Supplementary Information

Chondroitin sulphate-modified neuropilin 1 is expressed in human tumour cells and modulates 3D invasion in the U87MG human glioblastoma cell line through a p130Cas-mediated pathway

Paul Frankel, Caroline Pellet-Many, Pauliina Lehtolainen, Giovanna M D'Abaco, Michelle L. Tickner, Lili Cheng, Ian C. Zachary

Supplementary Methods

Retroviral infection

Retroviral stocks were generated by transfection of pVSVG (Clontech), pGag-pol (Clontech) and pBabe puro (Empty or expressing (WT) NRP1 or S612A NRP1 into HEK 293 cells. Generation of stable cell lines was achieved by infection (Fiorentino et al, 2000) of U87MG cells followed by selection with 1ug/ml puromycin. Expression of WT and S612A NRP1 were verified by western blot analysis and immunofluorescence. U87MG pBabe, WT NRP1 and S612A NRP1 cells were routinely grown in 0.5ug/ml puromycin to maintain expression of the transgene. For cell signalling assays cells were grown on collagen I coated plates and shifted to DMEM medium containing1% FCS for 18hr prior to harvesting cells.

Antibodies, Immunoblotting and Immunofluorescence

Antibodies to NRP1 (C-19), GAPDH (V—18), c-Src (N-16), FAK (C-20) were from Santa Cruz Inc; ERK, phospho-ERK, AKT, phospho-AKT, phospho-Src (Y416), phospho-p130Cas (Y249) antibodies from Cell Signalling Technologies Inc; Phospho-FAK (Y397) from Biosource; p130Cas Mab from BD Transduction labs; Chondroitin Sulphate MAb (CS-56) from Sigma; β1 Integrin from Chemicon Inc; Alexa-Fluor (486) donkey anti goat and Alexa-Fluor (555) donkey anti mouse were from Molecular Probes. For immunoblotting, cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, complete protease inhibitor (Roche) and phosphatse inhibitors I & II (Sigma) and analysed by SDS PAGE using 10% Bis-Tris gels; Nupage; Invitrogen) followed by electrotransfer onto Invitrolon PVDF membranes (Invitrogen). Membranes were blocked with 5% w/v non-fat dry milk (5% BSA for phospho-specific Ab) and 0.1% v/v Tween-20 in TBS, for 1hr at room temperature, before being probed with the primary antibody by overnight incubation at 4°C. Detection was via a horseradish peroxidase-linked secondary antibody (Santa-Cruz) incubated for 1hr at room temp and ECL reagents (GE Healthcare), following the manufacturer's protocol. In some cases immunoblots were quantitated by scanning of autoradiograms along with a calibration strip and analysed by densitometry using Image J (US National Institutes of Health; http://rsb.info.nih.gov/ij/). For immunofluorescent staining, cells were fixed in 4% formaldehyde in PBS before permeabilization in 0.2% Triton-X100 in PBS; antibody incubations were performed for 1 h at 37 °C in 1% BSA, 0.1% Tween20 in PBS. confocal imaging was performed using a BIO-RAD Radiance 2100 laser and an upright Nikon Eclipse E1000 microscope running LaserSharp 2000 software.

Tunicamycin treatment

A549 cells were seeded in 6 well-plates 24hr prior to the treatment, Tunicamycin was added to a final concentration of 5 μg/ml and incubated at 37°C, 5% CO_2 for 16 hrs. Cells were lysed with RIPA buffer (30 mM TrisHCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 2mM ETDA, Complete protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails 1 and 2 (Sigma) dilution 1:1000) and the samples were analysed by SDS PAGE and immunoblotting.

Chondroitinase and Heparinase treatments

Chondroitinase ABC (Sigma) was reconstituted in a buffer containing 0.01% BSA and heparinase I and III (heparitinase; Sigma) were prepared in a buffer containing 20 mM Tris-HCl, pH 7.5, containing 0.1mg/ml BSA and 4 mM $CaCl_2$,. The enzymes were added to the culture medium of the A549 cells at a final concentration of 1 unit per ml and cells were incubated for 2 hours at 37°C, in the presence of 5% CO_2. Cells were harvested in RIPA buffer and the samples were analysed by SDS PAGE and immunoblotting.

3D collagen I invasion assay

Collagen I plugs (2.1mg/ml) were prepared from fibrillar bovine collagen I (3.1 mg/ml; PureCol) by dilution in DMEM in accordance with the manufacturer's protocol (Nutacon, The Netherlands). 2.5ml collagen solution was placed in a 24mm diameter, 8μm pore transwell insert (Corning, Schiphol-Rijk, The Netherlands) and allowed to set for 60min at 37^0C. Cells (2.5×10^4) were plated in 1.5ml of serum-free DMEM medium (SFM) on

top of the plug. To establish a chemo-attractant gradient, 2.5mL of 20% FCS in DMEM was added to the lower chamber. Cells were allowed to invade for 3 days. To maintain the gradient the medium from both the top and bottom chambers was replaced every 10 to 12 hours. Cells were fixed in 4% formaldehyde, washed three times in PBS, permeabilised in 0.2% Triton X-100 and stained with Sytox green 1:5000 and AF546-Phalloidin (Molecular Probes). Confocal imaging was performed using a BIO-RAD Radiance 2100 laser and an upright Nikon Eclipse E1000 microscope (10 X LWD objective) running LaserSharp 2000 software. Analysis was performed by taking sections at 6μm intervals through 500μm of the collagen plug. Invading cells were determined to be those that had invaded more then 3 cell lengths (~80um). Cells were scored as amoeboid when the polarity index (long axis/short axis ratio) was less than two, with no apparent cellular protrusions. The percentage invading cells was determined by dividing the sum of the fluorescence in invading sections by the total fluorescence of all sections. The invasion index was determined by normalizing the percentage invading cells to the control. Levels of fluorescence were determined by Image J. 3D reconstructions were performed with LSM Browser (Zeiss) and Volocity (Improvision) imaging software.

^{125}I-VEGF-A_{165} binding assay

The U87MG cell lines were seeded in 24-well collagen I coated plates 24hr prior to analysis such that they were ~80% confluent (with few spindles). The cells were washed twice with phosphate-buffered saline. Binding medium (Dulbecco's modified Eagle's medium, 25 mM HEPES pH 7.3 containing 0.1% bovine serum albumin) was added, followed by addition of 0.1nM of ^{125}I-VEGF-A_{165} (1200-1800 Ci/mmol, GE Healthcare

Plc). After 2 h incubation at 4°C, the cells were washed four times with cold phosphate-buffered saline. The cells were lysed with 0.25 M NaOH, 0.5% SDS, and bound radioactivity was measured in a gamma counter. Non specific binding was determined in the presence of 100-fold excess unlabeled VEGF-A_{165} (R & D Systems).

Sema 3A binding assay

U87MG cells were washed with PBS before being detached with cell dissociation buffer (Sigma). Cells were counted and aliquoted into tubes and spun down at 4^0C. Cells were re-suspended in binding buffer (1X PBS /1% BSA/ 20 mM HEPES/ 2 ug/ml Heparin) on ice. Binding of Sema3A to cells was measured after incubation with 2 ug/mL Sema3A/Fc in binding buffer at 4°C for 50 minutes. After washing, Sema3A/Fc bound to cells was detected with FITC-conjugated $F(ab')_2$ goat anti–human IgG Fc (Jackson Immuno-Research Laboratories, West Grove, PA). As a control, we used human B7-1/Fc. Data were collected using a FACScalibur cytofluorometer (Becton Dickinson, San Jose, CA) and analyzed using CELLQuest software (Becton Dickinson).

Cell proliferation assay

U87MG pBabe, WT NRP1 and S612A NRP1 cells were seeded (0.5×10^5cells) on collagen I coated six well plates in 5ml of 1% FCS in DMEM. After 1–3 days incubation, the cells were trypsinized and cell counts were determined using a cell counter (CDA-500, Sysmex, UK).

Tissue Specimens

The glioma samples corresponding to Fig 7A were processed as previously described by (D'Abaco et al, 2006). The Glioma samples corresponding to Fig 7B were a kind gift from Prof S. Brandner, Institute of Neurology, UCL, London, UK. All samples were lysed in RIPA Lysis Buffer: Glycerol 10%, Tris-pH 7.5 20mM, NaCl 137mM, SDS 0.1%, NP40 (IGEPAL) 0.5%, Tx-100 1.0%, EDTA 2mM supplemented with protease/phosphatase inhibitors and stored at -80 degrees until used.

Statistical analysis

The data displayed on the graphs are means, with error bars representing the standard error of the mean (SEM). Statistical analysis was performed either by one-way analysis of variance with Bonferroni post test. Statistical significance was tested with a one-way ANOVA and validated at $P<0.05$.

References

D'Abaco GM, Ng K, Paradiso L, Godde NJ, Kaye A, Novak U (2006) ADAM22, expressed in normal brain but not in high-grade gliomas, inhibits cellular proliferation via the disintegrin domain. *Neurosurgery* **58**(1)**:** 179-186; discussion 179-186

Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, Alema S, Benedetti P, Segatto O (2000) Inhibition of ErbB-2 mitogenic and transforming activity

by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. *Mol Cell Biol* **20**(20)**:** 7735-7750

Supplementary Figure Legends

Supplementary Figure 1: Homogenous expression of WT NRP1 and S612A NRP1 in U87MG stable cell lines (**A**), Representative images of U87MG cells stably expressing WT NRP1, S612A NRP1 or control vector (pBabe puro) stained for NRP1 and AF555-Phalloidin. (**B**) Expression of WT NRP1 and S612A NRP1 in U87MG stable cell lines was determined by western blot analysis. Images and blots shown are representative of at least three separate experiments.

Supplementary Figure 2: Effect of WT NRP1 and S612A NRP1 expression on U87MG cell proliferation. U87MG pBabe, WT NRP1 and S612A NRP1 cells were seeded (0.5×10^5cells) on collagen I coated six well plates in 5ml of 1% FCS in DMEM. After 1–3 days incubation, the cells were trypsinized and counted. Data are expressed as the means +/- s.e.m of three experiments. $P< 0.05$ versus pBabe

Supplementary Figure 3: Effect of WT and S612A NRP1 expression on specific binding of ^{125}I-VEGF-A_{165} and Sema 3A to U87MG cells. (**A**), WT NRP1 but not S612A NRP1 expression in U87MG cells leads to an increase in ^{125}I-VEGF-A_{165} binding. U87MG pBabe, WT NRP1 and S612A NRP1 cells (~80% confluent, with few spindles) were incubated for 2 h at 4^0C with 0.1 nM ^{125}I-VEGF-$A_{165.}$ (B), WT NRP1 and S612A NRP1 expression in U87MG cells leads to an increase in Sema 3A binding. U87MG

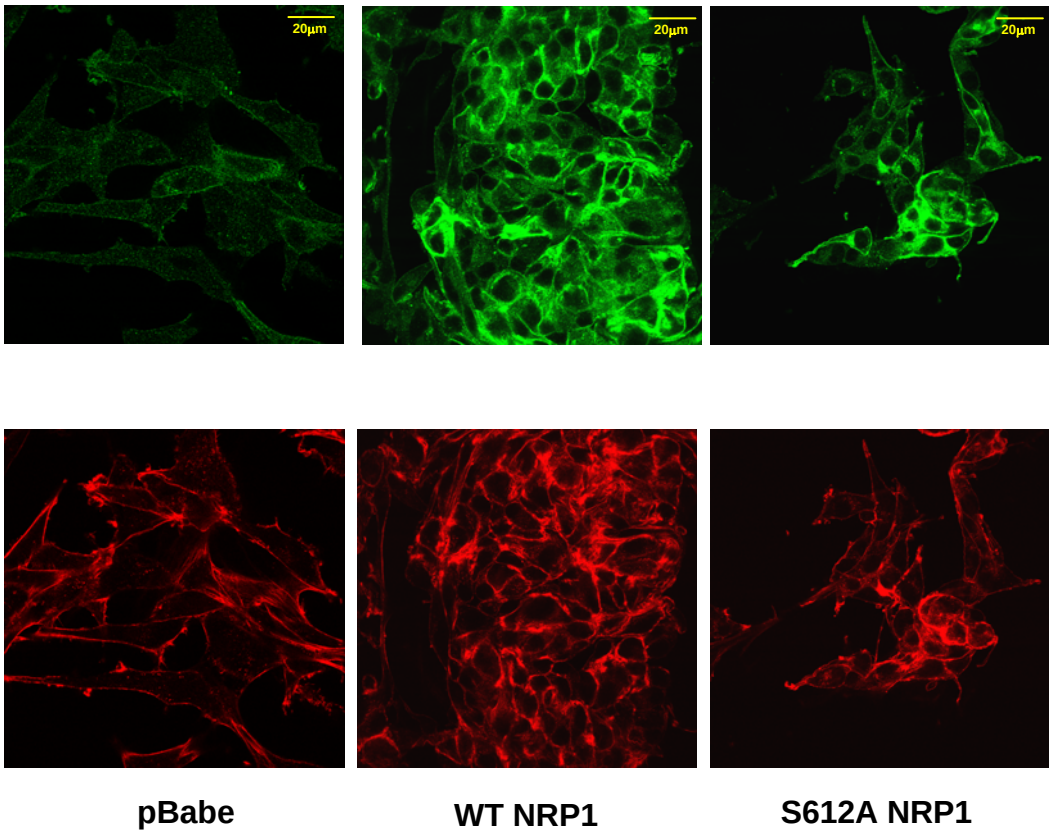
pBabe, WT NRP1 and S612A NRP1 cells were incubated for 50min at 4^0C with 2.0ug/ml of Sema3A. Values represent the means +/- s.e.m. of specific ^{125}I-VEGF-A_{165} and Sema 3A binding calculated from the results of three independent experiments each performed in triplicate. P< 0.05 versus pBabe

Supplementary Figure 4: Effect of WT and S612A NRP1 expression on signalling in U87MG cells. U87MG pBabe, WT NRP1 and S612A NRP1 cells were grown on collagen I coated plates and incubated in 1% FCS overnight (~18hr), whole cell lysates were analysed by SDS PAGE and probed for expression of phosphorylated and total forms of ERK1/2, AKT (**A**); FAK and Src (**B**). Blots shown are representative of at least three separate experiments.

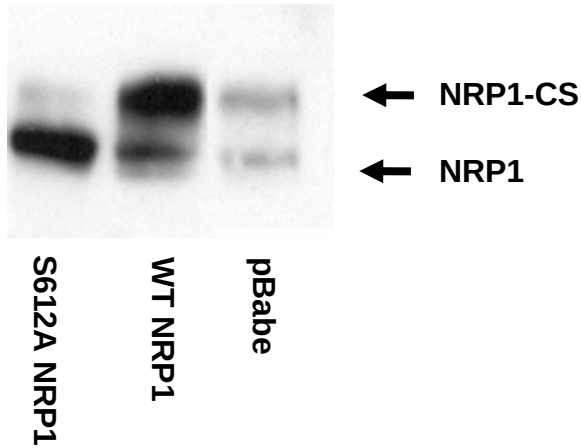
Supplementary Figure 5: β1 Integrin silencing has little effect on S612A NRP1 mediated invasion in 3D. (**A**), U87MG pBabe and S612A NRP1 cell lysates were immunoblotted for β1 Integrin, NRP1 and GAPDH 72h after transfection of cells with scrambled siRNA (Scr) or β1 Integrin siRNA. (**B**), Invasion of U87MG pBabe and S612A NRP1 cells, 24h after transfection with the indicated siRNAs. Data are expressed as the means +/- s.e.m from three experiments. P< 0.05 versus pBabe Scr

Supplementary Figures

Supplementary Fig 1A

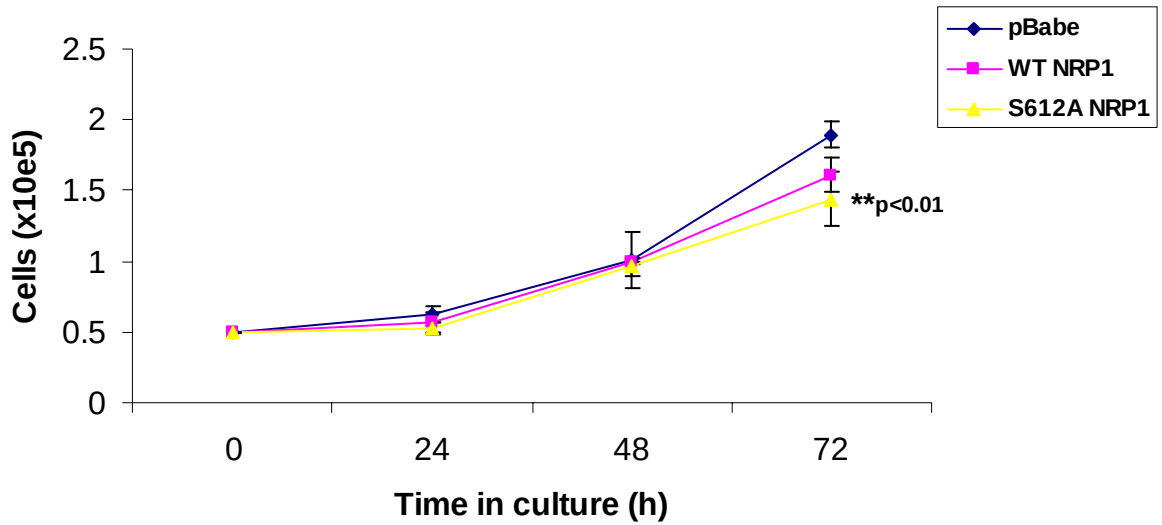


Supplementary Fig 1B



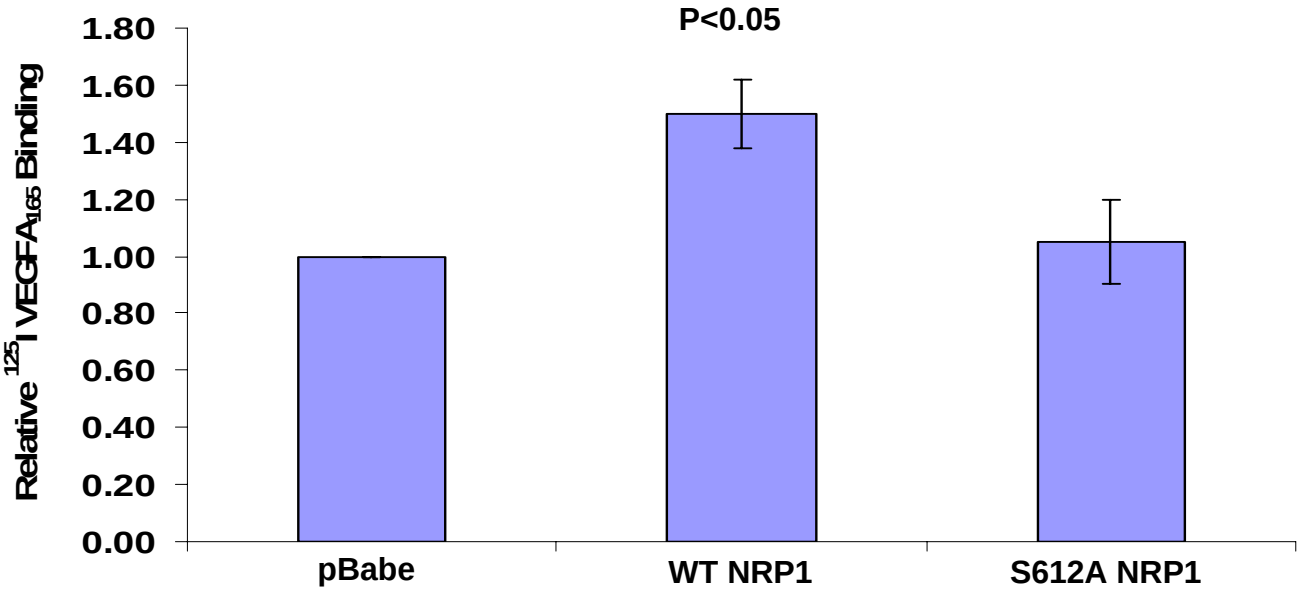
Frankel, et al.

Supplementary Fig 2

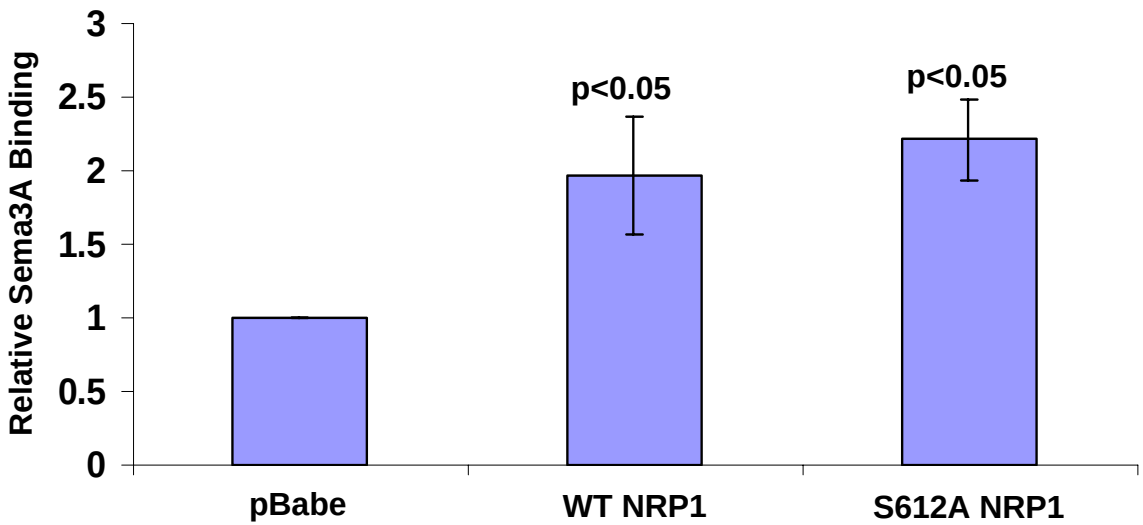


Frankel, et al.

Supplementary Fig 3A

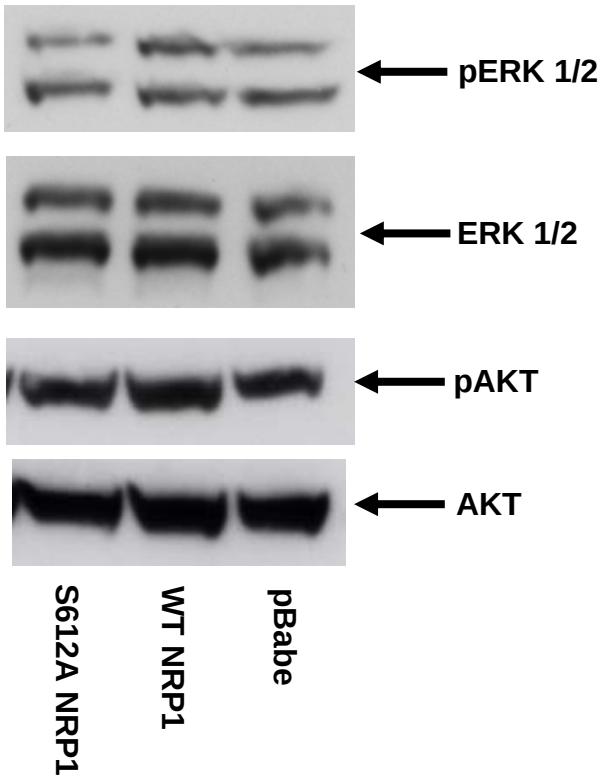


Supplementary Fig 3B

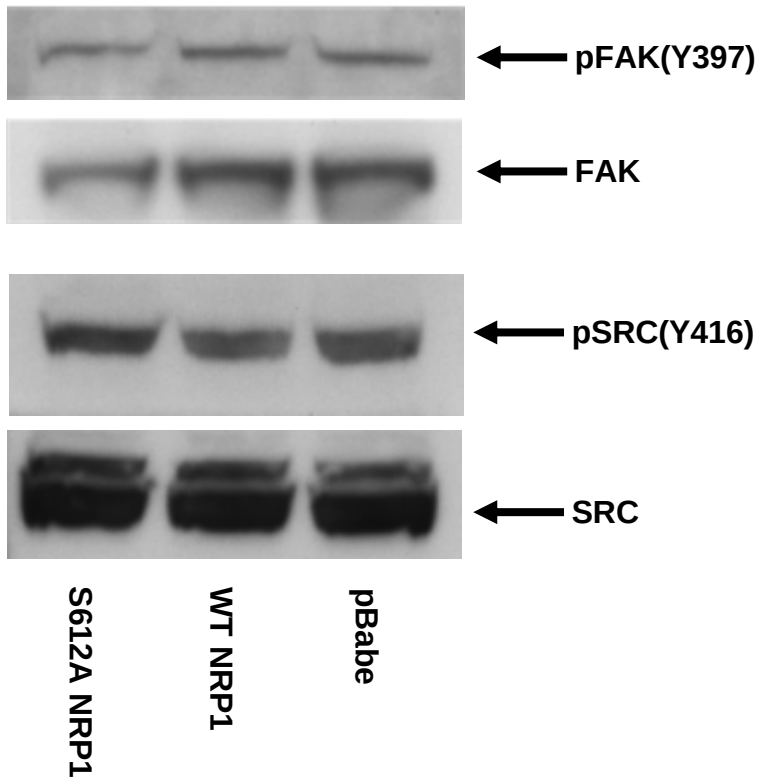


Frankel, et al.

Supplementary Fig 4A

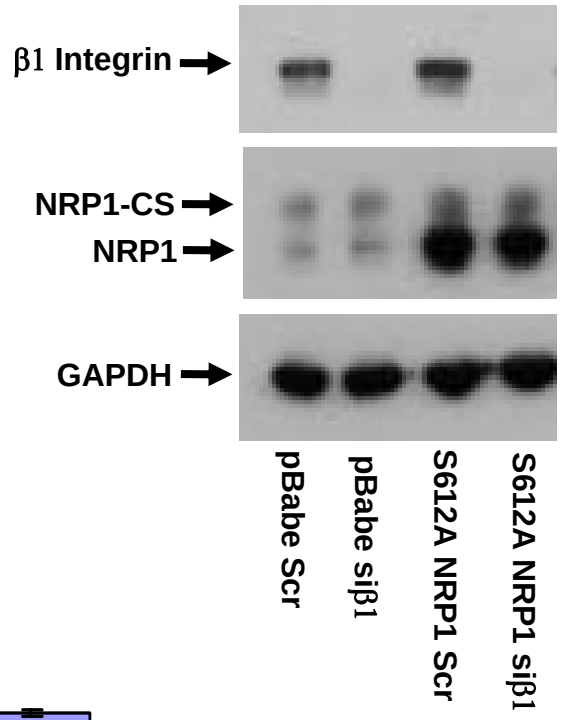


Supplementary Fig 4B

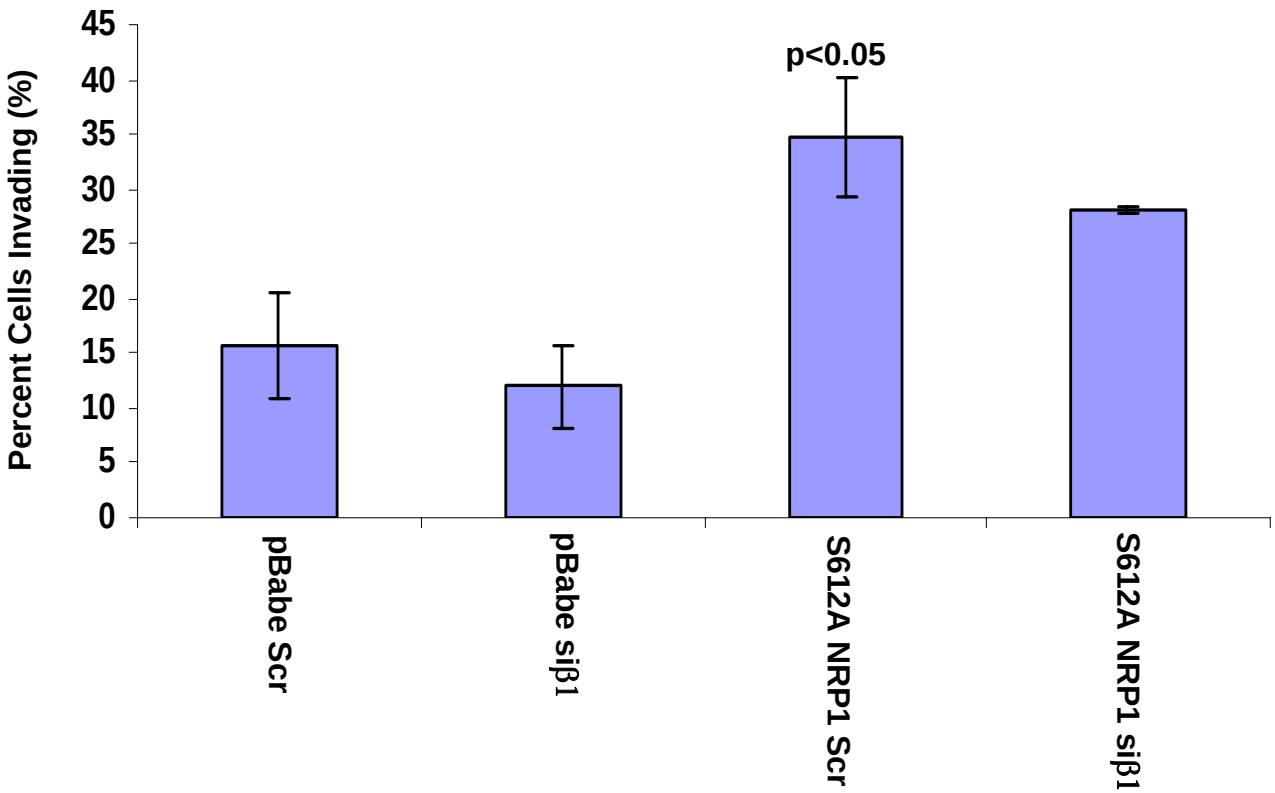


Frankel, et al.

Supplementary Fig 5A



Supplementary Fig 5B



Frankel, et al.