A guide to selecting high-performing antibodies for Rab27A (UniProt ID: P51159) for use in western blot, immunoprecipitation, and immunofluorescence

Vera Ruíz Moleón[1], Charles Alende[†1], Maryam Fotouhi[†1], Riham Ayoubi[1], Kathleen Southern[1], Carl Laflamme[1]*, NeuroSGC/YCharOS/EDDU collaborative group and ABIF consortium

[†] Authors contributed equally and are listed alphabetically
[1] Department of Neurology and Neurosurgery, Structural Genomics Consortium, The Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada
* Corresponding author: carl.laflamme@mcgill.ca

Abstract

Rab27A is a small Rab GTPase essential for vesicle exocytosis and exosome release, processes which facilitate cellular communication. Involved in various pathological conditions including immune deficiencies and cancer, highlights the need for the availability of high-performing antibodies to progress Rab27A related research, offering significant benefits to the scientific community. Here we have characterized fourteen Rab27A commercial antibodies for western blot, immunoprecipitation, and immunofluorescence using a standardized experimental protocol based on comparing read-outs in knockout cell lines and isogenic parental controls. These studies are part of a larger, collaborative initiative seeking to address antibody reproducibility issues by characterizing commercially available antibodies for human proteins and publishing the results openly as a resource for the scientific community. While use of antibodies and protocols vary between laboratories, we encourage readers to use this report as a guide to select the most appropriate antibodies for their specific needs.

Keywords:

UniProt ID: P51159, *RAB27A*, Rab27A, antibody characterization, antibody validation, western blot, immunoprecipitation, immunofluorescence

Introduction

The family of small Rab GTPase modulates membrane transport processes (1, 2). Among these, the Rab27A protein plays a central role in exosome secretion by regulating the fusion of the multivesicular body with the plasma membrane, facilitating intercellular communication through the transmission of signal molecules (3-6). Highly expressed in melanocytes and lymphocytes, mutations to the *RAB27A* gene are associated with Griscelli syndrome type 2, characterized by hypopigmentation and immune deficiency (7, 8). It has also been reported to be involved in cancer development (9, 10). As such, Rab27A is a critical research focus, with implications in understanding and treating various diseases.

This research is part of a broader collaborative initiative in which academics, funders and commercial antibody manufacturers are working together to address antibody reproducibility issues by characterizing commercial antibodies for human proteins using standardized protocols, and openly sharing the data (11-13). Here we evaluated the performance of fourteen commercial antibodies for Rab27A for use in western blot, immunoprecipitation, and immunofluorescence, enabling biochemical and cellular assessment of Rab27A properties and function. The platform for antibody characterization used to carry out this study was endorsed by a committee of industry academic representatives. It consists of identifying human cell lines with adequate target protein expression and the development/contribution of equivalent knockout (KO) cell lines, followed by antibody characterization procedures using most commercially available antibodies against the corresponding protein. The standardized consensus antibody characterization protocols are openly available on Protocol Exchange (DOI: 10.21203/rs.3.pex-2607/v1) (14).

The authors do not engage in result analysis or offer explicit antibody recommendations. Our primary aim is to deliver top-tier data to the scientific community, grounded in Open Science principles. This empowers experts to interpret the characterization data independently, enabling them to make informed choices regarding the most suitable antibodies for their specific experimental needs. Guidelines on how to interpret antibody characterization data found in this study are featured on the YCharOS gateway (15).

Results and discussion

Our standard protocol involves comparing readouts from WT (wild type) and KO cells (16, 17). The first step was to identify a cell line(s) that expresses sufficient levels of a given protein to generate a measurable signal using antibodies. To this end, we examined the DepMap transcriptomics database (Cancer Dependency Map Portal, RRID:SCR_017655) to identify all cell lines that express the target at levels greater than 2.5 $\log_2$ (transcripts per million "TPM" + 1), which we have found to be a suitable cut-off (11). The commercially available HAP1 cell line from the vendor Horizon Discovery expresses the *RAB27A* transcript at 3.3 $\log_2$ (TPM+ 1) and was selected for preliminary antibody testing. However, the signal-to-noise ratio for most antibodies was low, indicating that HAP1 may not express the Rab27A protein at adequate levels for detection by antibodies (DOI:10.5281/zenodo.10891238). To improve antibody detection, we sought to identify cell lines with higher endogenous Rab27A protein levels. Using DepMap, we selected cell lines with high RNA expression levels (Table 1) and compared Rab27A protein levels

across these lines using western blot analysis. The Rab27A antibody 95394** was selected because of its ability to specifically recognize Rab27A, as indicated by the signal in the HAP1 WT lysate that was absent in the HAP1 KO lysate (Figure 1, lanes 1 and 2). All screened cell lines showed higher Rab27A endogenous protein levels than HAP1 (Figure 1). The U87 MG line was chosen for further experimentation due to its adherent growth characteristics and large flat morphology, which are beneficial for immunofluorescence screening. Additionally, U87 MG can be easily transfected with siRNA for gene knockdown (KD) purposes. Consequently, *RAB27A* KD in U87 MG was used as a negative control to screen for Rab27A antibodies in western blot and immunofluorescence, while HAP1 WT was used for immunoprecipitation.

For western blot experiments, U-87 MG WT and *RAB27A* KD protein lysates were ran on SDS-PAGE, transferred onto nitrocellulose membranes, and then probed with fourteen Rab27A antibodies in parallel (Figure 2).

We then assessed the capability of all fourteen antibodies to capture Rab27A from HAP1 protein extracts using immunoprecipitation techniques, followed by western blot analysis. A specific Rab27A antibody identified in the previous step (refer to Figure 2) was selected for immunoblotting. Equal amounts of the starting material (SM), the unbound fraction (UB), as well as the whole immunoprecipitate (IP) eluates were separated by SDS-PAGE (Figure 3).

For immunofluorescence, the fourteen Rab27A antibodies were screened using a mosaic strategy. First, U-87 MG WT and *RAB27A* KD cells were labelled with distinct fluorescent dyes in order to distinguish the two cell lines, and the Rab27A antibodies were evaluated. Both WT and KD lines were imaged in the same field of view to minimize staining, imaging and image analysis bias (Figure 4). Quantification of immunofluorescence intensity in hundreds of WT and KD cells was performed for each antibody tested, and the images presented in Figure 4 are representative of this analysis (14).

In conclusion, we have compared antibody performance of fourteen Rab27A commercial antibodies in western blot, immunoprecipitation, and immunofluorescence using either human U-87 MG WT and *RAB27A* KD cells or the HAP1 cell line. To assist viewers in interpreting antibody performance, Table 3 outlines various scenarios in which antibodies may perform in all three applications. Several high-quality and renewable antibodies that successfully detect Rab27A were identified in all applications. Researchers who wish to study Rab27A in a different species are encouraged to select high-quality antibodies, based on the results of this study, and investigate the predicted species reactivity of the manufacturer before extending their research.

Limitations

Inherent limitations are associated with the antibody characterization platform used in this study. Firstly, the YCharOS project focuses on renewable (recombinant and monoclonal) antibodies and does not test all commercially available Rab27A antibodies. YCharOS partners provide approximately 80% of all renewable antibodies, but some top-cited polyclonal antibodies may not be available through these partners.

Secondly, the YCharOS effort employs a non-biased approach that is agnostic to the protein for which antibodies have been characterized. The aim is to provide objective data on antibody performance without preconceived notions about how antibodies should perform or the molecular weight that should be observed in western blot. As the authors are not experts on the Rab family of proteins or vesicle trafficking, a brief overview of the protein's function and its relevance in disease is provided. Rab27A experts are responsible for analyzing and interpreting observed banding patterns in western blots and subcellular localization in immunofluorescence.

Thirdly, YCharOS experiments are not performed in replicates primarily due to the use of multiple antibodies targeting various epitopes. Once a specific antibody is identified, it validates the protein expression of the intended target in the selected cell line, confirming the lack of protein expression in the KO cell line and supports conclusions regarding the specificity of the other antibodies. All experiments are performed using master mixes, and meticulous attention is paid to sample preparation and experimental execution. In immunofluorescence, the use of two different concentrations serves to evaluate antibody specificity and can aid in assessing assay reliability. In instances where antibodies yield no signal, a repeat experiment is conducted following titration. Additionally, our independent data is performed subsequently to the antibody manufacturers internal validation process, therefore making our characterization process a repeat.

Lastly, as comprehensive and standardized procedures are respected, any conclusions remain confined to the experimental conditions and cell line used for this study. The use of a single cell type for evaluating antibody performance poses as a limitation, as factors such as target protein abundance significantly impact results (14). Additionally, the use of cancer cell lines containing gene mutations poses a potential challenge, as these mutations may be within the epitope coding sequence or other regions of the gene responsible for the intended target. Such alterations can impact the binding affinity of antibodies. This represents an inherent limitation of any approach that employs cancer cell lines.

Methods

The standardized protocols used to carry out this KD cell line-based antibody characterization platform was established and approved by a collaborative group of academics, industry researchers and antibody manufacturers. The detailed materials and step-by-step protocols used to characterize antibodies in western blot, immunoprecipitation and immunofluorescence are openly available on Protocol Exchange, (DOI: 10.21203/rs.3.pex-2607/v1) (14).

Cell lines and antibodies used

Cell lines used and primary antibodies tested in this study are listed in Table 1 and 2, respectively. To ensure that the cell lines and antibodies are cited properly and can be easily identified, we have included their corresponding Research Resource Identifiers, or RRID (18, 19). HAP1 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-glutamine (Wisent cat. number 609-065, 100 IU penicillin and 100 µg/ml streptomycin (Wisent cat. number 450201). THP-1 cells were treated with 200 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Abcam, cat. number ab147465) for 2 days (Figure 1, lane 5) (20). For knockdown, U-87 MG cells were treated twice with 10 nM of *RAB27A* SMARTpool siRNA (Horizon Discovery, cat. number L-004667-00-0005). Lipofectamine RNAiMAX (Thermo, cat. number 13778030) was used to transfect the siRNA following the manufacturer's protocol. All other cell types were cultured as recommended by the provider.

Peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies are from Thermo Fisher Scientific (cat. number 65-6120 and 62-6520). Alexa-555-conjugated goat anti-rabbit and anti-mouse secondary antibodies are from Thermo Fisher Scientific (cat. number A21429 and A21424). Peroxidase-conjugated Protein A for IP detection is from MilliporeSigma, cat. number P8651.

Antibody screening by western blot

U-87 MG WT and *RAB27A* KD cells were collected in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Fisher Scientific, cat. number 89901) supplemented with 1x protease inhibitor cocktail mix (MilliporeSigma, cat. number P8340). Lysates were sonicated briefly and incubated for 30 min on ice. Lysates were spun at ~110,000 *x g* for 15 min at 4°C and equal protein aliquots of the supernatants were analyzed by SDS-PAGE and western blot. BLUelf prestained protein ladder (GeneDireX, cat. number PM008-0500) was used.

Western blots were performed with precast midi 10% Bis-Tris polyacrylamide gels from Thermo Fisher Scientific (cat. number WG1201BOX) ran with MES SDS buffer (Thermo Fisher Scientific, cat. number NP000202), loaded in LDS sample buffer (Thermo Fisher Scientific, cat. number NP0008) with 1x sample reducing agent (Thermo Fisher Scientific, cat. number NP0009) and transferred on nitrocellulose membranes. Proteins on the blots were visualized with Ponceau S staining (Thermo Fisher Scientific, cat. number BP103-10) which is scanned to show together with individual western blot. Blots were blocked with

5% milk for 1 hr, and antibodies were incubated overnight at 4°C with 5% milk in TBS with 0,1% Tween 20 (TBST) (Cell Signalling Technology, cat. number 9997). 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 were incubated with Pierce ECL (Thermo Fisher Scientific, cat. number 32106) or Clarity Western ECL Substrate (Bio-Rad, cat. number 1705061) prior to detection with the iBright™ CL1500 Imaging System (Thermo Fisher Scientific (cat. number A44240).

Antibody screening by immunoprecipitation

Antibody-bead conjugates were prepared by adding 2 µg to 500 µl of Pierce IP Lysis Buffer (Thermo Fisher Scientific, cat. number 87788) in a microcentrifuge tube, together with 30 µl of Dynabeads protein A- (for rabbit antibodies) or protein G- (for mouse and sheep antibodies) (Thermo Fisher Scientific, cat. number 10002D and 10004D, respectively). Tubes were rocked for ~1 hr at 4°C followed by two washes to remove unbound antibodies.

HAP1 WT cells were collected Pierce IP buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) supplemented with protease inhibitor. Lysates were rocked for 30 min at 4°C and spun at 110,000 *x g* for 15 min at 4°C. 0.5 ml aliquots at 2.0 mg/ml of lysate were incubated with an antibody-bead conjugate for ~1 hr at 4°C. The unbound fractions were collected, and beads were subsequently washed three times with 1.0 ml of IP lysis buffer and processed for SDS-PAGE and western blot on a precast midi 10% Bis-Tris polyacrylamide gels. Protein A: HRP was used as a secondary detection system at a dilution of 2.0 µg/ml.

Antibody screening by immunofluorescence

U-87 MG WT and *RAB27A* KD cells were labelled with a green and a far-red fluorescence dye, respectively (Thermo Fisher Scientific, cat. number C2925 and C34565). The nuclei were labelled with DAPI (Thermo Fisher Scientific, cat. Number D3571) fluorescent stain. WT and KO cells were plated on 96-well plate with optically clear flat-bottom (Perkin Elmer, cat. number 6055300) as a mosaic and incubated for 24 hrs in a cell culture incubator at 37ºC, 5% CO_2. Cells were fixed in 4% paraformaldehyde (PFA) (Beantown chemical, cat. number 140770-10ml) in phosphate buffered saline (PBS) (Wisent, cat. number 311-010-CL). Cells were permeabilized in PBS with 0,1% Triton X-100 (Thermo Fisher Scientific, cat. number BP151-500) for 10 min at room temperature and blocked with PBS with 5% BSA, 5% goat serum (Gibco, cat. number 16210-064) 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 Rab10 antibodies overnight at 4°C. Cells were then 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 with DAPI. Cells were washed 3 × 10 min with IF buffer and once with PBS.

Images were acquired on an ImageXpress micro confocal high-content microscopy system (Molecular Devices), using a 20x NA 0.95 water immersion objective and scientific CMOS cameras, equipped with 395, 475, 555 and 635 nm solid state LED lights (lumencor Aura III light engine) and bandpass filters to excite DAPI, Cellmask Green, Alexa-555 and Cellmask Red, respectively. Images had pixel sizes of 0.68 x 0.68 microns, and a z-interval of 4 microns. For analysis and visualization, shading correction (shade only) was carried out for all images. Then, maximum intensity projections were generated using 3 z-slices. Segmentation was carried out separately on maximum intensity projections of Cellmask channels using CellPose 1.0, and masks were used to generate outlines and for intensity quantification (21). Figures were assembled with Adobe Illustrator.

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Competing interests

For this project, the laboratory of Peter McPherson developed partnerships with high-quality antibody manufacturers and KO cell line providers. The partners provide antibodies and KO cell lines to the McPherson laboratory at no cost. These partners include: - Abcam-ABCD antibodies- ABclonal- Aviva Systems Biology -Bio Techne -Cell Signalling Technology -Developmental Studies Hybridoma Bank - GeneTex – Horizon Discovery – Proteintech – Synaptic Systems –Thermo Fisher Scientific.

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ABIF consortium: Claire M. Brown and Joel Ryan

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Table 1: Summary of the cell lines used

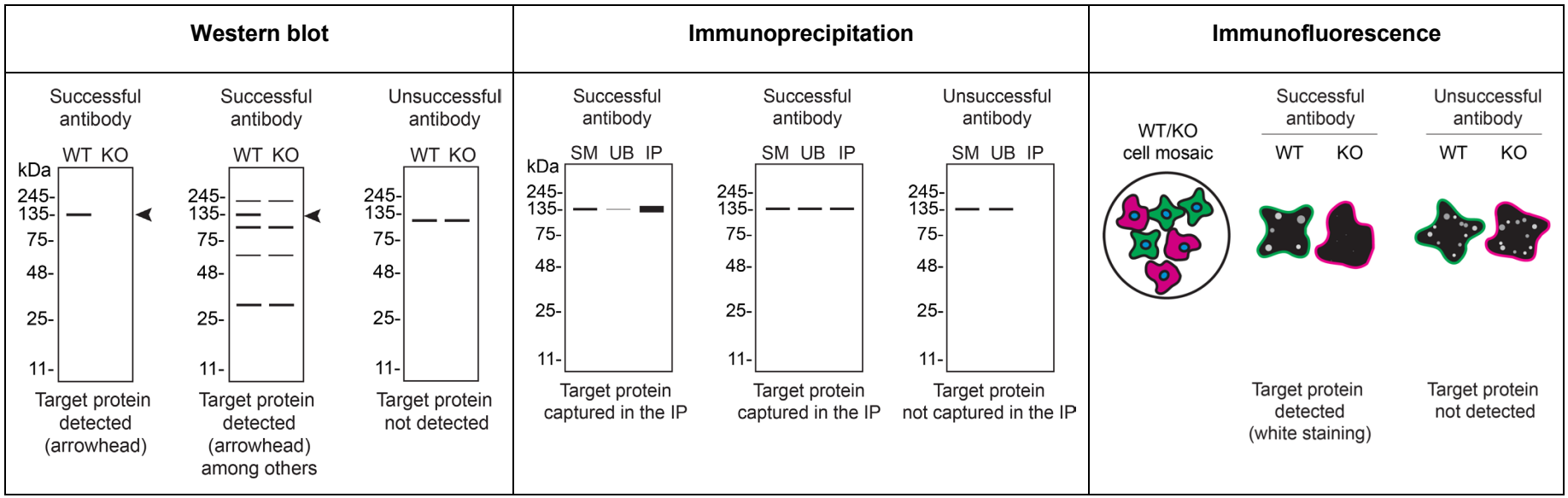
Institution	Catalog number	RRID (Cellosaurus)	Cell line	Genotype
Horizon Discovery	C631	CVCL_Y019	HAP1	WT
Horizon Discovery	HZGHC001240c001	CVCL_TI06	HAP1	*RAB27A* KO
ATCC	CCL-243	CVCL_0004	K-562	WT
ATCC	TIB-202	CVCL_0006	THP-1	WT
ATCC	HTB-14	CVCL_0022	U-87 MG	WT
ATCC	CCL-121	CVCL_0317	HT-1080	WT
ATCC	HTB-96	CVCL_0042	U-2 OS	WT
Abcam	ab255448	CVCL_0030	HeLa	WT
ATCC	HTB-26	CVCL_0062	MDA-MB-231	WT

Table 2: Summary of the Rab27A antibodies tested

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (µg/µl)	Vendors recommended applications
Abcam	ab313575**	1061329-3	AB_3101812	recombinant mono	EPR27120-68	rabbit	0.50	Wb, IP, IF
Abcam	ab55667*	1074760-2	AB_945112	monoclonal	1G7	mouse	1.00	Wb, IP, IF
ABclonal	A23993**	6100003559	AB_3083442	recombinant mono	ARC61834	rabbit	1.91	Wb, IF
Bio-Techne	AF7245	CFVM0120121	AB_10973494	polyclonal	-	sheep	0.20	Wb
Bio-Techne	MAB7245**	CMPX0119121	AB_3075307	recombinant mono	2537A	rabbit	0.50	Wb
Cell Signaling Technology	69295**	2	AB_2799759	recombinant mono	D7Z9Q	rabbit	0.08	Wb, IP, IF
Cell Signaling Technology	95394**	10180028	AB_2800247	recombinant mono	D7V6B	rabbit	0.01	Wb, IP, IF
Proteintech	16868-1-AP	9045	AB_2176716	polyclonal	-	rabbit	0.13	Wb
Proteintech	17817-1-AP	48869	AB_2176728	polyclonal	-	rabbit	0.33	Wb, IP, IF
Proteintech	66058-1-Ig*	10005592	AB_11042596	monoclonal	1C4B8	mouse	1.60	Wb
Synaptic Systems	168 013	08-Jan	AB_887766	polyclonal	-	rabbit	1.00	Wb, IP
Thermo Fisher Scientific	MA1-172*	RD232787	AB_2608617	monoclonal	4D3F11	mouse	1.00	WB
Thermo Fisher Scientific	PA5-102522	YA3806213A	AB_2851924	polyclonal	-	rabbit	1.00	Wb, IF
Thermo Fisher Scientific	PA5-79904	YA3806947A	AB_2747019	polyclonal	-	rabbit	0.50	Wb, IF

Wb=western blot; IF= immunofluorescence; IP=immunoprecipitation, *= monoclonal antibody, **= recombinant antibody

Table 3: Illustrations to assess antibody performance in all western blot, immunoprecipitation and immunofluorescence



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95394**

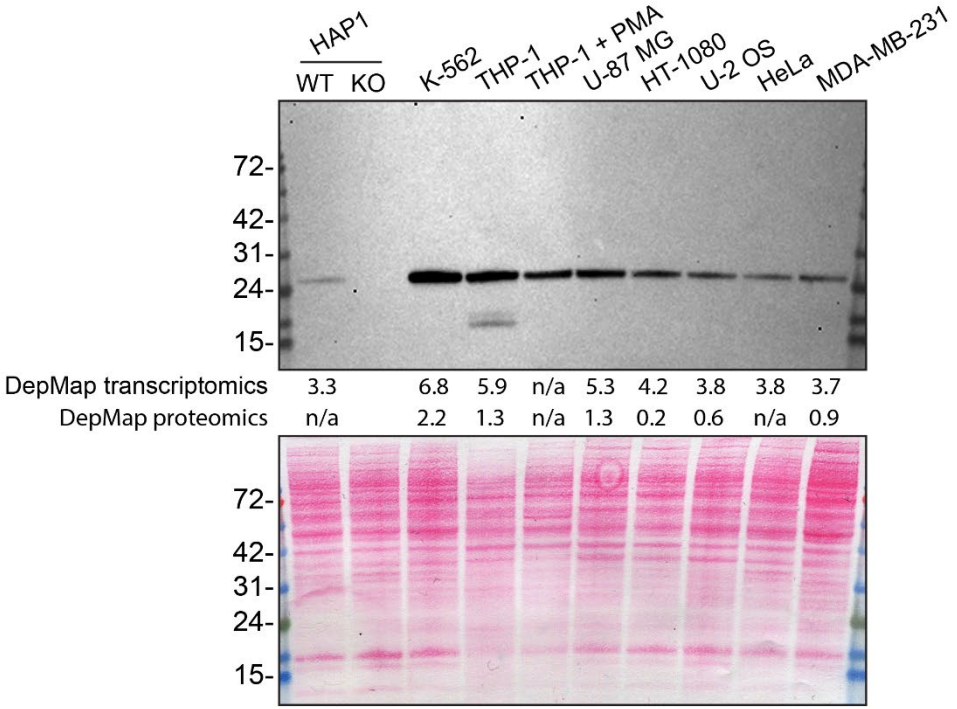

Figure 1: Rab27A western blot on various cell lysates

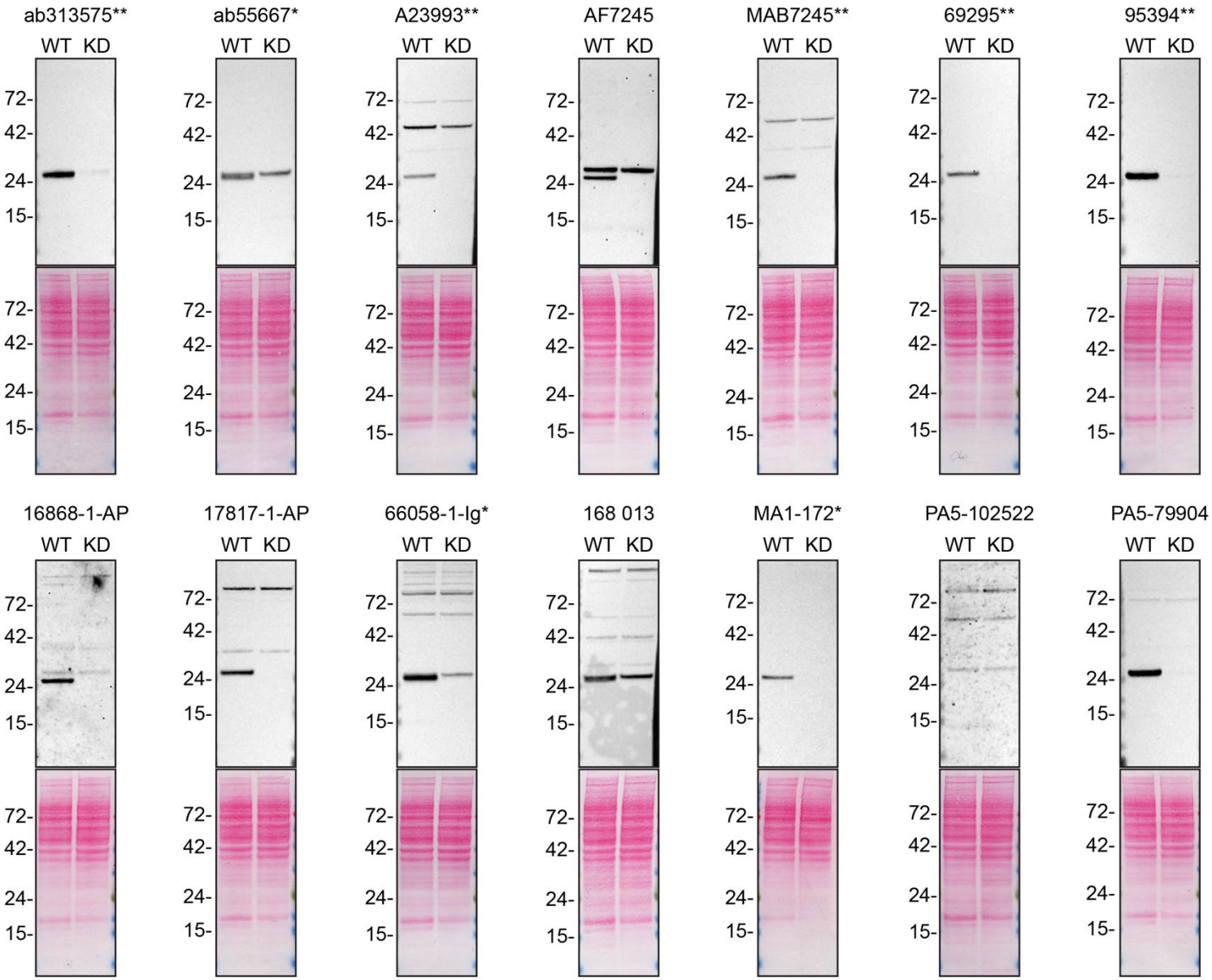

Figure 2: Rab27A antibody screening by western blot

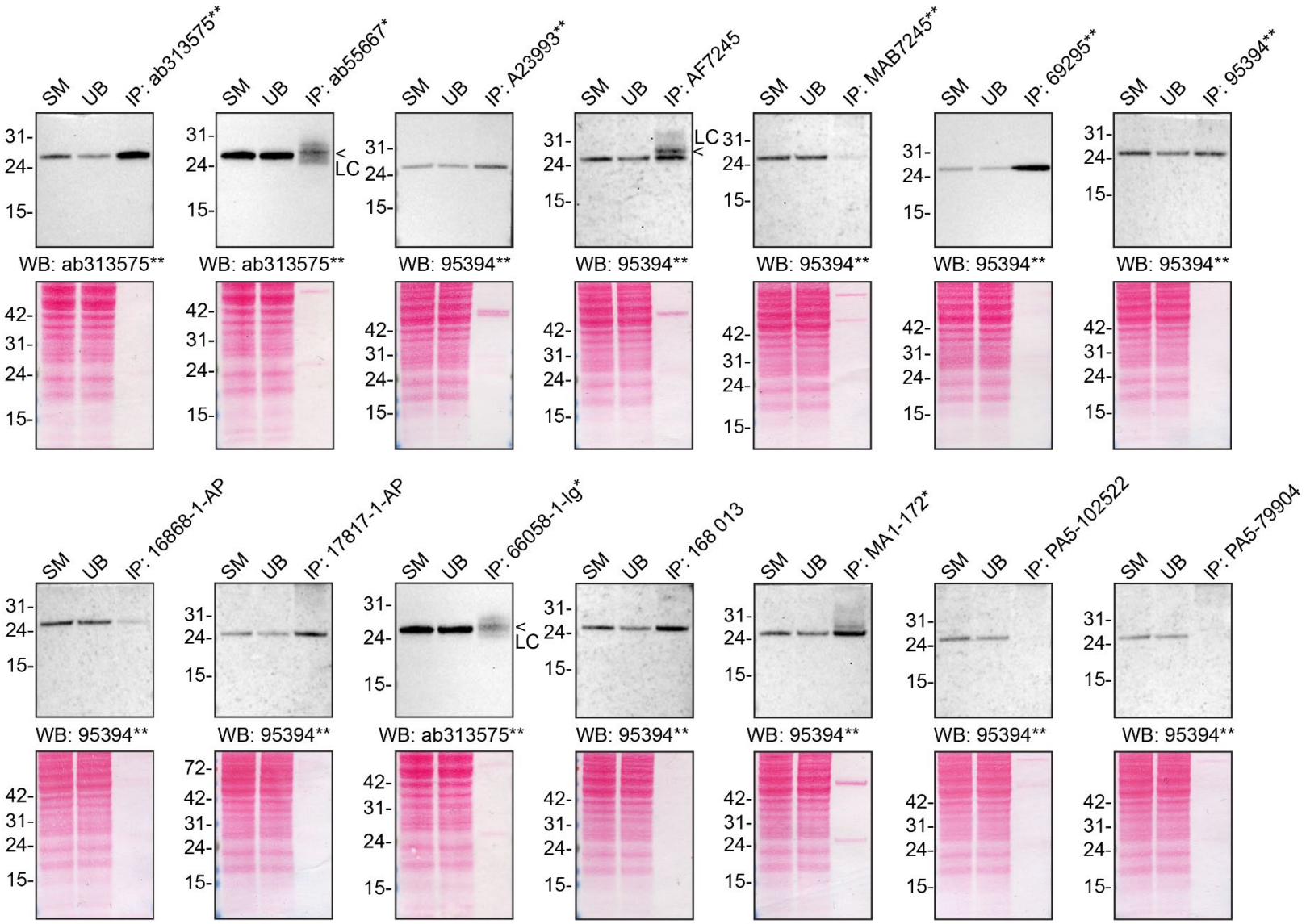

Figure 3: Rab27A antibody screening by immunoprecipitation

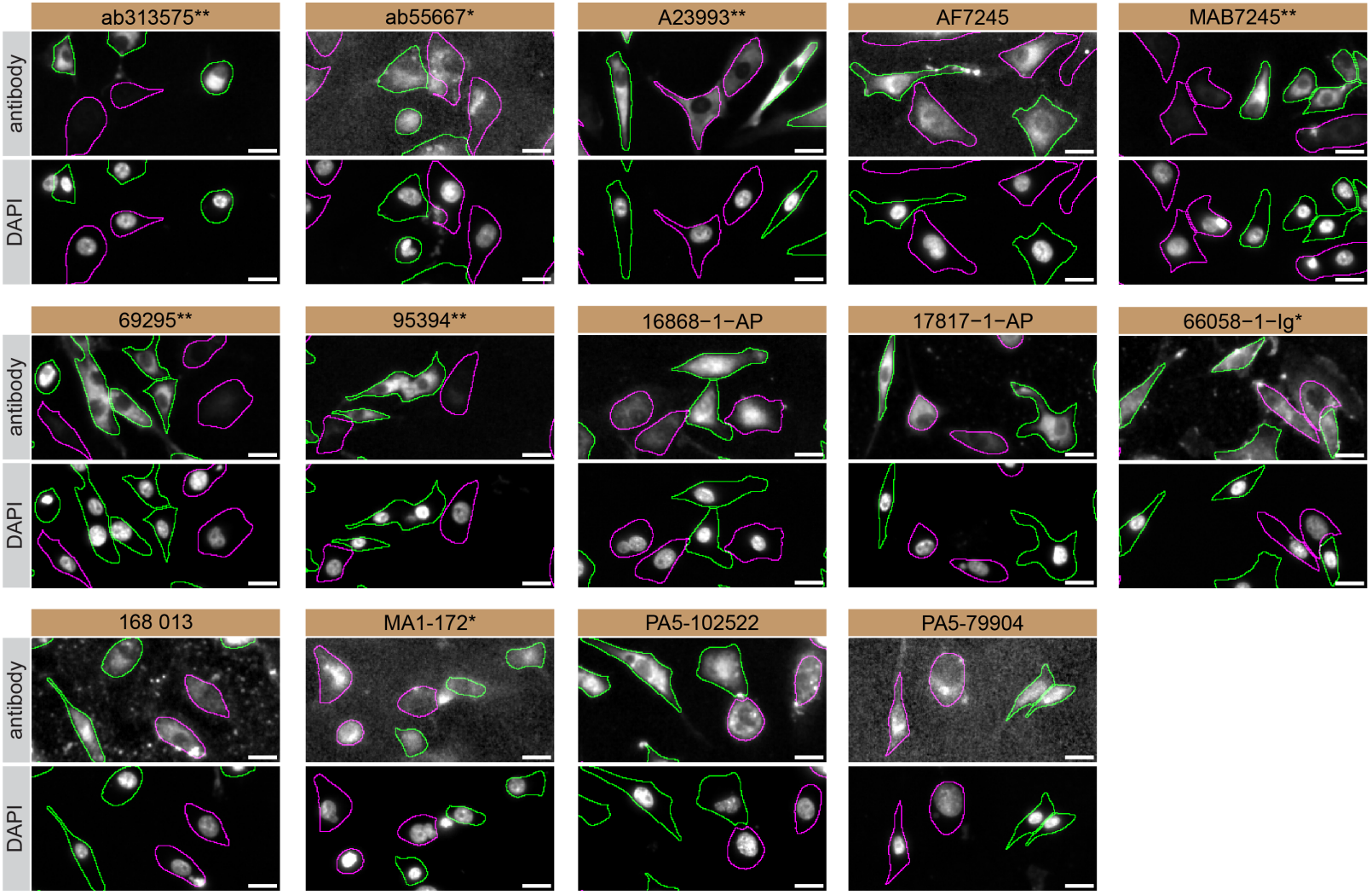

Figure 4: Rab27A antibody screening by immunofluorescence

FIGURE LEGENDS

Figure 1: Rab27A western blot on various cell lysates.

Lysates from HAP1 (WT and *RAB27A* KO), K-562, THP-1 (untreated or treated with 200 ng/ml PMA), U-87 MG, HT-1080, U-2 OS, HeLa, MDA-MB-231 were prepared, and 20 µg of protein were processed for western blot. The Ponceau stained transfers is presented to show equal loading of WT and KO lysates and protein transfer efficiency from the acrylamide gels to the nitrocellulose membrane. The antibody 95394** was used for western blot at 1/200. Predicted band size: 24.8 kDa. **= recombinant antibody, PMA= Phorbol myristate acetate, n/a= not available.

Figure 2: Rab27A antibody screening by western blot.

Lysates from U-87 MG WT and *RAB27A* KD were prepared, and 40 µg of protein were processed for western blot with the indicated Rab27A antibodies. The Ponceau stained transfers of each blot are presented to show equal loading of WT and KD lysates and protein transfer efficiency from the acrylamide gels to the nitrocellulose membrane. Antibody dilutions were chosen according to the recommendations of the antibody supplier. Exceptions were given for antibodies AF7245 and 95394** which were titrated because the signal was too weak when following the supplier's recommendations. Antibody dilution used: ab313575** at 1/1000, ab55667* at 1/1000, A23993** at 1/2000, AF7245 at 1/200, MAB7245** at 1/200, 69295** at 1/1000, 95394** at 1/200, 16868-1-AP at 1/1000, 17817-1-AP at 1/1000, 66058-1-Ig* at 1/5000, 168 013 at 1/1000, MA1-172* at 1/1000, PA5-102522 at 1/1000 and PA5-79904 at 1/1000. Predicted band size: 24.8 kDa, *= monoclonal antibody, **= recombinant antibody.

Figure 3: Rab27A antibody screening by immunoprecipitation.

HAP1 lysates were prepared, and immunoprecipitation was performed using 1 mg of lysate and 2.0 µg of the indicated Rab27A antibodies pre-coupled to Dynabeads protein A or protein G. Samples were washed and processed for western blot with the indicated Rab27A antibody. For western blot, ab313575** and 95394** were used at 1/1000 and 1/200, respectively. The Ponceau stained transfers of each blot are shown. SM=4% starting material; UB=4% unbound fraction; IP=immunoprecipitate, LC= antibody light chain. *= monoclonal antibody, **= recombinant antibody.

Figure 4: Rab27A antibody screening by immunofluorescence.

U-87 MG WT and *RAB27A* KD cells were labelled with a green or a far-red fluorescent dye, respectively. WT and KD cells were mixed and plated to a 1:1 ratio on coverslips. Cells were stained with the indicatedRab27A antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody including DAPI. Acquisition of the blue (nucleus-DAPI), green (WT), red (antibody staining) and far-red (KD) channels was performed. Representative images of the merged blue and red (grayscale) channels are shown. WT and KD cells are outlined with green and magenta dashed line, respectively. When an antibody was recommended for immunofluorescence by the supplier, we tested it at the recommended dilution. The rest of the antibodies were tested at 1 and 2 µg/mL and the final concentration was selected based on the

detection range of the microscope used and a quantitative analysis not shown here. Antibody dilution used: ab313575** at 1/500, ab55667* at 1/X, A23993** at 1/1000, AF7245 at 1/200, MAB7245** at 1/150, 69295** at 1/100, 95394** at 1/400, 16868-1-AP at 1/50, 17817-1-AP at 1/150, 66058-1-Ig* at 1/800, 168 013 at 1/1000, MA1-172* at 1/1000, PA5-102522 at 1/500 and PA5-79904 at 1/250. Bars = 10 µm. *= monoclonal antibody, **= recombinant antibody.