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 H2O.
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 & LYSIS

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.
7. Growth medium was removed and 40 µl lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS) was added per well. Plate was incubated for 30 min on ice and vortexed regularly.

SDS-PAGE & TRANSFER

8. The plate was centrifuged and lysate was mixed with reducing buffer and boiled at 95°C for 5 min.
9. Molecular weight marker (PageRuler Plus Prestained Protein Ladder, Thermo Fisher Scientific) and sample preparation was loaded on gel (4-20% Criterion™ TGX Stain-Free™ Precast Gels, Bio-Rad Laboratories) for SDS-PAGE.
10. The gel was imaged using a ChemiDoc MP and Image Lab 5.2 (Bio-Rad Laboratories) to obtain a total protein image prior to transfer.
11. Transfer was performed using the High MW program on Trans-Blot Turbo Blotting System (Bio-Rad Laboratories). The membrane was incubated in blocking buffer (1 mM Tris pH 7.5, 150 mM NaCl, 5% skim milk, 0.5% Tween20) for 45min at RT.

ANTIBODY PROBING

12. Rabbit polyclonal antibody, specific for each target, was diluted to 0.9 µg/ml in blocking buffer and the membrane was incubated with the antibody solution for 1h at RT, followed by washing (1 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20).
13. The membrane was incubated with a 1:3000 dilution of HRP-conjugated swine anti-rabbit (Dako) for 45 min at RT, followed by washing.

IMAGING & DATA ANALYSIS
14. Substrate for chemiluminescence (Immobilon Western Chemiluminescence HRP Substrate, Millipore) was used and image was acquired using a ChemiDoc MP and Image Lab 5.2.
15. Relative signal intensity of band in the siRNA lane compared to band in negative control was used to measure protein downregulation. Relative lane signal intensity between the siRNA lane and negative control lane was obtained to compare total protein between lanes.

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.