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Isolation of mCherry-LC3B and 97Q-GFP vesicles for LC-MS/MS

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ABSTRACT

Aggregation of proteins containing expanded polyglutamine (polyQ) repeats is the cytopathologic hallmark of a group of dominantly inherited neurodegenerative diseases, including Huntington's disease (HD). Huntingtin (Htt), the disease protein of HD, forms amyloid-like fibrils by liquid-to-solid phase transition. Macroautophagy has been proposed to clear polyQ aggregates, but the efficiency of aggrephagy is limited. Here, we used cryo-electron tomography to visualize the interactions of autophagosomes with polyQ aggregates in cultured cells *in situ*. We found that an amorphous aggregate phase exists next to the radially organized polyQ fibrils. Autophagosomes preferentially engulfed this amorphous material, mediated by interactions between the autophagy receptor p62/SQSTM1 and the non-fibrillar aggregate surface. In contrast, amyloid fibrils excluded p62 and evaded clearance, resulting in trapping of autophagic structures. These results suggest that the limited efficiency of autophagy in clearing polyQ aggregates is due to the inability of autophagosomes to interact productively with the non-deformable, fibrillar disease aggregates.

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Isolation of mCherry-LC3B and 97Q-GFP vesicles for LC-MS/MS

1 Isolation of mCherry-MAP1LC3B and polyQ-GFP positive autophagic vesicles

1.1 The vesicle isolation was carried out as following (Gao et al., 2010) with modifications.
Gao, W., Kang, J.H., Liao, Y., Ding, W.X., Gambotto, A.A., Watkins, S.C., Liu, Y.J., Stolz, D.B., and Yin, X.M. (2010). Biochemical isolation and characterization of the tubulovesicular LC3-positive autophagosomal compartment. Journal of Biological Chemistry.

1.2 HEK293 LC3B knock out cells or Neuro2a cells were grown in 6-well plates at starting cell density of 5x10^6. HEK293 was co-transfected for 97Q-GFP and LC3B-RFP (total 4ug) with lipofectamine 3000, and Neuro2a was transfected with LC3B-RFP and induced for 150Q-GFP with Muristerone A (1uM) for 48 hours. Autophagy was induced with 200 nM rapamycin and 150 mM trehalose (24 hours). Prior to harvest, chloroquine was added at 100μM for 5 hours.

1.3 Cells were harvested by centrifugation at 4°C, and were homogenized 15 times with 22-gauge needle in 1.5ml cold isotonic buffer (0.25M sucrose, 1mM EDTA, 20mM HEPES pH 7.4) with protease inhibitor on ice.

1.4 Lysates were centrifuged at 800g at 4°C for 10 min to pellet the nuclei.

1.5 The supernatant was then centrifuged at 10,000g for 20 min at 4°C to pellet the vesicles, the pellet was collected and washed in PBS to remove free mCherry-LC3B and then pellet again.

1.6 The pellet was then resuspended in PBS and filtered to remove clumps prior to vitrification, analyses by confocal microscope or by flow cytometry sorting.

1.7 Flow cytometry sorting was performed with a 70 µm nozzle on a BD FACSAria III with standard calibrations by fluorescence beads. A negative control without the tagged proteins was included as background control. Vesicles < ~1µm diameter were sorted and collected in PBS, and were applied to dot blots or processed for LC-MS/MS in triplicates.

2 Label-free quantitative LC-MS/MS

2.1 Proteins were reduced and alkylated in SDC buffer (1% Sodium deoxycholate, 40 mM 2-Cloroacetamide (Sigma), 10 mM TCEP (Thermo) in 100 mM Tris pH 8.0) for 20 min at 37 °C.

2.2 The samples were diluted with MS grade water (VWR) and digested overnight at 37 °C with 1 µg Lys-C (Labchem-wako) and 2µg trypsin (Promega), followed by acidification with Trifluoroacetic acid (Merck) to 1% (pH < 2).

2.3 The samples were purified via Sep-Pak Vac 1cc (50mg) tC18 Cartridges (Waters GmbH) with 0.1M acetic acid (Roth) wash and eluted with 80% Acetonitrile and 20mM acetic acid (Roth), and vacuum dried for resuspension in Buffer A (0.1% (v/v) Formic acid (Roth)).

2.4 Peptides were then loaded onto a 30-cm column (packed with ReproSil-Pur C18-AQ 1.9-micron beads, Dr. Maisch GmbH) via the Thermo Easy-nLC 1200 autosampler (Thermo) at 60°C.

2.5 Using the nano-electrospray interface, peptides were sprayed onto the Orbitrap MS Q Exactive HF (Thermo) in buffer A at 250 nl/min, and buffer B (80% Acetonitril, 0.1% Formic acid) was ramped to 30% in 60 min, 60% in 15 min, 95% in 5 min, and finally maintained at 95% for 5 min.

2.6 MS was operated in a data-dependent mode with survey scans 300 - 1650 m/z (resolution of 60000 at m/z =200), and up to 10 top precursors were selected and fragmented using higher energy collisional dissociation (HCD with a normalized collision energy value at 28).

2.7 The MS2 spectra were recorded at a resolution of 30000 (at m/z = 200). AGC target for MS and MS2 scans were set to 3E6 and 1E5 respectively within a maximum injection time of 100 and 60 ms for MS and MS2 scans respectively. Dynamic exclusion was set to 30ms.

2.8 Raw MS data were processed using the MaxQuant platform (Cox and Mann, 2008) with standard settings, and searched against the reviewed Human or Mouse Uniprot databases, as well as mCherry-LC3B and polyQ-GFP sequences, allowing precursor mass deviation of 4.5 ppm and fragment mass deviation of 20 ppm.

2.9 MaxQuant by default enables individual peptide mass tolerances. Cys carbamidomethylation was set as static, and Ub, Met oxidation and N-terminal acetylation as variable modifications.

2.10 Protein abundances within a sample were calculated using iBAQ intensities (Schwanhausser et al., 2011) and were quantified over the samples using the LFQ algorithm (Cox et al., 2014) for analysis with the Perseus software (https://maxquant.net/perseus/).