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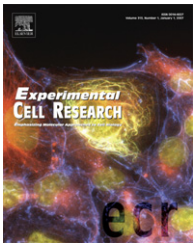

Research Article

Ubiquitin ligase Hrd1 enhances the degradation and suppresses the toxicity of polyglutamine-expanded huntingtin

Hui Yang[a,1], *Xiaoyan Zhong*[a,1], *Petek Ballar*[b], *Shouqing Luo*[c], *Yuxian Shen*[a], *David C. Rubinsztein*[c], *Mervyn J. Monteiro*[a,b], *Shengyun Fang*[a,b,*]

[a]*Medical Biotechnology Center, University of Maryland Biotechnology Institute, 725 W. Lombard Street, Baltimore, MD 21201, USA*
[b]*Program in Molecular Medicine, University of Maryland School of Medicine, 725 W. Lombard Street, Baltimore, MD 21201, USA*
[c]*Department of Medical Genetics, Cambridge Institute for Medical Research, Wellcome Trust/Medical Research Council Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK*

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ABSTRACT

E3 ubiquitin ligases catalyze the conjugation of ubiquitin onto proteins, which acts as a signal for targeting proteins for degradation by the proteasome. Hrd1 is an endoplasmic reticulum (ER) membrane-spanning E3 with its catalytic active RING finger facing the cytosol. We speculated that this topology might allow Hrd1 to ubiquitinate misfolded proteins in the cytosol. We tested this idea by using polyglutamine (polyQ)-containing huntingtin (htt) protein as a model substrate. We found that the protein levels of Hrd1 were increased in cells overexpressing the N-terminal fragment of htt containig an expanded polyQ tract (httN). Forced expression of Hrd1 enhanced the degradation of httN in a RING finger-dependent manner, whereas silencing of endogenous Hrd1 expression by RNA interference stabilized httN. Degradation of httN was found to be p97/VCP-dependent, but independent of Ufd1 and Npl4, all of which are thought to form a complex with Hrd1 during ER-associated degradation. Consistent with its role as an E3 for httN, we demonstrate that Hrd1 interacts with and ubiquitinates httN. Subcellular fractionation and confocal microscopy revealed that Hrd1recruits HttN to the ER and co-localizes with juxtanuclear aggregates of httN in cells. Interaction of Hrd1 with httN was found to be independent of the length of the polyglutamine tract. However, httN with expanded polyglutamine tracts appeared to be a preferred substrate for Hrd1. Functionally, we found that Hrd1 protects cells against the httN-induced cell death. These results suggest that Hrd1 is a novel htt-interacting protein that can target pathogenic httN for degradation and is able to protect cells against httN-induced cell death.

* *Corresponding author*. Medical Biotechnology Center, University of Maryland Biotechnology Institute, UMBI Building, N359, 725 W. Lombard Street, Baltimore, MD 21201, USA. Fax: +1 410 706 8184.
E-mail address: fangs@umbi.umd.edu (S. Fang).

Abbreviations: ER, endoplasmic reticulum; htt, huntingtin; httN, htt exon I or the N-terminal 588 amino acids with various numbers of glutamines; ERAD, ER-associated degradation; GST, glutathione S-transferase; HA, hemagglutinin; IB, immunoblotting; IP, immunoprecipitation; RING, really interesting new gene; GFP, green fluorescent protein; E3, ubiquitin ligase; GFP-polyQ, GFP fusion of huntingtin exon I with various numbers of glutamines; CHIP, carboxyl terminus of Hsc70-interacting protein; CHX, cycloheximide

[1] These authors contribute equally to this work.

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Introduction

Accumulation of misfolded protein is a common feature of many diseases, particularly neurodegenerative disorders [1–3]. Cells utilize a stringent protein quality control system to monitor the status of protein folding [4–8]. Irreversibly misfolded proteins are conjugated with polyubiquitin chain, a process catalyzed by ubiquitin ligases (E3s), and then targeted to the proteasomes for degradation [4,9–15]. The ubiquitinated proteins are delivered to the proteasomes where they are degraded. However, if the function of the ubiquitin proteasome system is compromised or overwhelmed, proteins accumulate, frequently forming aggregates [1–3].

Misfolded proteins in the ER are degraded by a process called ER-associated degradation (ERAD) [4,9,16]. Studies have shown that ER membrane-spanning E3s play an essential role in ERAD. The first E3 to be identified in ERAD was Hrd1/Der3, a yeast ER-resident E3 [4,9], that has five transmembrane domains and a RING finger. The RING finger renders Hrd1/Der3 E3 activity and is localized on the cytosolic surface of the ER [17]. Another ER membrane-spanning RING finger E3, Doa10, was subsequently discovered to function in ERAD [18]. Importantly, Hrd1/Der3 and Doa10 have distinct substrate specificities, although they share significant overlap in terms of ER substrates [18]. This overlapping role could ensure the removal of misfolded proteins from the ER in cases where the function of one E3 is compromised. Interestingly, Doa10 but not Hrd1/Der3 also mediates ubiquitination and degradation of non-ER substrates [19]. In the past few years, three ER-localized RING finger E3s: namely gp78, Hrd1, homologous to yeast Hrd1/Der3, and TEB4, homologous to Doa10, have been identified in mammals [10,15,20]. All of them are involved in ERAD. Like Hrd1/Der3 and Doa10, the RING finger of these three mammalian E3s is predicted to be exposed towards the cytosolic side of the ER. This topology makes it possible for ER membrane-spanning E3s to interact with cytosolic proteins, thereby facilitating their ubiquitination.

Apart from the ER-localized E3s, other E3s involved in protein quality control are present in the cytosol and nucleus [4,9–11,21]. The cytosolic E3s recognize misfolded proteins that are present in the cytosol as well as those that are anchored to the ER membrane. For example, the cytosolic E3 Parkin has been demonstrated to mediate ubiquitination and proteasomal degradation of cytosolic polyglutamine (polyQ)-expanded ataxin-3 [22]. Parkin can also be recruited to the cytosolic surface of the ER and ubiquitinates the misfolded ER transmembrane protein pael R (parkin-associated endothelin receptor-like receptor), a putative G protein-coupled receptor that is associated with Parkinson's disease [23]. Similarly, cytosolic E3 CHIP is capable of ubiquitinating CFTRΔF508, a mutant that is localized to the ER and causes cystic fibrosis [24]. CHIP also mediates ubiquitination of several cytosolic misfolded proteins, such as expanded polyQ htt and ataxin-3 [25], tau [26–28], and familial amyotrophic lateral sclerosis-causing mutant Cu/Zn-superoxide dismutase [29]. Moreover, CHIP has been shown to dimerize with parkin and to enhance parkin-mediated ubiquitination of pael R [13].

In this study, we found that the levels of ER-anchored E3 Hrd1 are increased in cells expressing httN protein. We demonstrate that Hrd1 acts as an E3 and can target pathogenic httN for degradation, thereby protecting cells against httN-induced cell death.

Materials and methods

Plasmids, antibodies and cells

pEGFP-C1-huntingtin exon I with 72 CAG repeats, namely pEGFP-72Q, and pCIneo-gp78, as well as pCIneo-Hrd1-FLAG have been previously reported [10,30–32]. pcDNA3.1-GFP-huntingtin exon I with 25, 47, and 103 CAG repeat was a gift from the Hereditary Disease Foundation. pcDNA3.1-wt Hrd1, pcDNA3.1-Hrd1C1A, and pcDNA3.1-Hrd1-C1A/C3A were generously provided by Dr. Emmanuel Wiertz [15]. Plasmids pCI 3X Flag-httN-588aa-17Q and pCI 3X Flag-htt N-588aa-138Q have been previously reported [33].

Mouse monoclonal antibodies against HA, FLAG, GFP, tubulin, and actin were purchased from Sigma. Mouse monoclonal anti-ubiquitin was acquired from Santa Cruz. Monoclonal anti-p97/VCP was obtained from Affinity BioReagents. Alexa Fluor-594 labeled anti-mouse IgG was from Molecular Probes. Polyclonal anti-gp78 antibodies have been described previously [10]. Rabbit polyclonal anti-Hrd1 antibodies against a synthetic peptide spanning amino acids 493–509 of human Hrd1 were generously provided by Dr. Allan M. Weissman. Rabbit polyclonal antibodies against Npl4 were raised using purified recombinant protein. Monoclonal anti-Ufd1 antibody was acquired from BD Biosciences. HRP-conjugated anti-mouse or anti-rabbit IgG was purchased from Pierce.

SHSY5Y and 293 cells were cultured in DMEM with 10% FBS. To make 293 cell line that stably expresses CD3δ, pBABE vector encoding puromycin-resistant gene was co-transfected with pCIneo-HA-CD3δ. Twenty four hours after transfection, 2.5 μg/ml puromycin was added to eliminate non-transfected cells. Culture medium was refreshed every 3 days until cell colonies become evident. Positive clones expressing HA-CD3δ were determined by immunoblotting (IB) for HA.

Immunoblotting (IB)

293 or SHSY5Y cells were seeded at 5×10^5 to 6×10^5 per well in 6-well plates prior to the day of transfection. Transfection was done with lipofectamine 2000 (Invitrogen) or by calcium phosphate precipitation. Twenty-four hours after transfection, cells were lysed in 2% SDS by boiling for 10 min. The resulted lysates were used for blotting of total proteins. To determine the solubility of GFP-tagged httN, cells were lysed in Triton X-100 lysis buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1% Triton X-100 and 1× protease inhibitor cocktail). After centrifuging for 10 min, the supernatant was used as the soluble fraction and the pellet was further solubilized in 2% SDS as the insoluble fraction. To evaluate the effects of Hrd1 on degradation of httN, 100 μg/ml cycloheximide (CHX) was added to the medium 16 to 20 h after transfection and the cells were subject to chase for 0 to