High-mobility group box 1 links sensing of reactive oxygen species by huntingtin to its nuclear entry
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Huntington’s disease (HD) is a neurodegenerative, age-onset disorder caused by a CAG DNA expansion in exon 1 of the HTT gene, resulting in a polyglutamine expansion in the huntingtin protein. Nuclear accumulation of mutant huntingtin is a hallmark of HD, resulting in elevated mutant huntingtin levels in cell nuclei. Huntingtin is normally retained at the endoplasmic reticulum via its N17 amphipathic α-helix domain but is released by oxidation of Met-8 during reactive oxygen species (ROS) stress. Huntingtin enters the nucleus via an importin β1– and 2–dependent proline–tyrosine nuclear localization signal (PY-NLS), which has a unique intervening sequence in huntingtin. Here, we have identified the high-mobility group box 1 (HMGB1) protein as an interactor of the intervening sequence within the PY-NLS. Nuclear levels of HMGB1 positively correlate with varying levels of nuclear huntingtin in both HD and normal human fibroblasts. We also found that HMGB1 interacts with the huntingtin N17 region and that this interaction is enhanced by the presence of ROS and phosphorylation of critical serine residues in the N17 region. We conclude that HMGB1 is a huntingtin N17/PY-NLS ROS-dependent interactor, and this protein bridging is essential for relaying ROS sensing by huntingtin to its nuclear entry during ROS stress. ROS may therefore be a critical age-onset stress that triggers nuclear accumulation of mutant huntingtin in Huntington’s disease.

Huntington’s disease (HD) is a monogenic, neurodegenerative, age-onset disorder caused by a CAG DNA expansion in exon 1 of the HTT gene, resulting in a polyglutamine expansion in the 3144-amino acid huntingtin protein (1). One of the classic observations in the HD brain is the presence of neuronal nuclear inclusions (2). These regions of high levels of nuclear polyglutamine-expanded mutant huntingtin may explain the elevated huntingtin levels seen in HD, as nuclear huntingtin evades degradation by the cytoplasmic proteasome (3).

Huntingtin has biological functions as a scaffold protein in the cytoplasm, at endosomal vesicles and the nucleus. At the endoplasmic reticulum (ER), we previously defined huntingtin as bound to outer ER lipids (4), but released from the ER upon various stresses, including ER stress and heat shock (4). The interaction of huntingtin with the ER is mediated by the first 17 amino acids of the protein adjacent to the polyglutamine tract, termed N17, and this region is post-translationally modified at serines 13 and 16 (5, 6) by phosphorylation (7). N17 has previously been defined, by multiple methods, as an amphipathic α helix (4) with a hydrophobic face and a charged face (8). In HD, serines 13 and 16 are hypophosphorylated (7), and phosphomimetic mutants of serines 13 and 16 in transgenic bacterial artificial chromosome HD mice (BACHD) prevent disease phenotypes (9).

At the center of the N17 motif is a single, highly conserved Met-8, which acts as a ROS sensor by sulfoxidation, leading to increased soluble huntingtin with release from the ER and phosphorylation of N17 (10). The hydrophobic face of N17 has two functions: interaction with ER lipids in the cytoplasm, and when soluble within the nucleus, interaction with CRM1/exportin-1 to mediate nuclear export as a nuclear export signal (11).

We previously defined the nuclear localization signal in huntingtin (amino acids 174–207) as a PY-NLS, a class of nuclear import signal that functions via direct interaction with importin β1 and β2 (transportin) nuclear transport factors (12), which can also mediate entry of huntingtin to the primary cilium (13, 14). PY-NLS sequences are typically defined as having interaction epitopes within an unstructured region that mediate interaction with importins. One aspect of the huntingtin PY-NLS is a unique 18-amino acid sequence located between 174KEIKK178 and arginine 200 basic epitopes, and flanked by prolines (13).

In response to ROS, we have previously shown that endogenous human huntingtin from both mutant and WT cells can localize to sites of DNA damage (15). Members of the ataxia telangiectasia mutated (ATM) DNA repair complex can be found at these sites, including HMGB1, also known as HMG1 or amphoterin. Here, via pulldown of the huntingtin PY-NLS peptide, and reciprocal co-immunoprecipitation with endogenous proteins, we demonstrate that HMGB1 binds both the PY-NLS region and the N17 domain. Impor-

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This article contains Figs. S1 and S2.

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1 The abbreviations used are: HD, Huntington’s disease; ER, endoplasmic reticulum; PY-NLS, proline–tyrosine nuclear localization signal; ATM, ataxia telangiectasia mutated; IVS, intervening sequence; HMG, high-mobility group; hTERT, human telomerase reverse transcriptase; YFP, yellow fluorescent protein; ROS, reactive oxygen species; 3-NP, 3-nitropropionic acid; SCA, spinocerebellar ataxia; FBS, fetal bovine serum; PFA, paraformaldehyde; CSK, cytoskeleton; IP, immunoprecipitation.

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**Results**

**HMGB1 binds to the intervening sequence (IVS) within huntingtin PY-NLS**

The unique sequence of the huntingtin IVS, between two critical basic epitopes of the PY-NLS (\(^{174}\)KEIKK\(^{178}\), arginine (Arg-200), and proline–tyrosine (PY 206–207) epitopes, in green. The 18-amino acid IVS is outlined in blue. Proline (P) and tryptophan (W) residues that are observed in HMGB1-binding peptides are red. B, peptide affinity chromatography using WT PY-NLS peptide, or the PY-NLS K177A/K178A mutant, was performed with STHdh\(^{Q7/Q7}\) cell lysates. Interacting proteins were separated by SDS-PAGE followed by anti-HMGB1 Western blotting. C, STHdh\(^{Q7/Q7}\) cells expressing the indicated YFP fusions were cross-linked in 1% PFA and lysed in CSK buffer. Lysates were incubated with Protein A–agarose beads with or without the YFP-specific antibody, anti-GFP. Co-IP products were separated by SDS-PAGE and immunoblotted with anti-HMGB1. Images are representative of three independent experiments. Size markers at 25 and 35 kDa are indicated.

Figure 1. HMGB1 binds to the unique IVS within huntingtin PY-NLS. A, the PY-NLS sequence is located between amino acids 174 and 207 and is characterized by three epitopes: the basic charged (\(^{174}\)KEIKK\(^{178}\)), arginine (Arg-200), and proline–tyrosine (PY 206–207) epitopes, in green. The 18-amino acid IVS is outlined in blue. Proline (P) and tryptophan (W) residues that are observed in HMGB1-binding peptides are red. B, peptide affinity chromatography using WT PY-NLS peptide, or the PY-NLS K177A/K178A mutant, was performed with STHdh\(^{Q7/Q7}\) cell lysates. Interacting proteins were separated by SDS-PAGE followed by anti-HMGB1 Western blotting. C, STHdh\(^{Q7/Q7}\) cells expressing the indicated YFP fusions were cross-linked in 1% PFA and lysed in CSK buffer. Lysates were incubated with Protein A–agarose beads with or without the YFP-specific antibody, anti-GFP. Co-IP products were separated by SDS-PAGE and immunoblotted with anti-HMGB1. Images are representative of three independent experiments. Size markers at 25 and 35 kDa are indicated.

As seen in Fig. 2, by laser confocal microscopy, we observed a natural variance from cell to cell in the amount of nuclear HMGB1, as well as in the levels of phospho-N17 huntingtin and unphosphorylated N17 huntingtin. However, only the phospho-N17 levels showed a correlation with nuclear HMGB1 levels (Fig. 2B). We performed these experiments in nontransformed human cell lines immortalized by the use of human telomerase reverse transcriptase (hTERT). Both WT- and HD-derived human lines were used, with the HD line defined as a Q43Q17 heterozygote, which is representative of average clinical alleles in HD. Although we could see a significant correlation between HMGB1 and phospho-N17 signals, we did not note any difference between HD and WT cells. This result is also consistent with nuclear huntingtin being primarily associated with phospho-N17 (7).

**HMGB1 nuclear levels correlate to phospho-N17 Huntingtin nuclear levels**

We previously demonstrated by Forster resonance energy transfer studies in live cells that the N17 domain in huntingtin could fold back toward the PY-NLS region (16). Because the correlation of HMGB1 to nuclear huntingtin was specific to the phosphoepitope at N17, we tested whether N17 could also bind HMGB1. We used N17 as a fusion to yellow fluorescent protein (YFP), which we have previously used for N17 structure/function studies (4, 10, 11). In a stable cell line expressing N17-YFP (Fig. 3A), we could co-immunoprecipitate HMGB1 (Fig. 3A) in a manner similar to the peptide experiment (Fig. 1B). A similar result was seen by transient expression of N17-YFP and co-immunoprecipitation with an anti-GFP antibody, which recognizes YFP variants of GFP (Fig. 3B). Using this system, and knowing that HMGB1 is a highly charged protein, we asked whether the N17 charged face aspartic acid mutant (E5A/E12A) or lysine mutant (K6A/K9A) could still interact with HMGB1. In previous stud-
ies, E5A/E12A had a stronger affinity for vesicles and ER lipids, whereas K6A/K9A had no obvious phenotype (4). We additionally tested the proline substitution mutant (M8P), which is known to disrupt the N17 helical structure, but not affect charge (4).

Although we did see a weak interaction with E5A/E12A, we did not detect any interaction with either K6A/K9A or M8P (Fig. 3, B and C). These data indicated that both the charged face and the helical structure of huntingtin N17 were essential for HMGB1 interaction.

Figure 2. Correlation between huntingtin and HMGB1 localization. A, hTERT-immortalized TruHD-Q21Q18 (WT) and TruHD-Q43Q17 (HD) human fibroblasts were co-stained with anti-phospho-N17 (pN17) or anti-unmodified-N17 (N17) and anti-HMGB1 antibodies. Greater nuclear phospho-huntingtin (green channel) is seen when there is greater nuclear HMGB1 fluorescence (red channel), whereas less nuclear phospho-huntingtin is seen with less nuclear HMGB1. This correlative relationship can be observed in both WT and HD lines using the anti-phospho-N17 antibody but is not seen in cells stained with anti-unmodified-N17. Images were captured by confocal microscopy with a ×60 oil-immersion lens. Scale bar is 10 μm. B, the correlation of nuclear huntingtin and HMGB1 was quantified using Pearson correlation and computed using a Python script written on PyCharm CE 2016.1.2. An average correlation value of 0.744 for WT and 0.677 for HD represents a very strong correlation between phospho-N17 and HMGB1 (circles). Correlation values for N17 and HMGB1 were 0.260 for WT and 0.310 for HD, indicating a weak relationship (squares). >500 cells were captured and measured. Error bars represent S.D. ****, p value < 0.0001 and ***, p value = 0.0007 by two-way ANOVA. n = 3 independent replicates.

Figure 3. HMGB1 co-immunoprecipitates with the N17 domain. A, ST/HdhQ7/Q7 cells stably expressing N17-YFP and B, ST/HdhQ7/Q7 cells transfected with WT N17-YFP and mutants N17 E5A/E12A-YFP, N17 K6A/K9A-YFP, and N17 M8P-YFP were used for co-IP. Lysates were incubated with Protein A-agarose beads with or without the YFP-specific antibody, anti-GFP. Co-IP products were separated by SDS-PAGE and immunoblotted with anti-HMGB1. Images are representative of three independent experiments. C, protein levels were quantified for WT N17 and mutants E5A/E12A, K6A/K9A, and M8P. The pixel density of each band was calculated and normalized to WT N17. Error bars represent S.D. ****, p values < 0.0001 by one-way ANOVA. n = 3 independent replicates. Size markers at 25 and 35 kDa are indicated.
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**A**

| Protein | Input | Beads | EPR5526 | kDa |
|---------|-------|-------|---------|-----|
| Huntingtin | -245 | -63 | -48 |
| α-YFP | -245 | -35 |

**B**

| Protein | Input | Beads | EPR5526 | kDa |
|---------|-------|-------|---------|-----|
| Huntingtin | -245 | -35 | -25 |
| α-YFP | -245 | -35 |

**Figure 4.** Huntingtin interacts with full-length HMGB1, but not its individual boxes. A, STHdhQ7/Q7 cells overexpressing HMGB1-YFP were lysed and incubated with or without huntingtin-specific antibody, EPR5526, followed by Protein A-agarose precipitation. Protein marker bands at 245 kDa, huntingtin, and anti-YFP at 25 and 35 kDa are indicated. B, co-immunoprecipitation of HMGB1 box A-YFP, or box B-YFP, was carried out as in A. Bottom panel: human HMGB1 amino acid sequence, box A highlighted in green, box B highlighted in red. Blue highlights the acidic tail of the full-length HMGB1 protein.

**HMG1 interaction with endogenous, full-length huntingtin requires both box A and box B fragments of HMGB1**

Having demonstrated an interaction between HMGB1 and synthetic peptides or N17-YFP fragments of huntingtin, we next tested whether endogenous, full-length huntingtin interacts with HMGB1-YFP expressed in STHdhQ7/Q7 cells (Fig. 4A). HMGB1 co-immunoprecipitated with the huntingtin protein using the validated rabbit monoclonal anti-huntingtin antibody, EPR5526 (20), similar to the interaction we previously described using the classic anti-huntingtin monoclonal mouse antibody MAB2166 (15).

We then tested if endogenous huntingtin could pulldown HMGB1 fragments corresponding to two defined DNA-binding motifs in HMGB1: box A and box B (Fig. 4B). These two regions make up most of HMGB1, with a remaining acidic tail carboxyl region comprised entirely of aspartic and glutamic acid residues. In contrast to full-length HMGB1-YFP, we did not detect any HMGB1 box A-YFP or box B-YFP in anti-huntingtin immunoprecipitates. A common caveat of many protein domain interaction experiments is that the domains may not fold properly in isolation. Although the crystal structure of box A has been solved, indicating that it can fold properly as a fragment (21), box B is known to be unstructured (22). We can therefore conclude that huntingtin cannot interact with the folded box A domain alone, nor with the unstructured box B alone, and that huntingtin-HMGB1 interaction requires the entire HMGB1 protein.

**HMG1-Huntingtin interaction is enhanced by reactive oxygen species stress**

Both HMGB1 (23) and huntingtin (10) are known to be responsive to ROS stress. In previous work, we identified HMGB1 as part of a ROS-dependent huntingtin interactome that included a number of proteins involved in DNA repair (15). We have also shown that huntingtin N17 phosphorylation is regulated by Met-8 oxidation (10). As shown in Fig. 5, the interaction between HMGB1 and N17-YFP was augmented by treatment with 3-nitropropionic acid (3-NP), a potent mitochondrial inhibitor that causes ROS stress in cells and HD-like symptoms in mice (24). Mutation of Met-8 to leucine, or serines 13 and 16 to alanine, abrogated HMGB1 interaction with or without stress.

We next tested whether we could detect an interaction between endogenous full-length huntingtin and HMGB1 by co-immunoprecipitation with endogenous proteins at the correct biological stoichiometry (Fig. 5, C and D). In contrast to HMGB1-YFP overexpression conditions (Fig. 4A), in which the interaction with huntingtin was detected even in the absence of ROS, we found that the interaction between endogenous proteins at normal expression levels was ROS-dependent.

To further inform the mechanism by which oxidized huntingtin interacts with HMGB1, we tested whether box A-YFP and box B-YFP could bind huntingtin under ROS conditions. As with the overexpression conditions in Fig. 4B, we did not detect an interaction between the individual domains and huntingtin even in the presence of ROS (Fig. S2). Thus, increased ROS, which leads to modification of HMGB1 (23, 25) and huntingtin N17 (10), facilitates the interaction of these two proteins.

**Discussion**

Recent HD genome-wide association studies have identified modifiers of disease onset, as the presence of expanded CAG in the HTT gene only accounts for about 50% of the variance of age of onset of disease (26–28). These studies have predominantly highlighted genes involved in DNA repair, as well as genes involved in redox control and mitochondrial health. These modifiers have also been cited as relevant to the spinocerebellar ataxias (SCAs), caused by CAG expansion in gene open reading frames (26). Thus, this interaction between HMGB1 and huntingtin is intriguing as it overlaps with the known roles of HMGB1 in ROS stress, inflammation, and DNA repair. HMGB1 is also an interactor of ataxin-1 and critical in the SCA1 disease in animal models, defining it as a potential target in SCA1 therapy (29–31). HMGB1 is also noted as protective in ataxin-1 mutant mice by improving mitochondrial DNA damage repair (30).

Huntingtin protein is localized to the ER by the N17 amphipathic α helix. This domain appears to be the master regulator of huntingtin intracellular location and is critical to the development of HD-like pathology in model animals. The huntingtin nuclear localization signal is a PY-NLS (13), which is also seen in proteins that can localize to the primary cilium (32, 33). This type of NLS can directly interact with importin β1 or β2, without the need for an adapter importin α (12). PY-NLSs are inherently unstructured regions where two basic epitopes, and one proline–tyrosine PY epitope, interact with the importin components. The NLS on huntingtin N17 is triggered by elevated ROS, which affects N17 helicity through oxidation of Met-8, and this transduces the
ROS-sensing signal back to the huntingtin PY-NLS to facilitate nuclear entry via a molecular bridge mediated by HMGB1. This concept is supported by our previous studies in which N17 was described to fold back in space to regions in the N terminus of huntingtin near the PY-NLS (16).

Although HMGB1/2 itself is too critical a protein for cell health to be a target for disease therapy (34), the huntingtin IVS sequence is unique to huntingtin, yet extremely conserved in vertebrate species, and may be more amenable to therapeutic targeting. Among its many functions, HMGB1 has roles in mitochondrial and nuclear DNA repair in response to elevated ROS (35). This overlaps HMGB1 function with almost all the pathways highlighted in HD GWAS (28). This would suggest that disruptors of the HMGB1-huntingtin IVS interaction could potentially modulate the ability of mutant huntingtin to enter the nucleus and dominantly inhibit DNA repair mechanisms. Although there is no consensus sequence for HMGB1-protein interactions, known HMGB1-binding peptides do contain proline and rare tryptophan residues in short peptides, similar to the huntingtin IVS (37).

In the nucleus, HMGB1 has well characterized activities in DNA repair (38), and notably, activates the P53 tumor suppressor (39), which in turn is known to activate huntingtin transcription (40). Mutant huntingtin is known to decrease soluble levels of HMGB1, as is mutant ataxin-1 (31). We have previously shown that upon induction of DNA damage, huntingtin is removed from the soluble phase during DNA damage repair (15), and is likely removing HMGB1 and other DNA repair factors from the soluble phase onto chromatin, in its role as a HEAT-repeat scaffold at DNA damage repair.

ROS enhancement of the HMGB1-huntingtin interaction, as well as the requirement of full-length HMGB1 to bind huntingtin, are consistent with the known response of HMGB1 to redox conditions. Under oxidative conditions, cysteine disulfide bridges mediate HMGB1 quaternary structure from dimer to tetramer, with DNA recognition thought to favor the dimeric state (41).

Figure 5. The interaction between HMGB1 and huntingtin increases with oxidative stress. A, STHdhQ17/Q17 cells overexpressing WT N17-YFP or the indicated N17 mutants were treated with or without 10 mM 3-NP for 1.5 h. Lysates were incubated with Protein A-agarose beads with or without anti-GFP. Western blots were immunoblotted with anti-HMGB1. B, pixel density of each co-IP band was calculated and normalized to WT N17 control (without treatment). Error bars represent S.D. ***, p value = 0.0007 comparing untreated conditions and ****, p value <0.0001 comparing treated conditions by one-way ANOVA. *, p value = 0.0120, comparing treated and untreated for WT by unpaired t test. n = 3 independent replicates. C, endogenous huntingtin was immunoprecipitated from STHdhQ17/Q17 cells using antibody EPR5526. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-HMGB1. D, proteins were quantified as in B. *, p value = 0.0318 by unpaired t test. n = 3 independent replicates. Size markers at 25 and 35 kDa are indicated for YFP fusions, marker at 245 kDa for full-length huntingtin.
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In our studies, we did not note any significant difference in HMGB1 interaction with the polyglutamine expanded mutant or WT huntingtin in relationship to nuclear entry. This is consistent with our previous work that did not show any inhibition of huntingtin nuclear localization in response to stress, but instead showed inhibition of relief of that response (42). In other neurological diseases, recent publications have noted trapped DNA repair complexes, and in particular, complexes that involve ATM kinase (43, 44). Thus, future work will focus on the role of HMGB1 within huntingtin-ATM DNA repair complexes and the potential disruption of the N17/HMGB1 or IVS/HMGB1 interface to prevent mutant huntingtin nuclear entry.

Experimental procedures

Tissue culture

T-antigen immortalized, WT mouse striatal STHdhQ7/Q7 cells (kind gift from M. E. MacDonald, MGH) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) at 33 °C with 5% CO2. hTERT-immortalized WT TruHD-Q21Q18 and HD mutant TruHD-Q43Q17 patient-derived fibroblast cells (36) were cultured in minimal essential medium with 15% FBS and 1% GlutaMax at 37 °C with 5% CO2. All media and supplements were from Life Technologies unless stated otherwise.

Antibodies

The following primary antibodies were used in this study: anti-HMGB1 rabbit polyclonal ab18256 (Abcam), anti-huntingtin EPR5526 (Abcam), anti-GFP (Abcam ab6550, ab5450); and anti-HMGB1 mouse monoclonal H00003146-M08 (Abnova). Huntingtin phosphorylation was detected using a previously characterized and validated antibody against huntingtin Ser-13 and Ser-16 phospho epitopes of the N17 domain (anti-phospho-N17) (7). The antibody specific for unmodified huntingtin and Ser-13 phospho epitopes of the N17 domain (anti-unmodified-N17) was also characterized and validated. Secondary antibodies against rabbit, mouse, and goat IgG, directly conjugated to Alexa Fluor 488, 594, or Cy3 (Invitrogen) were used for immunofluorescence and anti-rabbit IgG (ab97051), and anti-mouse IgG (ab97046) horseradish peroxidase conjugates (Abcam) were used for Western blot analysis.

Plasmid constructs

Double-stranded synthetic DNA oligonucleotides (Integrated DNA Technologies) encoding the PY-NLS and IVS sequence of huntingtin containing BspEI and Acc65I overhangs were cloned between BspEI/Acc65I sites of peYFPN1 (BD Biosciences/Clontech) to generate huntingtin PY-NLS-eYFPN1 and huntingtin IVS/eYFPN1 plasmids. Additional huntingtin plasmids of WT N17 and its mutants (E5A/E12A, K6A/K9A, M8L, M8P, S13A, S16A) were created as previously described (4). HMGB1 fragments (box A and box B) were generated by first amplifying the gene via PCR from plasmid mCherry-HMGB1 using oligonucleotides as specified below. The PCR product was cleaved with BspEI and Acc65I and subcloned into BspEI/Acc65I sites of peYFPN1. All DNA manipulation enzymes and buffers were purchased from New England Biolabs. All plasmid constructs were verified by nucleotide sequencing (McMaster MOBIX facility).

Oligonucleotides

PCR amplons were generated from plasmid mCherry-HMGB1 using oligonucleotides with BspEI and Acc65I overhangs. The following constructs were created using their respective forward and reverse primers: HMGB1 box A-peYFP (forward primer, AGTCtccgaATGGGCAAAGGAGATCC; reverse primer, GATGgtaccGATATAAGGTTTCTTCCTCT); HMGB1 box B-peYFP (forward primer, AGTCtccgaGT- TCAAGGATCCCAATGC; reverse primer, CATGggtacc- ATATGCAGCAATATCCTTCTTCT).
into a pipette tip column stuffed with glass wool. Columns were washed four times with binding buffer and once in washing buffer (0.3 M NaCl, 10 mM HEPES, pH 7.6, 10% (v/v) glycerol). Bound proteins were then eluted in 0.5 M NaCl. All affinity chromatography was conducted at 4 °C and analyzed by Western blotting for the presence of HMGB1 as described below.

**Mass spectrometry (MS) analysis**

All MS was conducted at University of Western Ontario Mass Spectrometry Laboratory. MS analysis was performed on a Micromass QToF Global, mass spectrometer equipped with a Z-spray source operating in the positive ion mode with the following parameters: data range, MS survey: 400–1800; data range, MS/MS: 50–1800; cone voltage: 100 V; source temperature: 80 °C; nano-ESI, DDA mode. UPLC was via a Waters nanoUPLC. Data were acquired using MassLynx 4.1 and PLGS 2.2.5.

**Co-immunoprecipitation and Western blot analysis**

STHdf<sup>Q7/Q7</sup> cells were transfected with YFP fusion proteins as outlined above. At 24 h post-transfection, cells were either treated with or without 10 mM 3-NP (Sigma) for 1.5 h, or 100 mM KBrO<sub>3</sub> for 30 min in Hank’s balanced salt solution (Life Technologies). Obtained cell pellets were re-suspended and incubated in 1% PFA at room temperature for 10 min and then quenched in 125 mM glycine (BioShop) at room temperature for 5 min. Cells were washed in ice-cold PBS twice and lysed in cytoskeleton (CSK) lysis buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% (v/v) Triton X-100) containing 1× protease and phosphatase inhibitor cocktails (Roche) for 3 min. Supernatants were incubated with GFP-Trap magnetic beads according to the manufacturer’s instructions (Chromotek), or pre-cleared in Protein A-agarose beads (Sigma) rotating for 1 h at 4 °C. A total of ~400–500 μg of solubilized proteins was collected and ~5% of precleared supernatants (input) was set aside. The remaining pre-cleared products were incubated in fresh Protein A-agarose beads with anti-GFP or anti-huntingtin EPR5526 for 2 h rotating at 4 °C. Samples were washed thoroughly in CSK lysis buffer and denatured in 1× DTT + 3× SDS loading dye (New England Biolabs) for 10 min at 100 °C. All samples were resolved on a 4–12% SDS-polyacrylamide gradient gel (Bio-Rad) and electroblotted onto a PVDF membrane (Millipore). Membranes were treated in methanol for 8 min and washed three times in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) for 10 min each prior to blocking in TBST containing 5% (w/v) skim milk powder and room temperature for 2 h. Membranes were then incubated with primary antibodies diluted 1:2500 in the same blocking buffer overnight at 4 °C. Membranes were washed six times in TBST + 2.5% (w/v) milk prior to the incubation of secondary antibodies diluted at 1:50,000 in blocking buffer at room temperature for 45 min. Membranes were washed as previously described and visualized using ECL (Millipore) and Microchemi (DNR Bio-Imaging Systems). The Gel Analyzer functions on ImageJ were used for densitometric analysis of Western blots.

**Pearson correlation and nuclear intensity quantification**

TruHD-Q21Q18 and TruHD-Q43Q17 fibroblast cells were treated and fixed for immunofluorescence and wide field epifluorescence microscopy as outlined above. A homemade Python script written on PyCharm CE 2016.1.2 was used to calculate the correlation of nuclear intensity of phospho-N17 and HMGB1. Raw, unprocessed images, captured at 16-bit depth, were thresholded using the intersect of the Hoechst stain and YFP (520 nm) or far red (680 nm) fluorescence to detect phospho-N17 or endogenous HMGB1, respectively. Images were separated via a morphological closing and connectivity-based segmentation and mean nuclear intensity was computed for each segmented region. Regions were excluded if their mean nuclear intensity was greater or less than 2 standard deviations from the mean for each image. Using Pearson’s correlation coefficient, the correlation of mean nuclear intensity of phospho-N17 and endogenous HMGB1 was calculated in ~500 cells from 3 trials. Results were displayed in a bar graph with standard error bars.

**Statistics**

All statistical analysis was completed using the Real Statistics Excel add-in (Microsoft). If data passed the Shapiro-Wilk normality test, statistical significance was determined using a Student’s t test. If data were not normal, the Mann-Whitney test was used.

**Author contributions** — S. S., C. R. D., and R. T. conceptualization; S. S., L. E. B., C. L. H., and R. T. data curation; S. S., L. E. B., C. L. H., and R. T. formal analysis; S. S., L. E. B., C. L. H., C. R. D., and J. X. investigation; S. S. visualization; S. S., L. E. B., C. L. H., and J. X. methodology; S. S., L. E. B., T. M., C. L. H., and R. T. writing-original draft; S. S., L. E. B., T. M., C. L. H., and R. T. writing-review and editing; C. L. H. validation; J. X. and R. T. supervision; R. T. funding acquisition; R. T. project administration.

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