Antibody Characterization Report for Sequestosome-1

YCharOS Antibody Characterization Report

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Target:

Protein name: Sequestosome-1

Alternative protein names: EBI3-associated protein of 60 kDa, EBIAP, p60, Phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa, Ubiquitin-binding protein p62, p62

Gene name: *SQSTM1*

Uniprot: Q13501

This report guides researchers to select the most appropriate antibodies for Sequestosome-1. We used an antibody characterization pipeline [1] based on knockout (KO) cells to perform head-to-head comparisons of commercial antibodies for Sequestosome-1 by immunoblot (Western blot), immunoprecipitation and immunofluorescence. U2OS was selected based on evidence of appropriate Sequestosome-1 protein expression determined through public proteomics databases, namely PaxDB [2] and DepMap [3]. U2OS was modified with CRISPR/Cas9 to knockout [4] the corresponding *SQSTM1* gene.

The authors do not provide an assessment of the quality of the tested antibodies as their respective performances are limited to our finite experimental conditions. The readers should interpret the present findings based on their own scientific expertise. The authors acknowledge that an antibody that demonstrates specificity in the stated test conditions can be suboptimal in a different experimental format or in cell lines that differ from those directly tested here.

1. Laflamme, C., et al., *Implementation of an antibody characterization procedure and application to the major ALS/FTD disease gene C9ORF72.* Elife, 2019. **8**.
2. Wang, M., et al., *Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines.* Proteomics, 2015. **15**(18): p. 3163-8.
3. Nusinow, D.P., et al., *Quantitative Proteomics of the Cancer Cell Line Encyclopedia.* Cell, 2020. **180**(2): p. 387-402 e16.
4. Shlaifer, I., et al. *Generation of Knockout Cell Lines Using CRISPR-Cas9 Technology*. February 24, 2020; Available from: https://zenodo.org/record/3738361#.YIyeDu2SlaR.

Table 1: Summary of the Sequestosome-1 antibodies tested

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (µg/µl)
GeneTex	GTX629890	41470	AB_2885144	monoclonal	GT1478	mouse	1.00
GeneTex	GTX629888	41470	AB_2885143	monoclonal	GT239	mouse	1.00
GeneTex	GTX100685	42893	AB_2038029	polyclonal	-	rabbit	0.67
Thermo	701510	2315239	AB_2532489	recombinant-mono	11HC14LC25	rabbit	0.50
Thermo	710539	RF229394	AB_2532735	recombinant-poly	11HCLC	rabbit	0.50
Thermo	MA5-32835	VL3152616	AB_2802482	monoclonal	10-E10	mouse	2.00
Proteintech	66184-1-Ig	not provided	AB_2881579	monoclonal	1H5C1	mouse	1.33
Proteintech	18420-1-AP	not provided	AB_10694431	polyclonal	-	rabbit	0.35
Proteintech	55274-1-AP	not provided	AB_11182278	polyclonal	-	rabbit	0.43
Bio-Techne	MAB80281	CMRM0120031	AB_2888658	recombinant-mono	2533b	rabbit	0.50
Bio-Techne	NBP2-23490	A-1	AB_2885153	monoclonal	5H7E2	mouse	1.00
Bio-Techne	MAB8028	CHZL0520071	AB_2885150	monoclonal	864807	mouse	0.50
Bio-Techne	MAB8028R	CLJP0118091	AB_2885151	recombinant-mono	864807R	mouse	0.50
Abcam	ab109012	GR3241806-8	AB_2810880	recombinant-mono	EPR4844	rabbit	0.43
Abcam	ab56416	GR3374761-2	AB_945626	monoclonal	Not provided	mouse	1.00
Abcam	ab207305	GR323335-4	AB_2885112	recombinant-mono	EPR18351	rabbit	0.21
Abcam	ab240635	GR3314160-2	AB_2885121	recombinant-mono	EPR23101-103	rabbit	0.49

Table 2: Summary of the cell lines used

Institution	Catalog number	RRID (Cellosaurus)	Cell line	genotype
Montreal Neurological Institute	-	CVCL_0042	U2OS	WT
Montreal Neurological Institute	-	CVCL_A6LP	U2OS	*SQSTM1* KO

Figure 1: Sequestosome-1 antibody screening by immunoblot.

Lysates of U2OS (WT or *SQSTM1* KO) were prepared and 25 µg of protein were processed for immunoblot with the indicated Sequestosome-1 antibodies. The Ponceau stained transfers of each blot are shown. Antibody dilution used: GTX629890 at 1/1000; GTX629888 at 1/1000; GTX100685 at 1/1000; 701510 at 1/1000; 710539 at 1/200; MA5-32835 at 1/200; 66184-1-Ig at 1/1000; 55274-1-AP at 1/1000; 18420-1-AP at 1/1000; MAB80281 at 1/1000; NBP2-23490 at 1/1000; MAB8028 at 1/1000; MAB8028R at 1/1000; ab109012 at 1/10000; ab56416 at 1/1000; ab207305 at 1/1000; ab240635 at 1/1000. Predicted band size: 47 kDa. Observed band size: ~62 kDa.

Figure 2: Sequestosome-1 antibody screening by immunoprecipitation.

Lysates were prepared and IP was performed using 1.0 µg of the indicated Sequestosome-1 antibodies pre-coupled to either protein G or protein A Sepharose beads. Samples were washed and processed for immunoblot with the indicated Sequestosome-1 antibody. For immunoblot, MAB80281 at 1/3000, 66184-1-Ig at 1/3000, ab56416 at 1/5000, ab 207305 at 1/10000 and GTX629890 at 1/5000 were used. The Ponceau stained transfers of each blot are shown. SM=10% starting material; UB=10% unbound fraction; IP=immunoprecipitate; HC=antibody heavy chain.

Figure 3: Sequestosome-1 antibody screening by immunofluorescence.

U2OS WT and *SQSTM1* KO cells were labelled with a green or a far red fluorescent dye, respectively. WT and KO cells were mixed and plated to a 1:1 ratio on coverslips. Cells were stained with the indicated Sequestosome-1 antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody. Acquisition of the green (WT), red (antibody staining) and far-red (KO) channels was performed. Representative grayscale images of the red channel are shown. WT and KO cells are outlined with yellow and magenta dashed line, respectively. Schematic representation of the mosaic strategy used is shown on the bottom-middle panel. Antibody dilution used: GTX629890 at 1/1000; GTX629888 at 1/1000; GTX100685 at 1/700; 701510 at 1/500; 710539 at 1/500; MA5-32835 at 1/2000; 66184-1-Ig at 1/300; 55274-1-AP at 1/1300; 18420-1-AP at 1/300; MAB80281 at 1/500; NBP2-23490 at 1/1000; MAB8028 at 1/500; MAB8028R at 1/500; ab109012 at 1/500; ab56416 at 1/1000; ab207305 at 1/200; 1/500; ab240635 at 1/500. Bars = 10 µm.

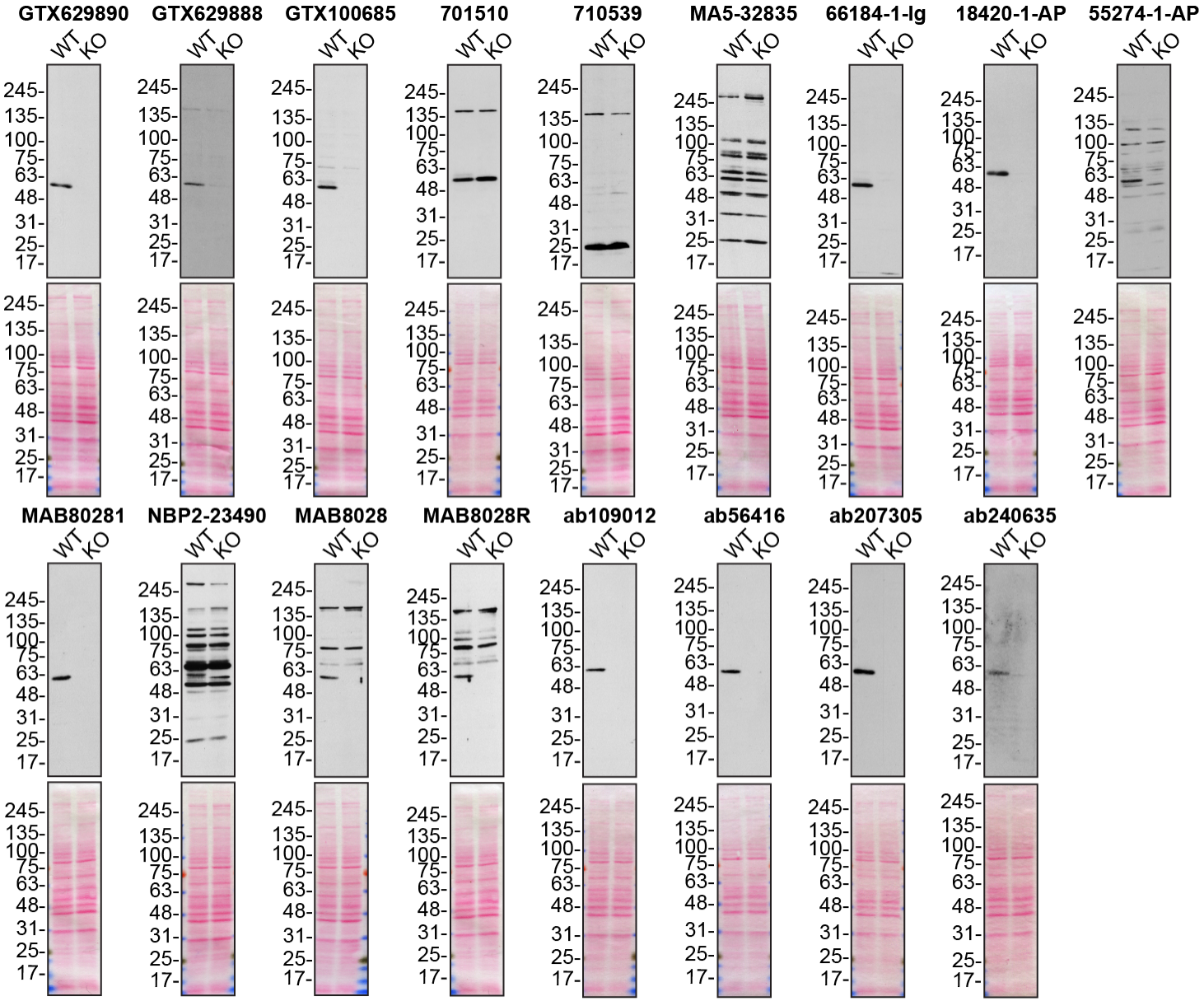

Figure 1 : Sequestosome-1 antibody screening by immunoblot

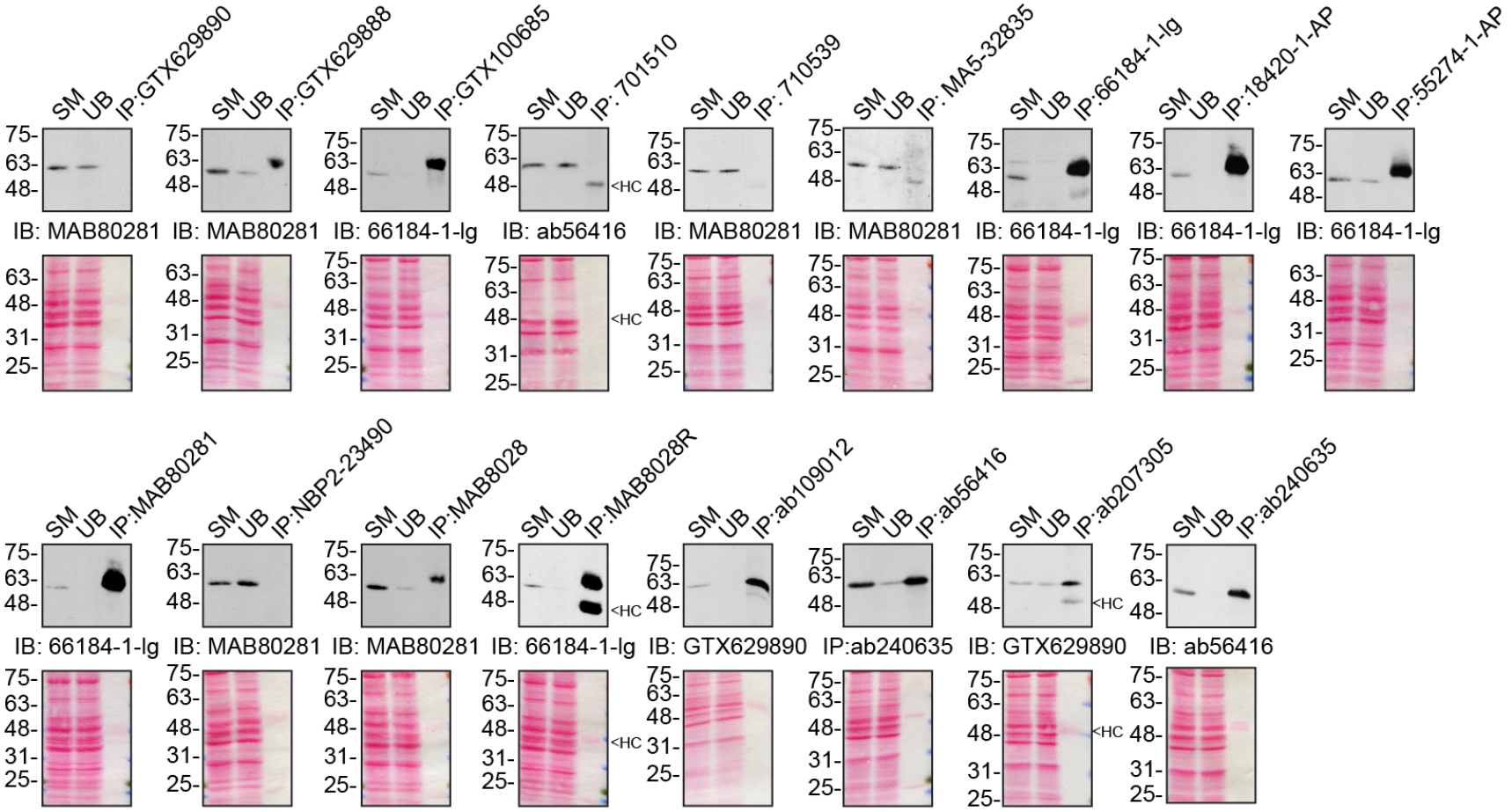

Figure 2 : Sequestosome-1 antibody screening by immunoprecipitation

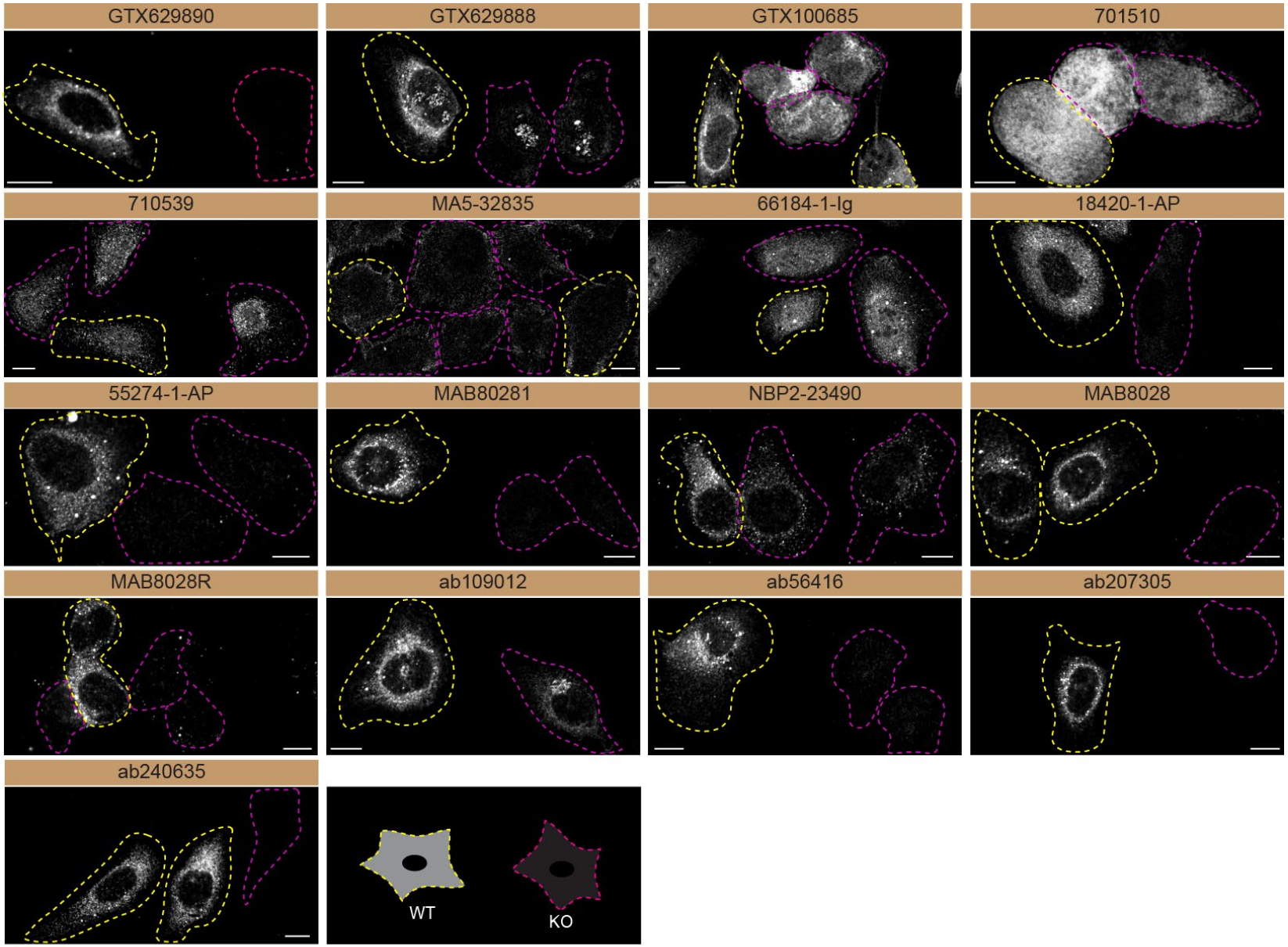

Figure 3 : Sequestosome-1 antibody screening by immunofluorescence

Materials and methods

Antibodies

All Sequestosome-1 antibodies are listed in Table 1. Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies are from Thermo Fisher Scientific (cat. number 65-6120 and 62-6520). Alexa-555-conjugated goat anti-mouse and anti-rabbit secondary antibodies are from Thermo Fisher Scientific (cat. number A21424 and A21429).

CRISPR/Cas9 genome editing

Cell lines used are listed in Table 2. U2OS *SQSTM1* KO clone was generated with low passage cells using an open-access protocol available on Zenodo.org: https://zenodo.org/record/3875777#.X9uE11VKjIX. Two guide RNAs were used to introduce a STOP codon in the *SQSTM1* gene (sequence guide 1: CCACCGCCCACCGUGUGCUC, sequence guide 2: AUGCGAGCUUGGUGUGCCCC).

Cell culture

Cells were cultured in DMEM high-glucose (GE Healthcare cat. number SH30081.01) containing 10% bovine calf serum (GE Healthcare cat. number SH30072.03), 2 mM L-glutamate (Wisent cat. number 609065, 100 IU penicillin and 100 µg/ml streptomycin (Wisent cat. number 450201).

Antibody screening by immunoblot

U2OS (WT and *SQSTM1* KO) were collected in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor. Lysates were sonicated briefly and incubated 30 min on ice. Lysates were spun at ~110,000xg for 15 min at 4°C and equal protein aliquots of the supernatants were analyzed by SDS-PAGE and immunoblot. BLUelf prestained protein ladder from GeneDireX (cat. number PM008-0500) was used.

Immunoblots were performed with large 4-15% gradient polyacrylamide gels and transferred on nitrocellulose membranes. Proteins on the blots were visualized with Ponceau staining which is scanned to show together with individual immunoblot. Blots were blocked with 5% milk for 1 hr, and antibodies were incubated O/N at 4°C with 5% bovine serum albumin in TBS with 0,1% Tween 20 (TBST). Following three washes with TBST, the peroxidase conjugated secondary antibody was incubated at a dilution of ~0.2 µg/ml in TBST with 5% milk for 1 hr at room temperature followed by three washes with TBST. Membranes are incubated with regular ECL

(cat. number 32106) or with super signal West Femto (cat. number 34096) from Pierce prior to detection with HyBlot CL autoradiography films from Denville (cat. number 1159T41).

Antibody screening by immunoprecipitation

Antibody-bead conjugates were prepared by adding 1.0 µg of antibody to 500 ul of PBS with 0,01% triton X-100 in a microcentrifuge tube, together with 30µl of protein A- (for rabbit antibodies) or protein G- (for mouse antibodies) Sepharose beads. Tubes were rocked O/N at 4°C followed by several washes to remove unbound antibodies.

U2OS WT were collected in HEPES buffer (20 mM HEPES, 100 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, pH 7.4) supplemented with protease inhibitor. Lysates are rocked 30 min at 4°C and spun at 110,000xg for 15 min at 4°C. One ml aliquots at 1.0 mg/ml of lysate were incubated with an antibody-bead conjugate for ~2 hrs at 4°C. Following centrifugation, the unbound fractions were collected, and beads were subsequently washed three times with 1.0 ml of HEPES lysis buffer and processed for SDS-PAGE.

Antibody screening by immunofluorescence

U2OS WT and *SQSTM1* KO were labelled with a green and with a deep red fluorescence dye from Abcam (cat. number ab176735 and ab176736), respectively. WT and KO cells were plated on glass coverslips as a mosaic and incubated for 24 hrs in a cell culture incubator. Cells were fixed in 4% PFA (in PBS) for 15 min at room temperature and then washed 3 times with PBS. Cells were permeabilized in PBS with 0,1% Triton X-100 for 10 min at room temperature and blocked with PBS with 5% BSA, 5% goat serum and 0.01% Triton X-100 for 30 min at room temperature. Coverslips were incubated face down on a 50 µl drop (on paraffin film in a moist chamber) with IF buffer (PBS, 5% BSA, 0,01% Triton X-100) containing the primary Sequestosome-1 antibodies O/N at 4°C. Cells were washed 3 × 10 min with IF buffer and incubated with corresponding Alexa Fluor 555-conjugated secondary antibodies in IF buffer at a dilution of 1.0 µg/ml for 1 hr at room temperature. Cells were washed 3 × 10 min with IF buffer and once with PBS. Coverslips were mounted on a microscopic slide using fluorescence mounting media (DAKO).

Imaging was performed using a Zeiss LSM 880 laser scanning confocal microscope equipped with a Plan-Apo 40x oil objective (NA = 1.40). Analysis was done using Image J. All cell images represent a single focal plane. Figures were prepared using Adobe Photoshop to adjust contrast, apply 1 pixel Gaussian blur and then assembled with Adobe Illustrator.