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Cell line construction and maintenance for Lyso-IP and Endo-IP analysis of amyloid precursor protein processing, version 2 V.2

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We use this protocol and it's working

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Disclaimer

Abstract

Lyso-IP is a method that allows for the isolation of lysosomes for proteomics and metabolomics (**dx.doi.org/10.17504/protocols.io.bybjpskn**; **dx.doi.org/10.17504/protocols.io.bx9hpr36**). We have developed an analogous approach for purification of early/sorting endosomes (Endo-IP). In addition, we have found that endolysosomal purification via Lyso-IP and Endo-IP can be coupled to a quantitative proteomics workflow to obtain snapshots of Amyloid Precursor Protein (APP) processing to its Aβ products (Park et al. 2022). Here, we describe methods for cell line construction and maintenance of 293 cells with TMEM192-3xHA and 3xFLAG-EEA1, which are used for lysosome and endosome purification, respectively. of patient mutations to APP promotes processing. Cells with endogenously-tagged TMEM192 and stably expressing FLAG-EEA1 are referred to as 293^{EL} cells, for **E**ndo-IP and **L**yso-IP. These cells were also prepared in a form that has a deletion of the APP gene (293^{EL} $APP^{-/-}$) and the same cells reconstituted with a lentivirus stably expressing a patient mutant form ($APP^{Sw;T700N}$) which promotes APP processing and allows functional analysis of APP processing.

Materials

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Chemicals, peptides, and recombinant proteins		
	Puromycin	Sigma-Aldrich	P9620
	G418 (Geneticin)	Invivogen	ant-gn-2
	Dulbecco's MEM (DMEM), high glucose, pyruvate	GIBCO / Invitrogen	11995
	Experimental models: Cell lines		
	293T cells	ATCC	CRL-3216
	293 cells	ATCC	CRL-1573
	293L: TMEM192-3xHA	This study	
	293L-APP-/-: TMEM192-3xHA; APP-/-	This study	
	293EL-APP-/-: TMEM192-3xHA; APP-/-; FLAG-EEA1	This study	
	293EL-APP∗: TMEM192-3xHA; APP-/-; FLAG-EEA1; APPSw;T700N	This study	
	Recombinant DNA		
	pSMART TMEM192-3xHA (targeting vector for genomic tagging)	35	Addgene #175777
	pHAGE-FLAG-EEA1	This study	Addgene #176491
	pHAGE-FLAG-RAB11A	This study	Addgene #176489
	pPHAGE-FLAG-TFR1	This study	Addgene #176490
	pPHAGE-FLAG-RAB5A	This study	Addgene #176488
	pX459-gRNA-APP (for making APP deletion	This study	Addgene #176487

	A	B	C
	by CRISPR/Cas9)		
	pENTR221-APP751	DNA Resource Core, Harvard Medical School	HsCD00431993
	pHAGE-APPSw;T700N	This study	Deposited in Addgene

Cell line maintenance

1 Maintain 293 cells in Dulbecco' Modifies Eagles Medium (DMEM) with 10% fetal bovine serum and optional 1% penicillin-streptomycin. Additionally, grow 293^{EL} cells in 1.2 µg/ml puromycin and 200 µg/ml G418 to maintain selection for 3xFLAG-EEA1 and TMEM192-3XHA, respectively.

Endogenous tagging of TMEM192 with 3xHA

2 For endogenous tagging of TMEM192 with 3xHA, co-transfect 293 cells with pX459 containing a gRNA (5'-AGTAGAACGTGAGAGGCTCA) targeting adjacent to the translational termination sequence in TMEM192 and pSMART containing 5' and 3' homology arms for TMEM192 in which the termination codon is replaced by a 3xHA epitope sequence followed by a TAA stop codon (Addgene #175777).

3 Identify homozygously targeted clones by immunoblotting cell extracts with α-HA and α-TMEM192. These are referred to as 293^{L} cells for Lyso-IP.

Stable expression of 3xFLAG-EEA1

4 Generate puromycin-resistant pHAGE lentiviral vectors expressing EEA1 by recombining open reading frames in pENTR vectors into a pHAGE-N-3xFLAG vector.

5 Infect 293^{L} cells with viral supernatants derived from transfection of 293T cells with pHAGE-3xFLAG-EEA1 vector (Addgene #176491) and appropriate packaging and envelope vectors. Select for viral integration with puromycin (1.2 µg/ml). Select a monoclonal population. These are referred to as 293^{EL} cells for Endo-IP and Lyso-IP.

APP knock-out

6 For APP knock-out, phosphorylate and anneal oligonucleotides (Top: 5'-CACCGGCGGAATTGACAAGTTCCGA, Bottom: 5'-AAACTCGGAACTTGTCAATTCCGCC), and clone into a pX459 vector.

7 Transfect 293^{EL} cells with the pX459-gRNA-APP plasmid (Addgene #176487) with Lipofectamine 3000, and select with 1.2 µg/mL of puromycin. Select monoclonal population, and confirm APP deletion by Western blotting.

Stable expression of $APP^{sw,T700N}$

8 To create an APP (isoform 751) open reading frame: PCR amplify pENTR-APP751 (open, no stop codon) to replace W752 with a stop codon using forward primer (5'-

GCAGAACTAGATCCACCCAGCTTTCTTG) and reverse primer (5'-GGGTGGATCTAGTTCTGCATCTGCTCAAAG).

9 Use two rounds of PCR to generate pENTR-APP^{Sw,T700N} using the following kits and primers: Sw (K651M/N652L): QuickChange II mutagenesis kit; Forward: 5'-tcggaattctgcatccagattcacttcagagatctcctccg; Reverse: 5'-cggaggagatctctgaagtgaatctggatgcagaattccga; T700N: Q5 mutagenesis kit; Forward: 5'-ATCGTCATCAACTTGGTGATG; Reverse: 5'- CACTGTCGCTATGACAAC. Transfer the APP^{Sw,T700N} open reading frame in pENTR to Gateway destination vector pHAGE-C-HA-FLAG-puro using Clonase to yield pHAGE-APP^{Sw,T700N}-puro.

Note: the stop codon in the APP open reading frame blocks translation into the HA-FLAG tag in this vector.

10 Prepare a stable cell line by lentiviral transduction of APP^{Sw,T700N} to 293^{EL} APP$^{-/-}$ cells followed by monoclonal selection.