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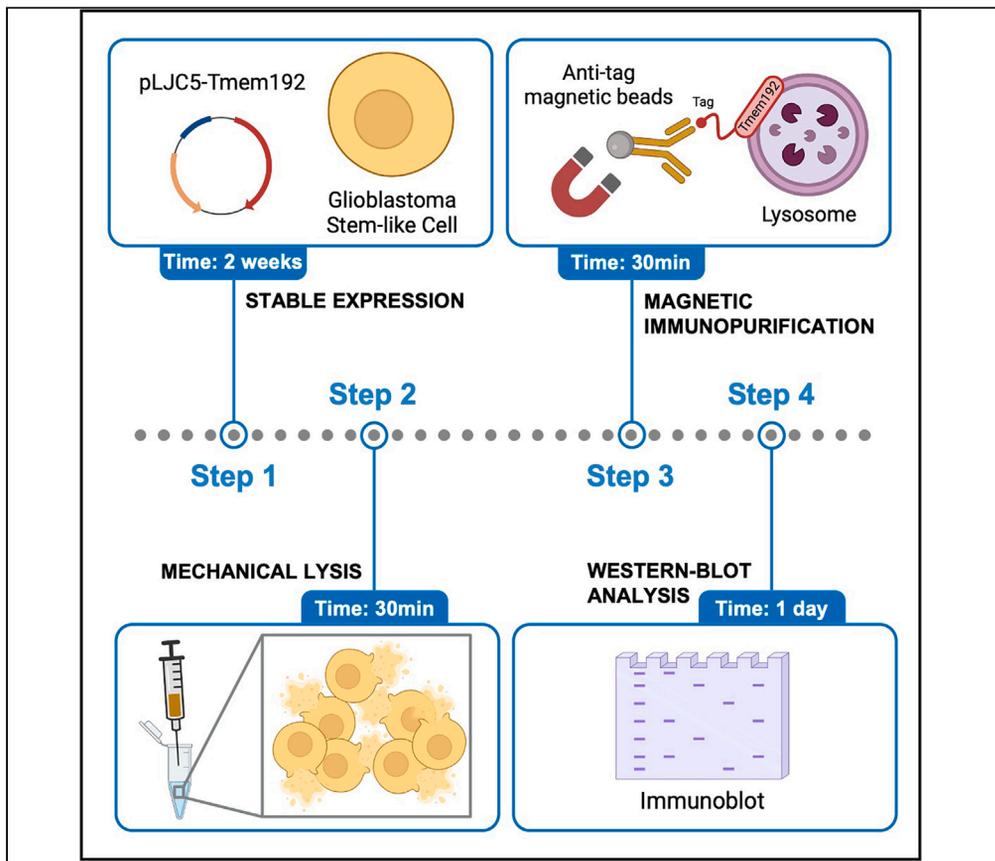
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Protocol

Protocol for qualitative analysis of lysosome immunoprecipitation from patient-derived glioblastoma stem-like cells



Lysosomes are critical for the sustenance of glioblastoma stem-like cells (GSCs) properties. We present a protocol to enrich and purify lysosomes from patient-derived GSCs in culture. We describe the steps required to stably express a tagged lysosomal protein in GSCs, mechanically lyse cells, magnetically immunopurify lysosomes, and qualitatively assess these organelles. We then detail the procedure for retrieving intact and purified lysosomes from GSCs. We also specify cell culture conditions, storage procedures, and sample preparation for immunoblotting.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generating patient-derived glioblastoma cells expressing lysosomal tagged proteins

Retrieving intact organelles from mechanical cell lysis

Isolating intact lysosomes with magnetic bead immunoprecipitation

Evaluating the qualitative features of purified lysosomes with western blots

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Protocol

Protocol for qualitative analysis of lysosome immunoprecipitation from patient-derived glioblastoma stem-like cells

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SUMMARY

Lysosomes are critical for the sustenance of glioblastoma stem-like cells (GSCs) properties. We present a protocol to enrich and purify lysosomes from patient-derived GSCs in culture. We describe the steps required to stably express a tagged lysosomal protein in GSCs, mechanically lyse cells, magnetically immunopurify lysosomes, and qualitatively assess these organelles. We then detail the procedure for retrieving intact and purified lysosomes from GSCs. We also specify cell culture conditions, storage procedures, and sample preparation for immunoblotting.

For complete details on the use and execution of this protocol, please refer to Maghe et al.¹

BEFORE YOU BEGIN

Operating at the crossroad between anabolism and catabolism, lysosomes play a critical role for maintaining cellular equilibrium. Mutations in lysosomal hydrolase or transporter genes generate a group of diseases called lysosomal storage disorders (LSDs).² Moreover, by influencing key signaling complexes on their surface, lysosomes have been linked to the onset of cancer development, metabolic diseases, and aging.³ Thus, defining their composition and alterations occurring during disease initiation and evolution emerges as imperative.

The empirical method for lysosome enrichment consists in the setup of a density gradient and differential centrifugation. Implemented in the mid-fifties, this approach allowed the pioneer visualization of lysosomes.⁴ While still in use to notably discriminate between different populations of similar organelles, this long lasting and heavy equipment-requiring method is not optimal in terms of purification yield and reproducibility. Alternatively, intra-lysosomal loading of iron nanoparticles can confer higher enrichment rates and purity, with the caveat of introducing chemicals with potential harmful actions on the native lysosomal environment.⁵ With the need for a rapid and specific method to isolate intact lysosomes, which remain suitable for further omics studies, the lysosome immunoprecipitation (LysolIP) technique was initially introduced in well-established cell lines such as epithelial HEK293T.⁶ This allowed for a qualitative and quantitative description of the lysosomal compartment.⁶

The protocol below details the different steps to immunopurify lysosomes (LysolIP) from patient-derived glioblastoma stem-like cells (GSCs). It is noteworthy that this protocol can be applied to



other human cell types, by adapting and adjusting specific cell culture, lysis, and immunoprecipitation conditions.

In addition to the institutional permissions, several materials and reagents need to be anticipated (including cells, plasmids, magnet and magnetic beads, and lab specific equipment).

Institutional permissions

This protocol requires prior subject consent and ethical approval for patient-derived human cell culture.

For the procedure described here, cells were cultured according to the French Ministry of Higher Education and Research rules under the #DUO10524 authorization. Moreover, all subjects have given their informed consent. This study was approved by the institutional review boards of Laennec Hospital, Nantes, France, and performed in accordance with the Declaration of Helsinki Protocol.

Culture of patient-derived GSCs

⌚ Timing: >30 min

1. Prepare the serum-free and growth factor-enriched “NS34” media for routine culture of GSCs.
 - a. In a sterile environment, filter (pore size of 0.22 μm) a mix of DMEM-F12, Glutamax, penicillin/streptomycin, amphotericin B, and N2, G5, and B27 supplements.
 - b. Store at 4°C and protected from light.

Note: NS34 media should be warmed at 37°C before use. The use of antibiotics is optional.

2. Routinely culture GSCs.
 - a. Harvest and centrifuge GSCs 3 min at 500 $\times g$, 20°C–22°C, every 2 or 3 days.
 - b. Leave 500 μL of previous culture medium on the top of cell pellets.
 - c. Resuspend GSC pellets by slowly pipetting up and down at least 20 times with a 1 mL filter-tip pipette.

Note: Make sure that the resuspension step is complete with the obtention of fully isolated cells and less than 5% cell death, as visualized with trypan blue. For initial isolation and characterization of patient-derived GSCs, please refer to Harford-Wright et al.⁷

Note: Cells are handled under safety cabinet class 2 and incubated at 37°C with 5% CO₂ in a standard tissue culture humidified incubator.

- d. Count GSCs using a hemacytometer or cell counter, such as Countess Automatic Cell Counter from Thermo Fisher Scientific.
- e. In a sterile T75 culture flask, add 1×10^6 GSCs per 10 mL NS34.

Note: Growth rate may vary and culture condition may need to be optimized in terms of cell number and passages.

Note: GSCs grow as non-adherent floating spheres.

Preparation of buffers

⌚ Timing: 30 min

3. Prepare the Triton lysis buffer for whole cell lysates.
 - a. Mix 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% NP-40, and 1% igepal, in a beaker.
 - b. Adjust the pH to 7.4.
 - c. Transfer the buffer to a volumetric flask and adjust to 500 mL with ddH₂O.
 - d. Transfer in a clean and sterile glass bottle and store at 4°C.

Note: Supplement the Triton lysis buffer with the 100× anti-protease cocktail before use. We recommend to prepare an aliquot in a 50 mL conic tube to be discarded at the end of the experiment.

4. Prepare the LysoIP elution buffer.
 - a. Dissolve 1 g of SDS in a beaker containing 40 mL ddH₂O using a magnetic stirrer.
 - b. Add 2.5 mL of Tris-HCl.
 - c. Adjust the pH to 8.5.
 - d. Transfer the buffer to a volumetric flask and adjust the volume to 50 mL with ddH₂O.
 - e. Transfer in a clean and sterile glass bottle and store at 4°C.

Note: Warm up at 20°C–22°C the LysoIP elution buffer before use to avoid crystallization.

5. Prepare the 2× HBS buffer.
 - a. Dissolve 1 g of NaCl in a beaker containing 80 mL ddH₂O using a magnetic stirrer.
 - b. Add 5 mL of HEPES and 1.5 mL of NaH₂PO₄.
 - c. Adjust the pH to 7.05.
 - d. Transfer the buffer to a volumetric flask and adjust the volume to 100 mL with ddH₂O.
 - e. Filter through a 0.22 μm pore size membrane in a sterile environment.
 - f. Transfer in an autoclaved glass bottle and store at 4°C.
6. Prepare the 2× SDS sample buffer.
 - a. Discard 2 mL from a 50 mL 2× SDS sample buffer bottle.
 - b. Add 2 mL of 2-mercaptoethanol and mix.
 - c. Prepare 2 mL aliquots.

Note: 2-mercaptoethanol-supplemented 2× SDS sample buffer can be stored at 4°C for two weeks and at –20°C for several months.

△ CRITICAL: 2-mercaptoethanol is harmful, necessitating the use of specific equipment such as gloves, lab coat, and eyes protection. Moreover, 2-mercaptoethanol has to be manipulated under a chemical hood.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CALRETICULIN 1:1,000	Cell Signaling Technology	Cat#12238; RRID:AB_2688013
CATHEPSIN D 1:1,000	BD Biosciences	Cat#610800; RRID:AB_398119
EEA1 1:1,000	BD Biosciences	Cat#610456; RRID:AB_397829
FLAG 1:1,000	Cell Signaling Technology	Cat#F1804; RRID:AB_262044
GM130 1:1,000	Abcam	Cat#ab52649; RRID:AB_880266
HA 1:10,000	Cell Signaling Technology	Cat#3724; RRID:AB_1549585
LAMP2 1:5,000	Santa Cruz Biotechnology	Cat#sc-18822; RRID:AB_626858
VDAC 1:1,000	Cell Signaling Technology	Cat#4661; RRID:AB_10557420
Goat anti-mouse IgG1, human ads-HRP 1:5,000	SouthernBiotech	Cat#1070-05

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Goat anti-rabbit IgG (H + L), mouse/human ads-HRP 1:5,000	SouthernBiotech	Cat#4050-05
Goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 546 1:500	Life Technologies	Cat#A-11035
Goat anti-mouse IgG1 cross-adsorbed secondary antibody, Alexa Fluor 488 1:500	Life Technologies	Cat#A-21121
Biological samples		
Patient-derived glioblastoma stem-like cells GSC#9	Harford-Wright E. et al. (2017)	N/A
Experimental models: Cell lines		
Human embryonic kidney cells HEK293T	ATCC	Cat#CRL-3216; RRID:CVCL_0063
Chemicals, peptides, and recombinant proteins		
Tris-HCl	Millipore	Cat#648313
Chloroquine	Sigma	Cat#C6628
NaCl	VWR	Cat#27810.295
Polybrene	Santa Cruz Biotechnology	Cat#sc-134220
EDTA	Millipore	Cat#324504
Triton X-100	Sigma	Cat#T9284
IGEPAL	Sigma	Cat#I8896
SDS 20%	Euromedex	Cat#EU0660-B
HEPES	Life Technologies	Cat#12104123
NaH ₂ PO ₄	Sigma	Cat#71507
CaCl ₂	Sigma	Cat#21115
2-mercaptoethanol	Carl Roth	Cat#4227
DAPI solution	Life Technologies	Cat#62248
Recombinant DNA		
pLJC5-Tmem192-3×HA	Addgene	Cat#102930; RRID:Addgene_102930
pLJC5-Tmem192-2×FLAG	Addgene	Cat#102929; RRID:Addgene_102929
psPAX2	Addgene	Cat#12260
pCMV-VSV-G	Addgene	Cat#8454
Other		
N2 supplement	Life Technologies	Cat#17502048
G5 supplement	Life Technologies	Cat# 17503012
B27 supplement	Life Technologies	Cat#17504044
100× Halt protease inhibitor cocktail	Life Technologies	Cat#78429
Pierce anti-HA magnetic beads	Life Technologies	Cat#88837
DMEM-F12	Life Technologies	Cat#10565018
DMEM	Life Technologies	Cat#11995
0.05% Trypsin-EDTA	Life Technologies	Cat#2500054
Penicillin/streptomycin solution	Life Technologies	Cat#15140130
Trypan blue stain 0.4%	NanoEntek	Cat#20230411
Amphotericin B	Life Technologies	Cat#15290026
Puromycin	Life Technologies	Cat#J67236.8EQ
GlutaMAX	Life Technologies	Cat#35050061
Magnet DynagMag-2	Thermo Fisher Scientific	Cat#12321D
SDS sample buffer	Life Technologies	Cat#LC2676
Fetal bovine serum (FBS)	Corning	Cat#35079CV
Paraformaldehyde	Electron Microscopy Sciences	Cat#15710; CAS: 30525-89-4
Triton X-100	Merck Millipore	Cat#T9284; CAS: 9036-19-5
Sterile 0.5/1 mL insulin syringe with 12.7 mm 29-gauge needle (33/100)	BD Micro-Fine	Cat#324892

MATERIALS AND EQUIPMENT

NS34 culture media

Reagent	Final concentration	Amount
Glutamax (100×)	200 μM	5 mL
Penicillin/streptomycin (100×)	100 units each	5 mL
Amphotericin B (250 μg/mL)	0.25 μg/mL	500 μL
N2 supplement (100×)	1×	5 mL
G5 supplement (100×)	0.2×	1 mL
B27 supplement (50×)	1×	10 mL
DMEM-F12	N/A	qsf 500 mL
Total	N/A	500 mL

Note: NS34 culture media can be stored at 4°C for several weeks.

HEK293T culture media

Reagent	Final concentration	Amount
DMEM	N/A	445 mL
Glutamax (100×)	200 μM	5 mL
Penicillin/streptomycin (100×)	100 units each	5 mL
Heat inactivated FBS	1×	50 mL
Total	N/A	500 mL

Note: HEK293T culture media can be stored at 4°C for several weeks.

Triton lysis buffer

Reagent	Final concentration	Amount
Tris-HCl (1 M)	50 mM	25 mL
NaCl (5 M)	150 mM	15 mL
EDTA (0.6 M)	2 mM	1.66 mL
Triton X-100	1%	5 mL
Igepal	1%	5 mL
ddH ₂ O	N/A	qsf 500 mL
Total	N/A	500 mL

Note: Triton lysis buffer can be stored at 4°C for several months.

LysolIP elution buffer

Reagent	Final concentration	Amount
Tris-HCl (1 M)	50 mM	2.5 mL
SDS	2%	1 g
ddH ₂ O	N/A	qsf 50 mL
Total	N/A	50 mL

Note: LysolIP elution buffer can be stored at 4°C for 6 months.

2× HBS buffer

Reagent	Final concentration	Amount
HEPES (1 M)	50 mM	5 mL
NaCl (5 M)	280 mM	1 g
NaH ₂ PO ₄ (100 mM)	1.5 mM	1.5 mL
ddH ₂ O	N/A	qsf 100 mL
Total	N/A	100 mL

Note: 2× HBS buffer can be stored at 4°C for 2 months.

Table 1. Transfection mix

Reagent	Final concentration	Amount
Sterile ddH ₂ O	N/A	150 μ L
pLJC5-TMEM-192xtag (HA or FLAG)	12.5 ng/ μ L	5 μ g
pPAX2	5 ng/ μ L	2 μ g
pVSV-g	3.75 ng/ μ L	1.5 μ g
CaCl ₂ (1 M)	125 mM	50 μ L
2 \times HBS buffer	1 \times	200 μ L
Total	N/A	400 μL

STEP-BY-STEP METHOD DETAILS

Stable expression of the tagged lysosomal protein TMEM-192

⌚ **Timing: 2 weeks**

The first step involves the stable expression of the tagged lysosomal protein TMEM-192 in GSCs. This allows the isolation of lysosomes, without major alteration in their quality.

Note: Alternate lysosome-resident transmembrane proteins can be tagged and expressed to isolate lysosomes. However, their ectopic expression may affect lysosomal and cellular functions. Here, we recommend the tagged lysosomal transmembrane protein TMEM-192, based on the original, pioneer work from Abu-Remaileh et al.⁶

⚠ CRITICAL: The production, use, storage, and elimination of the lentiviral particles have to be performed in compliance with the health and safety laws of the legal entities and institutions.

1. Prepare the media for routine culture of HEK293T.
 - a. In a sterile environment, mix DMEM, Glutamax, penicillin/streptomycin, and heat inactivated FBS.

Note: HEK293T media should be warmed at 37°C before use.

2. Routinely culture HEK293T in 10 cm dish.
 - a. When cells reach more than 80% confluency, typically every 2 or 3 days, remove media from the 10 cm dish by aspiration and wash with 5 mL of PBS.
 - b. Add 2 mL trypsin and put the plate in an incubator at 37°C for 2–3 min.
 - c. Add 10 mL of fresh heated media to a new 10 cm dish.
 - d. Visualize cell detachment from the plate under a microscope.
 - e. When cells are all detached, add 4 mL of fresh heated media to the re-suspended cells.
 - f. Dissociate cell aggregates with 5–10 up and down using a P1000 and transfer 1 mL of cells to the new 10 cm dish (0.5 mL if passage has to be performed 3 days later).
3. Prepare the lentiviral producing cells.
 - a. Seed two wells of a 6-well plate, each with 1×10^6 HEK293T in 2 mL complete media.
 - b. Incubate for 24 h.
4. Transfect HEK293T for lentiviral production.
 - a. In two separate 1.5 mL sterile tubes, prepare the transfection mixes (Table 1), one containing the HA-tag plasmid, the other the FLAG-tag plasmid, following strictly the indicated order.

Note: At this step, plasmid DNA precipitated with CaCl₂.

⚠ CRITICAL: Do not vortex the mix.

- b. Replace the HEK293T complete media with 2 mL media supplemented with chloroquine (25 μ M) to inhibit lysosomal degradation of the transfected plasmid.
- c. Add the transfection mix dropwise.
- d. Washout chloroquine-containing media after 16 h and replace with 2 mL complete media.

△ CRITICAL: At this step, cells are already producing lentiviral particles. Make sure to handle all the materials in agreement with health and safety rules.

- e. 24 h later, harvest media and centrifuge at 500 \times g for 5 min, 20°C–22°C, to pellet cells.
5. Infect GSCs with the supernatants containing lentiviral particles.
 - a. In three wells of a 12-well plate, seed 3 \times 10⁵ GSCs each in a maximum volume of 50 μ L NS34.
 - b. Add polybrene to a final concentration of 8 μ g/mL.
 - c. Add 500 μ L of the supernatant containing lentiviral particles to GSCs (wells 2 and 3), or 500 μ L of NS34 to control cells (well 1).

Note: The first condition (well 1) serves as a positive control for selection. No lentiviral infection is performed. The second and third wells received either FLAG- or HA-tagged TMEM-192 containing lentiviral particle supernatants.

Note: Lentiviral particles are usually not titrated. If needed, please refer to Star Protocols detailed by Brown et al.⁸

Note: Lentiviral particles are prepared freshly before each use.

- d. Centrifuge at 1,000 \times g for 1 h 30 min, 20°C–22°C.
 - e. Add 500 μ L of NS34 media to each well for 24 h.
 - f. Wash cells in 1 \times PBS by centrifugation at 500 \times g for 3 min, 20°C–22°C.
 - g. Resuspend the cell pellets in 1 mL of NS34 media for 24 h.
6. Select GSCs stably expressing the tag version of TMEM-192.
 - a. Replace media with NS34 media containing puromycin at a final concentration of 1 μ g/mL.
 - b. Culture infected GSCs with NS34 media containing puromycin until all control cells are dead.

Note: The selection process may take up to one week.

Optional: During the time of the cell selection process, conditions for the mechanical lysis can be set up (Please see step 6 in the '[glioblastoma stem-like cell harvesting, mechanical lysis, and organelle retrieval](#)' section).

7. Check for the expression and localization of the tagged TMEM-192 protein.
 - a. Check for the expression of the TMEM-tagged protein by western-blot using anti-HA and anti-FLAG antibodies ([Figure 1A](#)).

Note: Standard protocols for western-blot can be used. If needed, please refer to Star Protocols detailed by Andre-Gregoire et al.⁹

Note: The tagged protein should be resolved at around 37 kDa.

- b. Control the localization of the TMEM-192-tagged protein by immunofluorescent staining using anti-HA and anti-FLAG antibodies. Costain with LAMP1 or LAMP2 antibodies to ensure lysosomal localization ([Figure 1B](#)).

Note: Standard protocols for immunofluorescence staining of non-adherent cells can be used. If needed, please refer to detailed methods by Maghe et al.¹ Briefly, Triton

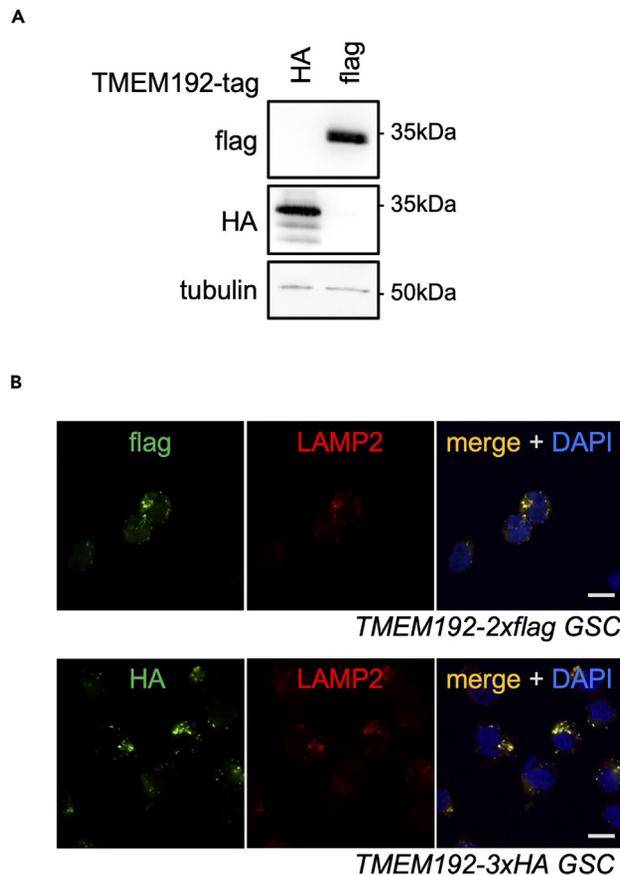


Figure 1. Stable expression and lysosomal localization of the TMEM192-tagged protein in GSCs

(A) Western-blot analysis of total cell lysates from GSCs stably expressing the TMEM192-tagged protein. Anti-flag and anti-HA antibodies were used to confirm the expression of the tagged protein. Tubulin served as a loading control. (B) Confocal analysis of LAMP2 (red) and flag/HA (green)-tagged TMEM192 in GSCs stably expressing the TMEM192-tagged protein. Nuclei are stained with DAPI (blue). Scale bars: 10 μ m.

X100-permeabilized and paraformaldehyde-fixed GSCs were incubated with primary antibodies at 1:200 in PBS-bovine serum albumin (BSA) 4% for 1 h at RT. Secondary Alexa Fluor-conjugated antibodies were incubated at 1:400 in PBS-BSA 4% for 1 h at RT.

Pause point: As selection was successful, puromycin can now be omitted for the expansion media. At that time, do not forget to expand GSCs stably expressing the tagged TMEM-192 protein and stock vials in liquid nitrogen. 3×10^6 GSCs can be frozen in 1 mL of a mix of FBS - 7.5% DMSO for several years. After defrosting vials, control that cells express the TMEM192-tag protein as in step 7.

Glioblastoma stem-like cell harvesting, mechanical lysis, and organelle retrieval

⌚ Timing: 30 min (excluding the setup of the lysis method)

In this step, we present a method to mechanically lyse glioblastoma stem-like cells to retrieve a consequent quantity of intact organelles, including lysosomes (Figure 2A).

8. Setup the ideal lysis method to retrieve the most intact organelles.

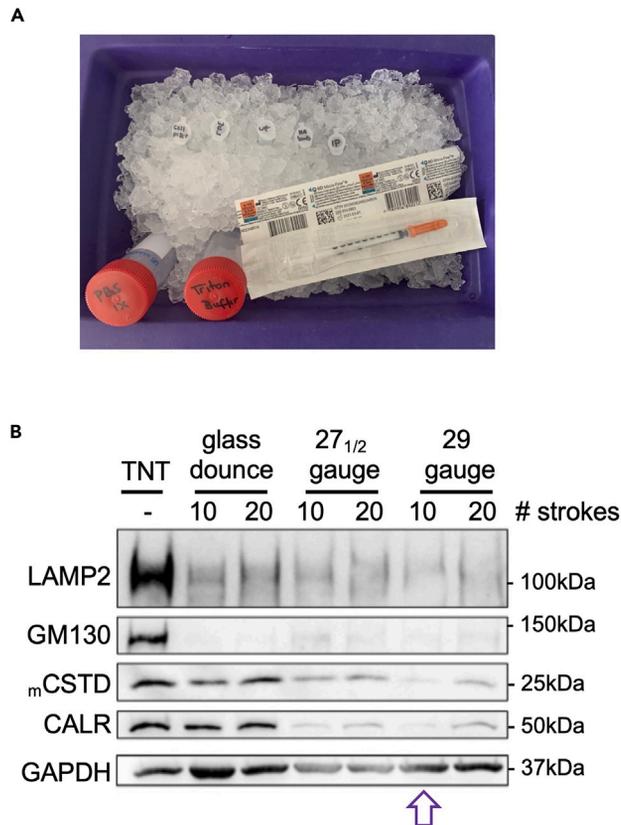


Figure 2. Mechanical lysis of GSCs

(A) Necessary materials for the mechanical lysis of GSCs.

(B) Western-blot analysis of the soluble fractions of mechanically lysed GSCs. GSCs were mechanically lysed as indicated, and the lysate was subjected to high speed (20,000 × g) centrifugation. Supernatants were collected and processed for western-blot analysis of the indicated proteins. The cytosolic GAPDH protein served as a loading control. Note that organellar proteins are almost absent from the 10 strokes, 29-gauge syringe condition.

△ **CRITICAL:** This step can be challenging and may require several trials. Make sure to anticipate ahead the time needed for the optimization.

△ **CRITICAL:** Buffers and tubes are kept on ice at all time to limit the degradation of protein samples.

- a. Harvest 15×10^6 GSCs per condition.
- b. Centrifuge GSCs at $1,000 \times g$ for 2 min, 4°C .
- c. Carefully remove the supernatant by pipetting it out or by slow aspiration from the opposite side of the tube.
- d. Resuspend the cell pellets in 1 mL $1 \times$ iced-cold PBS.
- e. Repeat steps b-c.
- f. Resuspend cell pellets in $500 \mu\text{L}$ $1 \times$ iced-cold PBS supplemented with anti-protease cocktail.
- g. Save $100 \mu\text{L}$ of the resuspended GSCs to serve as whole cell lysates and process them.
 - i. Centrifuge GSCs at $1,000 \times g$ for 2 min, 4°C .
 - ii. Carefully remove the supernatant by pipetting it out or by slow aspiration from the opposite side of the tube.
 - iii. Resuspend the cell pellets in $100 \mu\text{L}$ of iced-cold Triton lysis buffer supplemented with anti-protease cocktail.
 - iv. Incubate for 30 min on ice to ensure a complete lysis.

- v. Centrifuge cell lysates at $10,000 \times g$ for 10 min, 4°C , to pellet cellular debris.
- vi. Collect supernatants in a new 1.5 mL tube.
- vii. Samples can be stored at -20°C before analysis.

▮▮ Pause point: Samples can be stored at -20°C until processing for analysis.

- h. The conditions for lysing the remaining 400 μL of resuspended GSCs can be optimized with different tools and conditions. This includes glass dounce homogenizer, as described in the original publication of the LysolP method.⁶ Alternatively, syringes with varying needle diameter can be used. The number of strokes applied to the samples needs also to be optimized.

Note: We recommend starting with a range of 10–20 strokes.

- i. Perform a Trypan blue incorporation assay to ensure plasma membrane rupture.

Note: When more than 90% of cells are stained with Trypan blue, plasma rupture is considered effective enough to pursue.

- j. Centrifuge at $20,000 \times g$ for 10 min, 4°C .
- k. Collect the supernatant and store at -20°C before analysis.
- l. Perform western-blot analysis of the different lysates and assess lysis efficiency, as well as the integrity of intracellular organelles (Figure 2B).

Note: We recommend checking for the presence of cytosolic proteins (such as GAPDH, α -Tubulin) in the supernatant, indicative of cell rupture. The absence of lysosomal proteins (Cathepsin D, LAMP1, and LAMP2), as well as proteins associated with Golgi (GM130, Golgin-97), or Endoplasmic Reticulum (Calreticulin, PDI) provide evidence that the organelles were left intact and pelleted during the centrifugation.

As depicted in Figure 2B, the condition where 10 strokes of a 29-gauge syringe were applied to the cells was the most efficient as liberating GAPDH from the cell, without affecting intracellular organelles.

△ CRITICAL: The absence in the supernatant of intra-lysosomal proteases, such as Cathepsin D, is essential to validate the integrity of lysosomes.

- 9. Proceed to treatments and manipulations of the GSCs expressing the tagged TMEM-192 protein, according to the experimental design.

Note: FLAG-tagged TMEM-192 expressing GSCs serve as internal control of the HA immunoprecipitation. Cells do not thus need to be strictly manipulated as HA-tagged TMEM-192 GSCs (related to step 9).

△ CRITICAL: 15×10^6 GSCs per condition are required for the next step. Consider growing a maximum of 5×10^6 GSCs in 50 mL NS34 culture media in 3 independent T75 flasks (related to step 9).

Moreover, the percentage of cell viability resulting from step 9 may affect the efficiency of the mechanical lysis and result in a misinterpretation of the results. Do not allow cell death rate to reach up to 20%, as visualized with Trypan blue incorporation assay.

△ CRITICAL: Ensure that all subsequently required material remains at 4°C (related to step 10).

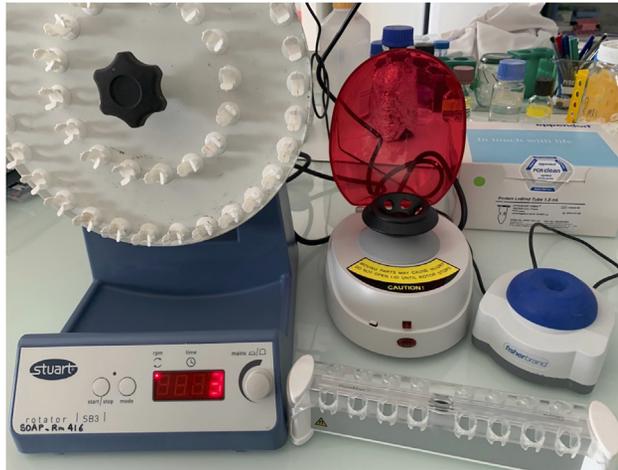


Figure 3. Equipment necessary to perform the magnetic immunoprecipitation of intact lysosomes from GSCs
Note that the procedure is performed in 4°C-cold room.

10. Process GSCs as in steps 8a–g.

Note: The mechanical lysis and immunoprecipitation (steps 11–13) should be launched during the 30 min of lysis.

11. Mechanically lyse the remaining 400 μ L of resuspended GSCs owing to the previously determined mechanical lysis method.

Note: We describe hereafter the optimized lysis method determined for GSCs.

Optional: To ensure a homogeneous temperature, the lysis step can be performed in a cold room maintained at 4°C.

a. Based on the results from step 8 optimization, apply 10 strokes of a 29-gauge syringe to the resuspended GSCs.

12. Pellet cellular debris.

a. Centrifuge at 1,000 \times g for 2 min, 4°C.

Note: Even if the pellet may appear consequent, the supernatant should remain cloudy, indicative of the presence of organelles.

Magnetic immunoprecipitation of the lysosomal compartment

⌚ **Timing:** 30 min

This step involves the use of magnetic anti-HA beads, allowing a rapid and specific isolation of lysosomes (Figure 3).

⚠ CRITICAL: To avoid a time-related degradation of the samples, prepare the beads before the mechanical lysis described in step 10. We recommend proceeding to the washes of the beads before the step 8 and save them in 1 \times iced-cold PBS supplemented with anti-protease cocktail until use.

13. Prepare the anti-HA magnetic beads.

- a. Per condition, dispense 75 μL of anti-HA magnetic beads in a new 1.5 mL tube.
- b. Add 500 μL 1 \times iced-cold PBS supplemented with anti-protease cocktail and vortex.
- c. Incubate the tubes on the magnet and wait for 10 s for the beads to be attracted, ([Methods video S1](#): Incubating on the magnet, related to step 13c).

△ CRITICAL: Ensure that the liquid is translucent, indicative that all the beads have attached to the side of the tube.

- d. Carefully remove the supernatant by pipetting it out from the opposite side of the tube, ([Methods video S2](#): Removing the supernatant, related to step 13d).
- e. Repeat steps b-d twice.

△ CRITICAL: Make sure to not let the magnetic beads dry for more than 10 min. Beads can stay in 1 \times iced-cold PBS supplemented with anti-protease cocktail during the time of mechanical cell lysis.

14. Mix the organelle-containing supernatant prepared in step 12 with the washed anti-HA magnetic beads.

Note: Collect 350 μL of the supernatant prepared in step 12 to add on to the magnetic beads to avoid withdrawing pelleted debris.

- a. Slowly pipette up and down to mix the beads with the organelles.
15. Incubate the mixed beads and organelle-containing supernatant on a rotating wheel for 15 min at 4°C.
16. Wash the magnetic beads.

- a. Place the tubes on the magnet and wait 10 s for the beads to be attracted.

△ CRITICAL: Work on ice, ideally in a cold room set at 4°C.

△ CRITICAL: Ensure that the liquid is translucent and that all the beads have attached to the side of the tube before pursuing.

- b. Carefully remove the supernatant by pipetting it out from the opposite side of the tube.
- c. Add 500 μL 1 \times iced-cold PBS supplemented with anti-protease cocktail and slowly pipet up and down to mix the beads.
- d. Repeat steps a-c two times.
17. Elute the immunopurified lysosomes.
- a. Place the tubes on the magnet and wait 10 s for the beads to be attracted.

△ CRITICAL: Work on ice, ideally in a cold room set at 4°C.

△ CRITICAL: Ensure that the liquid is translucent, indicative that all the beads have attached to the side of the tube.

- b. Carefully remove the supernatant by pipetting it out from the opposite side of the tube.
- c. Add 50 μL of preheated (20°C–22°C) LysolP elution buffer to the beads.
- d. Resuspend the beads by pipetting up and down.
- e. Heat the samples at 95°C for 5 min.
- f. Place the tubes on the magnet and wait 10 s for the beads to be attracted.

△ CRITICAL: Ensure that the liquid is translucent and that all the beads have attached to the side of the tube before pursuing.

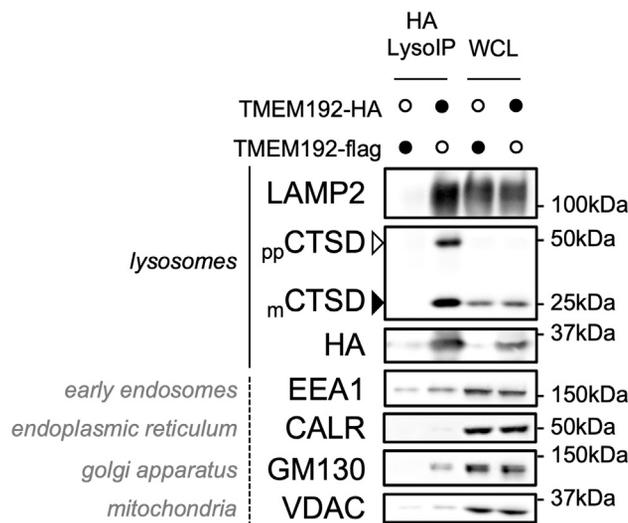


Figure 4. Enrichment and purity of immunoprecipitated lysosomes

Western-blot analysis of whole cell lysate (WCL) and immunoprecipitated lysosomes (HA LysolIP) from GSCs expressing TMEM192-3×HA or -2×flag, as indicated. The indicated proteins (with their intracellular localization annotated on the left) were detected to assess the enrichment and purity of immunoprecipitated lysosomes.

g. Collect the eluate in a new 1.5 mL tube and keep on ice.

Optional: Eluates can be collected in low-adhesion tubes for subsequent proteomic analysis. This ensures an optimal recovery of the samples.

h. Repeat steps c-g.

Note: The double elution allows a maximal recovery of proteins from the beads.

▮▮▮ **Pause point:** At that time, samples can be stored at -20°C before analysis, or at -80°C for long term storage.

18. Proceed to analysis.

Western-blot analysis

⌚ **Timing:** 1 day

This step describes the method that can be used to qualitatively define the immunopurified lysosomes by western-blot (Figure 4).

19. Prepare samples for western-blot analysis.

- For each condition, dispense 3.5 μL of whole cell lysates, as prepared in step 9 in new 1.5 mL tubes.
- For each condition, dispense 8 μL of eluates prepared in step 16 in new 1.5 mL tubes.
- In each tube, add 2× SDS sample buffer to a final concentration of 1×.
- Heat the samples at 95°C for 5 min.
- Quickly spin down the samples in a tabletop centrifuge to remove condensation.

Note: The volume of whole cell lysates to be loaded were optimized for western-blot analysis.

▮▮ **Pause point:** At that time, samples can be stored at -20°C until western-blot.

20. Resolve samples by western-blot.

Optional: We recommend resolving whole cell lysates and LysolP samples side-by-side to control efficiency of the immunoprecipitation.

Note: Standard western-blot methodologies are suitable. We recommend Tris-Acetate western-blot as detailed in the Star Protocols by Andre-Gregoire et al.⁹ Briefly, primary antibodies (Table 2) were incubated at 1:1000 in PBS-Tween 5% milk for 1 h at RT. Secondary HRP-conjugated antibodies were diluted at 1:5000, incubated for 1 h at RT, and revealed by chemiluminescence.

21. Assess the purity of immuno-isolated lysosomes.

- a. Probe the western-blot membranes with the following antibodies (Table 2) directed against lysosomal proteins and intracellular organelles.

Note: The western-blot analysis should show a strong enrichment of the lysosomal proteins in the LysolP samples, as compared to whole cell lysates.

Note: The mature form (mCSTD, 25 kDa) and the pre-pro form (ppCSTD, 50 kDa) of cathepsin D should be detected in the LysolP samples.

Note: The level of proteins from contaminant organelles should be low in the LysolP samples, as compared to lysosomal proteins.

22. Perform analysis of your protein target of interest.

- a. Probe western-blot membranes with antibodies directed against the target of interest.

EXPECTED OUTCOMES

This protocol provides a fast and efficient method to immunopurify lysosomes from patient-derived GSCs. By stably expressing a tagged-lysosomal protein followed by mechanical lysis and use of magnetic beads, lysosomes can be specifically isolated from other organelles.

We notably took advantage of this method in a previous study,¹ demonstrating the potential of this technique to characterize changes in the lysosomal proteome of patient-derived GSCs.

Western-blot analysis helped to characterize their protein composition (Figure 5). The data obtained with the LysolP procedures strengthened the findings that MALT1 inhibition might alter the lysosomal quality and unveil a possible interplay with autophagy processes.^{1,7}

LIMITATIONS

Limitations of this protocol include the quality of the purified lysosomes. While the enrichment in lysosomes is significant, contamination from other unwanted organelles is possible. Given that lysosomes serve as the endpoint of the autophagic degradative pathway and closely interact with other organelles, the presence of markers from undesired organelles may either indicate contamination or represent a specific phenotype. Therefore, incorporating controls becomes imperative to ensure the purity of lysosomes. For instance, treating GSCs with Bafilomycin A1, an inhibitor of the V-ATPase that blocks lysosome-autophagosome fusion, could facilitate the examination of the role of the autophagic pathway amidst specific markers in the lysosomal fraction. Hence,

Table 2. Recommended antibodies for assessment of LysolP efficiency and purity

Target	Source and catalog number	Concentration	Approximate running size
HA (Tag)	Cell Signaling Technology (Cat#3724)	1:10,000	37 kDa
LAMP2 (Lysosomes)	Santa Cruz Biotechnology (Cat#sc-18822)	1:5,000	100 kDa
CTSD (Lysosomes)	BD Biosciences (Cat#610800)	1:1,000	50–25 kDa
VDAC1 (Mitochondria)	Cell Signaling Technology (Cat#4661)	1:1,000	37 kDa
CALRETICULIN (Endoplasmic Reticulum)	Cell Signaling Technology (Cat#12238)	1:1,000	50 kDa
GM130 (Golgi Apparatus)	Abcam (Cat#ab52649)	1:1,000	130 kDa
EEA1 (Early Endosomes)	BD Biosciences (Cat#610456)	1:1,000	150 kDa

depending on the experimental design, it is essential to include specific controls to assess the purity of the lysosomes.

The quantity of immunopurified lysosomes can also be a limiting factor. Notably, determining the percentage of lysosomes immunopurified from a given number of cells is challenging. This factor must be considered when conducting quantitative studies. For instance, LysolP samples has to be qualitatively compared with whole cell lysate samples to appreciate the effects of a treatment on the lysosomal compartment. Alternatively, label free-proteomics can help in evaluating the enrichment factor in lysosomal proteins, as done in Maghe et al.¹ Moreover, for quantitative studies, it is recommended to use a lysosomal protein whose expression remains barely affected by the experimental conditions. This can be controlled at the RNA level with qPCR and at the protein level with immunofluorescence. However, this approach may not provide insights into the generation of different pools of lysosomes. To address this latter point, density gradient coupled with differential centrifugation can be a valuable technique to distinguish between various pools of the same organelles, thereby complementing LysolP experiments.

The use of lentiviruses to engineer transgenic cell lines can be a limitation. First, the manipulation of lentiviral particles requires special confinement, waste evacuation, and storage conditions. In addition, some parameters are hardly controllable. Indeed, the number of independent integrated copies may vary between conditions. Moreover, depending on the integration locus, inserted DNA might be subjected to silencing. It is therefore suggested to check several bulk and clones, in regards to parental cells.

Another limitation might come from the substantial number of cells required for this protocol. We recommend starting with small-scale experiments involving a limited number of conditions.

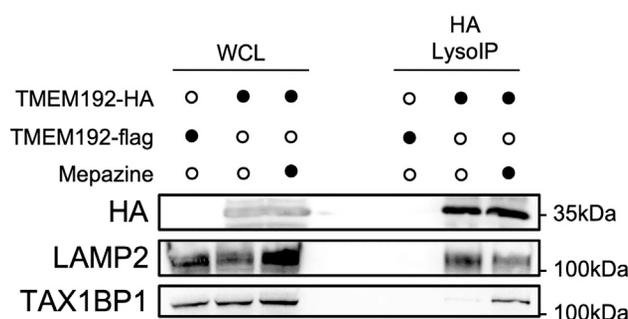


Figure 5. Expected outcomes

Western-blot analysis of whole cell lysate (WCL) and immunoprecipitated lysosomes (HA LysolP) from GSCs expressing TMEM192-3xHA or -2xflag, and treated with the MALT1 inhibitor Mepazine (20 μ M, 6 h), as indicated. LAMP2 lysosomal protein signal denotes the enrichment in lysosomes. HA tag serves as a loading control. Note the accumulation of the TAX1BP1 autophagic receptor in immunopurified lysosomes from MALT1-inhibited GSCs.

Furthermore, this protocol entails the overexpression of a lysosomal protein. Although no specific functions have been attributed to the TMEM-192 protein overexpression, we cannot rule out the possibility that it may affect the lysosomal compartment. Therefore, observed phenotypes in parental cells should be replicated in cells expressing TMEM-192, to ensure that its overexpression does not alter the expected outcomes.

TROUBLESHOOTING

Problem 1

GSCs adhere to the culture surface during the selection process (related to step 4).

Potential solution

Although plastic flasks are of standard use for maintaining GSCs, ultra-low adhesion plates (like Ultra-Low Attachment Culture Dish, #4615 from Corning manufacturer) might be required.

Problem 2

None of the GSCs survive the puromycin selection (related to step 4).

Potential solution

Puromycin concentration should be adjusted to ensure a specific toxicity against non-infected cells. Titration of the compound can be performed. If this does not resolve the problem, consider using a different viral vector adapted to your cells. Consider cloning the insert into another plasmid adapted to the required viral vector.

Problem 3

Impossibility of harvesting the required number of cells (related to step 8).

Potential solution

This protocol can be adapted to a lower number of cells but may require to adapt the lysis method and the immunoprecipitation step to the chosen number of cells. This may also affect the yield of immunopurified lysosomes, resulting in potential decrease in the sensitivity of the analysis method. If the lysosomal protein of interest is expressed at a low rate, consider increasing the number of cells, or concentrating the samples before analysis. Make sure not to alter the quality of the samples.

Problem 4

The mechanical lysis is affected by the treatment (related to step 10).

Potential solution

Some treatments can modify membrane fluidity/rigidity, affecting the efficiency of the mechanical lysis. Step 8 helps in optimizing the mechanical lysis condition, depending on the cell culture conditions and treatments required for experimentation.

Problem 5

A low number of lysosomal proteins is detected by western-blot (related to step 20).

Potential solution

The yield of lysosomal purification can be low under certain circumstances. The volume of LysoIP samples to be resolved by western-blot can be increased. However, this should not result in an increase in the level of contaminants proteins. The yield can also be enhanced with either a higher number of cells or elevated concentration of magnetic beads. This could however result in higher contamination.

Problem 6

High level of contaminant proteins in the analyzed samples (related to step 20).

Potential solution

Depending on their porosity, the type of beads used to perform the LysolP can affect the contaminants level. In this protocol, Pierce anti-HA magnetic beads showed the optimal yield of purification and the lowest level of contamination. Consider also additional washes of the beads after the immunoprecipitation step. This could however affect the yield of purification. It is important to make sure that a robust presence of a non-lysosomal protein may also reflect a specific phenotype induced by the culture/treatment conditions. Do not exclude exploring the effect of your experimental conditions on the trafficking and turn-over of the protein of interest.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julie Gavard, julie.gavard@inserm.fr.

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Clement Maghe, clementmaghe@gmail.com.

Materials availability

No newly generated materials were associated with this protocol.

Data and code availability

This protocol does not report original code. All data generated or analyzed during this protocol are included in this article.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2024.103121>.

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AUTHOR CONTRIBUTIONS

C.M. and J.G. designed the experimental approaches and coordinated the project. C.M. performed experiments, interpreted the data, prepared the figures, and wrote the manuscript. J.G. supervised the work, interpreted the data, and edited the text. All authors approved the submitted manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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