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Abstract

Lung cancer is the leading cause of cancer death worldwide. The epidermal growth factor receptor (EGFR) represents the main target for non-small cell lung cancer (NSCLC) therapy, as its overexpression or constitutive activation contributes to malignancy and correlates with poor prognosis. Our previous work demonstrated that in epithelial cells beta1 integrin is required for propagating EGFR signalling from the plasma membrane to the nucleus. Here, we silenced beta1 integrin in human NSCLC A549 cells. Beta1 integrin silenced cells show a defective activation of the EGFR signaling cascade, leading to decreased *in vitro* proliferation, enhanced sensitivity to Cisplatin and Gefitinib, impaired migration and invasive behavior. Inhibitory effects on tumor growth and on the EGFR pathway were also observed in *in vivo* experiments. Moreover, beta1 integrin silencing increases the amount of EGFR on the cell surface, suggesting that beta1 integrin is required for efficient constitutive EGFR turn-over at the cell membrane. Although the rate of EGF internalization and recycling is not affected in silenced cells, EGFR signaling is recovered only by expression of the Rab-coupling protein RCP, indicating that beta1 integrin sustains the endocytic machinery required for EGFR signaling. Overall, these results show that beta1 integrin is an essential regulator of EGFR signaling and tumorigenic properties of lung cancer cells, and that its silencing might represent an adjuvant approach to anti EGFR therapy.

Keywords: EGFR, beta1 integrin, lung cancer

Introduction

Lung cancer is the leading cause of cancer death worldwide and comprises diseases of diverse aethiology (Jemal *et al.*, 2006). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer and has an epithelial origin (Brambilla *et al.*, 2001). The epidermal growth factor receptor (EGFR) is overexpressed or constitutively activated in approximately 60% of NSCLC cases correlating with poor prognosis. Deregulation of the EGFR signalling contributes to malignancy conferring to transformed cells proliferative advantage, inhibiting apoptosis and favoring cell motility and invasiveness (Sharma *et al.*, 2007), thus rendering the EGFR the preferential molecule for NSCLC targeted therapies. However, the current use of EGFR kinase inhibitors present limitations mainly because nearly all patients become resistant to further treatment (Yoshida *et al.*) (Nguyen *et al.*, 2009).

Increasing evidences indicate that the integrin family of cell adhesion receptors play an essential role in tumour progression. It is well documented that integrins contribute to migration and invasion of cancer cells (Guo *et al.*, 2006); moreover it is becoming evident that they can promote intracellular signalling tipically in the context of activated growth factor receptors thus regulating cell proliferation and survival and sustaining tumor growth (Desgrosellier and Cheresh) (Walker and Assoian, 2005) (Klein *et al.*, 2007). In the context of the NSCLC, increased expression of alpha5/beta1 integrin has been recently reported to be a poor prognostic factor (Okamura *et al.*, 2007) (Adachi *et al.*, 2000), influencing patient survival (Dingemans *et al.*).

Our previous data show that in normal epithelial cells beta1 integrin is both sufficient to partially activate the receptor itself and required for the full activation of the EGFR in response to EGF leading to transcriptional activity (Moro *et al.*, 1998) (Moro *et al.*, 2002) (Cabodi *et al.*, 2009). Overall, our results contribute to the body of evidences showing that integrins and RPTKs have no longer to be considered as individual receptors, but rather as joint modules in which attachment to the matrix confers positional control to respond to soluble growth factors (Guo and Giancotti, 2004) (Desgrosellier and Cheresh) (Streuli and Akhtar, 2009) (Cabodi *et al.*). Therefore modulating beta1

integrin activation or expression might represent a way to interfere with EGFR-dependent signalling in cancer cells, in particular in those tumors where the efficacy of conventional therapeutic regimens aimed to block the EGFR activation is limited. To this regard beta1 integrin may represent a suitable target in lung cancer cells. Here we show that beta1 integrin has a prominent role in the maintenance of tumorigenic properties of A549 NSCLC cells by regulating EGFR signaling.

Results

Beta1 integrin silenced A549 lung cancer cells show a defective response to EGF

A549 cell line represents a model for NSCLC expressing high levels of the wt EGFR. To assess their dependence on EGFR, cells were treated with 5μM of the EGFR kinase inhibitor tyrphostin AG1478 and cell proliferation was assessed by counting cells at different time points. A549 proliferation was inhibited upon the treatment with the inhibitor (Suppl. Figure 1), confirming that the cells are addicted to EGFR in their ability to grow in standard culture conditions. We and others have already shown that integrins are able to support EGFR activation either in the absence or in the presence of EGF in normal epithelial cells (Miyamoto *et al.*, 1996) (Moro *et al.*, 1998) (Bill *et al.*, 2004) (Cabodi *et al.*, 2009). To evaluate whether beta1 integrin controls EGFR activation in tumor cells, A549 cells were plated on TS2/16 antibodies against the beta1 integrin subunit or kept in suspension in presence and absence of EGF. Consistent with the previous results, EGFR is phosphorylated by beta1 integrin-dependent adhesion even in absence of EGF. In addition, in adherent conditions, the treatment with EGF had a synergistic effect on EGFR phosphorylation (Figure 1A). A similar pattern of activation is observed also for the Erk1/2 MAPKs and Akt (Figure 1A), demonstrating that in A549 lung cancer cells beta1 integrin-mediated adhesion plays an essential role in regulating EGFR activation and its downstream signalling.

To assess whether EGFR activity might be modulated by beta1 integrin silencing, A549 cells were infected with pLKO.1 lentiviral particles carrying either a beta1 integrin specific shRNA sequence or an empty vector. Beta1 integrin expression was decreased up to 70% compared to control cells, as measured both by western blotting of total cell extracts (Figure 1B, upper panel) and FACS analysis on intact cells. Beta3 and beta4 integrin expression was also evaluated, showing that their expression on the cell surface remain unchanged in silenced cells (Figure 1B, lower panel).

In normal growth conditions silenced cells showed a delayed cell spreading accordingly with the requirement of integrin-activated signaling molecules in the spreading process. The defect was

recovered within 12 hrs of cell adhesion (Suppl. Figure 2), thus rendering silenced cells undistinguishable from the control ones in normal culture conditions.

To specifically assess the relevance of beta1 integrin in A549 response to soluble EGF, control and silenced cells were treated with 20 ng/ml of EGF for different times. Cell extracts were analysed for EGFR phosphorylation and for the activation of downstream molecules, such as Fak, Src, Erk1/2 MAPKs and Akt. As shown in Figure 1C, silenced cells showed decreased levels of EGFR activation on the signaling-related residues Tyr1068 and Tyr1173 compared to control cells all over the time course of stimulation. Consistently, downstream to EGFR decreased levels of Fak and Src phosphorylation as well as Akt and Erk1/2 were detected. Densitometric analysis (Figure 1D) indicated that beta1 integrin silencing does not impair the kinetics of EGF-dependent signaling, but rather affects the extent of the activation at each time point. A similar inhibition of the EGF signaling was also observed in the SK-MES1 lung squamous carcinoma cells silenced for beta1 integrin **(Suppl Figure 3A)**, further supporting that beta1 integrin controls EGFR activity in lung carcinoma cells, and that its silencing is an effective mechanism for modulating receptor activity.

Beta1 integrin silencing impairs lung tumor cell proliferation and enhances sensitivity to therapy

As shown above, A549 cell growth is dependent on EGFR activity. To evaluate the contribute of beta1 integrin silencing on their long term proliferation, A549 cells were plated at low density in normal culture conditions and counted daily until day 12. As shown in Figure 2A, control A549 cells reached the confluence earlier than silenced cells, which displayed a marked decrease in the proliferation rate. **Moreover beta1 integrin silencing affected cell proliferation at a similar extent also in SK-MES1 cells (Suppl Figure 3B)**. Interestingly, when A549 silenced cells were grown in soft agar, after two weeks of culture they gave rise to a number of colonies comparable to that observed in control cells. However, the mean area of colonies was significantly reduced in size and silenced cells never formed colonies larger than 1 mm (Figure 2B). These data indicate that

beta1 integrin silencing affects tumor cell proliferation both in anchorage-dependent and independent conditions, underlying the crucial role of beta1-dependent signaling in lung tumor cell growth.

Control and silenced A549 cells were then tested for their sensitivity to therapeutic agents. Cells were left untreated or treated for 72 hours with different doses of either cisplatin, a DNA damaging compound, or Gefitinib, a selective EGFR inhibitor (Arteaga and Johnson, 2001), which are currently in clinical use for NSCLC therapy. The graphs in Figure 2C show that, compared to control cells, silenced cells were more sensitive to Cisplatin and Gefitinib, thus indicating that beta1 integrin down-regulation significantly enhances the ability of lung tumor cells to respond to lower doses of pharmacological agents.

Beta1 integrin is required for A549 migration and invasive behavior

To assess the relevance of beta1 integrin in lung tumor cell migration, control and silenced A549 cells were subjected to Transwell migration assay. A549 cells do not require additional stimuli to migrate through the Transwell. However, while control cells efficiently migrate in basal conditions, silenced cells showed a 70% reduction in the number of migrating cells (Figure 3A).

Further, control and silenced cells were plated as single cells in a three dimensional (3D) basement membrane (Matrigel/Collagen I 1:1) and allowed to grow for two weeks. This assay enables to test the ability of cells to invade in a 3D environment, forming typical **sprouted** structures. When embedded into the matrix, control A549 cells gave rise to sprouted spheroids that extend massive invasive protrusions (Figure 3B, upper panels). In contrast, beta1 integrin silenced cells formed regular spheroids, without invasive features (Figure 3B, middle panels). **Similar results were also obtained treating wild-type A549 cells with the anti beta1 inhibitory antibody AIIB2 (29).** As EGF is a known inducer of tumor cell invasion (Xue *et al.*, 2006), to evaluate the co-operation between beta1 integrin and the EGFR in promoting the invasive program, cells embedded in 3D for 8 days were stimulated with 5 ng/ml EGF for additional 4 days. Upon EGF treatment, silenced cells

were still less invasive from the spheroids compared to control cells, as clearly shown in the images at lower magnification (Figure 3C). While in control cells a marked increase in cells escaping from the disorganized structures was observed (Figure 3C, upper panels), in silenced cells **as well as in A549 cells treated with the AIIB2 inhibitory antibody this event was much more limited (Figure 3C, middle and lower panels)**.

Cell extracts prepared from unstimulated or EGF-treated cells were analyzed for Fak and Src activation, as they are known integrin-dependent regulators of cell migration and invasion (Mitra and Schlaepfer, 2006). Western blot analysis of Fak and Src phosphorylation respectively on Tyr 397 and Tyr 416 revealed that beta1 integrin down-regulation strongly affects both Fak and Src activation (data not shown). Taken together, these data show that beta1 integrin silencing impairs the migratory and invasive behavior of lung tumor cells, affecting the activation of the motility-promoting signaling involving Fak and Src kinases.

Beta1 integrin affects *in vivo* tumorigenic properties of A549 cells

Cross-talk between integrin receptors and activated growth factor receptors has been demonstrated to play a critical role in the initiation and progression of cancer (Huck *et al.*) (Trusolino *et al.*, 2001) (Guo *et al.*, 2006) (Desgrosellier and Cheresh) (Streuli and Akhtar, 2009). Therefore we decided to investigate *in vivo* the role of beta1 integrin silencing in the process of A549 tumor growth. To this end we performed xenograft experiments injecting s.c. in SCID mice 3 x 10^6 control or silenced cells. Even though both cell types gave rise to palpable tumors, the ones derived from silenced cells were significantly smaller compared to those derived from control cells up to the end of the experiment (Figure 4A). **In another experimental set, mice bearing tumors derived from wild-type A549 cells were treated i.p. with two different beta1 integrin blocking antibodies, namely the above mentioned AIIB2 antibody and the BV7 (Cusinato *et al.*, 1999). As shown in Figure 4A the treatment with beta1 blocking antibodies inhibited tumor growth at the same extent of**

beta1 integrin silencing. These results represent the proof of concept of the potential therapeutic benefit of targeting beta1 integrin in established lung tumors.

Mice were then sacrificed **and tumors derived from control and silenced cells were** analyzed for protein expression. As shown in Figure 4B, beta1 integrin silencing was highly efficacious and durable also *in vivo*. The analysis of EGFR, Erk1/2 MAPKs and Akt phosphorylation showed that all these signaling molecules were less phosphorylated in tumors derived from silenced cells. Densitometric analysis (Figure 4B) of extracts from 13 control and 13 silenced tumors revealed that EGFR and Erk1/2 phosphorylation are significantly reduced in beta1 integrin silenced tumors. Overall these results indicate that beta1 integrin sustains *in vivo* proliferation of A549 cells, likely through an impaired activation of the EGFR signaling cascade.

Dual role for beta1 integrin in controlling EGFR turn-over and signaling

It has been recently demonstrated that integrins can regulate EGFR trafficking from the plasma membrane to the endosomes and back, thus modulating receptor activity (Caswell *et al.*, 2009).

Thus, to assess whether beta1 integrin-dependent control of EGFR turn-over **could explain the defective EGFR signaling observed in beta1 silenced A549 cells**, control and silenced cells were analyzed for EGF-dependent EGFR internalization and recycling using ^{125}I-EGF. As shown in Figure 5A, EGF was internalized with the same kinetics in control and silenced cells both at low (2 ng/ml) (left panel) and high (20 ng/ml) (right panel) EGF doses. Similarly, the rate of EGFR recycling on the cell surface was comparable between control and silenced cells (Figure 5B), thus showing that ligand-dependent EGFR turn-over is not affected by beta1 integrin silencing.

The experiments described above don't allow analyzing EGFR levels on the plasma membrane in basal conditions, i.e. in the absence of EGF stimulation. To this end cells were analyzed by FACS for EGFR levels on the plasma membrane. Interestingly, the amount of EGFR exposed on the cell surface in basal conditions was higher in beta1 integrin silenced cells, compared to control cells (Figure 5C). However, consistent with the data obtained with

^{125}I-EGF, FACS analysis confirmed that, in response to EGF, the rate of EGFR displacement from the plasma membrane was similar in control and silenced cells. Nevertheless silenced cells showed for each time point an absolute higher value than control cells due to the different basal amount. To confirm this observation, EGFR expression at the cell surface was analyzed by byotinilation of the plasma membrane. As shown in Figure 5D, streptavidin western blot of immunoprecipitated EGFR, showed that beta1 integrin silenced cells express an increased amount of cell surface EGFR.

Recent evidences point out that EGFR requires alpha5/beta1 integrin and the Rab-associated protein known as RCP for efficient basal endocytic recycling and signaling to Erk (Caswell *et al.*, 2008). To assess whether the defective signaling observed in beta1 silenced cells could be ascribed to an altered function of the beta1/RCP pathway, beta1 silenced cells were transfected with a plasmid expressing RCP. Indeed, upon RCP over-expression the level of Erk1/2 MAPK activation in response to EGF was partially rescued in silenced cells (Figure 5E). Thus these data show that in basal conditions beta1 integrin regulates EGFR turnover at the plasma membrane while EGFR internalisation and recycling in response to EGF are not affected by beta1 integrin silencing. However, beta1 integrin still controls the full propagation of signaling to Erk in response to EGF trough the RCP pathway.

Discussion

In this work we demonstrate that beta1 integrin is required for both *in vitro* and *in vivo* tumor growth and for migration and invasion of EGFR-dependent NSCLC cells. In addition we show that beta1 integrin controls EGFR signaling and EGFR turn-over at the cell membrane. Overall we provide evidence that beta1 integrin/EGFR crosstalk is a key element driving NSCLC tumorigenesis, thus rendering beta1 integrin a suitable target for adjuvant therapy in combination with anti EGFR agents currently used in clinic.

EGFR is a well known inducer of lung tumorigenesis and its expression and activity correlate with tumor onset and maintenance. Less is known on beta1 integrin relevance in lung tumors, although its expression has been shown to be aberrant and correlated with reduced patient survival (Dingemans *et al.*) (Okamura *et al.*, 2007). The results presented here are the first demonstration that beta1 and EGFR are functionally co-operating in lung cancer cells, giving rise to a crucial signaling platform required for primary tumor formation as well as for the invasive behavior of tumor cells. The relevance of the beta1 integrin/EGFR co-operation in the malignant transformation has been underlined in a normal breast line that becomes tumorigenic by acquisition of *de novo* integrin–EGFR interactions (Wang *et al.*, 1998). In these cells function-blocking antibodies to either beta1 integrin or EGFR led to a striking reversal to the normal phenotype (Weaver *et al.*, 1997). Therefore, beta1 integrin/EGFR cross-talk appears to be fundamental for cancers that co-express beta1 integrin and the EGFR.

Our data show that in A549 cells EGF treatment strictly requires beta1 integrin-dependent adhesion for fully activating the EGFR and its downstream signaling. Noteworthy, integrin-dependent adhesion is sufficient not only for triggering EGFR phosphorylation but also for activating downstream pathways. Conversely, in the absence of cell-matrix adhesion, even though EGF triggers a higher EGFR phosphorylation compared to adhesion *per se*, the signaling molecules Erk1/2 and Akt are not activated, further supporting the essential role of integrin-mediated adhesion in transducing EGF signalling. These data, consistent with previous observations in normal

epithelial cells (Boeri Erba *et al.*, 2005) (Cabodi *et al.*, 2009), demonstrate that in lung tumor cells addicted to EGF, signaling remains strictly dependent on cell-matrix adhesion.

Moreover our data provide evidence that beta1 integrin silencing is effective in impairing EGFR signaling to the same extent of the absence of cell matrix adhesion. In fact, beta1 integrin silenced cells that in standard culture conditions maintain normal adhesive properties, are largely defective in EGFR phosphorylation and in activation of downstream signaling molecules. Remarkably, even though the kinetics of activation is not affected, the extent of phosphorylation at each time point is strongly reduced, implying that reduction in beta1 integrin results in the inability of EGFR to fully respond to its ligand. Therefore, these data underline the essential role of beta1 integrin in determining the cell-matrix dependence of EGFR activity in transformed cells.

Our results clearly show that beta1 integrin silencing affects both *in vitro* and *in vivo* proliferation of A549 cells, resulting in decreased tumor growth. **A similar inhibition on *in vivo* growth of A540 cells was also observed by treating mice bearing tumor masses with anti beta1 inhibitory antibodies, further assessing the proof of concept of the potential therapeutic benefit of targeting beta1 integrin in established lung tumors.** Hystochemical analysis of tumors derived from silenced cells revealed that the morphology is not significantly different from the controls, and that the number of vessels is quite similar. Therefore beta1 integrin silencing does not affect the general architecture of these tumors neither their ability to recruit new blood vessels, but rather significantly slow down their proliferation, giving rise to tumors that are smaller compared to control. Interestingly, in tumors derived from silenced cells, EGFR phosphorylation and activation of downstream pathways is severely impaired, indicating that the inhibitory effect of beta1 down-regulation on EGFR signaling is persistent also *in vivo*. The observed inhibition of EGFR activity likely accounts for the decreased tumor growth. Therefore, this is the first demonstration that in EGFR–dependent tumors, beta1 integrin targeting is an efficient tool to negatively regulate EGFR signaling, thus affecting tumor development.

Tumor progression is a multi-step process involving not only cell proliferation but also migration and invasion of cancer cells to give rise to metastatic dissemination. Beta1 integrin is a well established player in cell motility (Friedl *et al.*, 2004) (Friedl and Wolf) (Cordes and Park, 2007) (Caswell *et al.*, 2009). Here, we show that its silencing**, as well its inhibition,** affects the invasive properties of A549 cells, both in terms of directional migration through Transwells and in the ability to invade into 3D matrix. In particular, silenced cells embedded into the 3D matrigel/collagen I matrix organize in spheroids smaller than control ones (data not shown), that do not extend protrusions while control cells give rise to sprouted structures. Consistently, also in the squamous carcinoma A431 cells, beta1 integrin silencing gave rise to less invasive tumors (Brockbank *et al.*, 2005). Although we were unable to perform lung colonization assays with the A549 cells, these in vitro data already clearly support the primary role of beta1 integrin in regulating lung cancer cell motility.

Recent work has underlined the relevance of beta1 integrins in the control of EGFR endosomal trafficking (Caswell *et al.*, 2009). It is commonly believed that EGFR internalization through canonical pathways is essential for EGFR signaling (Sigismund *et al.*, 2008) (Sorkin and von Zastrow, 2009). Our data suggest that beta1 integrin plays a dual role in EGFR internalization and signaling. Indeed, beta1 integrin silenced cells show an increased basal level of EGFR on the cell membrane and a decreased EGFR signaling. Therefore in the absence of beta1 integrin, the EGFR on the cell surface is less able to respond to EGF stimulus, not in terms of internalization, but only in terms of signaling. In silenced cells signaling capacity is partially rescued by over-expression of RCP (Caswell *et al.*, 2008), indicating that RCP/beta1 integrin endocytic route is sufficient to sustain EGFR signaling.

In conclusion our data indicate that silencing beta1 integrin on lung cancer cells **or treating tumor with anti beta1 inhibitory antibodies** leads to decreased cell proliferation and tumor growth. This effect is particularly important when cells are treated with therapeutic agents. Indeed silenced cells are sensitive to lower dose of either Cisplatin or Gefitinib compared to control cells. In clinic, the

conventional treatment of NSCLC lung cancer involves the use of surgery, radiotherapy, platinum-base chemotherapy or specific EGFR inhibitors. Although major advances have been made in this field, treatment outcomes for patients affected by advanced NSCLC is still non efficacious and need to be improved by experimenting novel therapies (Maione *et al.*). Therefore targeting beta1 integrin by selective down-regulation in combination with conventional therapy might represent a novel adjuvant approach in the context of NSCLC treatment.

Materials and Methods

Reagents and antibodies

mAbs TS2/16 to the beta1 integrin subunit and HB-8508 to the EGFR were purchased from ATCC. Polyclonal Abs anti EGFR and anti beta1 integrin were described previously (Moro *et al.*, 2002). Antibodies to Erk1 and to c-Src were from Santa Cruz Biotechnology (Palo Alto, CA, USA). mAbs to Fak were from Millipore (Billerica, MA, USA). Phospho-specific polyclonal antibodies to pEGFR (Tyr 1173), pEGFR (Tyr 1168), pFAK (Tyr 397), pSrc (Tyr 416), p-p42/p44 MAPK (Thr 202/Tyr 204) and pAkt (Ser 473) were purchased from Cell Signalling (Danvers, MA, USA). Human recombinant EGF, Cisplatin and secondary antibodies conjugated with peroxidase were from Sigma (St. Louis, MO, USA). Secondary antibodies conjugated with FITC were from Alexa Molecular Probes (Invitrogen, Carlsbad, CA, USA). Gefitinib was from SeqChem (Pangbourne, UK). Matrigel and collagen I were from BD Trasduction Laboratories (Franklin Lakes, NY, USA). AG1478 was from Calbiochem (San Diego, CA). **AIIB2, a β1 integrin function-blocking antibody was isolated and prepared from a hybridoma cell line (Developmental Studies Hybridoma Bank, Iowa, USA)(29). The BV7 antibody was prepared from a hybridoma cell line already present in the lab.**

Cell culture and transfection

Culture media, serum and Lipofectamine LTX were from Invitrogen (Carlsbad, CA). Human lung adenocarcinoma cell line A549 was purchased from ATCC and grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml and 100 microgram/ml, respectively) at 37°C in a 5% CO2 atmosphere. A549 cells grown to 80% confluence were transiently transfected with the plasmid pEGFP-C3 encoding for RCPwt (kindly provided by Dr. Jim Norman, Beatson Institute for Cancer Research, Glasgow, UK) by the Lipofectamine LTX reagent as described by the manufacturer. 24 hours after transfection cells were serum deprived and incubated for 24 hrs before treatment.

Knock-down of beta1 integrin by RNA interference

Beta1 integrin shRNA sequence in pLKO.1 lentiviral vector (5' AAACCCAGGGCTGCCTTGGAAAAG 3') (Thermo Scientific, Erembodegem, Germany) or an empty pLKO.1 vector were separately transduced into HEK293T cells along with lentiviral packaging vectors pCMV-dR8.74 and pMD2.G-VSV-G using Effectene reagent (Qiagen). Viral supernatants were collected 48 and 72 hours after transfection, pooled and filtered. A549 cells grown at 40% confluence in 6-well plates were infected either with shRNA or with the empty vector in the presence of 8 micrograms/ml polybrene (Sigma). Transduced cells were kept under Puromycin selection (1 microgram/ml).

Adhesion assay

Adhesion assay on TS2/16 anti beta1 integrin antibodies coated plates was performed as described in (Cabodi *et al.*, 2009).

Protein extraction, immunoprecipitation and Western blotting

Cell lysis, immunoprecipitation and Western blotting were performed as described in (Cabodi *et al.*, 2009). Protein extracts from tumors were obtained by homogenization in 50mMTris (pH 7.4), 10% glycerol, 1% NP-40, 150mM NaCl, 2mM EDTA, 2mM DTT, 0.4mM Na3VO4, 10mM NaF, 0.5mM PMSF, and 40-mg/mL protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA).Cells were extracted with 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8,5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and 40-mg/mL protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). Image J software (NIH) was used for blot quantification.

Cell migration

For migration assays, Transwell chambers were used. A total of $5x10^4$ cells were seeded on the upper side of the filters and incubated in 0.1% BSA RPMI in the presence, were indicated, of 100 ng/ml EGF (Sigma) in the bottom wells of the chambers. Cells migrating to the lower side were fixed, stained with Diff-Quick kit and counted under a phase-contrast microscope.

3D assay

3D assays were performed in agreement with protocols reported in: http://muthuswamylab.cshl.edu/protocols. Briefly, A549 cells were embedded as single cells in Matrigel/Collagen I 1:1 and let grow for 12 days. When indicated, EGF was added for the last 4 days. **AIIB2 inhibitory antibody was added to culture medium on alternate days.** Live images were collected by using Zeiss microscopy (Oberkochen, Germany) at 4X or 20X magnitude.

Cell proliferation and soft agar assay

A total of $5x10^3$ cells were seeded on 6 cm tissue culture dishes and let proliferate for 12 days in the presence of medium supplemented with 10% FCS. Every 2 days, cells were detached and manually counted in Burker chambers on triplicate dishes. For the clonogenic assay, $5x10^4$ cells were seeded in 0.4% agar on the top of a base layer containing 0.8% agar into six-well plates. After 2 weeks, colonies were counted under a phase-contrast microscope. ImageJ software (NIH) was used to calculate colonies' area. All of the experiments were performed in triplicate.

Cell surface biotynilation

Cell surface biotinylation was performed as described (Damiano *et al.*). Cell extracts were immunoprecipitated with EGFR HB8508 mAb and biotinylated fraction was detected by incubation with streptavidin HRP.

Flow cytometry

When indicated cells were treated with EGF. After PBS washing cells were put in trypsin for 2 min at RT and detached by pipetting; then medium was added to block trypsin; after PBS washing, cells were fixed in 1 ml of 1% formaldehyde for 20 min. After a blocking step in 1% BSA for 30 min, cell surface labeling of integrins beta1, beta3 or beta4 alternatively was performed using polyclonal rabbit antibodies (dilution 1:500). For EGFR labeling the anti-EGFR mouse monoclonal antibody HB8508 which recognizes the extracellular portion of the receptor was used (dilution 1:1000). Alexa 488-conjugated secondary antibodies were used for detection. 10^4 cells were analyzed at each experimental point by flow cytometry.

^{125}I-EGF internalization assay

Internalization assay of ^{125}I-EGF was performed as described (Tosoni *et al.*, 2005).

^{125}I-EGF recycling assay

Recycling assay of ^{125}I-EGF was performed as described (Kornilova *et al.*, 1996) (Sorkin *et al.*, 1991).

***In vivo* tumour growth**

Five-week-old female SCID mice (C.B-17TM/IcrCrl-scidBR) were purchased from Charles River Laboratories (Calco, Italy) and treated in accordance with the European Community guidelines. Mice were challenged subcutaneously bilaterally in the flank with $3x10^6$ **A549 wild-type, control (β1 ctrl) or silenced (β1 sh) cells suspended in 100 microliters of RPMI medium. BV7 and AIIB2 antibodies or non-specific IgG were injected at the indicated doses i.p. biweekly beginning on day 4 after cell implantation.**

The incidence and growth of tumours were evaluated twice weekly by measuring with calipers up to the eighth week.

Conflict of interest: non declared

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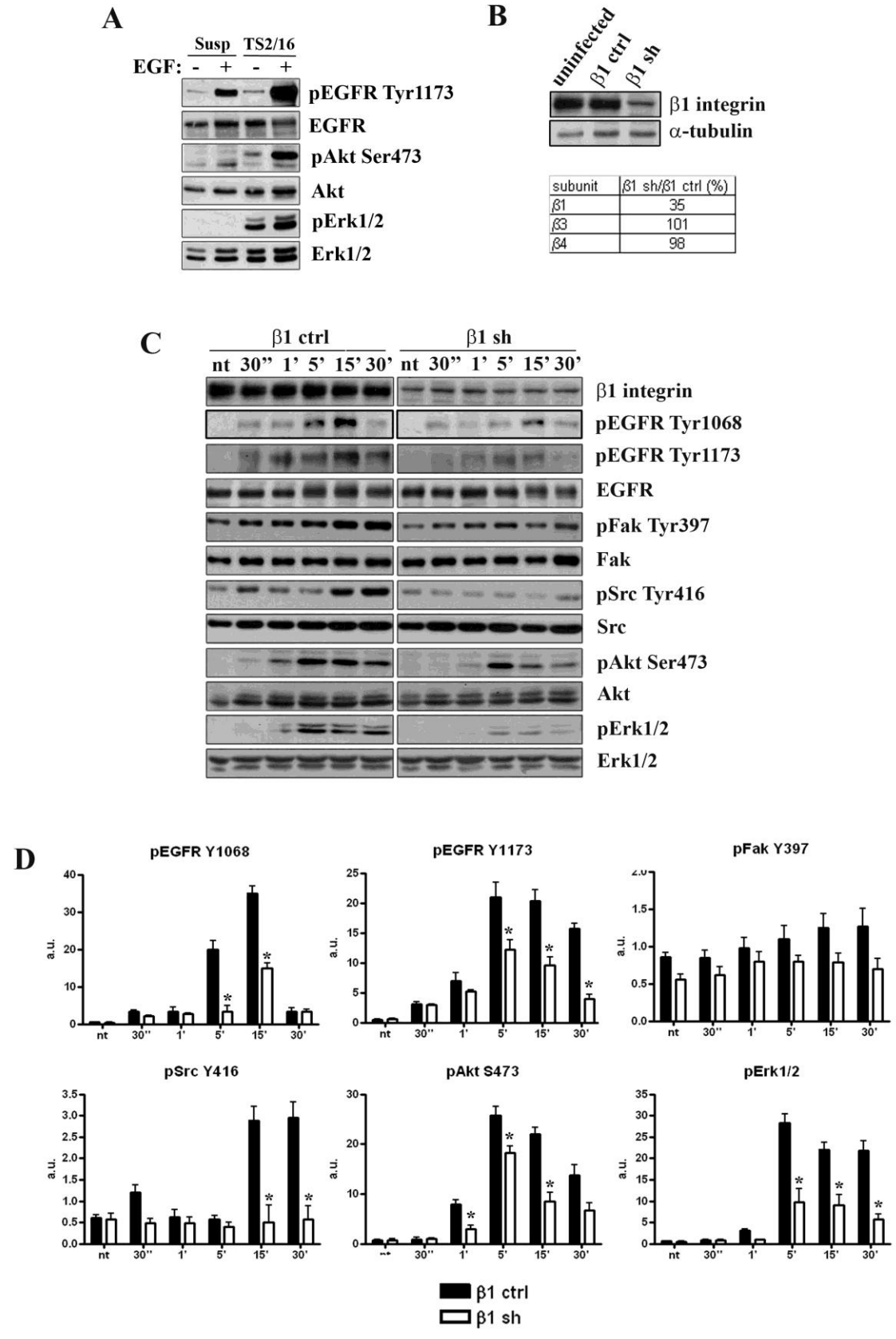
Figure 1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subunit | β1 sh/β1 ctrl (%)
β1 | 35
β3 | 101
β4 | 98
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β1 ctrl
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β1 integrin
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EGFR
pFak Tyr397
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pSrc Tyr416
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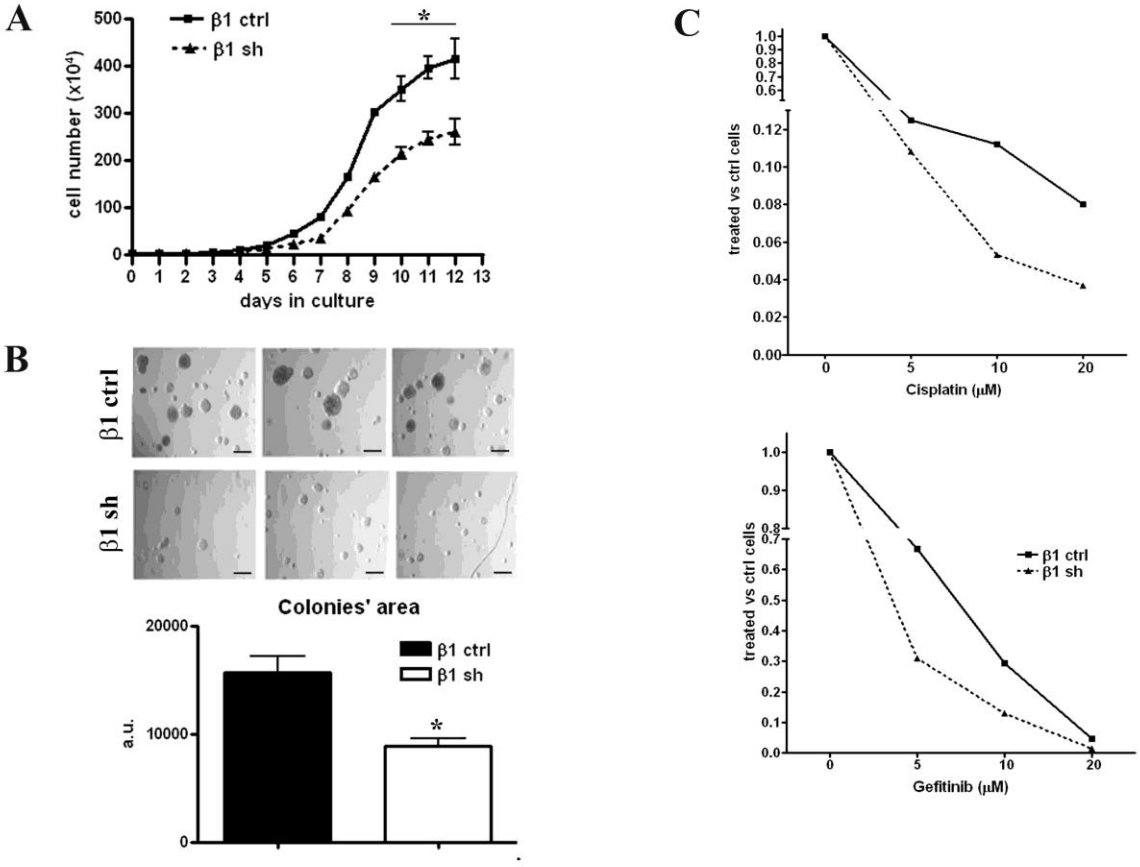
Figure 2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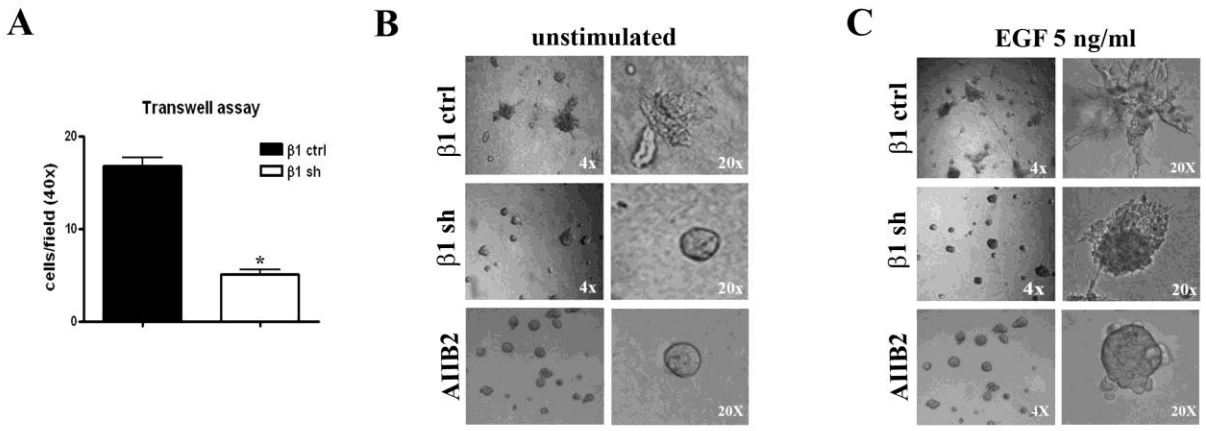
treated vs ctrl cells
Cisplatin (μM)
Gefitinib (μM)

Figure 3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EGF 5 ng/ml
β1 ctrl
β1 sh
AIIB2
4x
20X

Figure 4

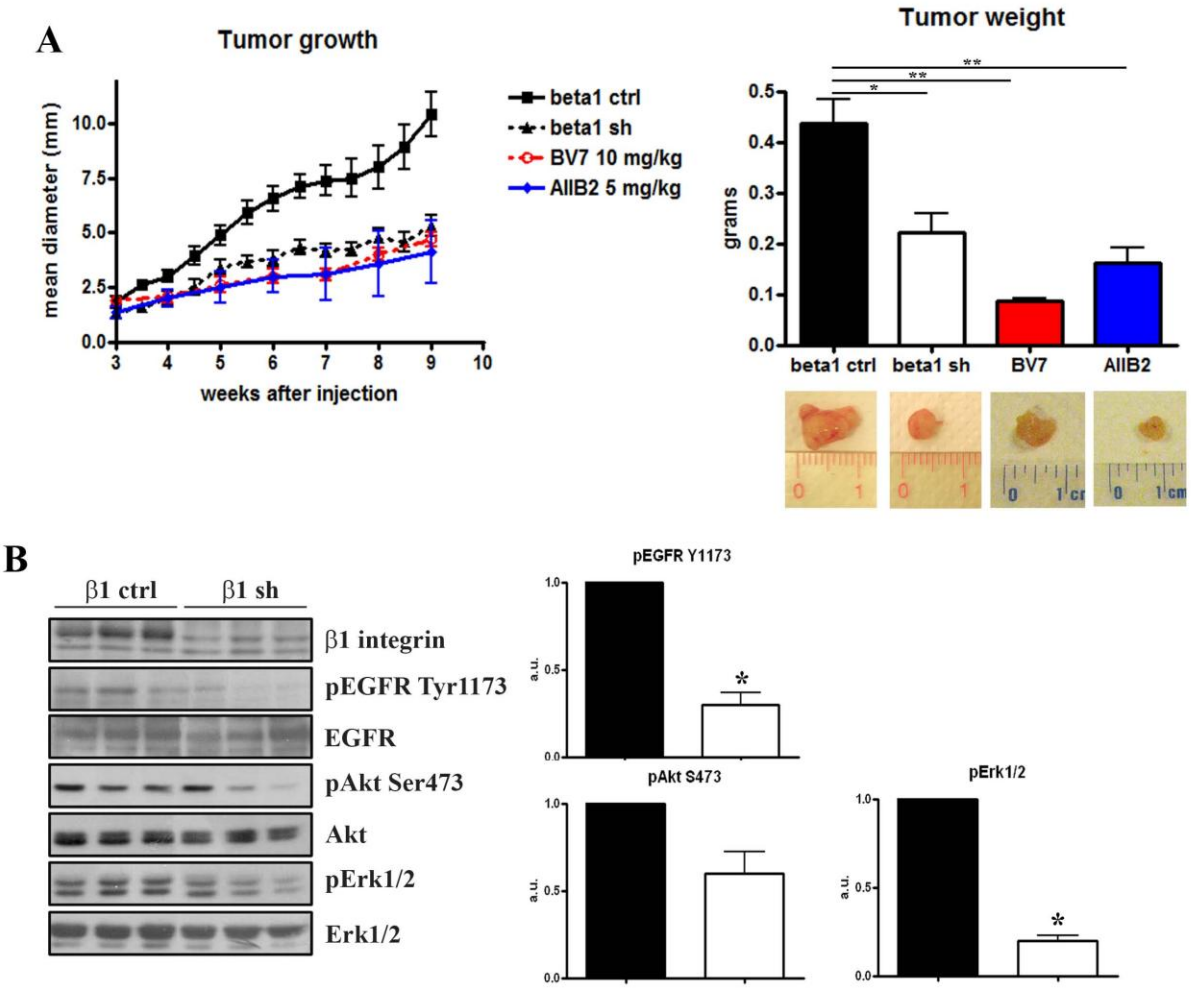

A
Tumor growth
mean diameter (mm)
weeks after injection
beta1 ctrl
beta1 sh
BV7 10 mg/kg
AIIB2 5 mg/kg
Tumor weight
grams
beta1 ctrl
beta1 sh
BV7
AIIB2
B
β1 ctrl
β1 sh
β1 integrin
pEGFR Tyr1173
EGFR
pAkt Ser473
Akt
pErk1/2
Erk1/2
pEGFR Y1173
pAkt S473
pErk1/2
a.u.

Figure 5

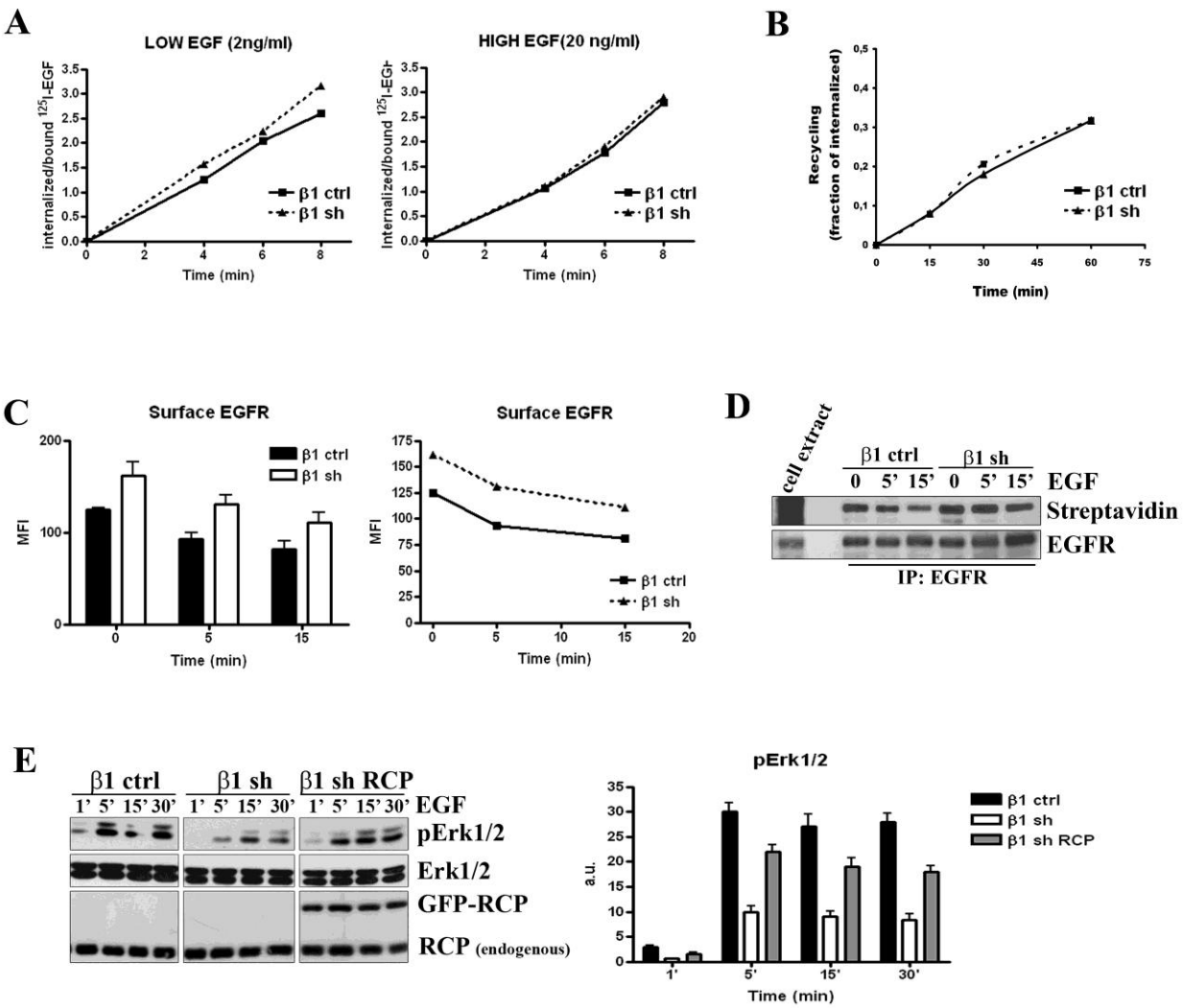

A
LOW EGF (2ng/ml)
HIGH EGF(20 ng/ml)
internalized/bound 125I-EGF
Time (min)
β1 ctrl
β1 sh
B
Recycling
(fraction of internalized)
Time (min)
C
Surface EGFR
MFI
Time (min)
D
cell extract
β1 ctrl
β1 sh
0 5' 15' 0 5' 15'
EGF
Streptavidin
EGFR
IP: EGFR
E
β1 ctrl
β1 sh
β1 sh RCP
1' 5' 15' 30'
EGF
pErk1/2
Erk1/2
GFP-RCP
RCP (endogenous)
pErk1/2
a.u.
Time (min)

Figure S1

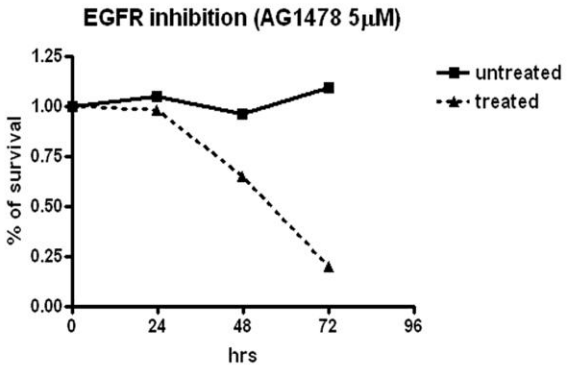


Figure S1. EGFR activation is required for A549 cell proliferation. 10^4 A549 cells were plated in standard culture conditions. After 12 hrs (t0) cells were either left untreated or treated with 5 μM AG1478 and then counted at the indicated timepoints. The graph reports on the y-axis the percentage of survived cells respect to cells in t0.

Figure S2

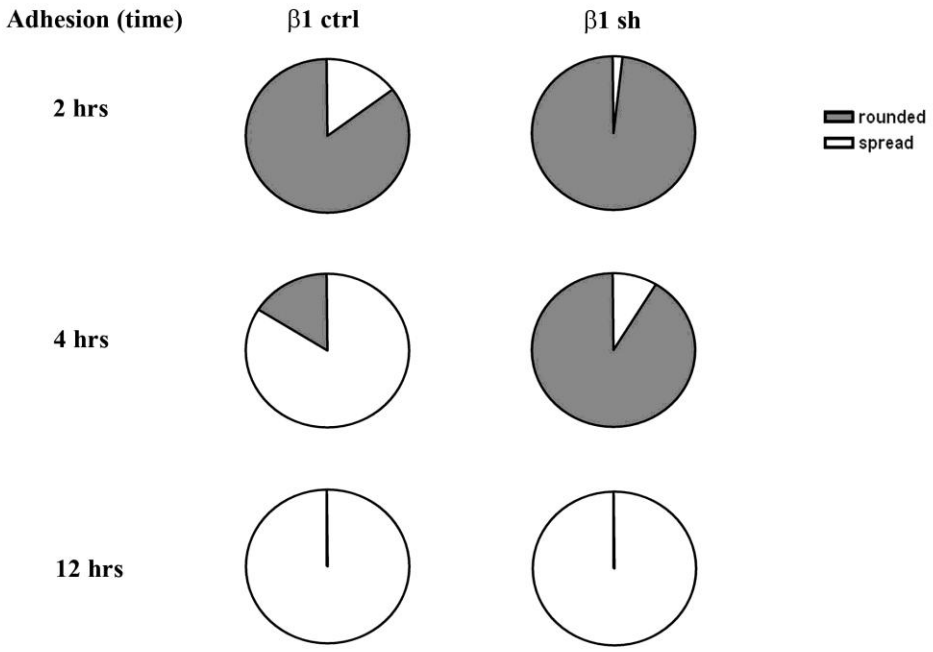
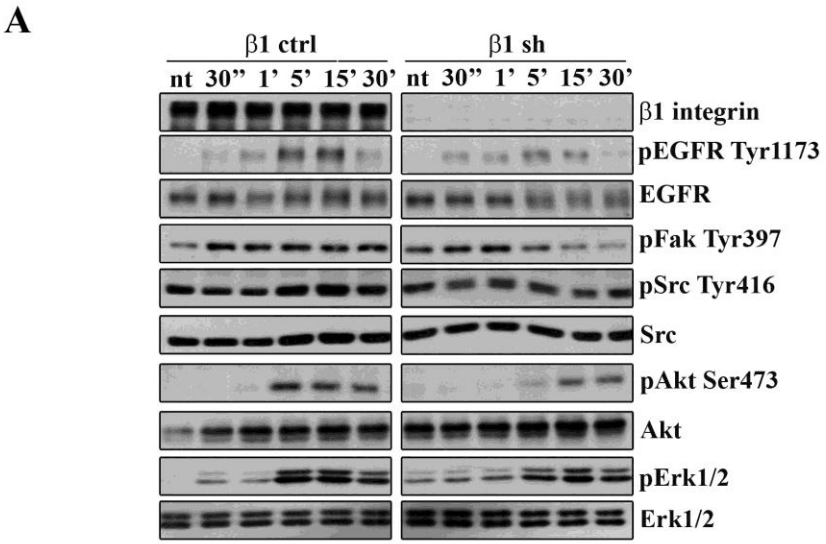


Figure S2. Beta1 integrin is required for early cell spreading. β1 ctrl and β1 sh cells were plated in standard culture plates. Cell spreading was evaluated under phase contrast microscope. The number of rounded vs spread cells at each time point is reported in the graph.

Figure S3



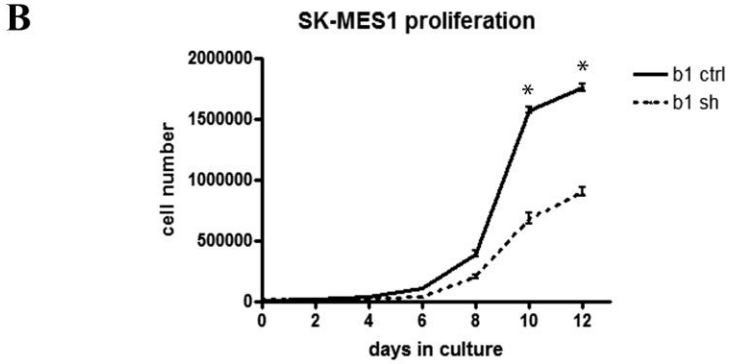


Figure S3. Beta1 integrin support EGF- dependent signalling and proliferation in SK-MES1 cells. (A)) SK-MES1 beta1 ctrl and beta1 sh cells were starved for 24 hrs and treated with EGF (20 ng/ml) for the indicated timepoints. EGF-dependent signalling was evaluated in Western blot with the indicated phospho-specific antibodies. For normalization purposes membranes were stripped and re-blotted with antibodies for total proteins. (B) 104 A549 beta1 ctrl and beta1 sh cells were grown in the presence of 10% FCS for 12 days. Every two days, cells were detached and counted. The mean number of cells from three separate experiments is reported on the y-axis (*$P<0.05$).

Figure legends

1) Beta1 integrin modulates EGFR signalling in A549 cells.

(A) Cells were starved for 24 hrs and then either kept in suspension (Susp) or replated on anti-beta1 integrin activating antibodies (TS2/16) for 30 min. Cell extracts were evaluated for EGFR activation and downstream signalling with the indicated antibodies. **(B)** A549 cells transduced either with empty vector (β1 ctrl) or with an shRNA sequence (β1 sh) were evaluated in Western blot for beta1 integrin levels. α-tubulin as loading control (upper panel). Beta1, beta3 and beta4 integrin expression on the cell surface on silenced cells relative to control ones was assessed by flow cytometry analysis (lower panel). **(C)** A549 β1 ctrl and β1 sh cells were starved for 24 hrs and treated with EGF (20 ng/ml) for the indicated timepoints. EGF-dependent signalling was evaluated in Western blot with the indicated phospho-specific antibodies. For normalization purposes membranes were stripped and re-blotted with antibodies for total proteins. **(D)** Densitometric analysis are representative of three independent experiments. The histograms show the ratio between active and total protein levels in arbitrary units (*$P<0.05$, Student's t-test).

2) Beta1 integrin support A549 cell proliferation and resistance to therapy.

(A) 10^4 A549 β1 ctrl and β1 sh cells were grown in the presence of 10% FCS for 12 days. Every two days, cells were detached and counted. The mean number of cells from three separate experiments is reported on the y-axis (*$P<0.05$). **(B)** 5×10^3 ctrl or sh cells were plated in soft agar. After 15 days live images were collected by using Zeiss microscope at 20X magnitude. Colonies' areas were measured using ImageJ software and the mean values are reported in the graph (n=100). **(C)** 10^4 A549 β1 ctrl and β1 sh cells were either grown in standard culture conditions or treated with different doses of Cisplatin (upper panel) or Gefitinib (lower panel). After 72 hrs cells were counted. Cell number was reported on the y-axis as percentage respect to untreated cells.

3) Beta1 integrin is required for A549 migration and invasive behaviour.

(A) Transwell assay. $5x10^4$ A549 β1 ctrl and β1 sh cells were seeded on the upper side of the filters and incubated in 0.1% BSA RPMI and let migrate for 6 hrs. To test the ability of cells to migrate in basal conditions, no additional stimuli were added in the lower chamber. Cells migrating to the lower side were fixed, stained with Diff-Quick kit and counted under a phase-contrast microscope. Numbers on the y-axes represent the number of cells migrated on the lower side of the Transwell. **(B)** and **(C) Invasion** assay. 10^3 A549 wild-type, β1 ctrl and β1 sh cells were embedded as single cells in matrigel/collagen I 1:1 and let grow for 12 days. When indicated, EGF was added for the last 4 days. **10 micrograms/ml AIIB2 was added to culture medium on alternate days.** Live images were collected by using Zeiss microscopy at 4X or 20X magnitude.

4) Beta1 integrin silencing affects *in vivo* tumorigenic properties of A549 cells.

(A) $3x10^6$ A549 β1 ctrl and β1 sh cells were injected bilaterally s.c. in 8 SCID mice per group, for a total of 16 tumors per group. For inhibitory antibodies treatment, 3 mice per group (BV7, red line, and AIIB2, blue line and control IgG, not shown) were challenged s.c. bilaterally with $3x10^6$ A549 wild-type cells, for a total of 6 tumors each. Beginning on day 4 mice were injected i.p. bi-weekly either with BV7 10 micrograms/kg or AIIB2 5 micrograms/kg in PBS. Tumour volume was measured twice a week within 8 weeks and reported on the y-axis (left panel). At the end of the experiment tumor masses were surgically resected and weighted. The average of tumor weights is reported on the y-axis (*P<0.05). **(B)** Tumor lysates were analysed by Western Blot. Beta1 integrin levels as well as EGF-dependent signalling was evaluated in Western blot with the indicated phospho-specific antibodies. For normalization purposes membranes were stripped and re-blotted with antibodies for total proteins. Densitometric analysis are reported in arbitrary units as average between different tumors (*P<0.05).

5) Beta1 integrin controls EGF-dependent turn-over and signalling through different mechanisms.

(A) Kinetics of EGF internalization. A549 β1 ctrl and β1 sh cells were incubated with either 2 ng/ml (low EGF, left panel) or 20 ng/ml (high EGF, right panel) ^{125}I-EGF. The rate of

internalization is expressed as internalized/surface-bound radioactivity. **(B)** EGF recycling. A549 β1 ctrl and β1 sh cells were incubated with 2 ng/ml ^{125}I-EGF. The amount of recycled EGF is expressed as fraction of internalized EGF. **(C) A549 β1 ctrl and β1 sh cells were starved for 24 hrs and treated with EGF (20 ng/ml) for the indicated timepoints. Flow cytometric analysis of EGFR expression on the cell surface was expressed as the mean fluorescence intensities (MFI) reported on the y-axis. (D) Cells treated like in (C) were cell-surface biotinylated and cell extracts were immunoprecipitated with EGFR mAb. Immunoprecipitates were blotted with HRP-Streptavidin and reprobed with anti-EGFR antibodies. (E) A549 β1 ctrl, β1 sh and β1 sh cells expressing exogenous RCP were starved for 24 hrs and treated with EGF (20 ng/ml) for the indicated timepoints. Erk1/2 activation was evaluated with phospho-specific antibodies. Filter was stripped and re-blotted for total Erk1/2. RCP blotting to assess transfected protein levels. Densitometric analysis reports the mean values from two independent experiments.**

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