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Characterizing spatial and temporal properties of ER-phagy receptors

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Melissa Hoyer[1], Harper JW[1]

[1]Harvard Medical School

Harper ASAP

Melissa Hoyer
Harvard Medical School

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We use this protocol and it's working

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Abstract

The endoplasmic reticulum (ER) has a vast proteomic landscape to preform many diverse functions including protein and lipid synthesis, calcium ion flux, and inter-organelle communication. The ER proteome is remodeled in part through membrane-embedded receptors linking ER to degradative autophagy machinery (selective ER-phagy). A refined tubular ER network is formed in neurons within highly polarized dendrites and axons. Autophagy-deficient neurons *in vivo*display axonal ER accumulation within synaptic ER boutons, and the ER-phagy receptor FAM134B has been genetically linked with human sensory and autonomic neuropathy. However, mechanisms and receptor selectivity underlying ER remodeling by autophagy in neurons is limited. Here, we combine a genetically tractable induced neuron (iNeuron) system for monitoring extensive ER remodeling during differentiation with proteomic and computational tools to create a quantitative landscape of ER proteome remodeling via selective autophagy.Using spatial sensors or ER-phagy we demonstrate receptor-specific autophagic capture of ER in axons.

Materials

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Cells lines		
	NGN2 inducible embryonic stem cells	See protocol dx.doi.org/10.17504/protocols.io.br9em93e	CVCL_9773 (modified from this source line)
	HEK293T cells	ATCC CRL-1573	CVCL_0045
	Chemicals		
	Dulbecco's MEM (DMEM), F12		
	Phosphate Buffered Saline 1X	Corning	21-031-CV
	E8 components	See protocol dx.doi.org/10.17504/protocols.io.bsacnaaw	
	ND1 and ND2 components	See protocol dx.doi.org/10.17504/protocols.io.br9em93e	
	Hygromycin B	Life Technologies	10687-010
	pAC150- FAM134C-GFP	This paper	Addgene 201932
	pAC150- TEX264-GFP	This paper	Addgene 201931
	pAC150- TEX264(deltaLIR, F273A)-GFP	This paper	Addgene 201930
	pCMV-hyPBase hyperactive piggyBac vector	Yusa et al 2011	Available upon request at the Sanger Institute Achives
	pHAGE-TEX264-GFP	An et al 2019	Addgene 201925
	pHAGE-TEX264(deltaLIR,F273A)-GFP	An et al 2019	Addgene 201926
	pHAGE-mCherry-LC3B	An et al 2019	Addgene 201924
	Software		
	Nikon Imaging Software Elements	5.21.3 (Build 1489)	https://cellprofiler.org/ SCR_014329

	A	B	C
	Cell Profiler	CellProfiler v4.0.6	https://cellprofiler.org/ SCR_007358
	Fiji	ImageJ V.2.0.0	https://imagej.net/software/fiji/ SCR_002285
	Hardware		
	Thermo Neon™ Transfection System	Thermo Fisher Scientific	MPK5000
	Yokogawa CSU-X1 spinning disk confocal on a Nikon Ti-E inverted microscope		

Troubleshooting

Generation of Stable Cell ES H9 Ngn2 line expressing ER receptors

1 Electroporation of PB vectors. Use ThermoFisher kit and ThermoFisher Neon Electroporator to electroporate ES cells with PB vector and PB helper vector.

2 Add 10microliter buffer R to a sterile 1.5ml tube. Add 1.0mg of pAC150- TEX264-GFP, pAC150-TEX264deltaLIR-GFP, or pAC150- FAM134C-GFP vectors and 1.0mg of pCMV-hyPBase hyperactive piggyBac vector. Pipet up and down to mix. Let it sit at RT for 10min. This is enough for 2 transfections (== one 6 well).

3 Individualize cells with Accutase. Neutralize Accutase with 5 times volume E8 with Rock inhibitor.

4 Count cells. You will need 2×10^5 for each transfection.

5 Spin down cells. Let it sit for a while so all the residue media can go down to the bottom of the tube. If the residue media is too much, take it out with a P200 pipet.

6 Resuspend cells to a concentration of 2×10^5 per 5 microliter (ie 4×10^7 per ml) using buffer R. You don't have to take all the residue media off but you will need to take into account the volume of residue media so you are not too much off.

7 Prepare a 6 well matrigel coated plate. Add 2mL of E8+ rock inhibitor (1:1000) to the wells you will use. Two transfections go into one well.

8 Wipe the Neon pipet station with EtOH and place it inside the hood.

9 Add 3ml of electrolytic buffer (buffer E) to the neon tube. Place the tube inside the station. You should feel a click before the tube is securely seated in the station.

10 Use program 13 from the optimization tab for electroporation parameter (Voltage: 1100. Pulse width: 20 Pulse number: 2). Program 9 should also work.

11 When everything is ready, mix 10-11microliters of resuspended cells with the plasmid containing R buffer. The final volume should be in the range of 21-22microliters.

12 Take up a neon tip, pipet 10microliters of the cell protein mix and electroporate with program 13. It is important to pipet slowly to avoid air bubble formation. It is also important to insert the pipet slowly into the station, especially during the end of the

insertion when you will feel a click. Help the pipet down slowly during the clicking so there is no sudden movement of the tip, which might create tiny air bubbles.

13 If you see air bubble in the tip, take it out, push everything out of the tip and re-pipet the mixture.

14 If you see sparking during the electroporation, your efficiency will reduce significantly.

15 Once electroporation is complete, push everything into one well of a 6 well plate. Do not pipet up and down with Neon tip.

16 Repeat the same procedure with the same tip and the left over cell mixture. Place the second electroporated mixture into the same well.

17 Disperse cells evenly in the well and place cells in a low O2 incubator. 17. Put electroporated cells into low oxygen incubator for 2 days

18 Start selection of cells with 50mg/mL hygromycin B 4 days post-electroporation. Grow cells in selection medium for 7-10 days until there is no longer any cell death and every cell has integration. Cells can also be sorted for equal amount of GFP expression.

Differentiation of Stable Cell ES to induced neurons (iN) for imaging

19 Differentiation to induced neurons (iN) is done by following the protocol “Neural differentiation of AAVS1-TREG3-NGN2 pluripotent stem cells (**dx.doi.org/10.17504/protocols.io.br9em93e**)

19.1 To help get spread out iNeurons for imaging, when cells get 90 percent confluent at any point in the day 5-7 range, cells are plated into onto 35 mm-glass bottom dishes (No. 1.5, 14 mm glass diameter, MatTek) at a low confluency (approximately 1×10^5 cells per 2mL dish).

Lentiviral iN transduction

20 LC3B tagged lentiviral pHAGE constructs (or if not using a stable piggyBac ER-phagy receptor line, co transduced with ER-phagy receptor lentiviral constructs) were packaged in HEK293T by cotransfection of pPAX2, pMD2 and the vector of interest in a 4:2:1 ratio using polyethelenimine (PEI). Normally a 6cm dish of 70-80% confluent HEK293T was transfected.

21 The next day the media on the transfected HEK293T cells is switched to ND2 no doxycycline media. If 6cm of HEK293T cells was transfected, 5mL of the ND2 no DOX

media is added.

22 The following day, virus containing supernatant (ND2 media with virus) was and filtered through a .22 micron syringe filter, aliquoted into epi tubes and frozen at -80C.

23 Target induced neurons are cultured to day10. After routine ND2 no doxycycline feeding cells were treated with varying amounts of the virus-containing media (for 20-40 percent iN confluent 2mL imaging dish, 300-500 microliters of ER-phagy receptor lentivirus media was added and 100-200 microliters of mCh-LC3B lentivirus media was added).

Analysis of autophagosomes and ER-phagy receptors in iN via confocal microscopy

24 Cells are imaged at various days in the differentiation live at 37C using a Yokogawa CSU-X1 spinning disk confocal on a Nikon Ti-E inverted microscope at the Nikon Imaging Center in Harvard Medical School. Nikon Perfect Focus System was used to maintain cell focus over time. The microscope is equipped with a Nikon Plan Apo 40x/1.30 N.A or 100x/1.40 N.A objective lens. 488nm (100mW) and 561nm (100mW) laser lines are controlled by AOTF.All images are collected with a Hamamatsu ORCA-ER cooled CCD camera (6.45 μm^2 photodiode) with Nikon Elements image acquisition software. Timelapses with various lengths and intervals were acquired.

25 Each channel time series are brightness and contrast adjusted equally and then converted to RGB for publication using FIJI software.

Protocol references

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