Antibody characterization report for Calpain-2 catalytic subunit

YCharOS Antibody Characterization Report

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Target:

Recommended protein name: Calpain-2 catalytic subunit

Gene name: *CAPN2*

Uniprot: P17655

This report guides researchers to select the most appropriate antibodies for the Calpain-2 catalytic subunit. We used an antibody characterization pipeline [1] based on *CAPN2* knockout (KO) cells to perform head-to-head comparisons of commercial antibodies for Calpain-2 catalytic subunit by immunoblot (Western blot), immunoprecipitation and immunofluorescence. An MDA-MB-231 *CAPN2* KO line was generated using methods described in Gao et al., 2019 [2].

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. Gao, Y., et al., *Genetic Models of Calpain Deficiency and Ectopic Expression.* Methods Mol Biol, 2019. **1915**: p. 261-274.

Table 1: Summary of the Calpain-2 catalytic subunit antibodies tested

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (µg/µl)	Vendors recommended applications
GeneTex	GTX102499	40163	AB_10618733	polyclonal	-	rabbit	1.00	Wb, IF
GeneTex	GTX111809	40387	AB_10727594	polyclonal	-	rabbit	0.47	Wb
Abcam	ab126600	GR3208503-1	AB_11128480	recombinant-mono	EPR5977	rabbit	1.31	Wb
Abcam	ab75994	GR109438-5	AB_1523255	recombinant-mono	EPR2562Y	rabbit	0.69	Wb
Abcam	ab39165	GR307292-11	AB_725844	polyclonal	-	rabbit	1.00	Wb
Cell Signaling Technology	2539	3	AB_2069843	polyclonal	-	rabbit	not provided	Wb
Thermo	PA5-17494	WD3265835	AB_10981526	polyclonal	-	rabbit	not provided	Wb
Thermo	PA5-82884	WE3266017A	AB_2790040	polyclonal	-	rabbit	0.10	Wb, IF
Thermo	PA5-27720	WD3265727	AB_2545196	polyclonal	-	rabbit	1.00	Wb, IF
Thermo	PA5-78912	WD3265574	AB_2746028	polyclonal	-	rabbit	0.50	Wb
Thermo	MA5-32719	WD3265258	AB_2809996	recombinant-mono	JA43-41	rabbit	1.00	Wb
Bio-Techne	NBP2-67202	HK0703	AB_2892119	recombinant-mono	JA43-41	rabbit	1.00	Wb, IF
Proteintech	11472-1-AP	00062414	AB_10755306	polyclonal	-	rabbit	0.30	Wb, IF

Wb=Western blot; IP= immunoprecipitation; IF=immunofluorescence

Table 2: Summary of the cell lines used

Institution	Catalog number	RRID (Cellosaurus)	Cell line	genotype
Queen's University	-	-	MDA-MB-231 pWPLXd (GFP positive)	WT
Queen's University	-	CVCL_A9AZ	MDA-MB-231 pWPLXd (GFP positive)	*CAPN2* KO
Abcam	ab255449	-	HEK293T	WT
Abcam	ab266628	-	HEK293T	*CAPN2* KO

Figure 1: Calpain-2 catalytic subunit antibody screening by immunoblot.

A) Lysates of MDA-MB-231 (WT and *CAPN2* KO) were prepared and 20 µg of protein were processed for immunoblot with the indicated Calpain-2 catalytic subunit antibodies. The Ponceau stained transfers of each blot are shown. Antibody dilution used: GTX102499 at 1/1000; GTX111809 at 1/500; ab126600 at 1/5000; ab75994 at 1/2000; ab39165 at 1/2000; 2539 at 1/1000; PA5-17494 at 1/1000; PA5-82884 at 1/1000; PA5-27720 at 1/1000; PA5-78912 at 1/1000; MA5-32719 at 1/1000; NBP2-67202 at 1/1000; 11472-1-AP at 1/1000. Predicted band size: 80 kDa. Observed band size: ~74 kDa.

B) Lysates of MDA-MB-231 and HEK293T (WT and *CAPN2* KO) were prepared and 20 µg of protein were processed for immunoblot as in A. The antibody ab126600 was used at 1/5000. A short (top panel) and long (middle panel) exposures are shown.

Figure 2: Calpain-2 catalytic subunit antibody screening by immunoprecipitation.

MDA-MB-231 lysates were prepared and immunoprecipitation was performed using 1.0 µg of the indicated Calpain-2 catalytic subunit antibodies pre-coupled to either protein G or protein A Sepharose beads. Samples were washed and processed for immunoblot with the indicated Calpain-2 catalytic subunit antibody. The Ponceau stained transfers of each blot are shown. SM=10% starting material; UB=10% unbound fraction; IP=immunoprecipitate.

Figure 3: Calpain-2 catalytic subunit antibody screening by immunofluorescence.

MDA-MB-231 WT and *CAPN2* KO cells are both GFP positive. The MDA-MB-231 *CAPN2* KO cell line was labelled with a violet fluorescent dye. WT and KO cells were mixed and plated to a 1:1 ratio on coverslips. Cells were stained with the indicated Calpain-2 catalytic subunit antibodies and with the corresponding Alexa-fluor 647 coupled secondary antibody. Acquisition of the violet (identification of the KO cells), green (identification of both WT and KO cells) and far-red (antibody staining) channels was performed. Representative images of the far-red (grayscale) channel is shown. WT cells are GFP positive only, where as KO cells are both violet and GFP positive. WT and KO cells are outlined in the figure with yellow and magenta dashed line, respectively. Antibody dilution used: GTX102499 at 1/1000; GTX111809 at 1/500; ab126600 at 1/1000; ab75994 at 1/700; ab39165 at 1/1000; 2539 at 1/500; PA5-17494 at 1/500; PA5-82884 at 1/100; PA5-27720 at 1/500; PA5-78912 at 1/500; MA5-32719 at 1/1000; NBP2-67202 at 1/1000; 11472-1-AP at 1/300. Bars = 10 µm.

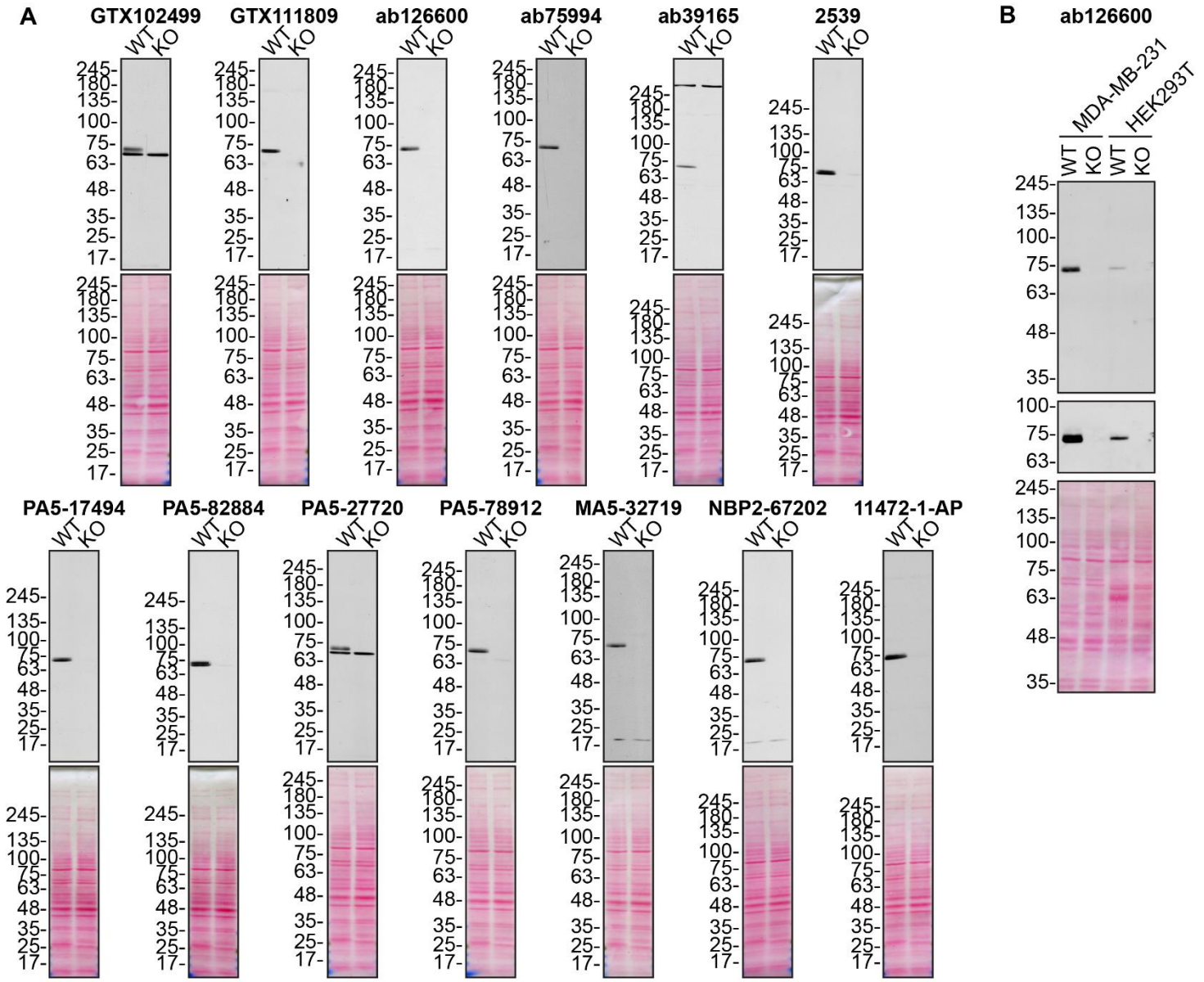

Figure 1: Calpain-2 catalytic subunit antibody screening by immunoblot

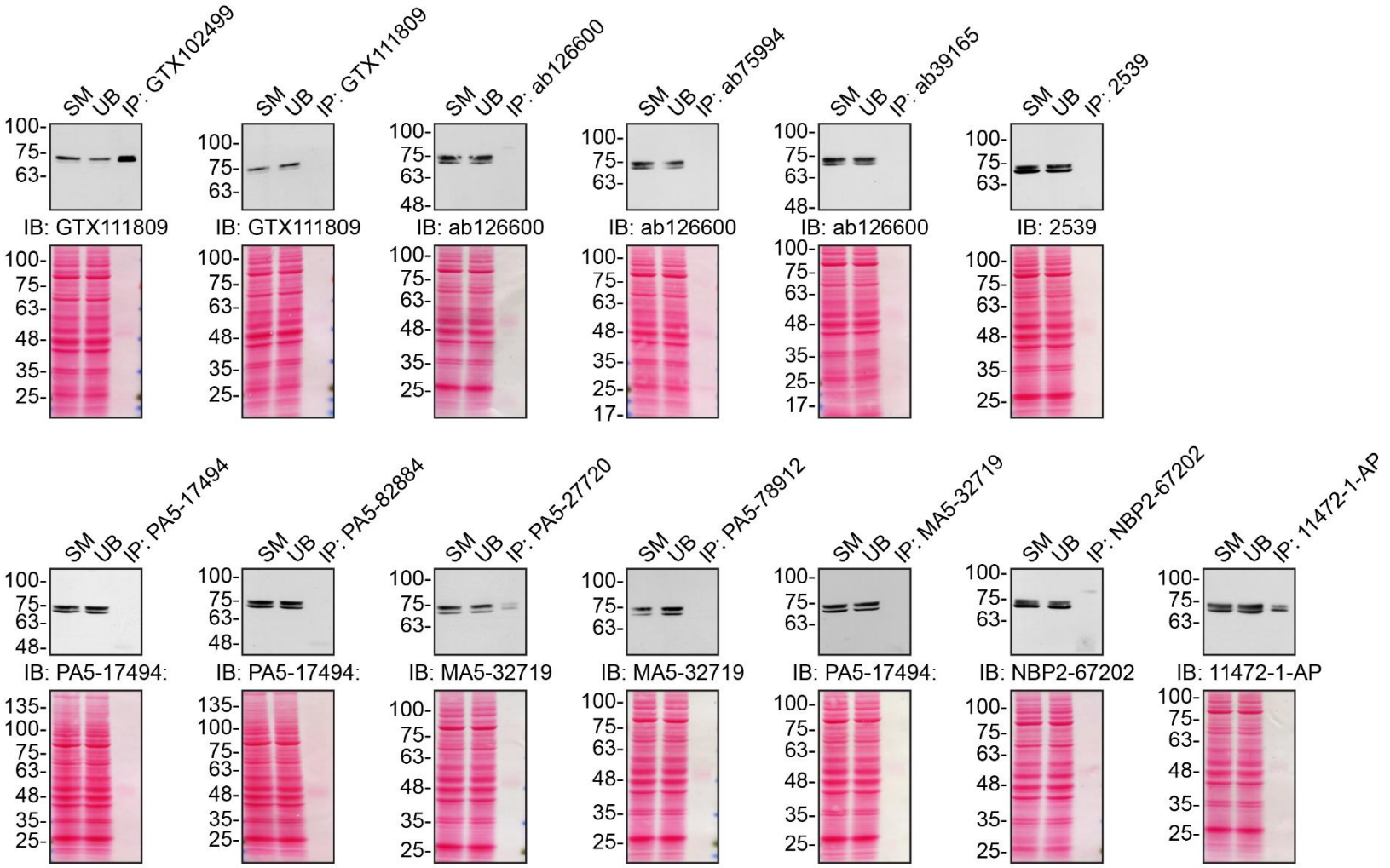

Figure 2: Calpain-2 catalytic subunit antibody screening by immunoprecipitation

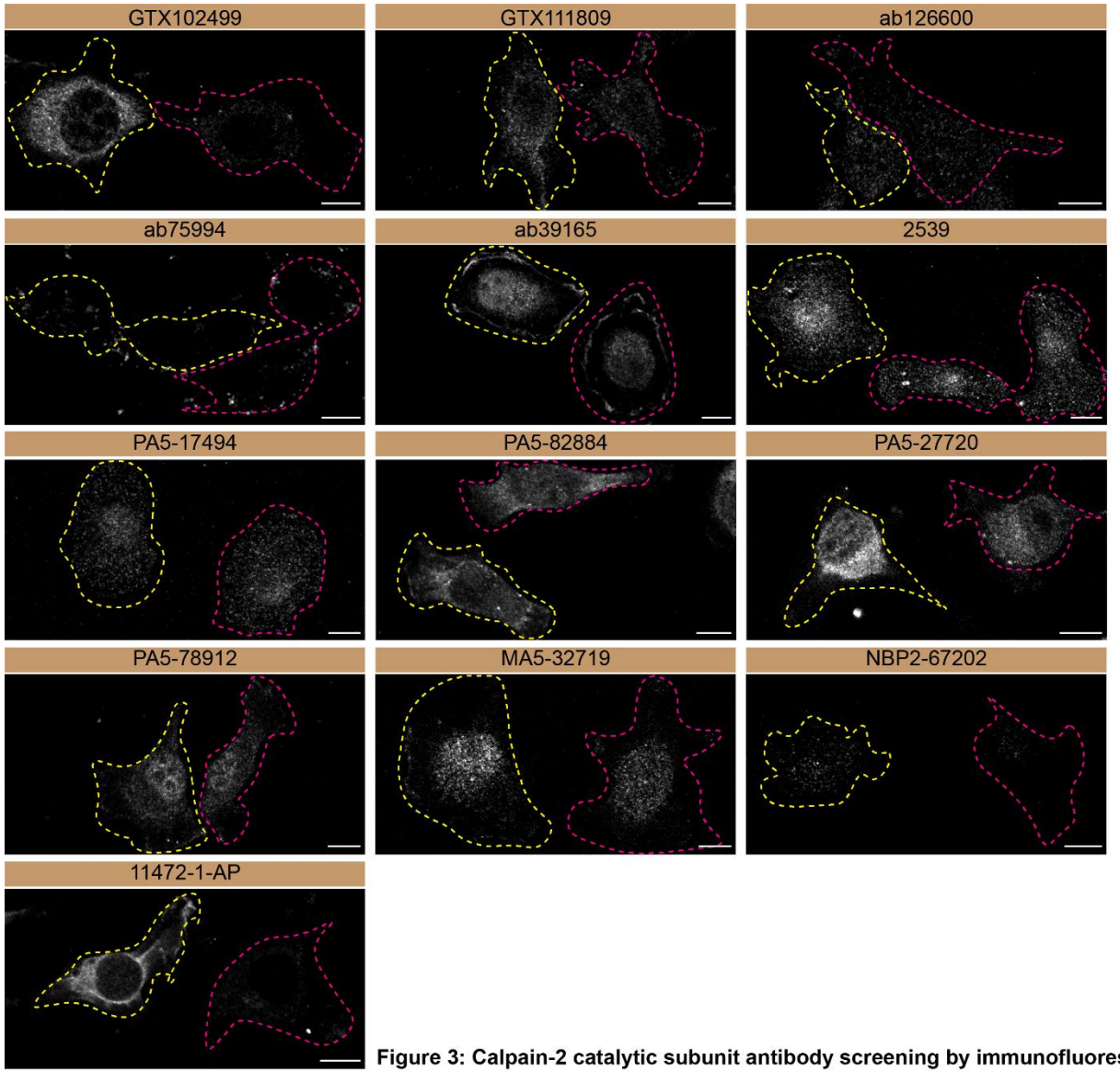

Figure 3: Calpain-2 catalytic subunit antibody screening by immunofluorescence

Materials and methods

Antibodies

All Calpain-2 catalytic subunit 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-647-conjugated goat anti-mouse and anti-rabbit secondary antibodies are from Thermo Fisher Scientific.

Cell culture

Cells were cultured in DMEM high-glucose (GE Healthcare cat. number SH30081.01) containing 10% fetal bovine serum (Wisent, cat. number 080450), 2 mM L-glutamate (Wisent cat. number 609065, 100 IU penicillin and 100 µg/ml streptomycin (Wisent cat. number 450201).

Antibody screening by immunoblot

MDA-MB-231 (WT and *CAPN2* 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 (Figure 1A) or 8% (Figure 1B) 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, then incubated with antibodies 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 ECL from Pierce (cat. number 32106) 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. For 2539 and PA5-17494, 5 µl of each antibody was used as the antibody concentration was not provided.

MDA-MB-231 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 and immunoblot on 4-15% polyacrylamide gels. Prot-A:HRP was used as a secondary detection system (MilliporeSigma, cat. number P8651) at a dilution of 0.4 µg/ml for an experiment where a rabbit antibody was used for both immunoprecipitation and its corresponding immunoblot.

Antibody screening by immunofluorescence

MDA-MB-231 *CAPN2* KO cell line was labelled with a violet fluorescence dye from Thermo Fisher Scientific (cat. number C10094). 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. Cells were incubated with IF buffer (PBS, 5% BSA, 0,01% Triton X-100) containing the primary Calpain-2 catalytic subunit antibodies O/N at 4°C. Cells were then washed 3 × 10 min with IF buffer and incubated with corresponding Alexa Fluor 647-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 the Zen navigation software (Zeiss). All cell images represent a single focal plane. Figures were assembled with Adobe Illustrator.