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Ubiquitin C-Terminal Hydrolase L1 regulates autophagy by inhibiting autophagosome formation through its deubiquitinating enzyme activity

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ABSTRACT

Ubiquitination modification has been shown to play a key role in autophagy. Increasing studies reported the involvement of de-ubiquitinating enzymes (DUBs) in autophagy pathway. To systematically search how DUBs manipulate autophagy, we utilized a double fluorescence tagged LC3 stable HeLa cell line, and did a genome wide screen of 55 human DUBs which is about 60% coverage of the DUB family. We found a bunch of DUBs have impact on autophagy by either changing the LC3 puncta formation or the autophagy flux. One of them, Ubiquitin C-Terminal Hydrolase L1 (UCHL1) correlated to Parkinson's disease, strongly affects autophagy by inhibiting autophagosome formation. We found UCHL1 overexpression inhibits LC3 puncta formation and is dependent on its DUB activity. Knockdown of UCHL1 significantly promotes LC3 puncta formation. Further study revealed that UCHL1 may affect autophagy by interacting with LC3 but not other autophagy related proteins. Interestingly, a Parkinson's disease related mutant UCHL1 I93 M defects its DUB activity and can no longer inhibit autophagosome formation. We further screened 22 commercially available DUB inhibitors and found two potent UCHL1 inhibitors LDN-57444 (LDN) and NSC632839 (NSC), when treating cells, both strongly induce LC3 puncta formation. Taken together, our results indicated a new insight into the manner in which DUB regulates autophagy and provided potential drugs for the Parkinson's disease.

1. Introduction

Autophagy-lysosome pathway is essential for cellular health and its dysfunction is associated with various diseases [1–4]. Autophagy is triggered by either environmental nutrition deprivation or internal protein aggregation. One well-studied type macroautophagy is a multistep process involves initiation and elongation of phagophore, fusion of autophagosome with lysosome and degradation of substrates [5,6]. It starts with the ULK1 complex to phosphorylate the downstream effectors such as AMBRA1 and Atg14 and induce the formation of phagophore [5]. LC3-I is then covalently conjugated to phosphatidylethanolamine (PE) on the phagophore to form LC-II executed by the ATG16L1/ATG5-ATG12 complex [7]. LC3-II is a widely used hallmark of autophagosome. p62/SQSTM1 is an adaptor protein that bridges LC3-II to the ubiquitinated substrates and reduction of p62 is usually linked to the activation of autophagy [8]. The phagophore then engulfs substrates and encloses itself to become the autophagosome. After fusion with lysosome, the whole compartment is degraded by the hydrolases in the lysosome.

Protein ubiquitination is a reversible post-translational modification process that regulates many vital signaling pathways. Ubiquitin conjugated on substrates can be removed by DUBs to reverse the functional effects of ubiquitination [9]. It has now been well recognized that autophagy substrates are ubiquitinated and some DUBs have been demonstrated to function in the pathway [10]. USP10, USP13 and A20 have been shown to de-ubiqutinate Beclin1 to negatively regulate autophagy [11,12]. In contrast, other report showed that USP10 de-ubiquitinates AMPKα to

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positively regulate autophagy [13]. USP8 specifically removes lys6-linked polyubiquitin chains from Parkin, and promotes the autophagic degradation of dysfunctional mitochondria [14]. Whereas USP30 negatively regulates mitophagy by de-ubiquitinating PINK, a kinase on mitochondria membrane [15]. USP33 de-ubiquitinates mono-ubiquitinated RALB, which allows RALB to associate with Beclin1-containing complexes to positively induce autophagy [16]. However, the detail mechanism of the majority of the DUBs is still unidentified.

UCHL1 is a DUB that has been widely studied for its relation to Parkinson's disease (PD) [17,18]. UCHL1 I93 M was reported in a familial autosomal dominant PD [19]. Sporadic reports suggested its involvement in autophagy. One study showed that UCHL1 suppresses autophagic degradation of $p21^{WAF1/Cip1}$ in cardiac fibroblasts via increasing mTOR activity [20]. Recently, it was reported that UCHL1 interacts with RPTOR in cells, which is not affected by growth factor signaling [21]. UCHL1 is also a key regulator of chaperone mediated autophagy, responsible for removing Parkinson's disease related alpha-synuclein [22]. UCHL1 inhibition by LDN activates autophagic pathway in both an alpha-synuclein overexpressed oligodendroglial cell line [23] and an alpha-synuclein transgenic mouse model [24]. However, the mechanism of how UCHL1 regulating autophagy is still a mystery.

To explore the detail function of DUBs in autophagy, we did a genome wide screen of 55 human DUBs and found a certain number of them affect autophagy in diverse aspects. One of them, UCHL1, interacts with LC3 and inhibits autophagosome formation relying on its DUB activity. These findings demonstrated that UCHL1 plays a critical role in regulating autophagy.

2. Materials and methods

2.1. Cell lines, plasmids and reagents

293FT and HeLa cells from ATCC were cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (Gemini, USA) and 1% Pen/strep (Life, USA) at 37 °C under 5% CO_2. mRFP-GFP-LC3 plasmid was provided by Prof Tamotsu Yoshimori (Tokyo Medical and Dental University, Japan). HeLa cells stably expressing mRFP-GFP-LC3 was maintained in complete medium containing 300 ng/μl G418 (Invitrogen CA, USA) [25]. EGFP-LC3 was constructed by inserting EGFP-LC3 fragment into pcDNA3.0. DUBs were either purchased from addegene website, or kindly gifted by Prof Zongping Xia from Zhejiang University. To generate UCHL1 C90S and I93 M, PCR-mediated site-directed mutagenesis was used based on the Flag-UCHL1 plasmid. The mutagenesis oligonucleotides are: C90S: (forward) 5′-GACCATTGGGAATAGCTGTGGCACAATCGG-3′ and (reverse) 5′-CCGATTGTGCCACAGCTATTCCCAATGGTC-3'. I93 M: (forward) 5′-TTCCTGTGGCACAATGGGACTTATTCACGCA-3′ and (reverse) 5′-TGCGTGAATAAGTCCCATTGTGCCACAGGAA-3'. His tagged UCHL1 was generated by inserting UCHL1 fragment into pet32a. For UCHL1 knockdown, target sequences UCHL1 shRNA-1: 5′-GGGAATTCCTGTGGCACAATC-3′ and UCHL1 shRNA-2: 5′-GGATGGCCACCTCTATGAACT-3′ are cloned into pSUPER. To induce autophagy pathway, sample cells were treated with 2 nM Torin1 (R&D systems company, USA) for 6 h.

2.2. Immunoprecipitation and immunoblotting

By using Lipofectamine 2000 transfection reagent (Invitrogen, USA), plasmids were transfected into cells according to the manufacturer's instructions. Cells were harvested 24 h after transfection, and were lysed in cell lysis buffer (Sigma-Aldrich, USA) containing protease inhibitor cocktail (Biotool, USA) for 30 min at 4 °C. After being centrifuged at 15,000 rpm for 10 min, the supernatant was incubated with *anti*-Myc affinity gel (Biotool, USA) and gently agitated sample for 2 h at 4 °C. Then the beads were washed three times with 1 ml IP wash buffer to remove nonspecific binding. Finally 50 μl 1 × loading buffer containing SDS were added into each sample and the interaction was detected by immunoblotting.

For immunoblotting, sample cells were lysed with RIPA buffer (Beyotime, China) for 30 min at 4 °C. Samples were spun down at 15,000 rpm for 15 min. Protein samples were separated by SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (Bio-Rad, USA) using semi-dry transfer unit (Bio-Rad, USA). After blocking with 5% non-fat milk in TBS containing 0.1% Tween 20 (TBST), proteins of interest were probed with corresponding primary antibodies. The following primary antibodies were used: LC3 (MBL, Japan), β-Actin (MBL, Japan), Flag (Sigma-Aldrich, USA), HA (Cell Signaling, USA), and UCHL1 (Abclonal, China). GFP, Myc, Ub and SQSTM1 are all from Santa cruz, USA. The membranes were washed three times in TBST and incubated with a HRP-conjugated secondary antibody (Jackson ImmunoResearch, USA). After being washed three times, protein signals were detected using ECL western blotting detection reagents (Perkin-Elemer, Finland).

2.3. Immunofluorescence staining

HeLa cells seeded on coverslips were transfected with desired plasmids. After 24 h, cells were washed with PBS and fixed with 16% PFA in PBS for 15 min. After four times wash with PBS, cells were blocked with staining solution (PBS, 0.1% NP40 and 4% FBS) for 10 min at room temperature. Then cells were incubated with primary antibody against Flag (Sigma-Aldrich, USA) for 1 h at room temperature. After being washed three times, cells were incubated with the secondary antibody conjugated with Dylight 568 (Thermo Fisher Scientific, USA) for 30 min. To show nucleus, cells were incubated with DAPI (Sigma-Aldrich, USA) for 10 min. Then cells were washed three times with PBS and once with deionized water. Coverslips with cells were mounted on a glass slide with Clear Mount™ Mounting solution (Invitrogen, USA). The images were detected under a confocal laser scanning microscope (ZEISS LSM 710, Germany), which were excited with 488 nm, 568 nm and 402 nm laser excitations.

2.4. Expression and purification of recombinant protein

His tagged UCHL1 was purified from *E. Coli* according to a previously described method [26]. Protein eluted from Ni-NTA beads (Qiagen, CA) were fractionated on a Superdex 200 HR (10/30) column in a buffer containing 50 mM Tris-HCl PH 7.5, 100 mM Sodium Chloride, and 2 mM DTT.

2.5. In vitro UCHL1 de-ubiquitination assay

The DUB activity was performed as previously described [27] with slight modifications. The DUB activities were measured by detecting the increase in fluorescence upon cleavage of Ubiquitin-AMC (Boston Biochem, MA). His-UCHL1 (5 nM) were added to 200 μl deubiquitinating assay buffer (20 mM Tris-HCl pH 7.4, 20 mM Potassium Chloride, 5 mM Magnesium Chloride, 1 mM DTT, including 2 nM Ub-AMC), and incubated at 37 °C. The fluorescence intensity was measured using a Molecular Devices (Perkin-Elemer, Finland) with excitation and emission wavelength set at 350 nm and 440 nm, respectively. For inhibition test, 10 μM DUB inhibitor LDN-57444 or NSC632839 (MCE, USA) was included in the DUB assay mixture.

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