The antibody validation experiments were essentially performed as described by Stadler et al (1), as briefly described below.

PREPARATION OF siRNA TRANSFECTION PLATES

A solid-phase transfection protocol (2) was used with Silencer Select siRNA from Ambion. For all genes, a scrambled siRNA sequence (Life Technologies, AM4635) was used as negative control.

1. For each target specific siRNA 1.75 µl Lipofectamine 2000 (Life Technologies) was mixed with 1.75 µl H_2O.
2. To this mix 3.5 µl sucrose/Opti-MEM solution (13,7% (w/v)) and 5 µl Silencer Select siRNA (3 µM) were added.
3. The solution was gently mixed and incubated for 20 min at RT before 7 µl 0.2% gelatin solution was added.
4. The siRNA transfection mix was stepwise diluted to a final concentration of 13 nM and subsequently transferred into the designated well of the 96-well plates (Greiner, SensoPlate).
5. The plates were dried in a speed-vac, GeneVac MiVac Quattro, initially for 90 min then 45 min and after that the required multiple of extra 30 minute runs until no solvent remained

After complete lyophilization, plates were stored with drying pearls in sealed boxes at room temperature for up to six months.

CELL CULTIVATION & TRANSFECTION

The U-2 OS human osteosarcoma cell line (ATCC-LGC Promochem, Borås, Sweden) was used. Cells were grown in McCoys growth media supplemented with 10% fetal bovine serum (FBS).

6. Cells were seeded (3500 cells per well) and incubated at 37°C in humidified air with 5.2% CO2, for 72 h.

IMMUNOSTAINING

All washes were performed at room temperature.

7. Growth medium was removed and the cells were washed in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2).
8. The cells were fixed for 15 minutes in ice-cold 4% paraformaldehyde pH 7.2 - 7.3 in growth medium supplemented with 10% fetal bovine serum (FBS).
9. The cells were permeabilized for 3x5 minutes with 0.1% Triton X-100 in PBS.
10. The cells were washed with PBS and incubated overnight at 4°C with primary antibodies diluted in PBS supplemented with 4% FBS. Rabbit polyclonal antibodies specific for each target protein were diluted to 2 µg/ml and a reference antibody for microtubules (Mouse anti-alpha Tubulin monoclonal antibody ab7291, Abcam) was diluted 1000x.

11. The following day the cells were washed 4x10 minutes with PBS and incubated for 1.5 hours in room temperature with secondary antibodies diluted 800x in PBS supplemented with 4% FBS (Alexa Fluor 555 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies).
12. The cells were counterstained for 4 minutes with the nuclear stain DAPI (Life Technologies) 0.6 μM in PBS.
13. The cells were washed 4x10 minutes with PBS and mounted in PBS containing 78% glycerol.

AUTOMATED CONFOCAL IMAGE ACQUISITION

Confocal image acquisition was automated using a Leica SP5 laser-scanning confocal microscope and the Leica Matrix Screener v.2.6.0 software (Leica Microsystems, Manheim, Germany). Two objectives were used, 10x /0.3 N/A dry objective and 40x/0.85 N/a dry objective, with the following settings; 8 bit acquisition, 600 Hz scan speed, line average 2 and an image size of 2048 x 2048 pixels. The detector gain settings were optimized for each antibody according to the staining of the well containing scrambled siRNA and kept identical for the specific siRNA well.

QUANTITATIVE IMAGE ANALYSIS

To process the quantitative and automated measurements of the fluorescence from the antibodies MATLAB was used. Segmentation masks using the fluorescent staining of the cells, nuclei and cytoplasm were applied prior to the fluorescence measurements. The Dapi staining enabled identification of primary objects (nuclei) with a size threshold of 30-100 and 150-400 pixels in diameter for 10x and 40x images respectively. Secondary objects were identified around the primary objects using the microtubule staining and a propagation algorithm to define cell borders. The overlay of the nucleus and the cell mask was used to generate the cytoplasm mask. For each object, the integrated fluorescence intensity (IFI) from the Atlas antibody was measured and normalized to the IFI of the common microtubule antibody to enable comparison between different samples. The median integrated fluorescence intensity (IFI) for each sample (siRNA and negative control) was calculated. To quantify the degree of siRNA efficacy and protein downregulation in the siRNA well, the median IFI was compared to the negative control to obtain the relative fluorescence intensity (RFI) between 0 and 100% where 100% indicate no decrease and 0% indicate complete disappearance of the signal.

More images and the results from the quantitative image analysis can be found at http://www.proteinatlas.org/subcellular for each antibody.

References

1. Stadler et al. Systematic validation of antibody binding and protein subcellular localization using siRNA and confocal microscopy. J Proteomics. 2012 Apr 3;75(7):2236-51.
2. Erfle H et al. Work flow for multiplexing siRNA assays by solid-phase reverse transfection in multiwell plates. J Biomol Screen 2008;13:575–80.