Antibody Characterization Report for CD44 antigen

YCharOS Antibody Characterization Report

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Target:

Protein name: CD44 antigen

Alternative protein names: CD44 molecule (Indian blood group), CDw44, Epican, ECMR-III, GP90 lymphocyte homing/adhesion receptor, HUTCH-I, Heparan sulfate proteoglycan, Hermes antigen, Hyaluronate receptor, PGP-I, CD44

Uniprot: P16070

Gene name: *CD44*

This report guides researchers to select the most appropriate antibodies for CD44 antigen. We used an antibody characterization pipeline[1] based on knockout cells to perform head-to-head comparisons of commercial antibodies for CD44 antigen by immunoblot (Western blot), immunoprecipitation and immunofluorescence. HAP1 *CD44* knockout cell line is available at Horizon Discovery and was selected for screening of antibodies. By performing immunoblot on a panel of several cell lines, we observed that HAP1 expresses *CD44* at very low level compared to other common cultured cell lines. Antibodies which demonstrate specificity for CD44 antigen in HAP1 are therefore highly selective for this target.

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* **8**, doi:10.7554/eLife.48363 (2019).

Table 1: Summary of the CD44 antigen antibodies tested

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (µg/µl)
Proteintech	15675-1-AP	not provided	AB_2076198	polyclonal	-	rabbit	0.27
Proteintech	60224-1	not provided	AB_11042767	monoclonal	6F4H2	mouse	1.27
GeneTex	GTX102111	42480	AB_1240596	polyclonal	-	rabbit	to add
GeneTex	GTX628472	41113	AB_2888037	monoclonal	GT981	mouse	1.00
GeneTex	GTX628895	41232	AB_2888071	monoclonal	GT462	mouse	0.70
Thermo	MA5-13890	VJ3094996B	AB_10986810	monoclonal	156-3C11	mouse	0.20
Thermo	14-0441-82	2183528	AB_467246	monoclonal	IM7	rat	0.50
Bio-Techne	NB600-1317	CKAF01-1	AB_10002839	monoclonal	OX-50	mouse	1.00
Bio-Techne	NBP1-47386	A-9	AB_10010339	monoclonal	8E2F3	mouse	1.00
Bio-Techne	BBA10	BGR0119021	AB_356933	monoclonal	2C5	mouse	0.50
Abcam	ab254530	GR3317001-2	AB_2885131	monoclonal	Hermes-3	mouse	0.54
Abcam	ab189524	GR3314218-1	AB_2885107	recombinant-mono	EPR18668	rabbit	0.47
Abcam	ab25340	GR3362083-1	AB_470456	monoclonal	KM201	rat	0.50
Abcam	ab264539	GR3341229-1	AB_2885133	monoclonal	C44Mab-5	mouse	1.27
Abcam	ab101531	GR3221323-3	AB_10710025	recombinant-mono	SP37	rabbit	not provided
Abcam	ab51037	GR3188832-9	AB_868936	recombinant-mono	EPR1013Y	rabbit	2.07
Abcam	ab157107	GR3317976-4	AB_2847859	polyclonal	-	rabbit	1.00
Human Protein Atlas	HPA005785	1484	AB_1078467	polyclonal	-	rabbit	to add
BioVision	A1078	2F141078	AB_2890074	monoclonal	Hermes-3	mouse	not provided
Cell Signaling Technology	5640	4	AB_10547133	monoclonal	8E2	mouse	not provided

Table 2: Summary of the cell lines used

Institution	Catalog number	RRID (Cellosaurus)	Cell line	genotype
Horizon Discovery	C631	CVCL_Y019	HAP1	WT
Horizon Discovery	HZGHC005968c010	CVCL_SH72	HAP1	*CD44* KO
ATCC	CRL-1573	CVCL_0045	HEK293	WT
ATCC	CCL-2	CVCL_0030	HeLa	WT
ATCC	CCL-185	CVCL_0023	A-549	WT

Figure 1: CD44 antigen antibody screening by immunoblot.

Lysates of HAP1 (WT or *CD44* KO) were prepared and 50 µg of protein were processed for immunoblot with the indicated CD44 antibodies. The Ponceau stained transfers of each blot are shown. Antibody dilution used: 15675-1-AP at 1/5000; 60224-1 at 1/5000; GTX102111 at 1/5000; GTX628472 at 1/500; GTX628895 at 1/500; MA5-13890 at 1/200; 14-0441-82 at 1/500; NB600-1317 at 1/500; NBP1-47386 at 1/1000; BBA10 at 1/250; ab254530 1/1000; ab189524 at 1/1000; ab25340 at 1/1000; ab264539 at 1/1000; ab101531 at 1/1000; ab51037 at 1/1000; ab157107 at 1/2000; HPA005785 at 1/3000; 5640 at 1/1000. The canonical CD44 isoform is predicted to run at 81 kDa.

Figure 2: CD44 antigen immunoblot on a panel of cell lines.

Lysates were prepared from HAP1 (WT and *CD44* KO), HEK293, HeLa and A549, and 50µg of protein were processed for immunoblot using the indicated CD44 antigen antibodies. A long (upper panel; full-length blot) and short (middle panel; cropped region) exposures are shown for each antibody. The Ponceau stained transfers of each blot are shown (bottom panel). Antibody dilution used: GTX102111 at 1/5000; MA5-13890 at 1/500; ab25340 at 1/2000; ab189524 at 1/2000; ab51037 at 1/5000; 5640 at 1/500.

Figure 3: CD44 antigen antibody screening by immunoprecipitation.

HAP1 lysates were prepared and immunoprecipitation was performed using 1.0 µg of the indicated CD44 antigen antibodies pre-coupled to either protein G or protein A Sepharose beads. **(A)** Ability of the antibodies to capture CD44 antigen was first assessed by comparing the level of CD44 antigen from the starting material (SM) to its level remaining in the unbound fractions (UB). CD44 antigen antibody ab254530 at 1/2000 was used for each immunoblot. **(B)** Analysis of the immunoprecipitates for antibodies (except HPA005785) that showed depleted CD44 antigen in the UB from (A). Antibodies ab189524 and ab254530 were both used at a dilution of 1/2000. The Ponceau stained transfers of each blot are shown. SM=10% starting material; UB=10% unbound fraction; IP=immunoprecipitate

Figure 4: CD44 antigen antibody screening by immunofluorescence.

WT and *CD44* 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 CD44 antigen antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody together with DAPI. Acquisition of the blue (DAPI-nucleus), green (WT cells), red (antibody staining) and far-red (KO cells) channels was performed and representative images are shown. WT and KO cells are outlined with yellow and magenta dashed lines, respectively. Representative images of the merged red (grayscale) and blue channels are shown. Antibody dilution used: 15675-1-AP at 1/300; 60224-1 at 1/1300; GTX102111 at 1/500; GTX628472 at 1/100; GTX628895 at 1/700; MA5-13890 at 1/200; 14-0441-82 at 1/500; NB600-1317 at 1/1000; NBP1-47386 at 1/1000; BBA10 at 1/500; ab254530 1/500; ab189524 at 1/500; ab25340 at 1/500; ab264539 at 1/1300; ab101531 at 1/1000; ab51037 at 1/2000; ab157107 at 1/1000; HPA005785 at 1/1000.

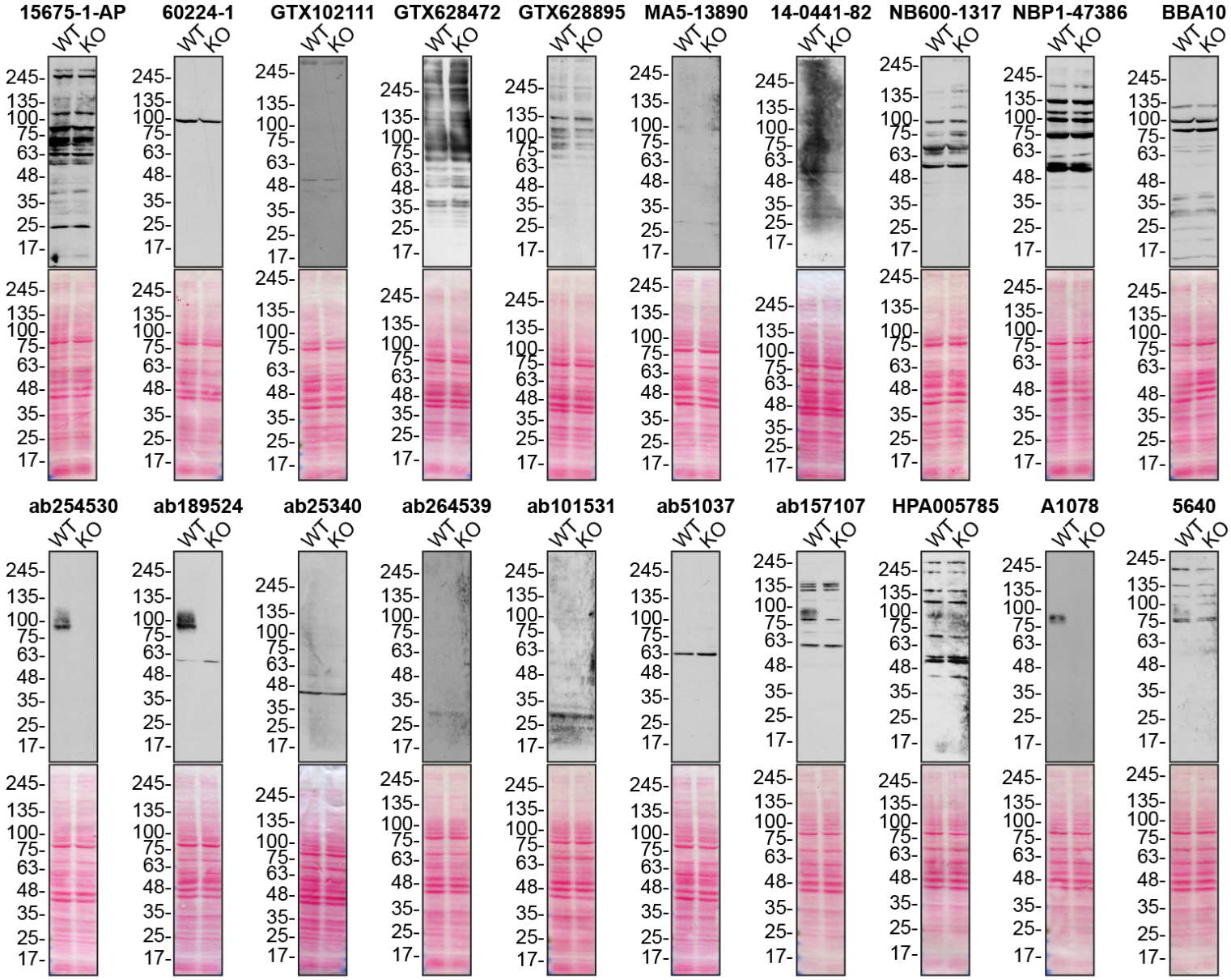

Figure 1: CD44 antibody screening by immunoblot

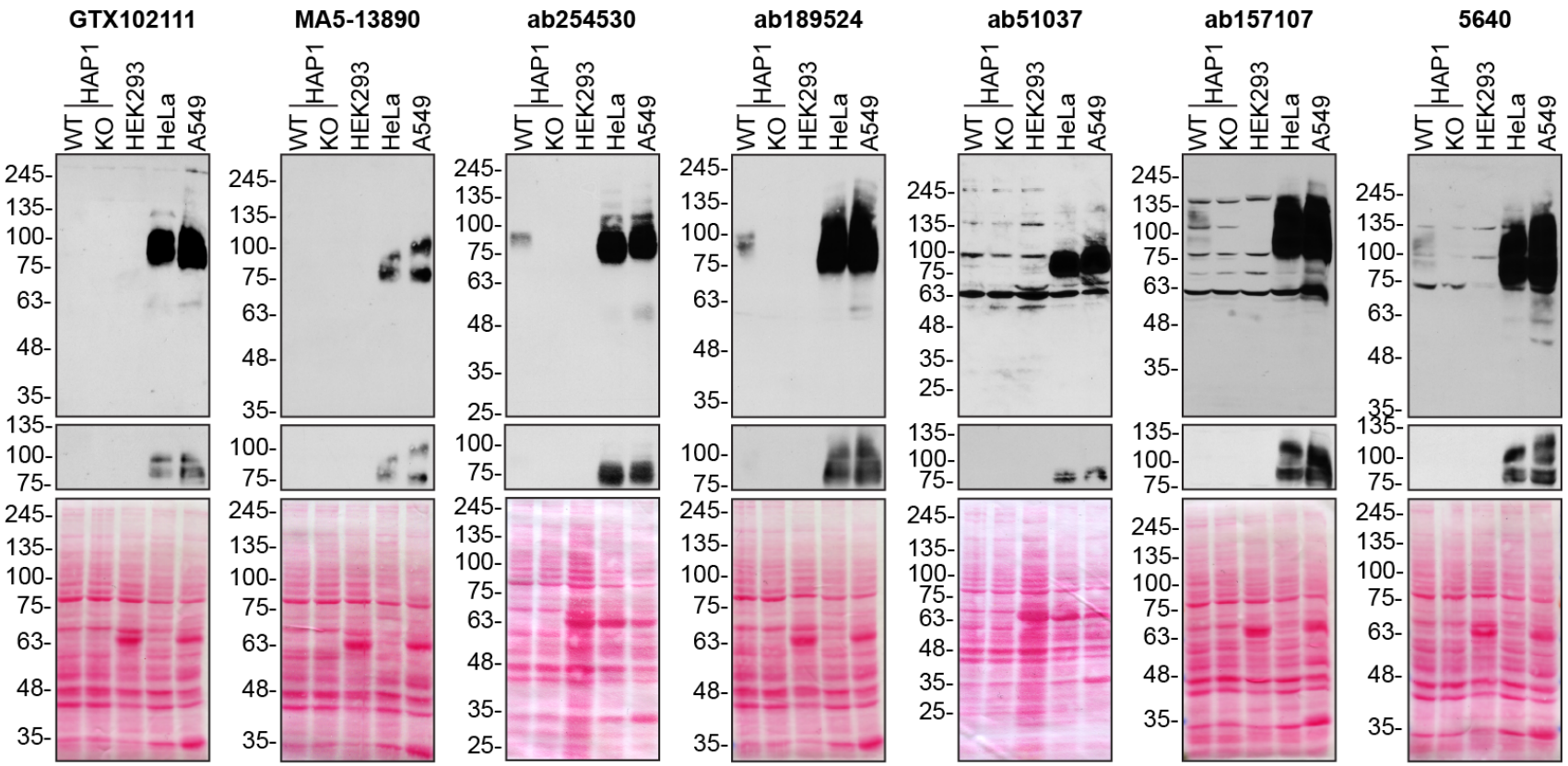

Figure 2: CD44 antigen immunoblot on a panel of cell lines

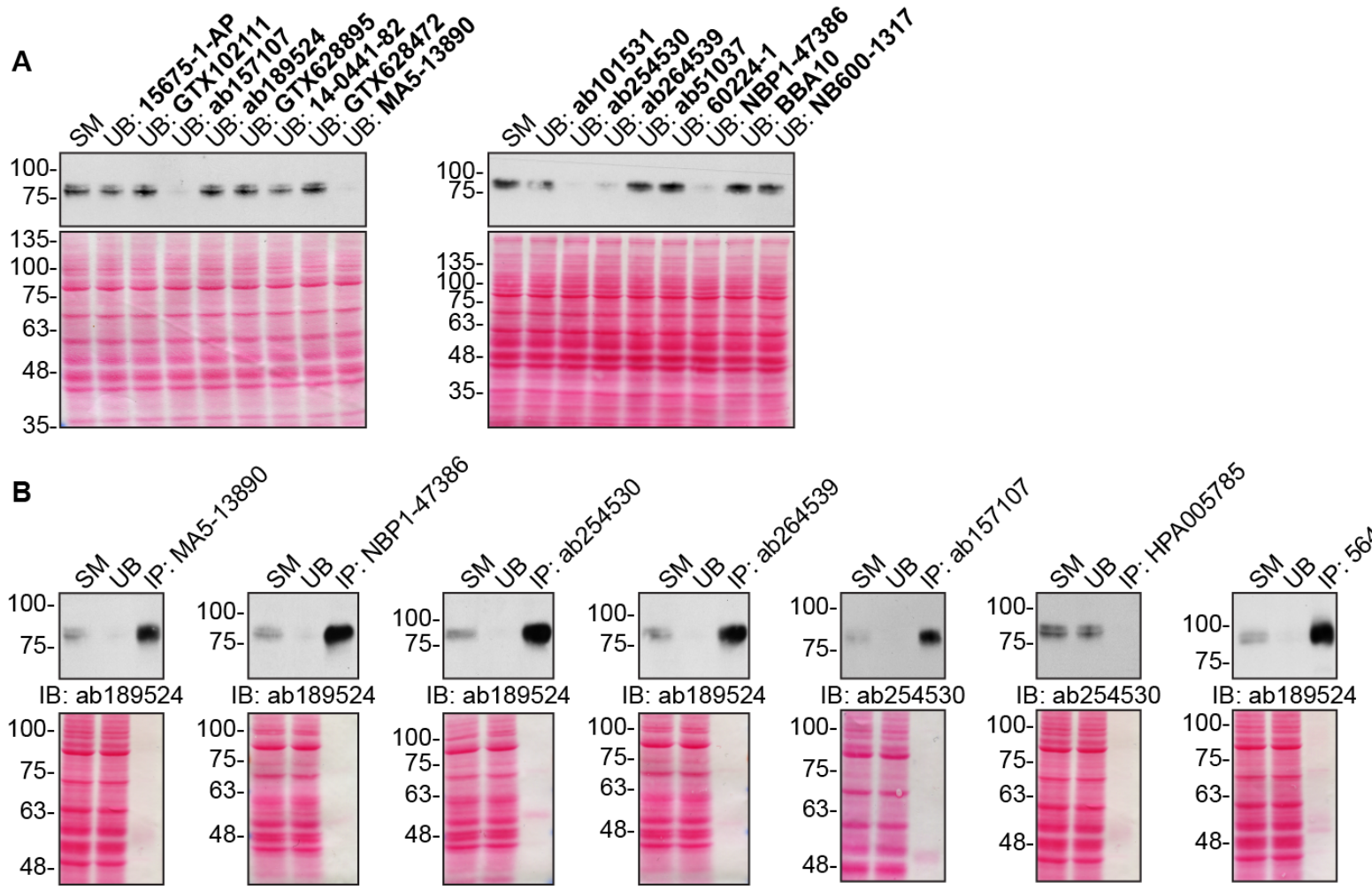

Figure 3 : CD44 antigen antibody screening by immunoprecipitation

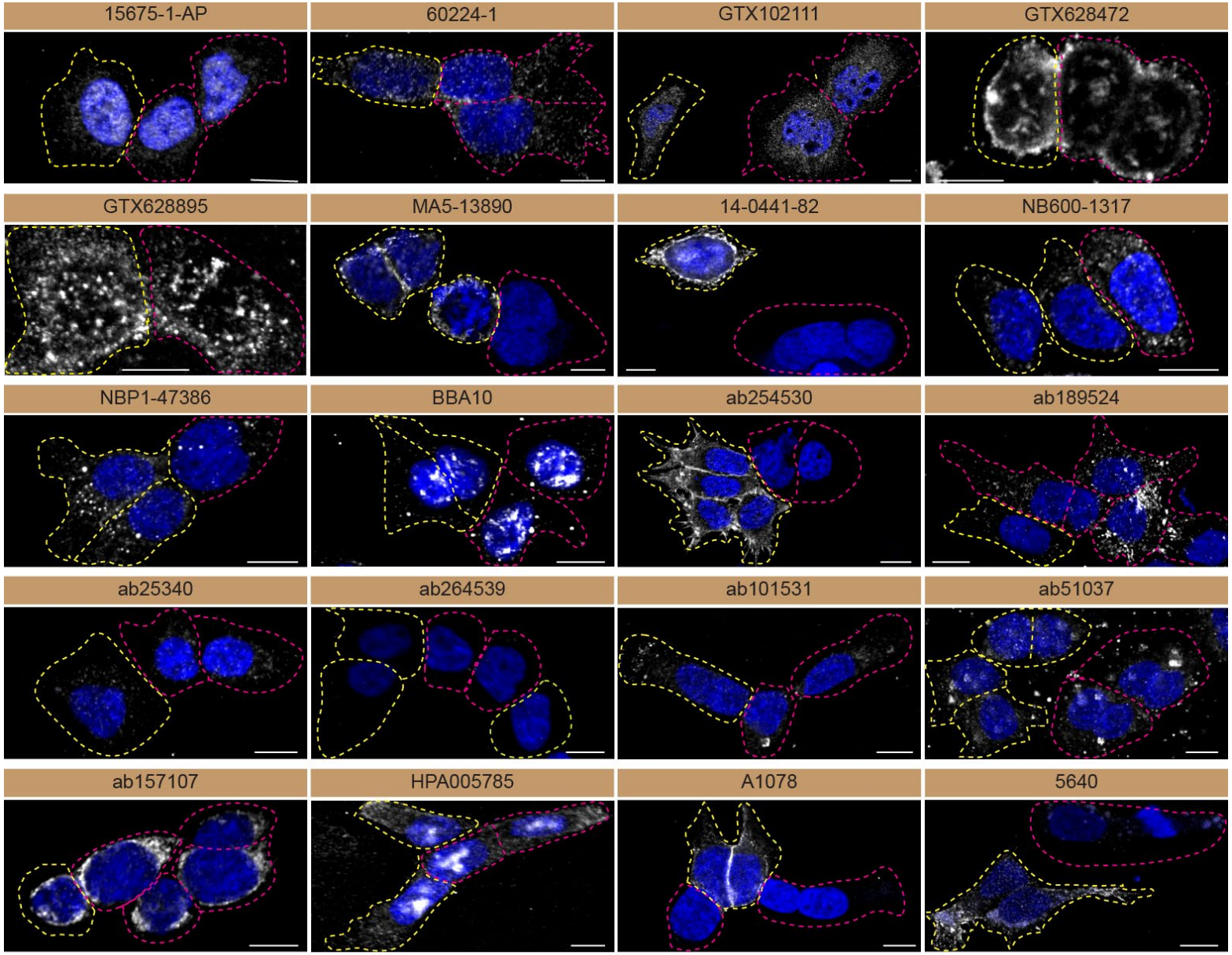

Figure 4 : CD44 antigen antibody screening by immunofluorescence

Materials and methods

Antibodies

All CD44 antigen 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).

Cell lines

All cell lines are listed in Table 2. 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).

Immunoblot

HAP1 (WT and *CD44* KO), HEK293, HeLa and A549 cells 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 1) or 8% (Figure 2) 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).

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.

HAP1 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.

Immunofluorescence

HAP1 WT and *CD44* KO were labelled with a green and with a deep red fluorescence dye from Abcam (cat. number ab176736 and ab176735), 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 CD44 antigen 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 0.5 µg/ml for 1 hr at room temperature with DAPI. 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.