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ORIGINAL ARTICLE

p140Cap suppresses the invasive properties of highly metastatic MTLn3-EGFR cells via impaired cortactin phosphorylation

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We have recently shown that the adaptor protein p140Cap regulates tumor properties in terms of cell motility and growth. Here, by using the highly metastatic rat adenocarcinoma cell line MTLn3-epidermal growth factor receptor (EGFR), we assess the role of p140Cap in metastasis formation. Orthotopic transplantation of MTLn3-EGFR cells over-expressing p140Cap in $Rag2^{-/-}\gamma_c^{-/-}$ mice resulted in normal primary tumor growth compared with the controls. Strikingly, p140Cap over-expression causes an 80% inhibition in the number of lung metastases. p140Cap over-expressing cells display a 50% reduction in directional cell migration, an increased number and size of focal adhesions, and a strong impairment in the ability to invade in a 3D matrix. p140Cap over-expression affects EGFR signaling and tyrosine phosphorylation of cortactin in response to EGF stimulation. Intriguingly, p140Cap associates with cortactin via interaction with its second proline-rich domain to the cortactin SH3 domain. The phosphomimetic cortactin tyrosine 421 mutant rescues migration and invasive properties in p140Cap over-expressing cells. Taken together, these data demonstrate that p140Cap suppresses the invasive properties of highly metastatic breast carcinoma cells by inhibiting cortactin-dependent cell motility.
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Keywords: p140Cap; cortactin; tumor invasion; metastasis; breast cancer

Introduction

Metastatic breast cancer is a leading cause of cancer death in Western countries. Metastasis formation is a multi-step process in which malignant cells spread from the primary tumor to colonize distant organs. Understanding the critical players involved in these processes is crucial to counteract cancer dissemination. In addition, it is important to develop good *in vivo* models to analyze each step of the metastatic spread. Rat mammary adenocarcinoma MTLn3 cells have been identified on the basis of gene profiling as a suitable model to study breast cancer progression and treatment (Neri and Nicolson, 1981; Marxfeld *et al*., 2006) and to follow actin cytoskeleton remodeling in live carcinoma cells (Lorenz *et al*., 2004). The epidermal growth factor receptor (EGFR) is often over-expressed in breast cancer, resulting not only in uncontrolled cell proliferation (Citri and Yarden, 2006; Normanno *et al*., 2006) but also in increased tumor cell motility and invasion (Condeelis and Segall, 2003). In particular, over-expression of EGFR in MTLn3 mammary adenocarcinoma cells results in increased cell motility *in vivo* in the primary tumor and intravasation and lung metastasis formation (Xue *et al*., 2006). We have recently established that the increased expression of the EGFR in the MTLn3 cell line is essential for efficient lung metastasis formation in the $Rag2^{-/-}\gamma_c^{-/-}$ mice model (Le Devedec *et al*., 2009).

p140Cap, also known as SNIP (Chin *et al*., 2000), is an adaptor protein that directly associates to Src and Csk kinases, and interferes with integrin/growth factor-dependent Src kinase activity, thus affecting tumor properties in cancer cells (Di Stefano *et al*., 2007; Cabodi *et al*., 2010; Damiano *et al*., 2010). We have recently reported that p140Cap is absent in 70% of human mammary carcinomas, including the aggressive Node + samples (Damiano *et al*., 2010), showing an inverse correlation with the state of malignancy. The modular structure of p140Cap is characterized by a tyrosine-rich and two proline-rich regions (Chin *et al*., 2000; Di Stefano *et al*., 2004; Damiano *et al*., 2010) that enable binding to effector molecules, such as Csk and Src, respectively (Di Stefano *et al*., 2007; Damiano *et al*., 2010). Mechanistically, p140Cap binds to and activates Csk kinase, phosphorylating Src on the inhibitory tyrosine 527, leading to its inactivation. Src inhibition results in impaired phosphorylation of the Fak (focal

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adhesion kinase) and p130Cas, and leads to inactivation of the GTPase Rac (Di Stefano *et al*., 2007; Damiano *et al*., 2010). p140Cap also controls EGFR signaling on at least two levels: the first one by inhibiting Src kinase activity, resulting in E-cadherin-dependent EGFR inactivation at the cell membrane. As a second event, p140Cap can directly affect Ras activation, thereby regulating cell proliferation. Therefore, p140Cap operates as an oncosuppressor, inhibiting *in vitro* and *in vivo* tumor growth (Di Stefano *et al*., 2007; Damiano *et al*., 2010).

We have already reported that p140Cap regulates cell spreading and *in vitro* cancer cell scattering, motility and invasion in MCF7 breast and in HT29 colon cancer cells (Di Stefano *et al*., 2007; Damiano *et al*., 2010). In this work, we have utilized the highly metastatic MTLn3-EGFR cells in order to investigate the involvement of p140Cap in lung metastasis formation. We provide evidence that high levels of p140Cap drastically reduce the number of lung metastasis induced after the injection of MTLn3-EGFR cells in Rag2$^{-/-}\gamma_c^{-/-}$ mice by affecting the early steps of cortactin-dependent motility and invasion.

Results

p140Cap over-expression inhibits anchorage-independent growth of MTLn3-EGFR cells

p140Cap was ectopically expressed in MTLn3-GFP-EGFR (MTLn3-EGFR) cells by viral transduction with an empty pBabe (Ctr) or with a pBabe-p140Cap (p140) retrovirus followed by selection with puromycin. As shown in Figure 1A, the basal level of p140Cap expression is increased by at least fourfold in p140 cells compared with the Ctr cells. In standard tissue culture conditions, p140 cells do not show a significant decrease in 2D cell proliferation compared with controls after 5 days in culture (Figure 1B). However, in soft agar assays, MTLn3-EGFR p140 cells formed 70% less colonies compared with Ctr cells. Moreover, the colonies formed by p140 cells had a significantly reduced area (Figure 1C). Interestingly, while Ctr cells formed colonies of heterogeneous shape, either round and compact or with extended protrusions, colonies formed by p140Cap over-expressing cells were all compact (Figure 1C, panels a–d). These data show that p140Cap over-expression reduces anchorage-independent growth of MTLn3-EGFR cells.

p140Cap over-expression inhibits lung metastasis formation

MTLn3-EGFR rat adenocarcinoma cells are an interesting model to study *in vivo* tumor metastasis. When injected into Rag2$^{-/-}\gamma_c^{-/-}$ mice, they efficiently form lung metastasis from primary tumors within 1 month from injection (Le Devedec *et al*., 2009). To assess the *in vivo* role of p140Cap in metastasis onset, MTLn3-EGFR Ctr and p140 cells were injected into the mammary fat pads of Rag2$^{-/-}\gamma_c^{-/-}$ mice and tumor growth was monitored for 4 weeks. Animals were then

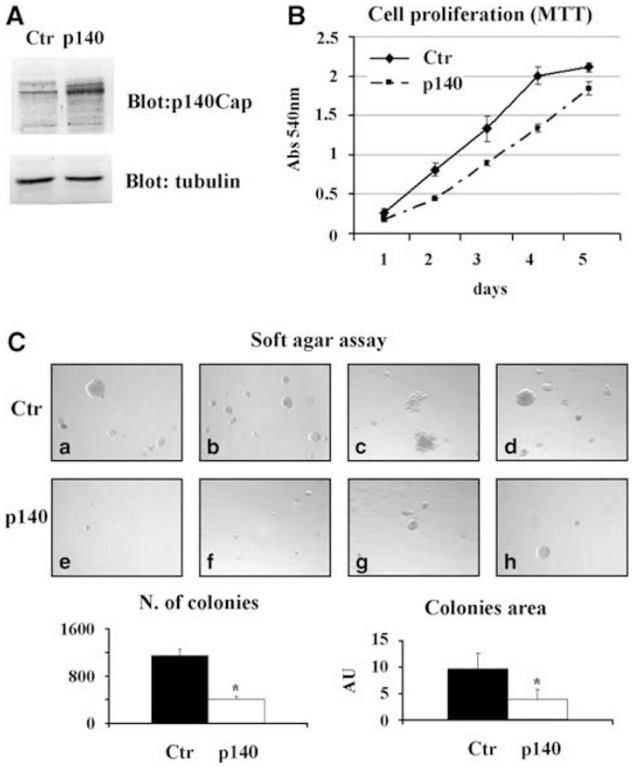

Figure 1 p140Cap over-expression affects anchorage-independent tumor growth. (**A**) Cell lysates from MTLn3-EGFR Ctr and p140 cells were analyzed by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot was performed using p140Cap and tubulin-specific antibodies. (**B**) In all, 3×10^3 MTLn3-EGFR Ctr and p140 cells were plated in 24-well plates and grown for 5 days. Cell proliferation was evaluated by 3(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay every day. Absorbance at 540 nm from three separate experiments is reported on the *y* axis. (**C**) In all, 8×10^3 Ctr and p140 cells were plated in soft agar and allowed to grow for 2 weeks. (Upper panels) Cells were photographed at $\times 4$ magnification. Eight representative pictures are shown. (Lower panels) Number of colonies (left histogram) and area (right) were analyzed using ImageJ and the mean data from three separate experiments is reported in the histograms (*$P < 0.05$).

killed at day 25, and lungs were analyzed for spontaneous lung metastasis formation. Tumors resulting from the Ctr and p140 cells were indistinguishable by volume and weight (Figures 2a and b). Also, imaging of the primary tumor with two-photon excitation did not reveal clear visible differences in tumor organization and structure of the collagen extracellular matrix (Supplementary Figure S1a). However, of particular interest, mice injected with p140Cap over-expressing cells show an 80% decrease in the number of lung metastases compared with control cells (Figures 2c and d). In addition, in the p140Cap group, the metastases were smaller in size compared with the control group when analyzed with confocal laser scanning microscopy (Supplementary Figure S1b). The reduction of lung metastases could be explained by either a defect in angiogenesis or a lower capacity of the p140Cap cells to be invasive. However, intravital imaging performed in the primary tumors indicated that the motility of p140Cap cells was reduced as we

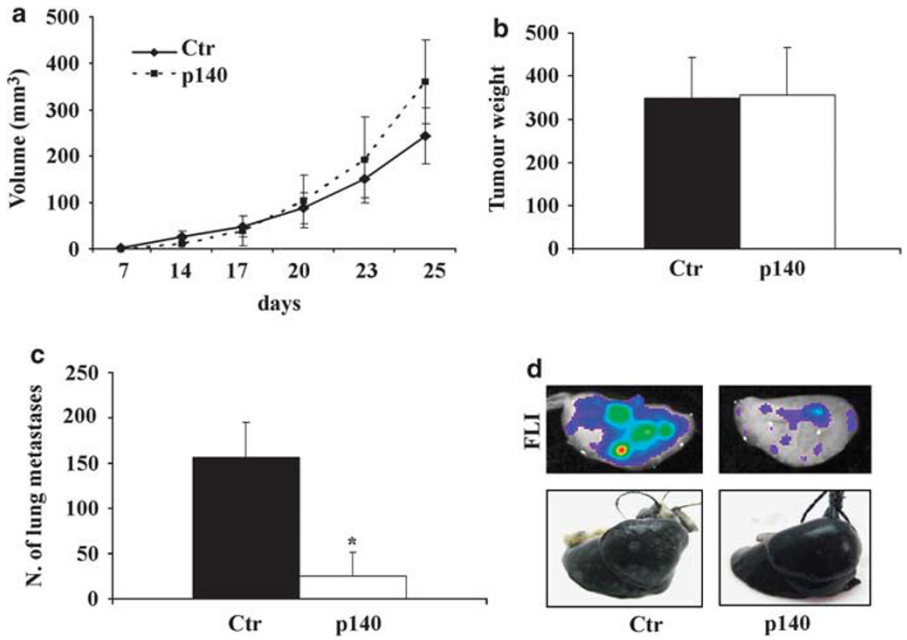

Figure 2 p140Cap over-expression inhibits spontaneous lung metastasis formation. (**a**) In all, 5×10^5 Ctr and p140 cells were injected subcutaneously in Rag2$^{-/-}$ $\gamma_c^{-/-}$ mice. Tumor volume was measured twice a week for 25 days and reported on the *y* axis ($n = 22$). (**b**) When the mice were killed, tumors were removed and weighed. The weight of tumors is reported on the *y* axis. (**c**) (Right panels) After killing the mice, lungs were colored with ink, metastasis were counted and the number of metastasis reported in the *y* axis of the histogram. Statistical significances were evaluated by Student's *t*-test: Ctr EGF vs p140 EGF (*$P < 0.05$). (**d**) (Upper panels) Two representative pictures of lung metastases visualized with the FLI (GFP detection) after spontaneous metastasis assay with the MTLn3-EGFR Ctr and p140 cells. (Lower panels) Two representative pictures of the lungs colored with ink are shown.

were unable to visualize cell movement in these tumors, whereas in the control group many migrating cells (Supplementary Figure S2a) and protrusion events (Supplementary Figure S2b) were observed in tumors. Thus ectopic expression of p140Cap expression does not affect MTLn3-EGFR tumor formation, but strongly inhibits the metastatic process of these highly invasive cells.

p140Cap inhibits directed cell migration

Motility is a fundamental property of cancer cells that escape from the primary tumor and invade into new tissues. To assess the role of p140Cap in cell motility, MTLn3-EGFR Ctr and p140Cap cells were serum starved for 4 h, stimulated with EGF and analyzed for random and directional cell migration at different times. The speed of cells in a random cell migration assay in response to EGF was similar between the two cell types (Figure 3a; Supplementary Figure S3). Yet, stable p140Cap knockdown in MTLn3-EGFR cells caused an increase in cell velocity under control conditions, indicative for a role of p140Cap in controlling random cell migration events (Supplementary Figure S4, panels c and d). EGF stimulation of these shp40Cap-MTLn3-EGFR cells was, however, not different from MTLn3-EGFR, suggesting that a maximal stimulation of cell migration is reached under these conditions. This enhanced migration of shp140Cap-MTLn3-EGFR cells was associated with a clear change in spindle-like elongated cell morphology in both culture conditions that upon EGF treatment, which is typical of motile tumor cells (Supplementary Figure S4, panels a, c and d). Similar findings were observed for transient small interfering RNA (siRNA)-based knockdown of p140Cap in MTLn3-EGFR cells (Supplementary Figure S5).

In the directional migration observed in wound-healing or transwell assays, p140 cells displayed a 50% reduction in the ability to migrate compared with control cells (Figures 3b and c), thus indicating that p140Cap affects directionality of cell migration, but not cell speed.

Cell migration requires dramatic changes in cell shape and in adhesion to the extracellular matrix, and both these processes must be spatiotemporally coordinated (Wolfenson *et al*., 2009; Gardel *et al*., 2010).To analyze focal adhesions and actin cytoskeleton, MTLn3-EGFR Ctr and p140Cap cells were plated on collagen, serum deprived for 4 h and stimulated with EGF for 5–15 min. As shown in Figure 4a, p140 cells show increased paxillin staining of focal adhesions compared with control cells. Remarkably, the difference in focal adhesions was highly evident in serum-starved cells (Figure 4a, right panels) and the quantification showed that both the area and the number of focal adhesions were increased both in basal conditions and following EGF stimulation (Figures 4c and d). To visualize the focal adhesions localized on the ventral cell surface, we used the evanescent field of total internal reflection fluorescence excitation. Total internal reflection fluorescence images revealed the presence of a higher number of punctuate focal adhesions in p140 cells, both unstimulated or stimulated with EGF, compared with

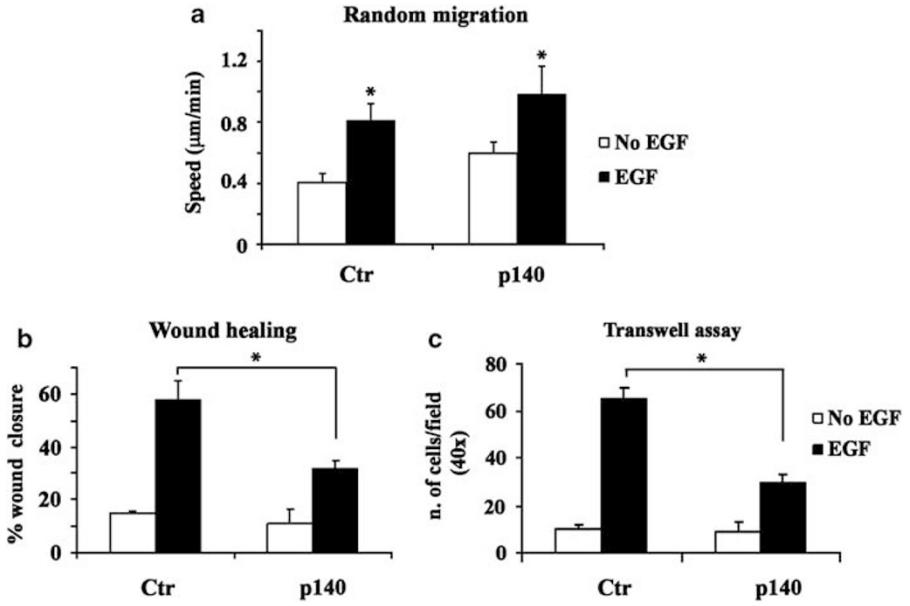

Figure 3 p140Cap over-expression affects cell migration. In all the experiments, the cells were serum deprived for 4 h and stimulated with 5 nM EGF. (**a**) Random migration of MTLn3-EGFR Ctr and p140 cells was visualized with both GFP fluorescence and DIC (differential interference contrast). Migration speed (μm/min) is indicated in the graph (*$P<0.05$). (**b**) Confluent monolayers were subjected to scratch wounding and incubated in serum-free media with or without EGF. After 10 h of migration, wound closure was photographed and quantified using ImageJ. Data were averaged from three independent experiments. Statistical significances were evaluated by Student's *t*-test: Ctr EGF vs p140 EGF (*$P<0.05$). (**c**) Ctr and p140 cells were tested for migration in a transwell assay. Cells were left to migrate for 5 h in the presence or the absence of EGF then fixed, stained and counted. Mean values were calculated on three independent experiments. Statistical significances were evaluated by Student's *t*-test: Ctr EGF vs p140 EGF (*$P<0.05$).

control cells (Figure 4b). Consistent with the increase in focal adhesion organization, phalloidin staining revealed that in p140 cells, actin was mainly organized in stress fibers (Figure 4a, right medium panels). Using total internal reflection fluorescence, p140 cells showed a marked amount of actin stress fibers on the ventral surface in both starved and EGF-stimulated cells (Figure 4b). To further support a role for p140Cap in regulating focal adhesion protein dynamics, decreased levels of tyrosine phosphorylation of Fak (Tyr925) and of paxillin (Tyr118) have been detected in p140Cap over-expressing MTLn3-EGFR cells compared with control cells (data not shown). Thus, p140Cap over-expression in MTLn3-EGFR cells imposes an increased organization of focal adhesions that correlates with enhanced formation of actin stress fibers.

p140Cap impairs cell invasion in 3D conditions

To assess the role of p140Cap in cell invasion, MTLn3-EGFR Ctr and p140Cap cells were analyzed using three different approaches. As a first step in the invasive process, metastatic tumor cells, cultured on a flat gelatin matrix substratum, extend specialized proteolytically active plasma membrane protrusions. These structures, termed invadopodia, are responsible for the focal degradation of the underlying substrate (Buccione *et al*., 2009). To evaluate the ability to degrade the substratum, cells were plated directly on crosslinked TRITC-labeled gelatin in the presence of a metalloprotease inhibitor (GM6001) and allowed to adhere and spread overnight. During this period no ECM degradation was observed. Upon GM6001 washout, cells resumed the ability to degrade the matrix. Areas of degradation underlying the cells were counted using a confocal microscope. As shown in Figure 5a, p140 cells gave rise to 50% less degradation compared with control cells.

In the second assay, cells were plated on classical matrigel-coated transwells and allowed to invade the 3D extracellular matrix for 48 h. As shown in Figure 5b, p140Cap over-expressing cells were dramatically impaired in their ability to invade compared with control cells. As a third alternative invasion assay, cells were grown as spheroids in 3D collagen matrix that were monitored within 3 days for cell crawl-out. While Ctr cells escaped from the central spheroids as single cells within 48 h, p140Cap over-expressing cells were significantly impaired in their ability to move out (Figure 5c). Consistently, shp140Cap-MTLn3-EGFR cells were more invasive than control MTLn3-EGFR cells in 3D collagen matrix spheroid invasion assays (Supplementary Figure S4, panel g). Taken together, these data indicate that p140Cap expression regulates the ability of MTLn3-EGFR cells to invade in a 3D matrix by controlling the initial degradation events at the cell–ECM interface and the subsequent invasive processes.

p140Cap regulates cell migration through cortactin interaction and phosphorylation

Our previous results have shown that increased expression of EGFR in the MTLn3 rat mammary tumor cell

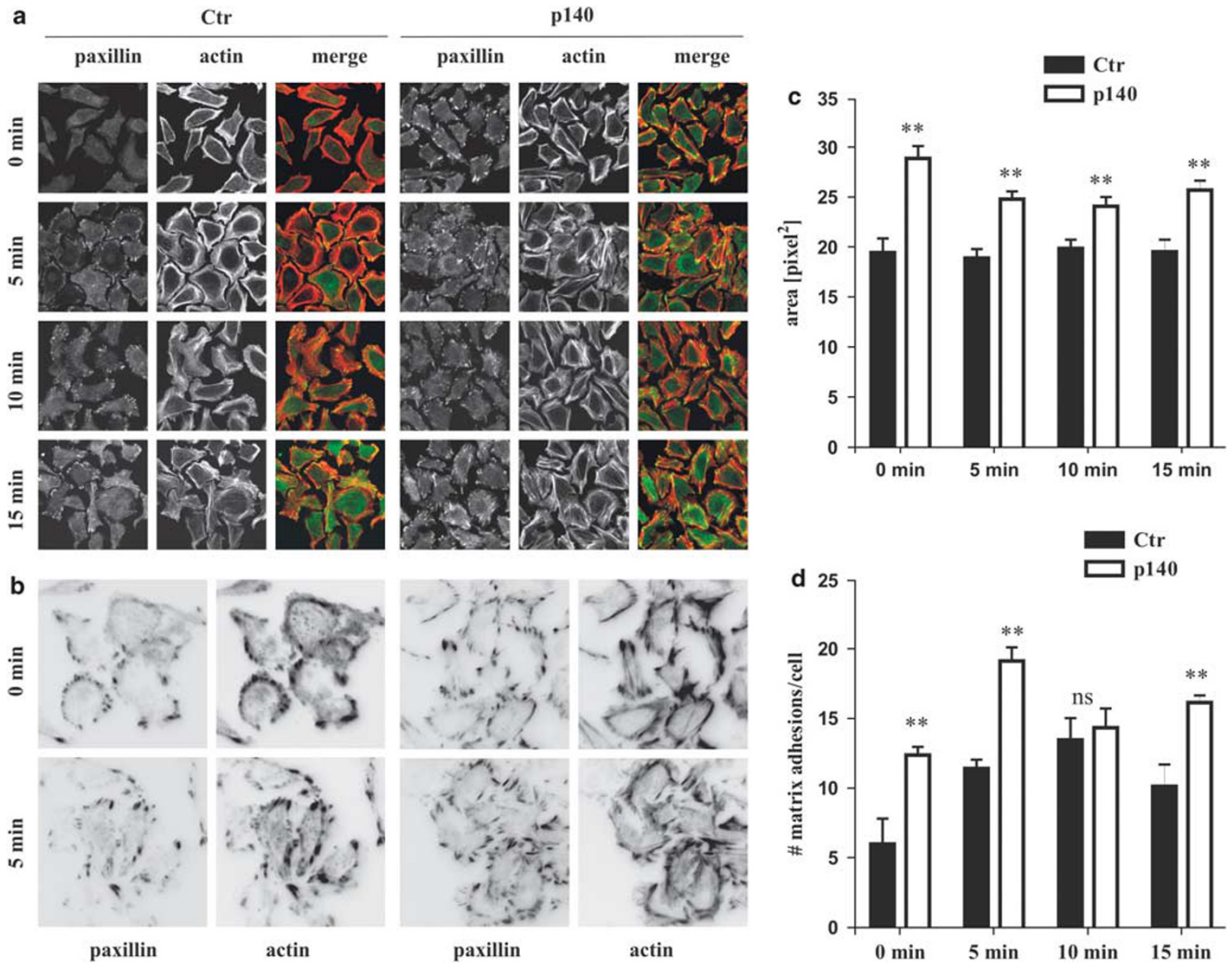

Figure 4 p140Cap regulates focal adhesions and actin organization and dynamics. (**a**) Ctr and p140 cells were fixed at different time points after exposure to EGF (10 nM) and stained for paxillin (green) and F-actin (red) and analyzed by confocal microscopy. (**b**) Ctr and p140 cells exposed or not to 10 nM EGF (5 min) were fixed and stained for paxillin and imaged by total internal reflection fluorescence microscopy. (**c**, **d**) Analysis of cell-matrix adhesions (average of matrix adhesion area and number of matrix adhesions per cell) in both cell types with Image J plugin (**$P<0.01$).

line is essential for efficient lung metastasis formation in the Rag mouse model, by strongly enhancing intravasation and lung metastasis (Le Devedec *et al*., 2009). Moreover, we have already demonstrated that p140Cap regulates c-Src and EGFR activation in MCF7 breast cancer cells (Di Stefano *et al*., 2007; Damiano *et al*., 2010). Therefore, to test whether p140Cap could affect EGFR signaling in this model, MTLn3-EGFR Ctr and p140Cap cells were stimulated with EGF, and EGFR activation was evaluated using a phosphospecific antibody that recognizes phopshorylated Tyr1173, one of the autophosphorylation sites of EGFR. p140 cells show a 60% decrease in EGFR Tyr1173 phosphorylation. Once activated, EGFR induces activation of downstream effectors, such as mitogenic-activated protein kinase Erk1/2 and c-Src. Indeed, in p140 over-expressing cells, Erk1/2 phosphorylation was decreased by 50% in response to EGF, and Src kinase activation was also completely impaired (Figure 6a). Therefore, in MTLn3-EGFR cells, p140Cap over-expression negatively regulates early activation of EGFR and downstream signaling.

A known Src substrate is cortactin, whose phosphorylation is necessary for regulating actin dynamics, cell motility and invasion (Head *et al*., 2003; Lua and Low, 2005). Moreover, cortactin has been shown to control the stages of invadopodia assembly and maturation (Ayala *et al*., 2008; Oser *et al*., 2009; Mader *et al*., 2011) and cortactin phosphorylation on tyrosines 421, 428 and 486 is a master switch during these processes. When MTLn3-EGFR Ctr and p140Cap cells were stimulated with EGF, cortactin was found to be phosphorylated on Tyr421 in response to EGF in Ctr cells. In p140 cells, however, cortactin phosphorylation was decreased by 50% (Figure 6a), indicating that p140Cap-dependent inhibition of Src kinase also leads to reduced cortactin tyrosine phosphorylation. Interestingly, p140Cap affects Src activation and cortactin phosphorylation in response to EGF also in highly metastatic mouse 4T1 and human 4175 (MDA-MB-231-Lung) breast tumor

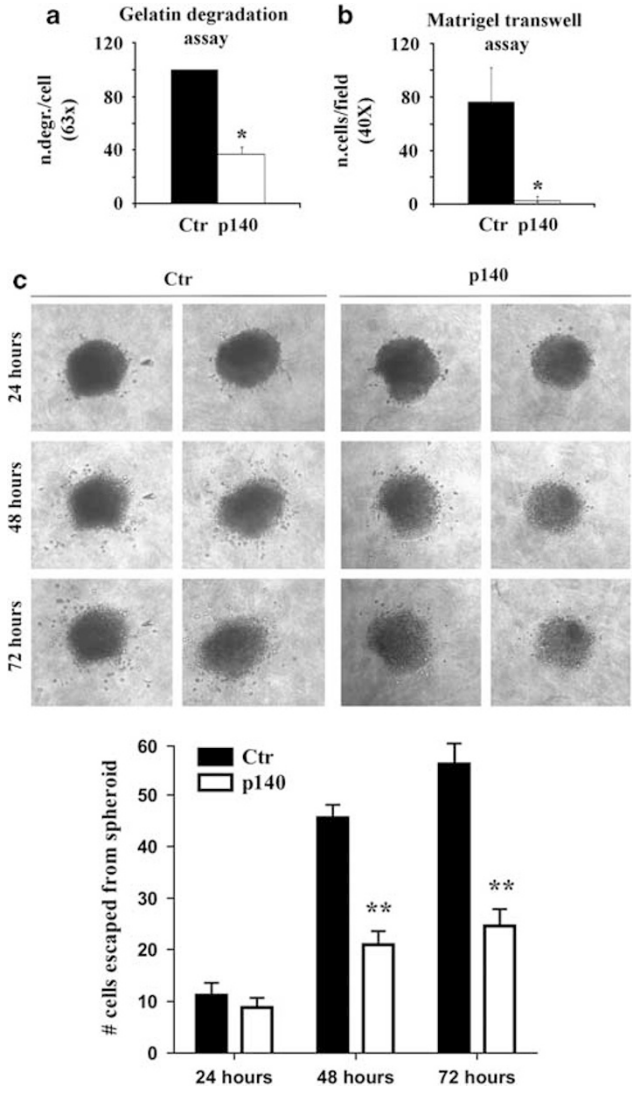

Figure 5 p140Cap impairs cell invasion in 3D conditions. (**a**) For gelatin degradation assays, Ctr and p140 cells were allowed to degrade TRITC gelatin for 30 min. Cells were then stained with Alexa633 phalloidin and photographed by confocal microscopy using a $\times 63$ magnification. Degradations were counted in 10 random fields/coverslips. The histograms represent the percentage of the number of degradation/cell. Data represent the mean of three independent experiments. Statistical significances were evaluated by Student's *t*-test ($^{*}P<0.05$). (**b**) Cells were plated on matrigel-coated transwells and allowed to invade for 48 h using complete medium. Cells on the lower part of transwells were fixed, stained and counted using a $\times 40$ magnification. In the histogram, the mean values of three independent experiments are shown. Statistical significances were evaluated by Student's *t*-test: Ctr EGF vs p140 EGF ($^{*}P<0.05$). (**c**) Tumor spheroids in collagen type I gels (eight per cell lines) were imaged every 24 h and pictured using a CCD camera and a $\times 10$ magnification. The number of cells outside the spheroid area was counted every day and plotted in a graph ($^{**}P<0.01$).

cells (Supplementary Figure S6). Moreover, p140Cap silencing also enhances Src activation and cortactin phosphorylation in response to EGF (Supplementary Figure S4, panel b).

To confirm that in MTLn3-EGFR cells, cortactin and its phosphorylation are crucial for the invasion process, cortactin was silenced, followed by re-expression of full-length (FL) cortactin and of the phosphomimetic cortactin mutant Y421D in which the primary SFK-phosphorylated residue 421 has been changed to aspartic acid. As shown in Supplementary Figure S6, siRNAs targeting endogenous cortactin induced an increase in F-actin stress fiber formation, which was associated with a reduced overall motility of cells (Supplementary Figure S7, panels a and c). While the subsequent expression of FL cortactin was unable to rescue this reduced cell motility, Y421D mutant cortactin recovered the ability of cells to properly migrate. This rescue of cell migration was related to the recovery of degradation of gelatin (Supplementary Figure S7, panels b, c and d).

Intriguingly, we have recently shown that p140Cap and cortactin immunoprecipitate in HeLa cells transfected with green fluorescent protein (GFP)-p140Cap cells (Jaworski *et al*., 2009). Therefore, p140Cap was immunoprecipitated from MTLn3-EGFR GFP Ctr cells, and p140Cap and cortactin were shown to co-immunoprecipitate (Figure 6b), indicating that they associate in the same complex in the invasive cells used in this study. Cortactin and p140Cap are both multi-domain proteins with many binding partners (Weaver, 2008; Cabodi *et al*., 2010). p140Cap has two proline-rich domains and cortactin has an SH3 domain that may be involved in this binding. To test this hypothesis, HEK293 cells were co-transfected with p140Cap and wild-type RFP-cortactin (Cort) or the DeltaSH3 mutant RFP-cortactin (Delta) and subjected to immunoprecipitation using anti-RFP monoclonal antibodies. As shown in Figure 6c, the p140Cap–cortactin interaction was dramatically impaired in the presence of the DeltaSH3 cortactin mutant, indicating that the SH3 cortactin domain is required for their association. Moreover, when HEK293 cells were transfected with a p140Cap-deleted mutant lacking amino acids 1000–1048, which include specifically the PPPPPRR sequence in the second proline-rich domain of p140Cap (DeltaPro) (Di Stefano *et al*., 2007), co-immunoprecipitation of p140Cap and endogenous cortactin was strongly impaired (Figure 6d). Moreover, expression of this mutant in the MTLn3-EGFR cells does not affect gelatin degradation ability compared with the FL p140Cap expression (Figure 6e). Overall, these data show that p140Cap interacts with cortactin through the second proline-rich domain and that p140Cap/cortactin binding correlates with the observed decrease in gelatin degradation.

To assess the involvement of cortactin in p140Cap-dependent inhibition of cell motility, a Y421D mutant was transfected in p140 cells. Transfected cells were subjected to both a transwell assay and a gelatin degradation assay. Expression of the Y421D mutant rescued transwell cell migration, recovering the ability of p140 cells to migrate to the same extent as control cells (Figure 6f). Moreover, the same mutant was also able to recover the ability of p140Cap cells to degrade the substratum (Figure 6g). Taken together, these data show that p140Cap-dependent inhibition of cortactin phos-

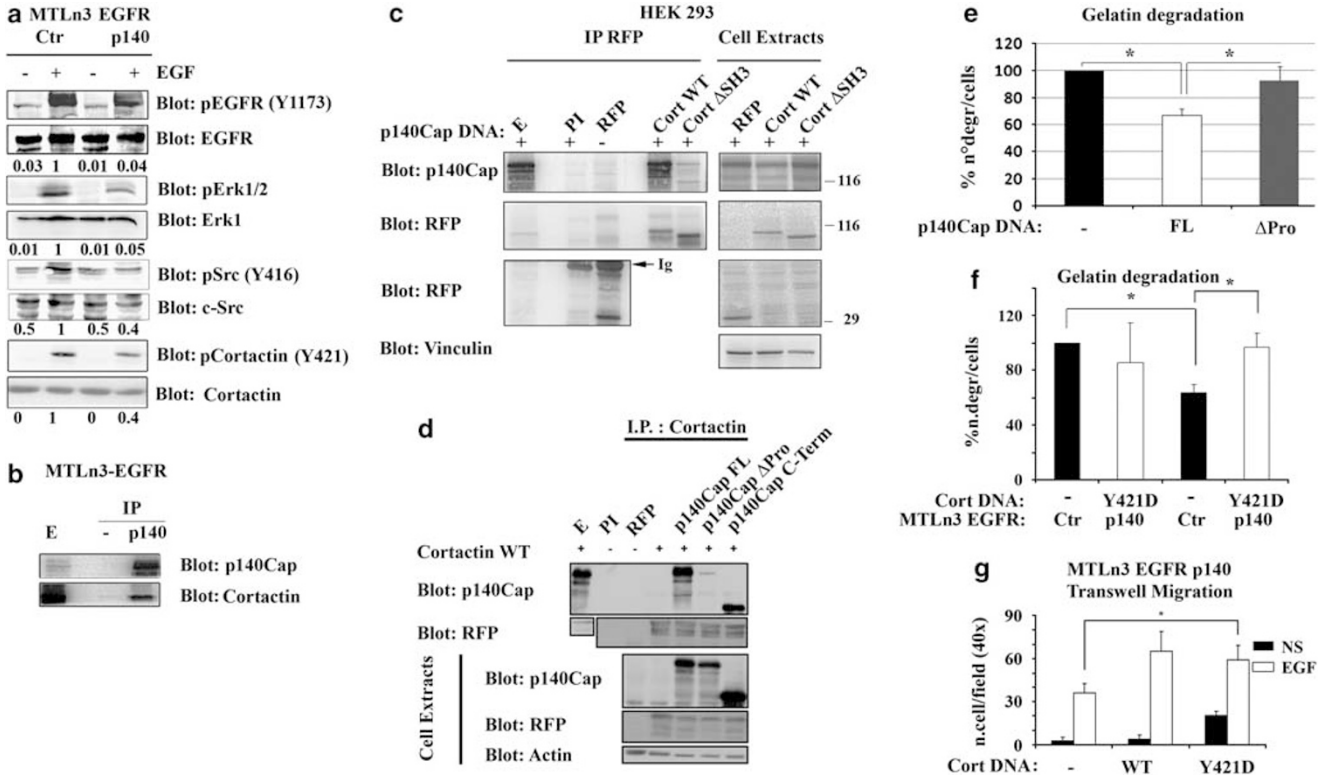

Figure 6 p140Cap regulates cell migration through cortactin interaction and phosphorylation. (**a**) Cells were serum starved and stimulated with EGF for 5 min. Cell extracts were analyzed on western blots using phospho-EGFR (Tyr1173), phospho-p42/44 Erk1/2, phospho-Src (Tyr416) and phospho-cortactin (Tyr421) antibodies. Membranes were then stripped and blotted with anti-EGFR, Erk1, Src and cortactin antibodies. Numbers below show the ratio between phospho and total proteins in arbitrary units. (**b**) MTLn3-EGFR Ctr cells were lysed and endogenous p140Cap was immunoprecipitated using a monoclonal purified antibody. Western blot was probed with anti-cortactin and p140Cap antibodies. (**c**) HEK293 cells were co-transfected with p140Cap and RFP-cortactin wild-type (Cort WT) or RFP-cortactin DeltaSH3 (ΔSH3). Cell extracts (right panels) were immunoprecipitated using mouse monoclonal preimmune (PI) and anti-RFP antibodies (left panels). Western blot was probed using anti-RFP, p140Cap and vinculin antibodies. Arrow indicates immunoglobulins. Molecular weights are shown on the right. (**d**) HEK293 cells were co-transfected with p140Cap FL, DeltaPro (ΔPro), C-terminal (C-Term) mutants and RFP-cortactin WT. Cell extracts (lower panels) were immunoprecipitated using mouse monoclonal PI and anti-cortactin antibodies (upper panels). Western blot was probed using p140Cap, RFP and actin antibodies. (**e**) MTLn3-GFP-EGFR Ctr were transfected with control vector (−), Myc p140Cap FL and Myc p140Cap DeltaPro mutant. After 48 h, cells were fixed and stained using Alexa633 phalloidin, anti-Myc antibodies followed by secondary Alexa405 antibodies and photographed by confocal microscopy using a ×63 magnification. Degradations were counted in 10 random fields/coverslips. The histograms represent the percentage of the number of degradation/cell. Data represent the mean of two independent experiments. Statistical significances were evaluated by Student's *t*-test (*$P<0.05$). (**f**) MTLn3-GFP-EGFR Ctr and p140 were transfected with a control vector (−) and a mutant FLAG-cortactin (Y421D). After 48 h, cells were analyzed for their ability to degrade TRITC gelatin for 30 min as shown in (**e**), using Alexa633 phalloidin, anti-FLAG antibodies followed by secondary Alexa405 antibodies. Statistical significances were evaluated by Student's *t*-test (*$P<0.05$). (**g**) MTLn3-GFP-EGFR p140 were transfected with a control vector (−), a wild-type cortactin (Cort WT) and a mutant cortactin (Y421D). After 48 h, the cells were serum deprived for 4 h, stimulated with 5 nM EGF for 5 h. Cells on the lower part of transwells were fixed, stained and counted using a ×40 magnification. In the histogram, mean values of three independent experiments are shown. Statistical significances were evaluated by Student's *t*-test: Ctr EGF vs p140 EGF (*$P<0.05$).

phorylation is a primary mechanism to interfere with invasive properties of highly metastatic cells.

Discussion

Our findings identify the adaptor protein p140Cap as a critical regulator of *in vitro* cell motility and invasion and *in vivo* metastasis formation of highly metastatic MTLn3-EGFR breast cancer cells. We also demonstrate that in these cells p140Cap, without affecting primary tumor growth, impairs the early steps of motility and invasion, by affecting cortactin tyrosine phosphorylation, thus resulting in the inhibition of cortactin-dependent invasive properties.

The formation of lung metastasis is a multi-step process, dependent on cancer cell autonomous biological programs that define migration, intravasation survival and extravasation of tumor cells (Condeelis and Segall, 2003). The data presented here show that increasing p140Cap expression in the highly aggressive MTLn3-EGFR cells results in an 80% decrease in *in vivo* lung metastasis formation, providing the first evidence that p140Cap acts as an effective negative regulator of the metastatic process. Consistently, p140Cap over-expressing MTLn3-EGFR cells show also reduced anchorage-independent cell growth, which is an *in vitro* characteristic that predicts the *in vivo* metastatic potential of many tumor cells (Mori *et al*., 2009).

To become metastatic, cancer cells have to detach from primary tumors, re-organize the actin cytoskeleton, migrate toward a stimulus and subsequently invade (Sahai, 2007). Interestingly, performing two-photon intravital imaging on both control and p140Cap-over-expressing tumors, we were able to easily detect and track a highly motile cell population only present in control tumors, and not in the p140Cap over-expressing tumors, which show poor motility. Moreover, p140Cap over-expressing cells were also more refractory than control cells to move out from cell spheroids embedded into a 3D collagen matrix. Consistently, p140Cap silenced MTLn3-EGFR cells were more invasive compared than MTLn3-EGFR control cells. Overall, these findings indicate that p140Cap is an important regulator of the early steps in metastasis onset, affecting the initial process of cell escape from the tumor.

Furthermore, detailed *in vitro* analysis of cell migratory and invasive abilities showed that p140Cap over-expressing cells have an impaired capacity to migrate in response to EGF. Interestingly, p140Cap cells only show a defect in directional cell migration, but not in random cell migration. A prerequisite for cell migration is the tight assembly and disassembly cycle of focal adhesions, mechanosensory signaling structures that are observed at the basal surface of the cells in 2D cultures (Webb *et al*., 2005; Gardel *et al*., 2010). Remarkably, p140Cap over-expressing cells display an increased number and area of focal adhesions, which correlate with the presence of actin stress fibers. While in MTLn3-EGFR untreated control cells, focal adhesions are poorly visible and organize only under the EGF stimulus, in p140Cap over-expressing cells, both focal adhesions and stress fibers are already present in unstimulated cells and treatment with EGF does not change their number or morphology. Upon knockdown of p140Cap in MTLn3-EGFR cells, the cells have a more elongated motile phenotype compared with control cells. Thus, high levels of p140Cap induce a re-organization of focal adhesions and actin stress fibers consistent with a less dynamic turnover of adhesive structures (Vicente-Manzanares *et al*., 2005; Webb *et al*., 2005). Consistent with a role for p140Cap in regulating focal adhesion protein dynamics, MTLn3-EGFR cells over-expressing p140Cap show decreased levels of tyrosine phosphorylation of FAK and paxillin (data not shown). Moreover, we have previously shown that p140Cap also regulates p130Cas phosphorylation and Rac activation in MCF7 cells (Di Stefano *et al*., 2007), indicating that p140Cap may be a crucial regulator of directional cell migration, by specifically controlling the extent of phosphorylation and activation of focal adhesion components relevant for focal adhesion assembly (Ilic *et al*., 1995; Lim *et al*., 2008).

In vitro and *in vivo* studies have shown that in MTLn3-EGFR cells, EGFR expression enhances invasiveness without affecting tumor growth (Xue *et al*., 2006), indicating that the contribution of the EGFR to metastasis is independent of its effect on proliferation. Overall, our data demonstrate that p140Cap does not impair primary tumor growth, but profoundly disrupts migratory and invasive properties of MTLn3-EGFR cells, by antagonizing early EGFR signaling in these cells. Indeed, the high levels of p140Cap in MTLn3-EGFR cells result in impaired activation of EGFR, Erk1/2 mitogenic-activated protein kinase and Src kinase in response to EGF. Similar results were also obtained in the highly metastatic mouse 4T1 cells. In our previous work we have already demonstrated that p140Cap interferes with integrin/EGF-induced Src kinase activity, also in cells that express endogenous levels of EGFR such as the luminal A MCF7 breast cancer and the HT29 colon cancer cells (Di Stefano *et al*., 2007; Damiano *et al*., 2010). Therefore, p140Cap behaves as a general regulator of EGF signaling, mostly affecting the Src kinase pathway.

Cortactin is a major substrate of Src kinase and localizes to cortical actin structures, where it regulates early cell migration and invasion by controlling actin assembly (Wu *et al*., 1991; Wu and Parsons, 1993; Weed *et al*., 2000), and its phosphorylation by Src is correlated with enhanced metastasis in breast cancer cells (Li *et al*., 2001). Cortactin tyrosine phosphorylation has been shown to regulate MTLn3 cells invadopodia assembly and maturation (Oser *et al*., 2009). Our results show that in p140Cap over-expressing cells, cortactin phosphorylation on Tyr421 in response to EGF is decreased. Interestingly, p140Cap affects Src activation and cortactin phosphorylation in response to EGF also in highly metastatic mouse 4T1 and human 4175 (MDA-MB-231-Lung) breast tumor cells (Supplementary Figure S6). Indeed, the expression of the phosphomimetic cortactin mutant Y421D is sufficient to completely rescue the defects in migration and invasion of MTLn3-EGFR p140Cap over-expressing cells. Moreover, confirming our previous data (Jaworski *et al*., 2009), p140Cap is a partner of cortactin in MTLn3-EGFR cells. Their association requires the second proline-rich domain of p140Cap and the cortactin SH3 domain, suggesting a direct interaction between the two proteins. The p140Cap mutant that does not associate cortactin is no more able to impair gelatine degradation as p140Cap does, suggesting that p140Cap binding to cortactin leads to decreased invasion. Overall, our results provide evidence that p140Cap regulates the extent of migration and invasion by affecting the EGFR/Src/cortactin pathway.

In conclusion, these findings identify p140Cap as a new regulator of the early steps of metastasis onset. Indeed, p140Cap associates with cortactin and regulates EGF-dependent cortactin phosphorylation, likely via inhibition of early EGF-dependent EGFR and Src signaling. These events result in defective directional cell motility, and 3D invasion leading to suppression of migratory and invasive properties of highly aggressive breast cancer cells.

Materials and methods

Reagents and antibodies
See Supplementary Information.

Cell lines and constructs
MTLn3-EGFR, GFP-positive cells, previously described (Xue *et al*., 2006), were maintained in α-minimal essential medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum and penicillin–streptavidin. MTLn3-EGFR Ctr and p140 cells were obtained infecting MTLn3-EGFR cells with empty pBABE and p140Cap-pBABE (Damiano *et al*., 2010) retroviruses and selecting using 1 μg/ml puromycin. Myc p140Cap FL and Myc p140Cap DeltaPro have been previously described (Di Stefano *et al*., 2007). Myc p140Cap C-Term mutant has been originated by digesting the FL with Nhe-Kpn and cloning the fragment in pcDNA3 Myc. RFP-cortactin wild-type, RFP-cortactin 3YF, RFP-cortactin DeltaSH3, cortactin-flag wild-type and cortactin-flag Y421D constructs were previously described (Ayala *et al*., 2008). They were transfected in MTLn3-EGFR cells using Lipofectamine LTX (Invitrogen, Paisley, UK) according to manufacturer's instructions. HEK293 cells were maintained in Dulbecco's Modified Eagle Medium 10% serum and penicillin/streptavidin and transfected using the calcium–phosphate method.

pGIPZ-p140Cap lentiviral shRNAmir were from Open Biosystem (Huntsville, AL, USA); p140Cap and cortactin siRNAs were Smart Pools from ThermoFisher Dharmacon (Waltham, MA, USA). siRNA were transfected with the Dharmafect reagent (ThermoFisher Dharmacon).

Proliferation and soft agar assays
See Supplementary Information.

Migration assays
For random migration assays, cells were cultured in 24 glass-bottom plates overnight, starved for 4 h followed by visualization with high-throughput microscopy for several hours on a Nikon TE2000-E microscope (Nikon, Tokyo, Japan) equipped with perfect focus system in a humid climate of 37°C and 5% CO_2. Subsequently, cells were treated with EGF and time lapses were recorded using both GFP fluorescence and differential interference contrast of the cells. Movies were captured 6 min per frame with ×20 objective. Cell speed was obtained by tracking the cells and calculating the distance between two sequential frames using an in-house developed Image J plugin (Yan K *et al*., unpublished data).

For wound-healing assays, cells were cultured until reaching confluence. Cells were serum deprived for 4 h and wounded using p200 pipette tip. Cell migration was followed for 10 h after EGF treatment by time lapse. Images were further quantitatively analyzed by using ImageJ software (NIH, Bethesda, MD, USA).

For the transwell assay, the lower side of transwells was coated with 10 μg/ml collagen. A total of 5×10^4 starved cells were seeded on the upper side of the filters (8 μm diameter, Corning, New York, NY, USA) and let to migrate to the bottom wells filled with 0.1% bovine serum albumin RPMI, and 5 nM EGF. Cells migrating to the lower side were fixed and stained with Diff-Quick kit. In all, 10 field/transwell were counted on the microscope using ×40 magnification.

Focal adhesion detection and analysis
To determine cell-matrix adhesion size, we used images of MTLn3 cells stained for paxillin at different time points following EGF stimulation. Images were segmented based on paxillin staining, and the area of all individual cell-matrix adhesions was determined using ImageJ software. The average cell-matrix adhesion size of up to 30 individual cells/treatment conditions is shown.

Gelatin degradation assay
Gelatin degradation assays were performed on fluorophore-conjugated gelatin prepared with Rhodamine B isothiocyanate (Sigma, St Louis, MO, USA) and porcine gelatin (Sigma) according to Ayala *et al*. (2008). Gelatin-coated coverslips were prepared and assays were carried out as described previously (Bowden *et al*., 2001; Baldassarre *et al*., 2006). Briefly, thin layers of Rhodamine B-conjugated gelatin were placed on coverslips, crosslinked with 0.5% glutaraldehyde for 15 min at 0°C, washed three times with PBS and incubated for 3 min at room temperature with 5 mg/ml $NaBH_4$. Finally, after a wash and 10 min incubation in 70% ethanol, coverslips were washed with complete medium for 1 h at 37°C before cell plating. Cells were plated directly on gelatin in the presence of 5 μM GM6001 (Millipore, Temecula, CA, USA). GM6001 was washed out to allow degradation for 30 min. Cells were then fixed in 4% paraformaldehyde and analyzed with appropriate antibodies. Experiments were observed using a Zeiss confocal microscope using ×63 magnification. Degradations were counted in 10 random fields/coverslips.

Invasion assays
For invasion assays, 5×10^4 cells were seeded on the upper side of transwells coated with matrigel and allowed to invade for 48 h using complete medium. Cells on the lower part of the filters were fixed and stained with Diff-Quick kit. In all, 10 field/transwell were counted using a microscope with a ×40 magnification. For the 3D invasion assay, collagen gels were prepared from type I collagen (Invitrogen) according to the manufacturer's procedure. Cells were placed in collagen, allowed to form spheroids, and images were taken every 24 h with a Nikon TE2000 microscope using ×10 magnification.

In vivo *tumor and metastasis formation*
For MTLn3-EGFR cells 6-week-old $Rag2^{-/-}\gamma_c^{-/-}$ mice were obtained from in-house breeding. Sterilized food and water were provided *ad libitum*. To measure spontaneous metastasis, MTLn3 tumor cells were grown to 70–85% confluence and 5×10^5 MTLn3 cells were injected into the right thoracic mammary fat pads of mice, in a volume of 100 μl of PBS without Ca^{2+} or Mg^{2+} using a 25-gauge needle. Tumor growth rate was monitored weekly. Horizontal (h) and vertical (v) diameters were determined, and tumor volume (V) was calculated ($V = 4/3\pi(1/2[\sqrt{(h \times v)}]3)$). After 3 or 4 weeks, animals were killed and lungs were excised. The right lung was used to count the tumor burden. For a rough estimation, right lungs were imaged with the FLI (fluorescent imaging) unit IVIS. For detailed quantification, the flat side of the right lung was analyzed with an immunofluorescence microscope. With a ×10 objective lens, we screened the flat surface of the lobe and counted the number of GFP-positive metastases. Subsequently, the right lung was fixed in 4% paraformaldehyde. The left lung was injected with ink solution, de-stained in water and fixed in Fekete's (4.3% (v/v) acetic acid, 0.35% (v/) formaldehyde in 70% ethanol).

Statistical analysis
Results are representative of at least three independent experiments and are expressed as mean values ± s.e.m. (standard error of the mean). Statistical analysis of the data was performed using a Student's *t*-test.

Conflict of interest

The authors declare no conflict of interest.

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