletion, and reactive oxygen stress (ROS; H2O2) to determine whether HSB formation is specific to heat shock. In all cases, we observed that cells formed HSBs, albeit on different time scales – requiring 60 minutes to form HSBs in response to ATP depletion and reactive oxygen stress, and 120 minutes in response to cold shock (Figure 1B). Subsequent work was done using heat shock stress as it
effectively recapitulates a variety of stress pathways, decreases cellular ATP levels, and results in increased calcium signaling from the ER\(^ {21, 22}\).

To ensure that HSB formation was not simply an artifact of immortalized, transformed ST\(Hdh\) cells, we replicated our experiments in differentiated ST\(Hdh^{Q7/Q7}\) cells, as well as wild-type primary human fibroblasts, HEK 293 cells (Supplementary Figure 1A), and mouse primary cortical neurons (Figure 1D). In all cases, we observed that cells reproducibly formed HSBs in response to stress, suggesting that this stress response is universal.

**Huntingtin stress body formation occurs upstream of canonical cell stress responses**

To determine the temporal dynamics of the HSB response in comparison to canonical cell stress responses, such as the heat shock response (HSR), we performed immunofluorescence against heat shock factor 1 (HSF1) in HeLa cells transfected with our 1-586 Q17-eYFP construct at steady state and following exposure to varying degrees of heat shock stress. Formation of nuclear HSF1 granules in response to stress was used as an indicator of activation of the HSR\(^ {23}\). As shown in Figure 1E, HSB formation was observed following only 10 minutes of heat shock stress, while formation of HSF1 granules required that cells be challenged with at least 45 minutes of heat shock stress. This therefore suggests that HSB formation occurs upstream of the HSR as it precedes HSF1 activation.

**Huntingtin stress bodies are distinct from stress granules and processing bodies**

To determine whether HSBs are distinct from other canonical stress structures, such as processing bodies, and stress granules, we co-transfected ST\(Hdh^{Q7/Q7}\) cells with 1-586 Q17-eYFP, a construct encoding DCP1A, a decapping enzyme found in processing bodies, fused to mRFP (DCP1A-mRFP), and a construct encoding TIA1, a protein involved in stress granule formation, fused to CFP (TIA1-CFP)\(^ {24}\). Following challenge with one hour of heat shock stress, we did not observe any co-localization between HSBs and DCP1A, or HSBs and TIA1 (Supplementary Figure 2C), indicating that these structures are distinct from HSBs.
N17 and huntingtin 81-171 are required for HSB formation

We next sought to determine which domain(s) of huntingtin are required for HSB formation. We transfected STHdhQ7/Q7 cells with constructs encoding different fragments of huntingtin fused to a carboxyl-terminal eYFP fluorophore, and following challenge with heat shock stress, quantified the number of cells with puncta. As smaller huntingtin fragments are prone to aggregation, we performed live cell imaging on cells expressing the various constructs, in parallel, to conclusively determine whether the puncta being observed were HSBs or huntingtin aggregates. We noted that the 1-171 and 1-586 fragments (and larger) formed HSBs most efficiently, with 1-117 forming HSBs at a lower frequency (Figure 2A, and 2B, and Supplementary Videos 1, 4, and 5). Cells expressing the N17 domain alone, or 1-81 (huntingtin exon1) did not form HSBs (Figure 2A, and 2B, Supplementary Videos 2 and 3). As cells transfected with a huntingtin fragment smaller than 1-586 displayed punctate protein localization in the absence of stress, all subsequent work was performed using the 1-586 fragment.

To further delineate the role of different huntingtin domains in HSB formation, we generated constructs encoding deletions of N17 and other critical regions, in the 1-586 context, fused at their carboxyl-terminus to eYFP. We then transfected these constructs into STThdhQ7/Q7 cells and observed whether cells formed HSBs in response to heat shock stress. We noted that deletion of N17 (Δ2-13) completely abrogated HSB formation, as did deletion of exon1 (Figure 2C and 2D). Deletion of the polyproline region flanking the polyglutamine tract (ΔpolyP), however, did not affect HSB formation efficiency (Figure 2C, and 2D).

N17 structure influences HSB formation

We have previously shown that the N17 domain of huntingtin is a highly structured amphipathic alpha-helix that functions as a membrane association domain(14), and a CRM1-dependent nuclear export signal (NES)(25). Furthermore, we have also demonstrated that the phosphorylation status of two serine residues (S13 and S16) within this region can modulate huntingtin localization through induction of structural changes(15). N17 has also been shown by solution and solid-state NMR to associate directly with the outer ER lipid.
To build on our previous work(14, 15) and results indicating that N17 is required for HSB formation (Figure 2C, and 2D), we further examined its role in this stress response. We generated constructs encoding various N17 mutations in the 1-586 context fused at their carboxyl-terminus to eYFP, transfected them into STHdhQ7/Q7 cells and quantified the number of cells that formed puncta in response to stress. We observed that cells expressing the methionine to proline (M8P) mutant construct, which results in disruption of the secondary structure of the domain(14), formed HSBs significantly less frequently than cells transfected with the wild-type construct (Figure 2E). Cells expressing the aspartic acid to alanine, (E5A) mutant construct, which increases huntingtin’s affinity for membranes(14), displayed an opposite effect, with 100% of cells forming puncta in response to stress across all trials (Figure 2E). Simulating post-translational modification of S13 and S16, or T3 through phospho-mimetic (S13E S16E, T3D, respectively) or phospho-resistant (S13A S16A, T3A, respectively) mutations did not significantly affect puncta formation (Figure 2E). Similarly, a leucine to alanine (L4A) mutation, which decreases the ability of huntingtin to associate with membranes(14), did not significantly affect HSB formation, but did reduce HSB formation efficiency (Figure 2E). To further investigate the potential for N17 phosphorylation in modulating HSB formation, we treated STHdhQ7/Q7 cells transfected with 1-586 Q17-eYFP with either BMS 345541 or DMAT, compounds we have previously shown to increase and decrease N17 phosphorylation, respectively(15) for 12 hours prior to induction of stress, and observed no effect on HSB formation dynamics (Supplementary Figure 2A). Thus, we conclude that N17 secondary structure is critical to HSB formation, while phosphorylation is not.

Huntingtin localizes to early endosomes in response to cell stress

Motivated by data demonstrating that modulating huntingtin’s affinity for membranes via N17 structure can affect HSB formation, and literature suggesting that huntingtin is closely involved in intracellular vesicular trafficking(5-8), we sought to examine whether HSBs co-localize with endosomes. STHdhQ7/Q7 cells expressing huntingtin 1-586 Q17-eYFP following challenge with heat shock stress were immunostained for early (Rab5C), late (Rab7), and recycling (Rab11) endosomal markers(27). Imaging revealed that huntingtin co-localizes primarily with Rab5C following stress, and does not co-localize with Rab7 or Rab11 (Figure 3A).
We next determined whether other proteins involved in vesicular trafficking are present at HSBs. Through immunofluorescence, we assayed STHdh^{Q7/Q7} cells transfected with huntingtin 1-586 Q17-eYFP, following HSB formation, for the presence of clathrin, dynamin1, huntingtin-associated protein 1 (HAP1), and through co-expression, huntingtin-associated protein 40 (HAP40) (Figure 3B). Clathrin, a protein responsible for forming meshwork-like coats on inbound vesicles(28), was not found to co-localize with HSBs (Figure 3B). Similarly, HAP1, which is involved in trafficking of late endosomes(29), did not co-localize with HSBs (Figure 3B). In contrast, we noted that dynamin1, which functions to sever vesicles at the plasma membrane and aids in vesicular trafficking(30), and HAP40, a protein postulated to be a Rab5 effector(31), both co-localized with HSBs (Figure 3B).

**HSB formation results in arrest of early-to-recycling and early-to-late endosome fusion**

As HSB formation requires huntingtin to localize to early endosomes, and we have previously shown huntingtin to inhibit high-energy processes in response to stress(16), we postulated that HSBs function to arrest energy-dependent vesicular trafficking processes.

Early endosomes serve as a sorting center for inbound cargo, branching into the recycling and late endosomal pathways, which subsequently lead to the plasma membrane, and lysosome, respectively(27). To analyze trafficking along the early-to-recycling pathway, we incubated STHdh^{Q7/Q7} cells transfected with huntingtin 1-586 Q17-eYFP with fluorescently labeled transferrin, a plasma glycoprotein that traffics from early endosomes to recycling endosomes(32-34). Following treatment with transferrin, cells were either kept at steady state, or challenged with heat shock stress. Subsequently, we performed immunofluorescence against either Rab5C or Rab11 to visualize the early and recycling endosomal compartments, respectively. In cells kept at steady state, transferrin trafficked normally into recycling endosomes – as shown by extensive localization to the Rab11-positive compartment (Figure 4A). In cells challenged with heat shock stress, however, we observed a block in transferrin trafficking, with co-localization being observed between HSBs, transferrin, and Rab5C (Figure 4B), but not transferrin and Rab11. This suggests that HSB formation resulted in sequestration of transferrin in the Rab5C-positive, early endosome compartment, preventing its progression into the Rab11-positive, recycling endosome compartment.
To analyze the effects of HSB formation on early-to-late endosome fusion, we replicated our trafficking assays using epidermal growth factor (EGF), which traffics from early endosomes to late endosomes(35), and performed immunofluorescence against Rab7. In cells kept at steady state, we noted, through co-localization with Rab7, that EGF transitioned from the early endosome to the late endosome (Figure 4C). In cells challenged with heat shock stress, however, we observed that EGF co-localized only with HSBs, and Rab5C, but not with Rab7 (Figure 4D), suggesting that HSB formation results in arrest of early-to-late endosome trafficking, mirroring our transferrin trafficking observations.

**Mutant huntingtin can form HSBs and arrest endocytic trafficking**

Next, we determined whether polyglutamine-expanded, mutant huntingtin could form HSBs and arrest endosomal trafficking in a similar manner. To determine whether mutant huntingtin differs from wild-type huntingtin in its ability to form HSBs, we transfected STHdh\textsuperscript{Q7/Q7} cells with constructs encoding huntingtin 1-586 in the context of different polyglutamine tract lengths, fused at their carboxyl-terminus to eYFP. We then quantified the number of cells with puncta following heat shock stress. Image analysis revealed no difference in HSB formation frequency across cells transfected with a wild-type huntingtin fragment (Q3 and Q17) when compared to cells transfected with polyglutamine-expanded huntingtin (Q138) (Supplementary Figure 2B).

Following this, we replicated both our transferrin and EGF trafficking assays in STHdh\textsuperscript{Q7/Q7} cells transfected with a construct encoding huntingtin 1-586 with an expanded polyglutamine tract of 138, fused at its carboxyl-terminus to eYFP (1-586 Q138-eYFP) to discern whether mutant huntingtin HSBs could arrest endocytic trafficking. Image analysis revealed that mutant huntingtin is able to arrest early-to-late and early-to-recycling endosome fusion in a manner similar to the wild-type protein (Figure 5). Thus, we conclude that HSB formation functions to arrest early-to-late and early-to-recycling endosome fusion processes in response to cell stress.

**Expression of mutant huntingtin results in defective recovery from HSB formation**

We next examined whether HSB formation is a transient event, and whether mutant huntingtin is altered in its ability to participate in this stress response. We transfected STHdh\textsuperscript{Q7/Q7} cells with huntingtin 1-586 Q17-
eYFP and following challenge with heat shock stress, imaged cells live at 33°C. Imaging revealed that recovery from HSB formation commences between one and three hours post-stress, with huntingtin localization becoming gradually diffuse over time (Supplementary Video 6). To determine whether mutant huntingtin expression alters the ability of cells to recover from HSB formation, we replicated our live cell imaging with \( STHdh^{Q7/Q7} \) cells transfected with huntingtin 1-586 Q138-eYFP. We observed that recovery from stress took longer in mutant huntingtin expressing cells, and in some cases, cells did not recover from stress, instead forming large aggregates prior to cell death (Supplementary Video 7). To quantify this difference, we transfected \( STHdh^{Q7/Q7} \) cells with either wild-type (1-586 Q17-eYFP) or mutant (1-586 Q17-eYFP) huntingtin and allowed them to recover for either three hours, or 24 hours at 33°C, post heat shock stress. We subsequently quantified the number of cells with puncta and noted that at the 3 hour mark, almost all of the cells expressing wild-type huntingtin had recovered, showing diffuse huntingtin localization, while most of the cells expressing mutant huntingtin had punctate protein localization (Figure 6A, and 6B). No significant differences were observed in cells expressing wild-type versus mutant huntingtin at the 24 hour mark, with both groups of cells having diffuse huntingtin localization. This therefore suggests that mutant huntingtin expression results in defective recovery from HSB formation.

**Discussion**

The cell stress response is traditionally viewed as a long-term event involving the action of various chaperones and isomerases(36, 37). These proteins correct the structure of peptides denatured as a result of stress, and facilitate clearance and sequestration of toxic species(38). While all of these processes require ATP(39), the source of this energy remains unclear. Increased transcription and available energy is noted in ER stress responses, but for some stresses, the ER must respond very quickly or the cell will die, leaving minimal time for canonical signals to boost ATP production. It is thus likely that this ATP is sourced from early stress responses, which function to modulate or arrest high-energy activities within the cell and rapidly increase available ATP, without relying on the time required for upregulation of cellular metabolism.
Here, we present one such early stress response involving huntingtin protein. We demonstrate that huntingtin rapidly localizes to early endosomes in response to stress, forming distinct puncta that we have termed HSBs. HSB formation occurs within 30 seconds of a stress event, while canonical stress responses, such as the HSR and the UPR, and processes involved in translational arrest, such as stress granules and processing bodies, require minutes to engage and even longer to exert their effects(23, 24, 40). The HSR, for instance, is initiated by the heat shock protein-promoting transcription factor, HSF1, which activates as late as 45 minutes following stress(23), and signal transduction in the UPR may take even longer(40). HSBs are positive for dynamin1, Rab5C, and HAP40, a Rab5 effector(31), but not clathrin or HAP1. This is consistent with our marker data, which shows that huntingtin localizes exclusively to early endosomes during stress, and not late, nor recycling endosomes. The absence of clathrin from HSBs can be attributed to the nature of clathrin-mediated endocytosis, whereby the clathrin coat is lost prior to maturation of an early endosome.

Formation of HSBs is associated with arrest of early-to-recycling and early-to-late endosomal trafficking. We postulate that this occurs in order to rectify an imbalance in cellular bioenergetics. Endosomal trafficking requires a large pool of energy as the Rab proteins that denote different endosomal compartments function as small, membrane-bound, Ras family monomeric GTPases(27). As HSB formation is associated with an arrest in endosomal trafficking, it is likely that this stress response substantially increases the levels of ATP available to the cell during stress. We propose that this energy is subsequently used by the cell to fuel longer-term stress pathways, such as the HSR, which lie temporally downstream of HSB formation. This is congruent with our previous work demonstrating that huntingtin is required for nuclear cofilin-actin rod formation(16), a stress response that arrests actin treadmilling in order to increase available ATP during stress(18).

Cell stress is also of great importance in the context of neurodegeneration. As the brain ages, metabolic stresses, such as reactive oxygen species (ROS), increase due to decreased mitochondrial efficiency(41), thereby placing emphasis on stress response pathways. Medium spiny neurons, the neuronal population most prominently affected in HD, are especially susceptible to these stresses as they require optimal cytoskeletal and vesicular trafficking dynamics(42). We demonstrate that mutant huntingtin expression results in a defective recovery process following HSB formation, with cells expressing mutant protein displaying a persistent HSB
phenotype. This suggests that in HD, cells may not be able to relieve this huntingtin-mediated stress response, resulting in prolonged arrest of endocytic trafficking, neuronal dysfunction, and eventually, cell death. This is congruent with previous studies that show that aberrant vesicular trafficking in the context of HD can lead to improper transport of nutrients, such as brain-derived neurotrophic factor (BDNF), leading to insufficient nourishment, and cell death(8, 43). This hypothesis is also consistent with the presence of huntingtin on moving vesicles and direct scaffolding of GAPDH for the production of ATP by glycolysis for fast axonal transport(7). Therefore, a defective HSB response may explain the late age-onset and specific neurodegeneration observed in HD.

Huntingtin 1-171 is required for optimal HSB formation, while huntingtin 1-117 is able to form HSBs in limited cases. This suggests that residues located within 117-171 of the protein play a key role in HSB formation dynamics. It has been shown that residues 128-138 of huntingtin can promote protein aggregation(44). Hence it is plausible that the absence of this region may limit the protein’s ability to nucleate to form HSBs.

HSB formation dynamics were also observed to be independent of microtubules and the actin cytoskeleton, which were disrupted using nocodazole, and latrunculin A, respectively (Supplementary Figure 3). As HSB formation is associated with an arrest in endosomal trafficking, it may simply be that huntingtin is sequestering vesicles from the cytoskeletal machinery involved in transport, thereby eliminating the need for optimal cytoskeletal dynamics.

The requirement of N17 for HSB formation further underscores criticality of this domain in modulating huntingtin function. Structurally, N17 is an amphipathic alpha-helix that is capable of inserting into membranes, normally tethering huntingtin by direct lipid association to the outer leaflet of the ER(26, 45, 46). Our data indicate that disrupting the domain’s secondary structure reduces the cell’s ability to form HSBs in response to stress. This suggests that the membrane-association properties of N17 are critical to HSB formation as an intact structure is required for interaction with biological membranes. The requirement of distal regions of huntingtin, in addition to N17, for HSB formation are also congruent with our recent work highlighting the spatial
orientation of N17 in relation to downstream regions of the protein, and the potential for intramolecular communication(47).

In summary, we have uncovered a novel, very early cell stress response mediated by huntingtin. We propose that this response functions to arrest endocytic trafficking in response to stress in order to increase available ATP for use by downstream stress pathways (Figure 6C). Future work will be directed at investigating the potential role of huntingtin at other high-energy sites within the cell to understand the exact mechanism of normal huntingtin function in cell stress.

Materials and Methods

Plasmid construction and molecular cloning

Plasmids encoding the N17, 1-81 Q17, 1-117 Q17, 1-171 Q17, 1-586 Q3, 1-586 Q17, 1-586 Q138, 1-586 Q17 Δ2-13, 1-586 M8P Q17, 1-586 S13A S16A Q17, 1-586 S13E S16E Q17, and 1-586 Q17 ΔpolyP fragments of huntingtin, all fused to eYFP at their C-terminus, as well as the mCh-6G-HAP40 construct, were cloned as described previously(14-16, 48). Site-directed mutagenesis was performed using inverse PCR and subsequent gel purification using the Qiagen QIAEX II kit (as per manufacturer’s instructions), phosphorylation and ligation. All constructs were sent to Mobix Lab at McMaster University for sequencing analysis prior to use in experiments.

Tissue culture and transfection

STHdhQ7/Q7 STHdhQ111/Q111 cells (a kind gift from M. E. MacDonald, Massachusetts General Hospital) derived from the murine striatum were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS; Sigma) at 33°C with 5% CO₂ in an air-jacketed incubator and were kept under clonal temperature selection using Geneticin G418 (Life Technologies) as described previously(49). HEK 293 cells (ATCC) were cultured in alpha-minimum essential medium (α-MEM) supplemented with 10% FBS at 37°C with 5% CO₂ in an air-jacketed incubator. HeLa cells (a kind gift from
Jon Draper, McMaster Stem Cell and Cancer Research Institute), were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS; Sigma) at 37°C with 5% CO₂ in an air-jacketed incubator. 24-hours prior to transfection, cells were split from 10cm dishes of 75-80% confluence and seeded into 35mm glass-bottom tissue culture dishes (Sarstedt) or Delta T Dishes (Bioptechs Inc.) using Trypsin-EDTA Solution (0.05% trypsin, 0.02% EDTA; Life Technologies). Seeded cells were transfected with 3µg of plasmid DNA using 4µL of TurboFect Transfection Reagent (Fermentas). Human wild-type (GM02149) and HD (GM01061) fibroblasts were obtained from Coriell Cell Repositories and cultured in Minimum Essential Medium (MEM; Sigma) supplemented with 15% FBS (Sigma) and 2mM L-Glutamine (Sigma) at 37°C with 5% CO₂ in an air-jacketed incubator. Cells were split into Delta T Dishes (Bioptechs Inc.) or 35mm glass-bottom tissue culture dishes (Sarstedt) as described above. 3µg of plasmid DNA was nucleofected into cells using a 4D Nucleofector Type-X Electroporator (Lonza). Primary cortical neurons cultured from E16 embryonic mouse brains (a kind gift from Karun Singh, McMaster Stem Cell and Cancer Research Institute), were grown in Neurobasal media (Life Technologies), supplemented with B27, P15 and Q at 37°C with 5% CO₂ in an air-jacketed incubator. Cells were cultured directly into 35mm glass-bottom tissue culture dishes (Sarstedt), and were transfected with 3µg of plasmid DNA using Lipofectamine LTX (Life Technologies).

**Differentiation of striatal progenitor cells**

$STHdh^{Q7/Q7}$ cells were seeded into 35mm glass-bottom tissue culture dishes and transfected with constructs of interest as described above. 12 hours following transfection, medium was replaced with serum-free DMEM (Life Technologies) supplemented with a differentiation cocktail (0.65nM L-Dopamine (Sigma), 50µM Forskolin (Sigma), 10ng/µL alphaFibroblast Growth Factor (α-FGF; Sigma), and 250µM 3-isobutyl-1-methylxanthine (IBMX; Sigma)). Cells were left at the growth conditions described above for 30 hours to allow for differentiation to occur.

**Heat shock stress challenge and cell fixation**

Cells were cultured in 35mm glass-bottom tissue culture dishes and transfected as described earlier. Following a 24-hour expression period, dishes were wrapped with parafilm and placed in a pre-warmed water
bath at 42.5°C for 10 minutes to heat shock cells. Cells were fixed immediately following heat shock using 4% paraformaldehyde (PFA; Sigma) in phosphate-buffered saline (PBS) for 20 minutes at room temperature. Cells were left in PBS for imaging.

**ATP depletion, cold shock, and reactive oxygen stress induction**

ATP depletion was induced by incubating cells in pyruvate-free DMEM (Life Technologies) supplemented with 0.25mM NaN₃ (Sigma) and 0.15mM 2-deoxyglucose (Sigma) at 33°C for 60 minutes to disrupt glycolysis. Cold shock stress was induced by placing cells at 4°C for 120 minutes. Reactive oxygen stress was induced by incubating cells with 400μM H₂O₂ (Sigma) in serum-free DMEM (Life Technologies) at 33°C for 60 minutes. Prior to induction of stress, cells were cultured and transfected as described previously and were permitted to express protein for 24 hours.

**Immunofluorescence**

*Paraformaldehyde/Triton X-100 Method*

Cells were fixed in 4% PFA (Sigma) in PBS for 20 minutes at room temperature and subsequently washed three times with PBS in 1-minute intervals. Cells were then permeabilized with 0.5% Triton X-100 (BioShop) and 2% FBS (Sigma) in PBS for 15 minutes at 4°C. Following permeabilization, cells were blocked for two hours in 30-minute intervals with 2% FBS (Sigma) in PBS. Primary antibody anti-clathrin (mouse monoclonal, Abcam 2731, 1/50), anti-dyamin1 (mouse monoclonal, Abcam 13251, 1/50), anti-huntingtin associated protein 1 (HAP1; goat polyclonal, Santa Cruz Biotechnology N18, 1/50), anti-huntingtin (mouse monoclonal, Millipore mAb2166, 1/100), anti-Rab11 (mouse monoclonal, Millipore 05-583/clone 47, 1/50), anti-Rab5C (rabbit polyclonal, Abcam 74854, 1/50), anti-Rab7 (rabbit polyclonal, Abcam 74906, 1/50), anti-HSF1 (rat monoclonal, Abcam 61382, 1/100), and anti-β-tubulin (mouse monoclonal, University of Iowa Developmental Studies Hybridoma Bank E7, 1/100) were applied in antibody dilution solution (1% FBS (Sigma), 0.02% Tween-20 (Sigma) in PBS) and incubated overnight at 4°C. Following overnight incubation, the primary antibody was aspirated and cells were blocked for 30 minutes in blocker solution (2% FBS (Sigma) in PBS) in 10-minute intervals. Cells were probed with secondary antibodies (Molecular Probes/Life
Technologies) conjugated to either Alexa594 (1/500) or Cy5 (1/350) dye for one hour at room temperature in antibody dilution solution (1% FBS (Sigma), 0.02% Tween-20 (Sigma) in PBS). Following one hour, the secondary antibody was aspirated, and cells were washed for 40 minutes with PBS at room temperature in 10-minute intervals. Cells were left in PBS for imaging.

**Methanol Method**

Cells were fixed-permeabilized in ice cold methanol for 12 minutes at -20°C. Following fixation-permeabilization, cells were washed in PBS for 20 minutes at room temperature in 10-minute intervals. Immunofluorescence was then performed as described above.

**Transferrin uptake assay**

Cells were cultured in 35mm glass-bottom tissue culture dishes and transfected as described above. Following a 24-hour expression period, cells were placed in serum-free DMEM (Life Technologies) for 60 minutes at the growth conditions described previously to remove any existing transferrin. Following this, medium was replaced with serum-free medium supplemented with 50μg/mL of human transferrin conjugated to Alexa594 dye (Molecular Probes/Life Technologies). Cells were then either immediately heat shocked or left at steady state in the growth conditions described previously. Following transferrin uptake and/or stress, cells were fixed and imaged immediately, or prepared for immunofluorescence analysis as described above.

**EGF uptake assay**

Cells were cultured in 35mm glass-bottom tissue culture dishes and transfected as described above. Following a 24-hour expression period, cells were placed in serum-free DMEM (Life Technologies) for 60 minutes at the growth conditions described above to allow for degradation of existing EGF. Following this, medium was replaced with a solution of 3μg/mL EGF labeled with TexasRed dye (Life Technologies), and 0.1% BSA (New England BioLabs) in serum-free DMEM. Cells were then either immediately heat shocked or left at steady state in the growth conditions described previously. Following EGF uptake and/or stress, cells were fixed and imaged immediately, or prepared for immunofluorescence analysis as described above.
**HSB recovery assay**

Cells were cultured in 35mm glass-bottom tissue culture dishes and transfected as described previously. Following a 24-hour expression period, cells were heat shocked as described above and placed back in the incubator at 33°C. Cells were permitted to recover from stress for either 3 hours or 24 hours and were subsequently fixed as described above.

**Nocodazole and latrunculin A treatment**

Cells were cultured in 35mm glass-bottom tissue culture dishes and transfected as described previously. Following a 12-hour expression period, cells were treated with 50ng/mL, 75ng/mL, or 100ng/mL nocodazole (Sigma) diluted in culture medium, for 12 hours. Cells treated with latrunculin A were allowed to express protein for 20 hours prior to being treated with 100nM, 500nM, or 1μM latrunculin A (Sigma) diluted in culture medium, for 1 hour. Following treatments, cells were challenged with heat shock stress, fixed, and imaged as described above.

**Microscopy**

Following a 24-hour expression period, cells transfected with protein(s) of interest were imaged live. Imaging was done using a Nikon Eclipse Ti inverted widefield epifluorescence microscope using either a 60X oil immersion N.A. 1.4 plan apochromat objective, or a 100X oil immersion N.A. 1.4 plan apochromat objective (Nikon, Japan). Images were acquired using a Hamamatsu ORCA Flash 4.0 digital camera (Hamamatsu Photonics, Japan). NIS Elements Advanced Research 4.1 was used for microscope controlling and image acquisition. Live cell videos were recorded with 10-second intervals between frames with acquisition of the first frame beginning prior to induction of heat shock at 42.5°C via a heated stage and heated dish lid (Bioptechs Inc.). Live cell imaging of recovery from HSB formation was performed by placing cells in a temperature controlled microscope chamber set at 33°C and 5% CO₂. Frames were captured every 30 seconds for three hours with acquisition of the first frame immediately following challenge with heat shock stress.

Fixed cells were imaged using either a 40X air N.A. 0.6 plan fluor, or 60X oil immersion N.A. 1.4 plan apochromat objective (Nikon, Japan) as described above. Live cells were imaged in growth medium, while fixed cells were imaged in PBS. A Spectra LED lamp served as the light source for microscopy, attenuated with
ND2 or ND4 filters as necessary. Filter sets and dichroic filters were supplied by Semrock in a filter wheel supplied by Sutter Instruments. Qualitative images were generated by obtaining a multichannel Z-stack and performing blind 3D non-iterative deconvolution using algorithms from AutoQuant (Media Cybernetics/Roper Industries Inc., Rockville, MD, USA) within NIS Elements 4.1. All images were captured in a 16-bit non-compressed tagged-image format (TIFF/.tif) and converted to JPEGs or bitmaps (BMP) in ImageJ64 (National Institutes of Health) prior to figure preparation. Scale bars represent the diameter of the nucleus (~10µm).

Figures were prepared in Adobe Illustrator CS6 and Corel Draw X6. Live cell imaging videos were captured in the Nikon proprietary .nd2 format and were converted to .avi using the NIS Elements Advanced Research 4.1 software.

**Statistics and Quantification**

Quantitative imaging was performed as described above for fixed cells. 20 images were taken per condition at 40X magnification and the number of cells with puncta counted. All quantitative experiments were performed in triplicate. Where applicable, a student’s t-test was performed to determine statistical significance between conditions. Error bars on all graphs represent the standard deviation and the bars represent the mean.

Co-localization was assessed qualitatively through observation of white signal in overlays of green/magenta and red/green/blue images, and quantitatively by determining the Pearson’s Correlation Coefficient (PCC) and Mander’s Overlap (MO) values in NIS Elements 4.1 software. Graphing and statistical analysis was performed in GraphPad Prism 6 for Mac OS (GraphPad Software Inc.)

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References


Figure Legends

Figure 1: Huntingtin is involved in a rapid cell stress response. (a) Immunofluorescence of STHdh\textsuperscript{Q7/Q7} and STHdh\textsuperscript{Q111/Q111} cells at steady state (33°C) and following challenge with 10 minutes of heat shock at 42.5°C using anti-huntingtin mAb2166. (b) Imaging of fixed STHdh\textsuperscript{Q7/Q7} cells transfected with 1-586 Q17-eYFP at steady state, and following challenge with 10 minutes of heat shock at 42.5°C, 120 minutes of cold shock at 4°C, 1 hour of ATP depletion, and 1 hour of treatment with 400μM H\textsubscript{2}O\textsubscript{2}. (c) Representative live-cell images of STHdh\textsuperscript{Q7/Q7} cells transfected with 1-586 Q17-eYFP undergoing challenge with heat shock stress. (d) Imaging of fixed primary cortical mouse neurons transfected with 1-586 Q17-eYFP following challenge with 10 minutes of heat shock at 42.5°C. (e) Immunofluorescence of HeLa cells transfected with 1-586 Q17-eYFP at steady state (33°C) and following challenge with varying amounts of heat shock stress using an anti-HSF1 antibody. Panels a-d show transfected protein and panels e-h show endogenous HSF1. Secondary antibody was Cy5. All scale bars, 10μm.

Figure 2: Huntingtin 1-171 is necessary for formation of HSBs. (a) Quantification of ability of different huntingtin fragments to form HSBs. STHdh\textsuperscript{Q7/Q7} cells transfected with indicated fragments fused C-terminally to eYFP were fixed after 10 minutes of heat shock, or at steady state, and 20 images were taken at 40X magnification. The number of cells with puncta were counted. Experiments were performed in triplicate. n = ~100 cells per triplicate, total n = ~300 cells. Bars represent the mean and error bars indicate standard deviation. (b) Representative images of graphical data presented in B. (c) Quantification of N17, exon1, and poly-proline region deletion on HSB formation was performed as described in A. n = ~100 cells per triplicate, total n = ~300 cells. (d) Representative images of graphical data presented in D. (e) Quantification of ability of different N17 mutants in the 1-586 context to form HSBs. STHdh\textsuperscript{Q7/Q7} cells transfected with indicated constructs, fused C-terminally to eYFP were fixed following challenge with 10 minutes of heat shock stress. 20 images were captured at 40X magnification and the number of cells with puncta counted. Experiments were performed in
triplicate. n = ~100 cells per triplicate, total n = ~300 cells. Bars represent the mean and error bars indicate standard deviation. *P-value < 0.0001. All scale bars, 10μm.

**Figure 3:** Huntingtin localizes to early endosomes upon induction of cell stress. (a) Immunofluorescence of STHdh\(^{Q7/Q7}\) cells transfected with 1-586 Q17-eYFP following challenge with 10 minutes of heat shock stress using antibodies against early (Rab5C), late (Rab7), and recycling (Rab11) endosomal markers. Secondary antibodies were Cy5. (b) Immunofluorescence of STHdh\(^{Q7/Q7}\) cells transfected with 1-586 Q17-eYFP following heat shock using antibodies against dynamin1, clathrin, and HAP1 (panels a-i). Secondary antibodies for clathrin and dynamin1 were Cy5, and Alexa594 was used for HAP1. Co-expression of 1-586 Q17-eYFP with mCh-6G-HAP40 in STHdh\(^{Q7/Q7}\) cells following 10 minutes of heat shock is shown in panels j-l. Co-localization is shown in white. *R*-values are representative of the Pearson’s Correlation Coefficient averaged over 10 cells in images that were not deconvolved. All representative images are post-deconvolution. All scale bars, 10μm.

**Figure 4:** HSB formation is associated with arrest of early-to-recycling and early-to-late endosome fusion. (a) Immunofluorescence of STHdh\(^{Q7/Q7}\) cells transfected with 1-586 Q17-eYFP and treated with Alexa594 transferrin at steady state using antibodies against Rab5C (panels a-d) and Rab11 (panels e-h). (b) Immunofluorescence of STHdh\(^{Q7/Q7}\) cells transfected with 1-586 Q17-eYFP and treated with Alexa594 transferrin during challenge with 10 minutes of heat shock using antibodies against Rab5C (panels a-e) and Rab11 (panels f-j). (c) Immunofluorescence of STHdh\(^{Q7/Q7}\) cells transfected with 1-586 Q17-eYFP and treated with TexasRed-EGF at steady state using antibodies against Rab5C (panels a-d) and Rab7 (panels e-h). (d) Immunofluorescence of STHdh\(^{Q7/Q7}\) cells transfected with 1-586 Q17-eYFP and treated with TexasRed-EGF during challenge with 10 minutes of heat shock using antibodies against Rab5C (panels a-e) and Rab7 (panels f-j). Secondary antibodies were Cy5. Three-channel co-localization is shown in white, and co-localization between green and blue channels is shown in aquamarine. All scale bars, 10μm.
Figure 5: Mutant huntingtin HSB formation is associated with arrest of early-to-recycling and early-to-late endosome fusion. (a) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1-586 Q138-eYFP and treated with Alexa594 transferrin at steady state using antibodies against Rab5C (panels a-d) and Rab11 (panels e-h). (b) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1-586 Q138-eYFP and treated with Alexa594 transferrin during challenge with 10 minutes of heat shock using antibodies against Rab5C (panels a-e) and Rab11 (panels f-j). (c) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1-586 Q138-eYFP and treated with TexasRed-EGF at steady state using antibodies against Rab5C (panels a-d) and Rab7 (panels e-h). (d) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1-586 Q138-eYFP and treated with TexasRed-EGF during challenge with 10 minutes of heat shock using antibodies against Rab5C (panels a-e) and Rab7 (panels f-j). Secondary antibodies were Cy5. Three-channel co-localization is shown in white, and co-localization between green and blue channels is shown in aquamarine. All scale bars, 10µm.

Figure 6: Mutant huntingtin expression leads to defective recovery from HSB formation. (a) Quantification of recovery of cells expressing mutant huntingtin versus wild-type huntingtin. STHdhQ7/Q7 cells transfected with 1-586 Q17-eYFP (Q17) or 1-586 Q138-eYFP (Q138) were fixed following challenge with 10 minutes of heat shock stress and either a 3 hour or 24 hour recovery period. 20 images were acquired at 40X magnification and the number of cells with puncta counted. Experiments were performed in triplicate, with n = ~100 cells per trial and total n = ~300 cells. Bars indicate the mean and error bars represent standard deviation. A student’s t-test was performed to test for statistical significance. *P-value < 0.001. (b) Representative images of graphical data presented in A. (c) Model depicting the rapid, huntingtin stress body response. Upon induction of cell stress, huntingtin releases from the ER, and rapidly localizes to early endosomes throughout the cell cytosol, resulting in arrest of vesicular trafficking in a matter of seconds. This results in an immediate increase in available ATP levels within the cell, allowing them to be funneled for use by the HSR and UPR, longer term stress responses regulated at the transcriptional level, without the need for increased energy metabolism. Relief from stress results in resumption of vesicular trafficking and return of huntingtin to the ER, except in the case of polyglutamine-expanded huntingtin, which pathologically continues to inhibit vesicular trafficking. All scale bars, 10µm.