

A guide to selecting high-performing antibodies for Rab6A (Uniprot ID: P20340) for use in western blot, immunoprecipitation, and immunofluorescence

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Abstract

Rab6A, a Rab GTPase, is involved in the regulation and transport of vesicles between the Golgi apparatus and endoplasmic reticulum. Here we have characterized twelve Rab6A 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 P20340, *RAB6A*, Rab6A, antibody characterization, antibody validation, western blot, immunoprecipitation, immunofluorescence

Introduction

Rab6A, a member of the Rab GTPase family, regulates vesicle trafficking between the Golgi apparatus and endoplasmic reticulum (1, 2). Dysfunction in Rab6A activity can lead to pathogenic conditions, including cancer and neurodegenerative diseases (3, 4). A deeper understanding of Rab6A's role in health and disease is a significant research focus, which would be facilitated with the availability of high-quality antibodies.

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 (5-7). Here, we evaluated the performance of twelve commercial antibodies for Rab6A for use in western blot, immunoprecipitation, and immunofluorescence, enabling biochemical and cellular assessment of Rab6A 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, a preprint server (DOI: [10.21203/rs.3.pex-2607/v1](https://doi.org/10.21203/rs.3.pex-2607/v1)) (8).

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 (9).

Results and discussion

Our standard protocol involves comparing readouts from WT (wild type) and KO cells (10, 11). 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 (5). The HAP1 cell line expresses the Rab6A transcript at $5.3 \log_2$ (TPM+1), which is above the average range of cancer cells analyzed. As such, *RAB6A* KO HAP1 cells were obtained from Horizon Discovery (Table 1).

For western blot experiments, WT and *RAB6A* KO protein lysates were ran on SDS-PAGE, transferred onto nitrocellulose membranes, and then probed with twelve Rab6A antibodies in parallel (Figure 1).

We then assessed the capability of all twelve antibodies to capture Rab6A from HAP1 protein extracts using immunoprecipitation techniques, followed by western blot analysis. For the immunoblot step, a Rab6A

antibody previously identified to be specific (refer to Figure 1) was selected. Equal amounts of the starting material (SM) and unbound fractions (UB), as well as the whole immunoprecipitate eluates were separated by SDS-PAGE (Figure 2).

For immunofluorescence, twelve antibodies were screened using a mosaic strategy. First, HAP1 WT and *RAB6A* KO cells were labelled with different fluorescent dyes in order to distinguish the two cell lines, and the Rab6A antibodies were evaluated. Both WT and KO lines imaged in the same field of view to reduce staining, imaging and image analysis bias (Figure 3). Quantification of immunofluorescence intensity in hundreds of WT and KO cells was performed for each antibody tested, and the images presented in Figure 3 are representative of this analysis (8).

In conclusion, we have screened twelve Rab6A commercial antibodies by western blot, immunoprecipitation, and immunofluorescence by comparing the signal produced by the antibodies in human HAP1 WT and *RAB6A* KO cells. 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 Rab6A were identified in all applications. Researchers who wish to study Rab6A 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 Rab6A 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, only a brief overview of the protein's function and its relevance in disease is provided. Rab6A 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 (8). 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 KO 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, a preprint server (DOI: [10.21203/rs.3.pex-2607/v1](https://doi.org/10.21203/rs.3.pex-2607/v1)) (8).

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 (12, 13). Peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies (Thermo Fisher Scientific, cat. number 65-6120 and 62-6520). Alexa-555-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Thermo Fisher Scientific, cat. number A21429 and A21424). Peroxidase-conjugated Protein A from MilliporeSigma, cat. number P8651 was used as a secondary system to detect the IPs.

Antibody screening by western blot

HAP1 WT and *RAB6A* KO 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 (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 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 or 10 µl NBP2-97111, provided at an unknown concentration, 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) (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 in 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 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

HAP1 WT and *RAB6A* KO 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 a 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 37°C, 5% CO₂. 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 Rab6A 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 (14). 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

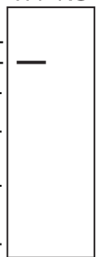
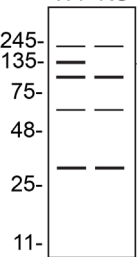
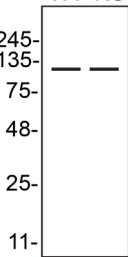
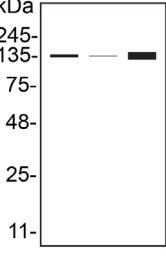

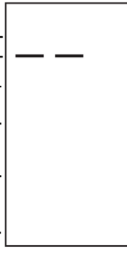



Institution	Catalog number	RRID (Cellosaurus)	Cell line	Genotype	Comments
Horizon Discovery	C631	CVCL_Y019	HAP1	WT	-
Horizon Discovery	HZGHC001233c001	CVCL_T122	HAP1	<i>RAB6A</i> KO	A signal observed at ~22 kDa in western blots using Rab6A antibodies suggests the presence of a truncated form of the Rab6A protein.

Table 2: Summary of the Rab6A antibodies tested

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (µg/µL)	Vendors recommended applications	Immunogen
Abcam	ab271094**	10161851	AB_3101984	recombinant mono	EPR2447 2-24	rabbit	0.59	Wb, IP, IF	proprietary
Abnova	H00005870-M01*	N6051-3G3	AB_463878	monoclonal	3G3	mouse	0.50	Wb	C-terminus
Cell Signaling Technology	9625**	1	AB_10971791	recombinant mono	D37C7	rabbit	0.50	Wb, IP, IF	C-terminus
GeneTex	GTX110646	44097	AB_1951582	polyclonal	-	rabbit	0.21	Wb, IF	full length
GeneTex	GTX635981**	44256	AB_2909932	recombinant mono	HL1047	rabbit	1.00	Wb, IF	full length
Novus Biologicals (Bio-techne)	NBP1-33110	44097	AB_2175468	polyclonal	-	rabbit	0.20	Wb, IF	full length
Novus Biologicals (Bio-techne)	NBP2-32386	000030562	AB_3086617	polyclonal	-	rabbit	0.10	Wb, IF	C-terminus
Novus Biologicals (Bio-techne)	NBP2-88116	QC50009-42200	AB_3086618	polyclonal	-	rabbit	0.50	Wb	C-terminus
Novus Biologicals (Bio-techne)	NBP2-88117	QC61657-42970	AB_3086619	polyclonal	-	rabbit	0.50	Wb	middle region
Novus Biologicals (Bio-techne)	NBP2-97111	HD09DE02 05-B	AB_3086771	polyclonal	-	rabbit	n/a	Wb, IP, IF	N terminus
Novus Biologicals (Bio-techne)	NBP3-13701**	44256	AB_3086655	recombinant mono	HL1047	rabbit	1.00	Wb, IF	full length
Proteintech	10187-2-AP	00104993	AB_2175463	polyclonal	-	rabbit	0.35	Wb, IP, IF	N terminus
Synaptic Systems	273003	273003/1-2	AB_2619999	polyclonal		rabbit	1.00	Wb, IP	N terminus

Wb=western blot; IF= immunofluorescence; IP=immunoprecipitation, *= monoclonal antibody, **= recombinant antibody, n/a=not available

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

Western blot			Immunoprecipitation			Immunofluorescence		
Successful antibody	Successful antibody	Unsuccessful antibody	Successful antibody	Successful antibody	Unsuccessful antibody	WT/KO cell mosaic	Successful antibody	Unsuccessful antibody
WT KO	WT KO	WT KO	SM UB IP	SM UB IP	SM UB IP	WT KO	WT KO	WT KO
								
Target protein detected (arrowhead)	Target protein detected (arrowhead) among others	Target protein not detected	Target protein captured in the IP	Target protein captured in the IP	Target protein not captured in the IP		Target protein detected (white staining)	Target protein not detected

This table was reproduced with permission from Ayoubi et al., Elife, 2023 (5)

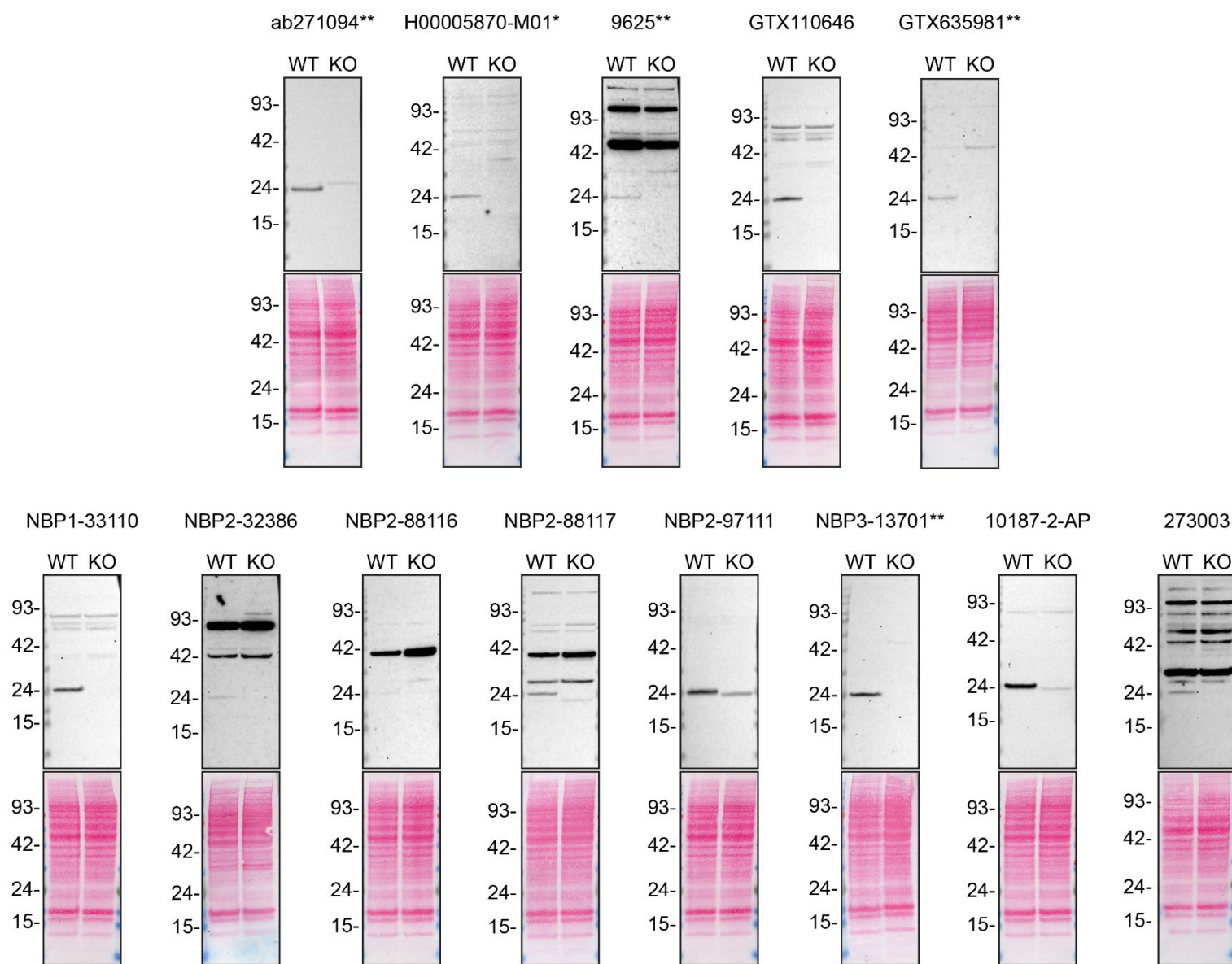


Figure 1: Rab6A antibody screening by western blot

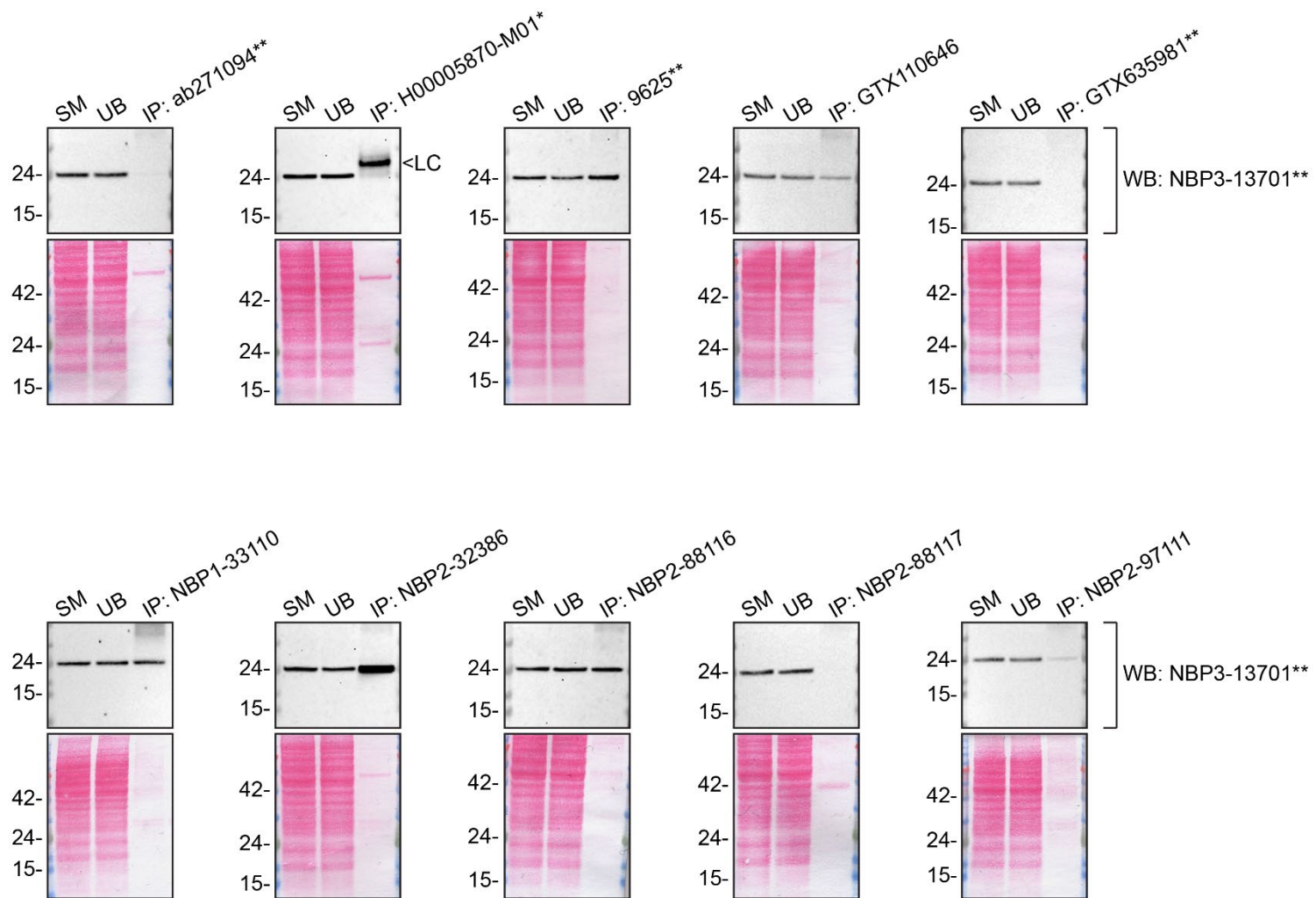


Figure 3: Rab6A antibody screening by immunoprecipitation (1/2)

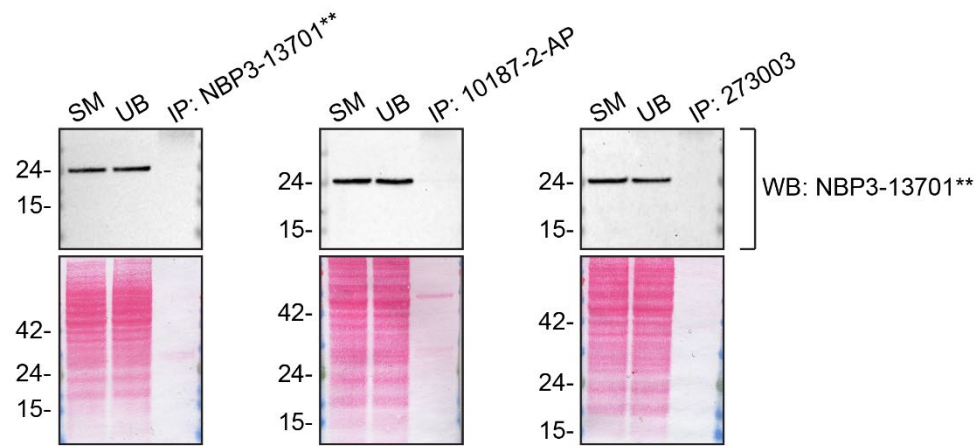


Figure 3: Rab6A antibody screening by immunoprecipitation (2/2)

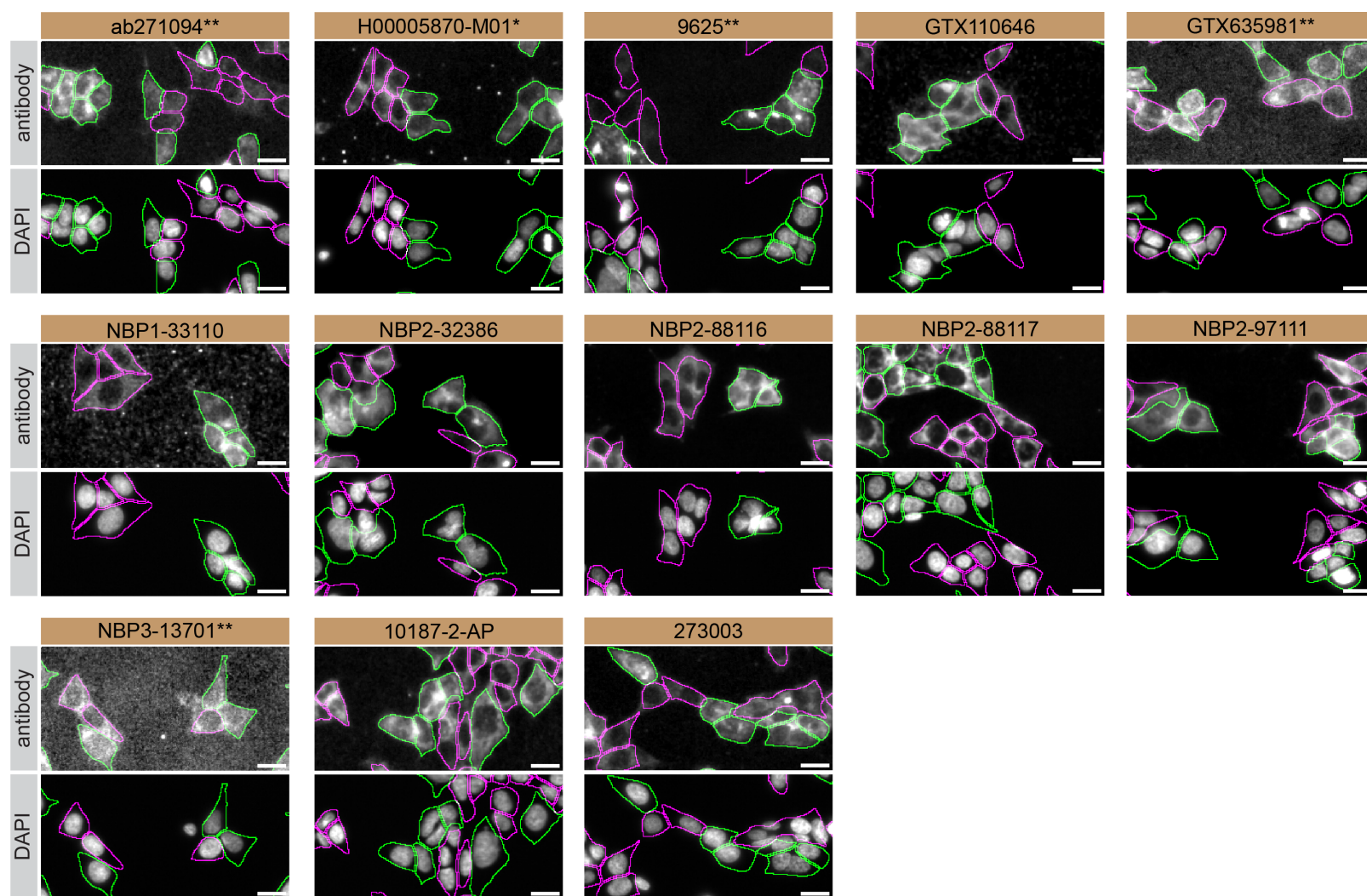


Figure 3: RAB6A antibody screening by immunofluorescence

FIGURE LEGENDS

Figure 1: Rab6A antibody screening by western blot.

Lysates of HAP1 WT and *RAB6A* KO were prepared, and 40 µg of protein were processed for western blot with the indicated Rab6A antibodies. The Ponceau stained transfers of each blot are presented to show equal loading of WT and KO 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. Antibody dilution used: ab271094** at 1/1000, H00005870-M01* at 1/1000, 9625** at 1/100, GTX110646 at 1/1000, GTX635981** at 1/200, NBP1-33110 at 1/1000, NBP2-32386 at 1/200, NBP2-88116 at 1/1000, NBP2-88117 at 1/200, NBP2-97111 at 1/1000, NBP3-13701** at 1/200, 10187-2-AP at 1/1000 and 273003 at 1/1000. Antibodies 9625**, GTX635981**, NBP2-88117, NBP3-13701** and 10187-2-AP were titrated as the signal was too weak when following the supplier's recommendations. Predicted band size: 23.5 kDa *= monoclonal antibody, **= recombinant antibody.

Figure 2: Rab6A antibody screening by immunoprecipitation.

HAP1 lysates were prepared, and immunoprecipitation was performed using 1 mg of lysate and 2.0 µg of the indicated Rab6A antibodies pre-coupled to Dynabeads protein A or protein G. The concentration of NBP2-97111 is unknown and therefore 10 µL of this antibody were tested. Samples were washed and processed for western blot with the indicated Rab6A antibody. For western blot, NBP3-13701** was used at 1/200. Bis-Tris 10% gels with MES-SDS buffer were used. 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 3: Rab6A antibody screening by immunofluorescence.

HAP1 WT and *RAB6A* 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 Rab6A 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 (KO) channels was performed. Representative images of the merged blue and red (grayscale) channels are shown. WT and KO 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: ab271094** at 1/500, H00005870-M01* at 1/500, 9625** at 1/500, GTX110646 at 1/200, GTX635981** at 1/1000, NBP1-33110 at 1/800, NBP2-32386 at 1/50, NBP2-88116 at 1/500, NBP2-88117 at 1/250, NBP2-97111 at 1/1000, NBP3-13701** at 1/1000, 10187-2-AP at 1/300 and 273003 at 1/1000. Bars = 10 µm. *= monoclonal antibody, **= recombinant antibody.