**Supporting online material**

## Materials and methods

Plasmid constructs

*Generation of talin-mRFP constructs*: Full-length mouse talin-1 cDNA was the gift of David Critchley (University of Leicester, UK). A 1080 base-pair fragment corresponding to amino acids 1984-2344 was amplified by PCR using the GGGGGGGAATTCGCTGTGTCTGGTATCATTGC (F) and AAAAAAGCGGCCGCATTGTTCCTCAAAGTTC (R) primers to generate a 5' EcoRI and a 3' NotI restriction site. This region in the talin rod domain has been reported to contain an integrin binding site ("Fragment G"; *23*). The PCR product was then cloned into the pcDNA-RFP-C vector (a gift of Roger Tsien, UCSD, CA, USA) to generate a C-terminal monomeric red fluorescent protein tag. Similarly, a 1299 base-pair fragment corresponding to amino acids 1-433 of talin was amplified by PCR and fused to a C-terminal monomeric RFP tag. This region in the head of the talin protein also contains an integrin-binding site (*24*). The primers used were GCAGAATTCCATGGTTGCGCTTTCGCTGAAGGCGCAG (F) and ACATTTGCGGCCGCTTCCTTTACCGTCCTGAAGGACTGTTGA (R).

*Generation of β1-GFP*: Full-length human β1A integrin cDNA was the gift of Ken Yamada (NIDCR, NIH, Bethesda, MD, USA). To mutate the stop codon prior to fusion with a 3' fluorescent tag, and generate a novel 3' ApaI restriction site, a fragment corresponding to amino acid 578-799 of the mature sequence was generated by PCR using the primers TTGCAAGTGTCGTGTGTGTG (F) and

GTGGATCCCGGGCCCCCCCCCCTCCTTTTCCC (R). The PCR product was then digested with SpeI and ApaI. The remaining 5' part of β1 was cut out of β1-pECE using KpnI and SpeI, and both fragments simultaneously ligated into pcDNA3 that had been digested with KpnI and ApaI. The fidelity of the resulting construct was confirmed by sequencing (GenBankTM code XM_005799.1). The mutated, full-length β1 integrin construct was then cloned into pEGFP N1 (Clontech) using KpnI and ApaI. The initial 12 amino acid linker between the end of the mature β1 integrin sequence and the start codon of the GFP tag proved insufficient for mammalian cell expression. Therefore an 18 amino acid linker was constructed. A top strand and a bottom strand oligonucleotide were designed and annealed together, digested with ApaI and Age I and used to replace the 3' end of the β1 integrin-pEGFP N1 construct. The resulting linker sequence between the 3' end of the mature β1 integrin coding sequence and the start codon of GFP was GGGGARRRGQAGDPPVAT. To enable greater flexibility with the colour of the fluorescent tags, the new construct was initially cloned into pHcRed N1 (Clontech) and when a GFP tag was required, the RFP fluorophore was cut out using AgeI and NotI, and the GFP sequence from pEGFP N1 cloned into the pHcRed backbone using the same restriction sites. The fidelity of the β1 integrin constructs was verified by sequencing. These constructs were successfully expressed transiently in MEF 7929 (β1-/-) cells. To maximise the level of stable incorporation of the β1 integrin-GFP DNA in MEF 7929 (β1-/-) cells, an additional retroviral construct was employed. The β1 integrin-pHcGreen coding sequence was cut out of the pHc backbone using EcoRI and NotI. This coding sequence was ligated into the pFBneo vector (Stratagene) using the same restriction sites, resulting in a construct that produced β1 integrin-GFP expressing retroviral particles

when expressed by amphotropic AM12 packaging cells (a gift of Ian Hart, Cancer Research UK, London, UK).

*Generation of α4-GFP*: A full-length clone of human α4 integrin in pBluescript (from Yoshi Takada, Dana-Farber Cancer Center, Boston, MA, USA) was used as a backbone. For the generation of α4-GFP, the coding region of the α4-pBluescript was removed and an AgeI (PinAI) site inserted at the 3' using PCR. The α4 cDNA already had a 5' SalI site and these sites were used to subclone α4 into pECFP-N1. The CFP cassette was then replaced with GFP-N1 to give α4-pEGFP-N1. This was then digested with NheI and NotI positioned 5' and 3' to the entire α4-pEGFP and the hyg+ cassette from pcDNA3.1 hyg+ ligated using the same restriction sites. PCR primers used to insert a 3' AgeI (PinAI) site into the vector backbone were CAA CAG TAA AAG CAA TGA TGA TGG CGG CGG CGG CGG ACC GGT GGA CTT CTT TCA AAT TGA GAG AAT GG and GGA TAG ATA TTA GCT TTC TCC.

*Generation of mRFP-paxillin*: Full-length paxillin in pEGFPC2 was a gift from Vic Small (IMBA, Vienna, Austria). PCR mutagenesis was employed using the primers below to insert an EcoRI site restriction site at the 3' end and a 5' HindIII site. The primers used were GGA TCC AAG CTT GCC GCC ATG GAC GAC CTC GAC GCC CTG C (F) and GGA TCC GAA TTC CTA GCA GAA GAG CTT GAG GAA GC (R). The PCR product was then sequentially digested and ligated into pcDNA3.1-mRFP-N (from Roger Tsien, UCSD).

*Generation of β1-/- MEFs*: Immortalised β1 integrin-/- murine embryonic fibroblasts were obtained by crossing Immorto mice (Charles River Laboratories, Wilmington, USA) carrying the thermolabile large T antigen H-2KtsA58 (*25*) with mice in which exon 1 of the β1 integrin gene was flanked by loxP sites (*26*; a gift of Uli Muller, Friedrich Miescher Institute, Basel, Switzerland), and genotyped by tail-tip PCR. Mice that were homozygous for the wild type or floxed β1 integrin allele and carried at least one Immorto allele were interbred and E13.5 embryos dissected for MEF preparation. Isolated MEFs were cultured at 33°C in the presence of IFNγ for five passages until all non-immortalised cells had senesced. Adenovirus-Cre (AdCreM1; Microbix Inc.) was added at an MOI of 40 overnight to subconfluent cultures of a random subset of cells derived from β1 (fl/fl)/Immorto embryos. Deletion of β1 integrin expression was monitored by flow cytometry with mAb KMI6 (BD Pharmingen) and confirmed by PCR and Western blot. Clones from the β1-/- MEFs were derived by limited dilution and deletion of β1 integrin was reconfirmed for each clone by flow cytometry.

Retroviral transduction

AM12 packaging cells were seeded overnight into 6 well plates and transfected with 1μg/well β1-GFP-pFBneo using FuGene 6 reagent (Roche) according to the manufacturer's instructions. 24 hours after transfection the medium was changed and the transfected cells allowed to condition the fresh medium for a further 48h. The conditioned medium was removed, filtered through a 2μm-pore filter, mixed 50:50 with MEF growth medium and placed onto 50% confluent β1-/- MEFs. 24-48 hours after adding the retrovirus-containing medium, transduced cells were selected with 0.5mg/ml neomycin for 10 days then assayed for GFP expression by flow cytometry using

untransduced cells as the negative control. In addition to the parent population expressing a mixed level of β1-GFP expression, a subpopulation of high expressors was isolated by flow cytometry and 9 individual clones were derived by limited dilution from these cells.

Cell culture and transient transfection

MEFs were routinely cultured in DMEM, 10% FCS (Sigma) supplemented with 20 U/ml mouse IFNγ (Sigma) at 33°C. For all experiments, MEFs were cultured in growth medium without IFNγ at 37°C to switch off the immortalising gene. B16F1 mouse melanoma cells and A375-SM human melanoma cells were both cultured in DMEM (Sigma) supplemented with 10% FCS, glutamine and penicillin/streptomycin at 37°C. For FLIM experiments, β1-GFP-MEFs, B16F1 or A375-SM melanoma cells were seeded overnight in 6 well plates and transfected with 1μg/well of indicated plasmid DNA using FuGene 6 according to the manufacturer's instructions. Cells typically were left to express constructs for 24-72h following transfection (as assessed by microscopy) and were used between 24-48h post-transfection for all FLIM studies. Prior to imaging, cells were plated on glass coverslips or Mattek dishes (for line experiments) coated with extracellular matrix proteins or poly-L-lysine (Sigma) as indicated.

Bead coating and incubation

4μm latex beads (Dynal) were washed in PBS and incubated with diluted proteins as stated in figure legends at indicated concentrations. Beads were left overnight at 4°C to allow even protein binding, and subsequently washed three times and resuspended in PBS. Plated cells were incubated with 5μl/ml of beads in growth media and incubated at

37°C for 30 minutes. Cells were then washed three times in medium and either fixed for FLIM analysis or imaged live in phenol-red free growth media at 37°C.

Fluorescence lifetime measurements by time-correlated single photon counting (TCSPC) FLIM

Time-domain FLIM was performed with a multi-photon microscope system as described previously (*27, 28*). The system is based on a modified Bio-Rad MRC 1024MP workstation, comprising a solid-state-pumped femtosecond Ti:Sapphire (Tsunami, Spectra-Physics) laser system, a focal scan-head and an inverted microscope (Nikon TE200). Enhanced detection of the scattered component of the emitted (fluorescence) photons was afforded by the use of fast response (Hamamatsu R7401-P) non-descanned detectors, developed in-house, situated in the re-imaged objective pupil plane. Fluorescence lifetime imaging capability was provided by time-correlated single photon counting electronics (Becker & Hickl, SPC 700). A 40x objective was used throughout (Nikon, CFI60 Plan Fluor N.A. 1.3) and data collected at 500 ± 20 nm through a bandpass filter (Coherent Inc. 35-5040). Laser power was adjusted to give average photon counting rates of the order $10^4 – 10^5$ photons s-1 (0.0001 to 0.001 photon counts per excitation event) to avoid pulse pile up. Acquisition times up to 300 s at low excitation power were used to achieve sufficient photon statistics for fitting, while avoiding either pulse pile-up or significant photobleaching. Excitation was at 890 nm. Widefield acceptor (mRFP) images were acquired using a CCD camera (Hammamatsu) at <200ms exposure times.

Analysis of data for FRET experiments

Data was analysed as previously described (*27*). Briefly, bulk measurements of FRET

efficiency (i.e. intensity-based methods) cannot distinguish between an increase in FRET efficiency (i.e. coupling efficiency) and an increase in FRET population (concentration of FRET species) since the two parameters are not resolved. Measurements of FRET based on analysis of the fluorescence lifetime of the donor can resolve this issue when analyzed using multi-exponential decay models. The assumption that non-interacting and interacting fractions are present allows the determination of the efficiency of interaction. The FRET efficiency is related to the molecular separation of donor and acceptor and the fluorescence lifetime of the interacting fraction by:

$$\eta_{fret} = \left( R_0^{\,6} / \left( R_0^{\,6} + r^6 \right) \right) = 1 - \tau_{fret} / \tau_d$$

Where *R0* is the Förster radius, *R* the molecular separation, *νfret* is the lifetime of the interacting fraction and *τd* the lifetime of the donor in the absence of acceptor. *nfret* and *τd* can also be taken to be the lifetime of the interacting fraction and non-interacting fraction, respectively. For measurements of bulk interactions (i.e. where only single exponential decays are fit to the data), measured efficiencies will appear significantly lower due to the assumption that all donors are associated with one or more acceptors. All data were analysed using TRI2 software (developed by P.Barber, Gray Cancer Institute, London UK). Histogram data presented here are derived from bi-exponential analysis and plotted as mean FRET efficiency from >10 cells per sample. For analysis of FRET at bead interface, a mask of constant pre-set pixel size was placed over individual bead areas and the efficiency calculated within that area alone. Average FRET efficiency is shown +/- SEM. ANOVA was used to test statistical significance between different populations of data.

Confocal microscopy

Cells were permeabilised with 0.2% (v/v) Triton-X-100/PBS following fixation in 4% (w/v) paraformaldehyde. Primary antibodies or phalloidin were diluted 1:200-1:500 in phosphate-buffered saline containing 1% (w/v) BSA. The FITC- and Cy3-labelled secondary conjugates were obtained from Jackson ImmunoResearch Laboratories. Confocal images were acquired on a confocal laser scanning microscope (model LSM 510 Meta, Carl Zeiss Inc.) equipped with both 40X/1.3Plan-Neofluar and 63X/1.4Plan-APOCHROMAT oil immersion objectives.

**Supplementary figure and table legends**

Supplementary Figure 1. Generation and characterisation of β1-GFP cells. A. FACS trace of MEF 7929 (floxed β1+/+) and MEF 7929 (β1-/-) cells. β1 integrin was detected with KMI6 rat anti-mouse β1 integrin. B. PCR of MEF 7929 (β1+/+), MEF 7929 (β1-/-) and MEF 4043 (with wild-type β1). The top panel shows PCR for β1 integrin, and the lower panel shows PCR of the Immorto allele. C. Western blot of MEF 7929 (floxed β1+/+) and MEF 7929 (β1-/-) cells using KMI6 or rat IgG. D. Ablation of β1 integrin expression prevented attachment to the α2β1 ligand, type I collagen. MEF 7929 carrying floxed β1 integrin attached normally to collagen I. MEF 7929 (β1-/-) cells were still able to attach to the 50 kDa fragment of fibronectin, most likely via αVβ3 integrin. E. FACS trace of MEF 7929 (β1-/-) cells retrovirally transduced with GFP-pFBneo or β1-GFP-pFBneo. F. Attachment of MEF 7929 (β1-/-) cells retrovirally transduced with GFP-pFBneo or β1-GFP-pFBneo to fibronectin. Both cell lines attached to fibronectin in a

dose-dependent manner. Attachment was cation-dependent (ablated by 20mM EDTA) and was reduced by a combination of function-blocking rat anti-mouse β3 integrin (2C9.G2) and rat anti-human β1 (mAb13) antibodies (10μg/ml each). G. Expression of GFP-tagged β1 integrin restores β1 KO MEF attachment to laminin-1 and collagen-1. Attachment to laminin-1 and collagen-1 was inhibited by rat anti-mouse α6 integrin antibody GoH3 and rat anti-mouse α2 antibody HMa2, respectively (10μg/ml each). H. GFP-transfected MEF 7929 (β1-/-) (top two panels) and β1-GFP-transfected MEF (bottom two panels) spread and formed adhesion contacts on fibronectin. Cells were co-stained with either TRITC-phalloidin for F-actin or anti-vinculin. Merged images are shown in far right panels. Scale bar = 20μm.

Supplementary Figure 2. S976162 induces LIBS and reduces LABS epitope expression in a dose-dependent manner. A. Binding of the mouse anti-human β1 integrin monoclonal antibody 12G10 [which detects a ligand-induced binding site (LIBS) epitope] to affinity-purified α4β1 in the presence of varying concentrations of S976162 (open circles), or equivalent dilutions of dimethylformamide solvent (DMF; black circles). B. Binding of the rat anti-human β1 antibody mAb13 [which detects a ligand-attenuated binding site (LABS) epitope]. Assays were performed as described by Newham *et al.* (*28*). Briefly, purified α4β1 was immobilised and BSA blocked in a 96-well ELISA plate format before incubation for 2 hours at 37°C with 0.1μg/ml 12G10 in the presence of compounds or DMF, or 0.3μg/ml mAb13 in the presence of 2mM $Mn^{2+}$ and small molecule inhibitors or DMF. Bound antibody was detected with anti-rat HRP and the substrate ABTS as previously described.

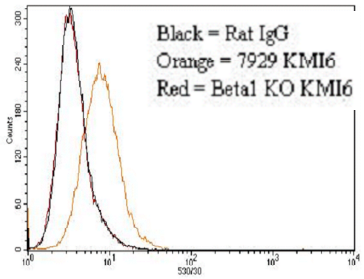
Supplementary Table 1.

| *Interaction Partners* | *IC50's for S97 6162 (BIO 1211)* | *IC50's for V0519* |
|---|---|---|
| α4β1 / VCAM-1 | 0.88 ± 0.15 nM | 24.5 ± 5.3 μM |
| α5β1 / Fibronectin | >100 μM | 260 ± 70 nM |
| α2β1 / Collagen | >100 μM | approx 100 μM |
| Molt4 (α4β1) / VCAM-1 | 119 ± 76 nM | ND |
| JY (αVβ3) / Vitronectin | approx 100 μM | <1 μM |
| KL4 (αLβ2 ) / ICAM-1 | >50 μM | ND |

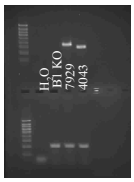
Specificity and potency of S976162 and V0519 for selected ligand-receptor interactions. Values represent means and standard deviations of a representative result from at least 3 independent assays. Assays were performed as originally described by Glasner *et. al.* (*22*). Where appropriate, cell types used are indicated, with the respective cell surface integrin heterodimer utilised in brackets. Otherwise affinity-purified integrins were used in direct protein-protein assays. S976162 was originally published as BIO-1211 (*29*). IC50 values were calculated using Sigmaplot versions 2.0 or 8.0 (SPSS Inc., IL, USA).

## Supplementary Fig 1

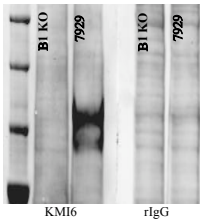
**1A**



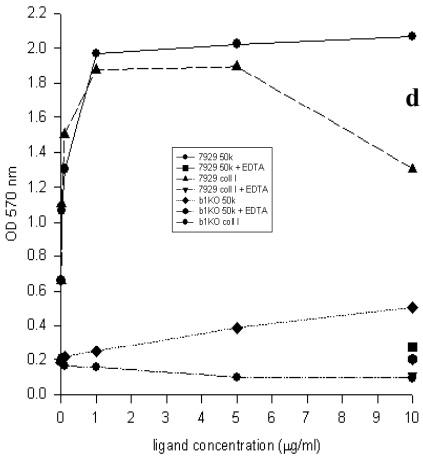

**1B**



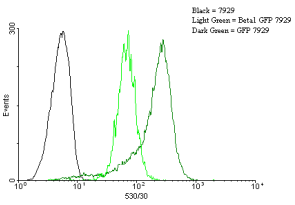

**1C**



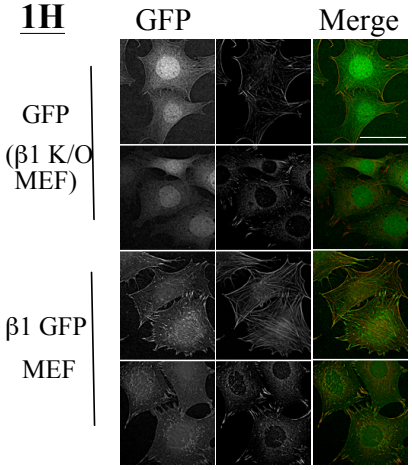

**1D**



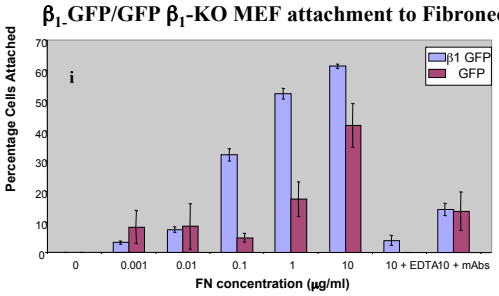

**1E**



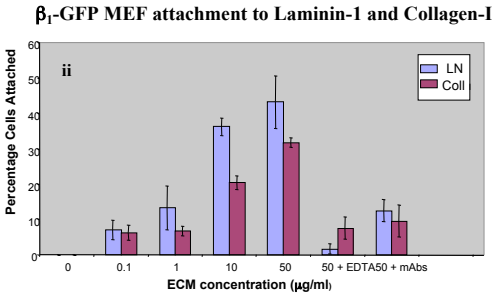

**1H**




**1F**




**1G**



# Supplementary Figure 2