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Original Article

Identification of two regions in the p140Cap adaptor protein that retain the ability to suppress tumor cell properties

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Abstract: p140Cap is an adaptor protein that negatively controls tumor cell properties, by inhibiting *in vivo* tumor growth and metastasis formation. Our previous data demonstrated that p140Cap interferes with tumor growth and impairs invasive properties of cancer cells inactivating signaling pathways, such as the tyrosine kinase Src or E-cadherin/EGFR cross-talk. In breast cancer p140Cap expression inversely correlates with tumor malignancy. p140Cap is composed of several conserved domains that mediate association with specific partners. Here we focus our attention on two domains of p140Cap, the TER (Tyrosine Enriched Region) which includes several tyrosine residues, and the CT (Carboxy Terminal) which contains a proline rich sequence, involved in binding to SH2 and SH3 domains, respectively. By generating stable cell lines expressing these two proteins, we demonstrate that both TER and CT domains maintain the ability to associate the C-terminal Src kinase (Csk) and Src, to inhibit Src activation and Focal adhesion kinase (Fak) phosphorylation, and to impair *in vitro* and *in vivo* tumor cell features. In particular expression of TER and CT proteins in cancer cells inhibits *in vitro* and *in vivo* growth and directional migration at a similar extent of the full length p140Cap protein. Moreover, by selective point mutations and deletion we show that the ability of the modules to act as negative regulators of cell migration and proliferation mainly resides on the two tyrosines (Y) inserted in the EPLYA and EGLYA sequences in the TER module and in the second proline-rich stretch contained in the CT protein. Gene signature of cells expressing p140Cap, TER or CT lead to the identification of a common pattern of 105 down-regulated and 128 up-regulated genes, suggesting that the three proteins can act through shared pathways. Overall, this work highlights that the TER and CT regions of p140Cap can efficiently suppress tumor cell properties, opening the perspective that short, defined p140Cap regions can have therapeutic effects.

Keywords: p140Cap, breast cancer, lung cancer, colon cancer, cell signaling, Csk, Src

Introduction

The p140Cap adaptor protein has been recently involved in tumor progression, as a negative regulator of integrin and growth factor-dependent carcinoma cell properties (for a review [1]). Screening of mammary breast cancers by IHC revealed that p140Cap expression in human breast tumours inversely correlates with tumour malignancy. Indeed p140Cap is expressed in 41% of tumor specimens characterized by G1/G2 index, as well in 41% of node negative patients. The percentage of p140Cap positive samples drops to 5% in G3 tumors and to 10% in node positive tumors [2], thus suggesting that p140Cap may contribute at least to slow tumor progression.

The p140Cap modular structure contains a Tyrosine Enriched Region (TER) which includes several tyrosine residues, a coil-coiled domain, two regions rich in charged amino acids, and a Carboxy Terminal which contains a proline rich sequence (CT) [3, 4]. p140Cap was originally identified to bind through coil-coiled interactions to the synaptic membrane protein SNAP-

25 [3], and, through the proline-rich region in the CT region, to Src kinase [5], Vinexin [6], and Cortactin [7, 8]. The CT domain of p140Cap also associates to EB3, a member of the microtubule plus-end tracking protein EB family [7]. Moreover, p140Cap directly binds Csk [5], through tyrosine residues inserted in the TER region, namely the sequences EPLYA and EGLYA, that associate to the Csk SH2 domain [9]. In the presence of p140Cap, Csk is more active and phosphorylates the inhibitory tyrosine on the C-terminal domain of Src allowing the closure of Src in an inactive conformation [5]. Overall p140Cap behaves as a molecular hub that may negatively control signaling, like the Src kinase activation, through direct association with specific partners.

Since it is increasingly interesting understanding how a cancer cell can be controlled from networks originating from modular domains present in "hub" proteins [10], we further dissected the functional role of specific regions of p140Cap, focusing on the TER and the CT regions. We show here that the TER (Tyrosine Enriched Region) and the CT regions retain the ability to associate Csk and Src and mimic full length p140Cap properties in down-regulating Src kinase activity and the tumourigenic features of different cancer cell lines. By mutating EGLYA and EPLYA tyrosine residues in the TER domains, and deleting the proline rich region in CT we highlighted the contribution of these residues to the inhibition of tumourigenic properties of breast cancer cells. Microarray analysis shows that p140Cap, TER and CT expressing cells share a great number of genes down- and up-regulated, implying that these three proteins can use common pathways for affecting tumor features.

Material and methods

Cell lines, reagents and antibodies

Human breast (MDA-MB-231) and colon (HT-29) carcinoma cell lines were obtained from ATTC and cultured in DMEM with 10% Fetal Calf Serum (FCS) and Penicillin/Streptomycin as previously described [2, 5, 8]. Antibodies to the activated Src (pSrc Y416), tyrosine phosphorylated Fak (pFak Y925) and vimentin were purchased from Cell Signaling (Beverly MA); antibodies to Src, Vinculin and Csk were from Santa Cruz Biotechnology (SantaCruz, CA). p140Cap monoclonal and polyclonal antibodies, GFP and Fak monoclonal polyclonal antibodies were produced in our laboratory as previously described [4]. Glutathione-Sepharose, Protein A-Sepharose, Protein G-Sepharose, Horse radish peroxidase, nitrocellulose and films were from GE Healthcare Bio-Science AB. Tissue culture media, serum and antibiotics, LTX lipofectamine, and neomycine (G418) were from Invitrogen. Matrigel was from Becton Dickinson.

Preparation of cDNA constructs and generation of stable populations of expressing cells

The CT construct was generated by digesting the full length p140Cap cDNA with NheI/XbaI restriction enzymes and by cloning the fragment in pEGFP N1. The TER construct was prepared by digesting the full length p140Cap cDNA with PstI enzyme and by cloning in pEGFP N1.

The mutants construct PG-TER encoding for the double point mutation of TER tyrosines 264 and 396 into phenylalanine (Y/F) was obtained by digesting with Pst1 the PG-FL p140Cap construct already described in [9]. The DeltaPro-CT was prepared by digesting with NheI and XbaI the DeltaPro-FLp140Cap already described in [5].

The different constructs were stably transfected in carcinoma cell line using Lipofectamine LTX, according to manufacturer's instruction (Invitrogen). For selection 800 microgram/ml of G418 (Gibco) were used and GFP-positive cells were sorted using fluorescence activated cell sorting machine.

Protein expression analysis

To analyse protein expression, cells were detergent extracted with lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) containing protease and phosphatase inhibitors (1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 100 mM NaF, and 200 mM NaVO4). The supernatants were collected and assayed for protein concentration using the Bio-Rad protein assay method (Bio-Rad). Fifty micrograms cell extracts were mixed with Laemmli's sample buffer, and boiled for 10 min. After 6% SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose (Amersham, Arlington

Heights, IL) and check for the protein expression using anti GFP monoclonal antibody.

For immunoprecipitation, Dynabeads (Invitrogen) were cross linked with the specific antibody, following manufacture protocol. Cell extracts (2 mg) were incubated with the Dynabeads-conjugated antibodies for two hours at 4°C. The immunoprecipitates were washed three times with 1 ml of lysis buffer, resolved on SDS-PAGE, transferred onto nitrocellulose and reacted with specific antibodies. As input, fifty micrograms of cell extracts were analysed by SDS-PAGE and Western Blotting.

For serum stimulation, confluent cells were starved overnight and stimulated with 20% FCS for 30 min.

Directional cell migration assay

For directional cell migration assays, Transwell chambers were coated with 10 microgram/ml fibronectin. A total of 5 x 10^4 cells were seeded on the upper side of the filters and left to migrate for 2 hr in presence or absence of 20% FCS. Cells migrating to the lower side were fixed and stained with Diff-Quick kit. The mean number of migrated cells was counted in 10 random fields.

Cell proliferation and soft agar assays

A total number of 1 x 10^4 stably transfected cells were seeded on 12 wells tissue culture dishes in the presence of culture medium. Every 2 days, cells were detached and manually counted in Burker chambers on triplicate wells. For the clonogenic assay, 5 x 10^5 cells were seeded in 0.35% agar on the top of a base layer containing 0.7% agar into six-well plates. After 3 weeks, colonies were counted in four random fields under a phase-contrast microscope at 20X magnification. All of the experiments were performed in triplicate.

In vivo tumor growth

Five week old female SCID mice were purchased from Charles River Laboratories (Calco, Italy) and treated in accordance with the European Community guidelines. 10 x 10^7 MDA-MB-231 or 1 x 10^6 HT-29 cells were mixed with 150 microliters DMEM and 150 microliters Matrigel and then injected subcutaneously into the left and right inguinal region of female SCID mice. The size of the tumors was evaluated twice weekly using calipers. In all the *in vivo* tumor growth experiment, the investigators were blind to the cell type the animal received. All the in vivo experiments were repeated three times. Tumor volumes were analyzed with the Anova test.

Gene expression analysis

Total RNA was isolated from cells using Absolutely RNA mRNA kit (Agilent Technologies, Palo Alto, CA). mRNA was amplified and labeled by Amino Allyl MessageAmp II aRNA Kit (Ambion, Austin, TX) using NHS ester Cy3 dye (Amersham Biosciences, Arlington Heights, IL). Total RNA quality and labeling was checked by means of RNA 6000 Nanochip assays and run on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA, amplified and labeled mRNA concentrations were calculated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Equal amounts (0.2 microgram) of labeled specimens were fragmented and hybridized to Human Whole Genome Oligo Microarrays 8x60K (Agilent Technologies). Each step was performed using the In Situ Hybridization Kit-Plus (Agilent Technologies) and following the 60-mer oligo microarray processing protocol. Slides were then washed with the SSPE wash procedure and scanned using an Agilent C dual-laser microarray scanner.

Images were analyzed using Feature Extraction software v10.5 (Agilent Technologies) and raw data processed within R statistical environment, using the limma library. The raw intensity values were background corrected with method "normexp", as provided by the function backgroundCorrect of the limma package. In order to have similar intensity distributions across all arrays, log2 transformed intensity values were then subjected to between array normalization with "quantile" method. For each condition, replicates were combined and the empirical Bayes method was applied to retrieve a list o modulated probes in treated vs control samples, using 0.01 as cut-off for the Benjamini-Hochberg adjusted *p*-value and 1 for absolute log fold change. Venn diagrams to depict the commonly regulated genes by the three constructs (FL, CT and TER) were created with the

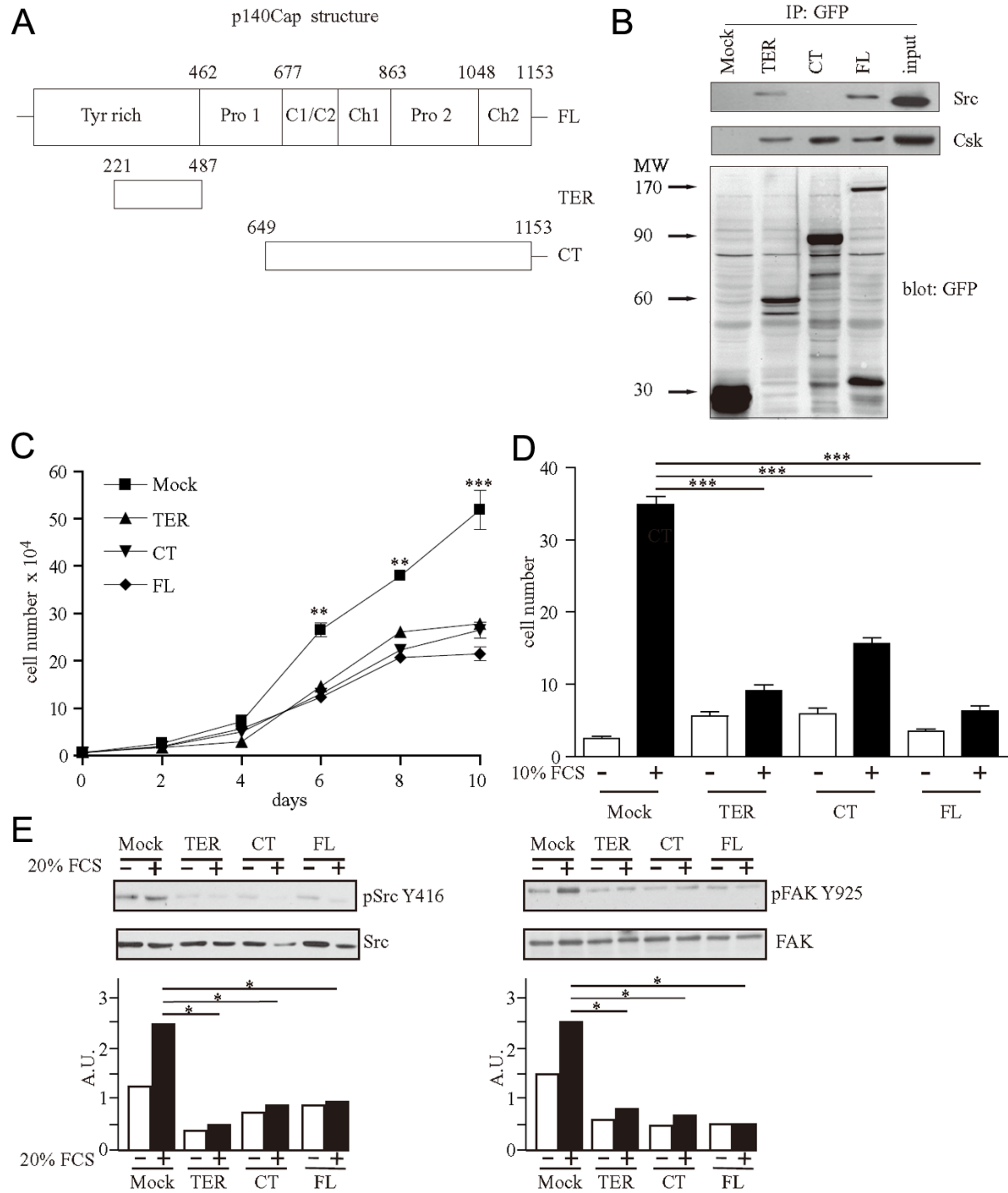

Figure 1. Expression of TER and CT modules affects MDA-MB-231 *in vitro* cell proliferation, migration, and Src and Fak phosphorylation. A. Schematic representation of p140Cap FL, NT, TER and CT protein structure. The position of relevant domains is indicated: Tyr = Tyrosine, Pro = Proline, C1/C2 coiled/coil, Ch = charged amino acids. The numbers indicate the amino acid position. B. Upper panels. To evaluate the association with Csk and Src, four mg extracts of MDA-MB-231 cells stably expressing Mock, NT, CT and FL p140Cap, all tagged with EGFP, were immunoprecipitated with antibodies to GFP. ¾ of the material was run on 8% SD-PAGE and western blotted with antibodies to Csk and Src. Input: fifty micrograms of FL p140Cap cell extract. Lower panel. For quantification of GFP-tagged fusion proteins expression, the remaining immunoprecipitate was run on 6% SDS-PAGE and blotted with antibodies to GFP. C. For assessing cell proliferation, 1 x 10^4 cells were plated in 12 wells dishes in 10% FCS DMEM culture medium. Each second day cells three wells were detached and counted manually. The mean number of cells is represented by *y*-axis. The results are representative of three independent experiment. The data were evaluated with two-way ANOVA followed by Bonferroni multiple comparison post hoc tests (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

D. For assessing cell migration, cells were starved overnight, detached and plated (5 x 10^4) on fibronectin-precoated Transwells. Cells were allowed to migrate with or without 20% FCS for 2 hours. Cells migrating to the lower side were fixed and stained with Diff-Quick kit. The mean number of migrated cells was counted in 10 random fields. Mean values were calculated on five independent experiment. The data were evaluated with one-way ANOVA followed by Bonferroni multiple comparison post hoc tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). E. Cells were starved over night and treated with 20% FCS for 30 min. Cell extracts were run on 8% SDS-PAGE and western blotted with antibodies to the activated Src (pSrc Y416), Src, tyrosine phosphorylated Fak (pFak Y925) and Fak. The histograms show the ratio between phosphorylated and total amount of protein in arbitrary units. (*$P < 0.05$, Student's *t*-test; ** $P < 0.01$).

help of the on-line tool Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Gene Ontology enrichment analysis

GO analysis was done either separately or together for up and down-regulated genes. In this case, the filter on log fold change was not applied.

Each list of modulated probes was loaded on DAVID tool for functional annotation analysis (http://david.abcc.ncifcrf.gov/summary.jsp), using the Agilent Whole Genome Oligo Microarray 8x60K as background. The Gene Ontology Biological Processes level 5 (BP5) overrepresented in all the three conditions (FL, TER and CT) were identified and the union of the genes modulated in at least one condition and involved in each overrepresented BP5 was used to create expression matrices containing the log fold change of the expression of each gene in FL, TER and CT compared to MOCK. The matrices of the most interesting BP5 were selected and loaded in Tigr MultiExperiment Viewer (TmeV version 4.6.1, http://www.tm4.org/mev/) to generate heatmaps by means of hierarchical clustering.

Results and discussion

The p140Cap TER and CT regions are sufficient to down regulate in vitro tumor cell features.

MDA-MB-231 cells are known as an aggressive model of basal-like breast cancer [11], and do not express endogenous p140Cap, thus representing an ideal model to evaluate whether exogenous expression of p140Cap or of its domains can suppress their intrinsic tumourigenic properties. We have already shown that expression of p140Cap in these cells suppress *in vivo* tumor growth [5]. In order to assess the ability of specific domains of p140Cap to negatively control tumor features, we stably expressed in MDA-MB-231 breast cancer cells constructs encoding for the tyrosine enriched region (TER) (from amino acids 221 to 487), and the C-terminal (CT) part of p140Cap (from aa 694 to aa 1153), as well as the p140Cap full length (FL), as fusion proteins with EGFP (**Figure 1A**). Empty vector expressing EGFP was used as a positive control (Mock). As shown in **Figure 1B**, immunoprecipitation experiments with antibodies to GFP revealed that Mock, TER, CT and FL expressing cells produced GFP-fusion proteins of the expected molecular weight (30, 57, 97 and 170 kDa, respectively) (**Figure 1B**, lower panels). As expected, the FL protein co-immunoprecipitated both Src and Csk [4, 5, 9]. The TER and CT protein retain the ability to associate Csk and Src, respectively, likely through the Csk and Src binding domains previously identified in the FL (**Figure 1B**, upper panels). Interestingly the TER protein also immunoprecipitated Src, suggesting that the TER region could also associate Src. This binding might be due either to direct association of the Src SH2 domain with a tyrosine residue inserted in the TER region, or indirectly through Csk. Therefore, the TER and CT regions, expressed as such, still retain a molecular conformation that confers the ability to associate with Src and Csk at a similar extent than the FL protein.

p140Cap has been identified as a critical regulator of *in vitro* cell proliferation and migration of breast cancer cells [2, 5, 8]. To assess the ability of TER and CT modules to interfere with cell proliferation, an equal number of Mock, TER, CT and FL expressing cells was plated in a 12 well plate and counted every 48 hours for 10 days. As shown in **Figure 1C**, cells overexpressing FL exhibited a significant decrease in cell proliferation compared with Mock cells. Both TER and CT cells showed an inhibition of cell proliferation comparable to that observed in FL expressing cells.

Motility is a fundamental property of cancer cells that escape from the primary tumor and

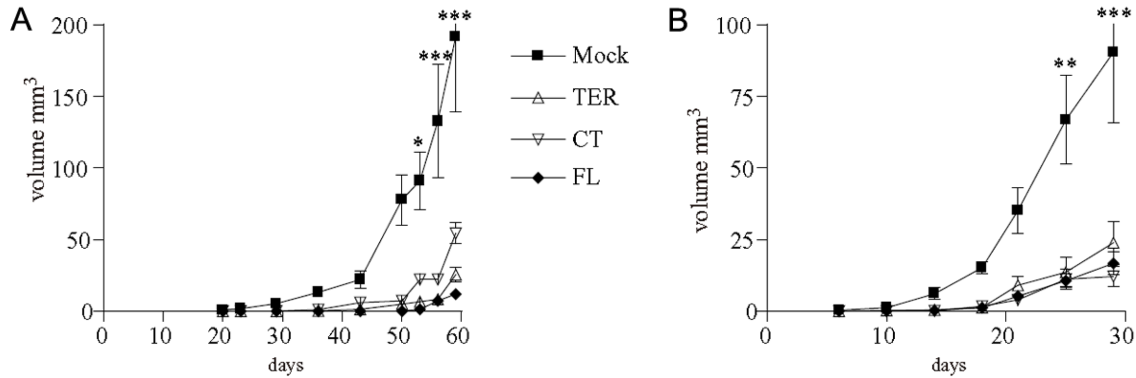

Figure 2. Expression of TER and CT modules affects MDA-MB-231, and HT-29 *in vivo* tumor growth. A. 1 x 10^7 MDA-MB-231 cells expressing TER, CT and FL p140Cap or Mock were mixed with Matrigel and injected subcutaneously in the two flanks of 5 week old female SCID mice. Four mice for each cell type wahe size of the tumors was evaluated twice a week using caliper for 60 days. B. 1 x 10^6 HT-29 cells over-expressing TER, CT and FL p140Cap or Mock were injected as in A. The size of the tumors was evaluated twice a week using calliper for 28 days. A-C: The results are representative of two independent experiments, with a number of six tumors for each cell type in each experiment. Differences in tumor volume were evaluated with two-way ANOVA followed by Bonferroni multiple comparison post hoc tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

invade into new tissues. To test directional migration in transwell assays, Mock, TER, CT and FL expressing cells were plated on fibronectin-coated Transwells and allowed to migrate towards serum for 2 hours. The mean of three experiments (**Figure 1D**) showed that upon stimulus TER and CT expressing cells migrated significantly less than Mock cells, as FL expressing cells. The difference in cell migration was not dependent on cell adhesion, since the number of cells adherent to fibronectin was similar in the four cell subtypes (data not shown). The data presented here indicate that in MDA-MB-231 cells the TER and CT regions are capable of interfering with *in vitro* tumor cell proliferation and migration at a similar extent than the FL protein.

TER and CT domains impair Src activation and Fak phosphorylation

Growth factor treatment as well as integrin-mediated adhesion leads to Src kinase activation which results in phosphorylation of Fak on the tyrosine (Y) 925 [12, 13]. We have previously shown that p140Cap over-expression inhibits Src activation leading to reduced Fak phosphorylation [2, 5]. To assess the ability of TER and CT domains to regulate cell signaling, MDA-MB-231 cells expressing Mock, TER, CT and FL proteins were starved overnight and treated with or without serum for 30 minutes. Cell extracts were evaluated with antibodies to the activated Src (pSrc Y416), and tyrosine phosphorylated Fak (pFak Y925). As expected [5], cells over expressing FL p140Cap were profoundly inhibited in Src activation, and in Fak tyrosine phosphorylation (**Figure 1E**, upper panels), both at basal and stimulated level, compared to Mock cells. Remarkably, cells expressing TER and CT proteins also presented a similar strong down-regulation of Src activation and consequently of Fak phosphorylation. Densitometric analysis of three independent experiments (**Figure 1E**, lower panels) show that the extent of TER and CT-mediated inhibition of Src and Fak phosphorylation is quite similar to that of FL expressing cells, indicating that TER, CT and FL p140Cap proteins share the ability to interfere with Src signaling.

So far p140Cap function in tumor epithelial cells has been mostly associated to its ability to inhibit Src kinase, indicating that p140Cap expression in tumors can rewire Src signaling ‘circuits’ resulting in inhibition of growth and metastasis formation [5, 8]. Therefore the capability of the TER and CT domains to down-regulate Src activity and Fak phosphorylation may represent a main mechanism through which these domains negatively affect tumor cell proliferation and migration. However, the ability of TER and CT proteins to down-regulate additional signaling pathways cannot be excluded and will be investigated in a next future.

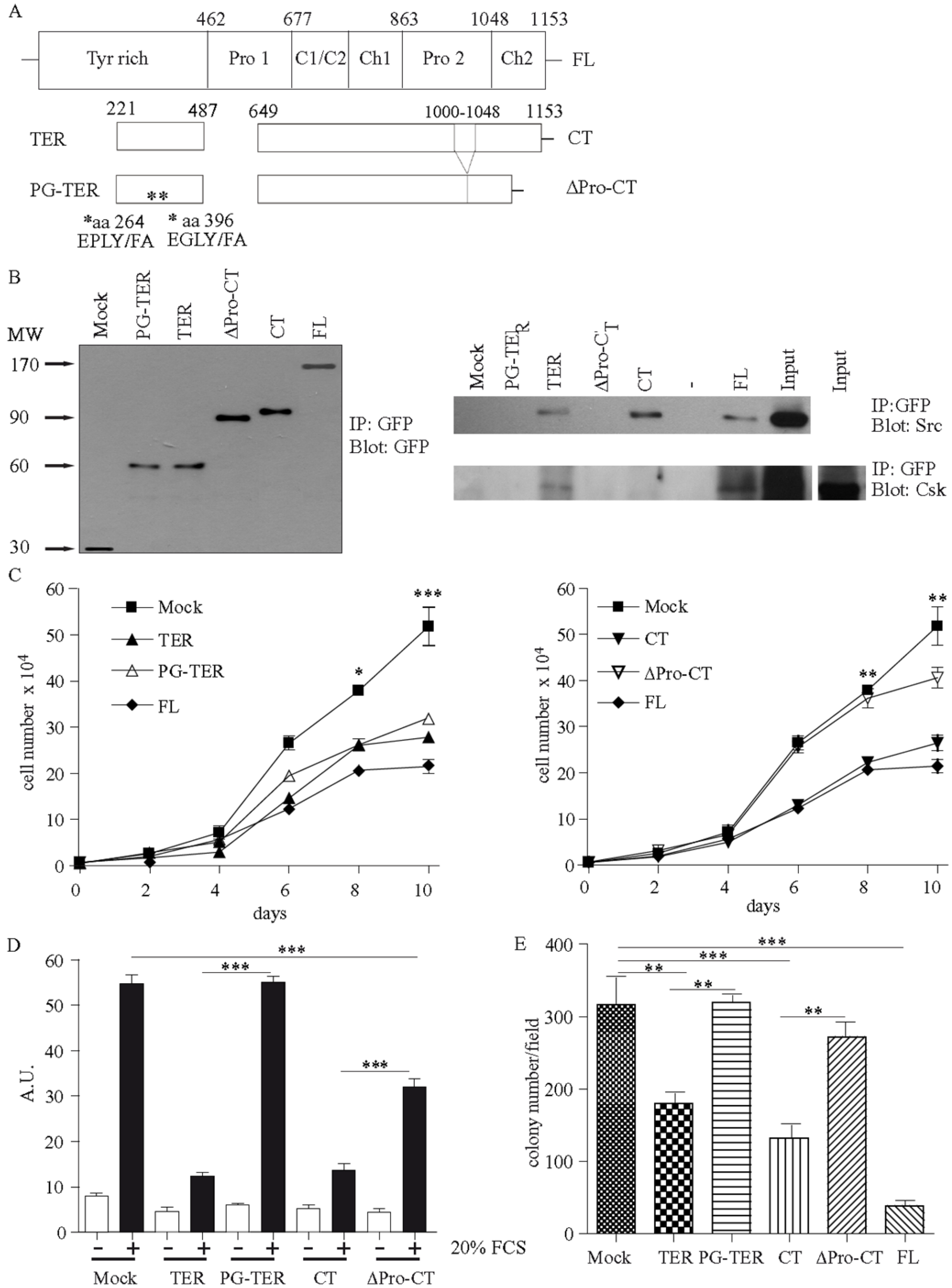

Figure 3. Expression of TER and CT mutants affects specific properties of TER and CT proteins. A. Left panel. Schematic representation of p140Cap FL, TER and CT protein structure and their respective mutants PG-TER and DeltaPro-CT. In PG-TER the tyrosines (Y) in 264 and 396 have been mutated in phenylalanine (F). In DeltaPro-CT a region spanning amino acid 1000-1048 has been deleted. Right panel. For quantification of GFP-tagged fusion

proteins expression, 50 microgram cell extracts were run on 6% SDS-PAGE and blotted with antibodies to GFP. B. To evaluate the association with Csk and Src, four mg extracts of MDA-MB-231 cells stably expressing the different GFP-tagged fusion proteins, were immunoprecipitated with antibodies to GFP, run on 8% SD-PAGE and western blotted with antibodies to Src and Csk. Input: fifty micrograms of FL p140Cap cell extract. A lower exposure for Csk is shown on the right. C. For assessing cell proliferation, 1×10^4 cells were plated in 12 wells dishes in 10% FCS, DMEM culture medium. Each second day cells three wells were detached and counted manually. The mean number of cells is represented by *y*-axis. The results are representative of three independent experiments. Left panel: Mock, TER, PG-TER and FL. The data were evaluated with two-way ANOVA followed by Bonferroni multiple comparison post hoc tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Mock cells were significantly different from TER, PG-TER and FL that do not differ each other. Right panel: Mock, CT, DeltaPro-CT and FL. The data from Mock cells significantly different from CT anf FL, and not for DeltaPro-CT. D. For assessing anchorage-independent growth, Mock, TER, PG-TER, CT, DeltaPro-CT, and FL cells were plated in a soft agar assay and let to grow for three weeks. The bars represent the mean number of colonies for each cell type, as counted in four random fields under a phase-contrast microscope at 20X magnification. The data were evaluated with one-way ANOVA followed by Bonferroni multiple comparison post hoc tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). E. For assessing cell migration, Mock, TER, PG-TER, CT, DeltaPro-CT, and FL cells were starved overnight, detached and plated (5×10^4) on fibronectin-precoated Transwells. Cells were allowed to migrate with or without 10% FCS for 2 hours. The mean number of migrated cells was counted in 10 random fields. The data were evaluated with one-way ANOVA followed by Bonferroni multiple comparison post hoc tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

TER and CT domains profoundly impair in vivo tumor growth in breast and colon cancer cell lines

Forced expression of p140Cap in MDA-MB-231 breast cancer cells strongly inhibits *in vivo* tumor formation [5]. In order to determine the respective role of TER and CT regions on *in vivo* tumor progression, immunocompromised mice were orthotopically injected with 1×10^7 Mock, TER, CT and FL MDA-MB-231 cells. As expected [5], Mock cells gave rise to tumors of 200 mm^3 volume within sixty days after subcutaneous injection, and FL expressing cells did not grow as detectable tumors (**Figure 2A**). Remarkably, injection of TER and CT expressing cells resulted in extremely reduced tumor growth. In particular, TER cells did not give rise to detectable tumors, while CT cells drastically delayed the appearance of tumors that at sixty days were very small (**Figure 2A**). Therefore both TER and CT regions retain the ability of FL p140Cap to profoundly modify tumor cell properties, down-regulating *in vivo* tumor growth of this aggressive breast cancer model.

To assess if the ability of TER and CT proteins to impair in vivo tumor growth is not restricted to a breast cancer model, but can be extended to other carcinoma cell types, we stably expressed the proteins in HT-29 cells, as a model of aggressive colon tumors. The levels of expression were comparable to that reached in MDA-MB-231 cells (data not shown). Subcutaneously injected p140Cap FL expressing cells grew very poorly *in vivo* compared to Mock cells, and TER and CT expressing HT-29 cells behave like the FL cells (**Figure 2B**). These cells express an endogenous p140Cap protein whose silencing leads to cells that grow as tumors of increased volume compared with control cells [2], showing that tuning p140Cap expression can profoundly affect HT-29 tumor cell growth. The results presented here indicate that the expression of TER and CT proteins further strengthens the negative regulation of tumor growth exerted by the endogenous protein p140Cap FL. These data also suggest that TER, CT and FL proteins may share the ability to affect key pathways that both breast and colon tumor cells exploit for *in vivo* growth. Although we can not prove at this stage that TER and CT proteins use mechanisms of inhibition common or alternative to those regulated by the FL protein, these data indicate that alone on in combination with the endogenous protein, the TER and CT proteins are highly effective for impairing *in vivo* growth of breast and colon carcinoma cells.

Specific residues in the TER and CT domains contribute to inhibition of cell migration and tumor cell growth

The TER and CT proteins still span over hundreds of amino acids, however they contain short amino acid stretches that on the basis of their binding capacities, might be sufficient for mediating the observed inhibition of tumor properties. In the FL protein, we have already demonstrated that mutation of the two tyrosine residues inserted in the EPLYA and EGLYA sequences impairs Csk binding [9]. Moreover, mutation of the carboxy-terminal consensus proline rich sequence SPPPPPPRRS affects Src

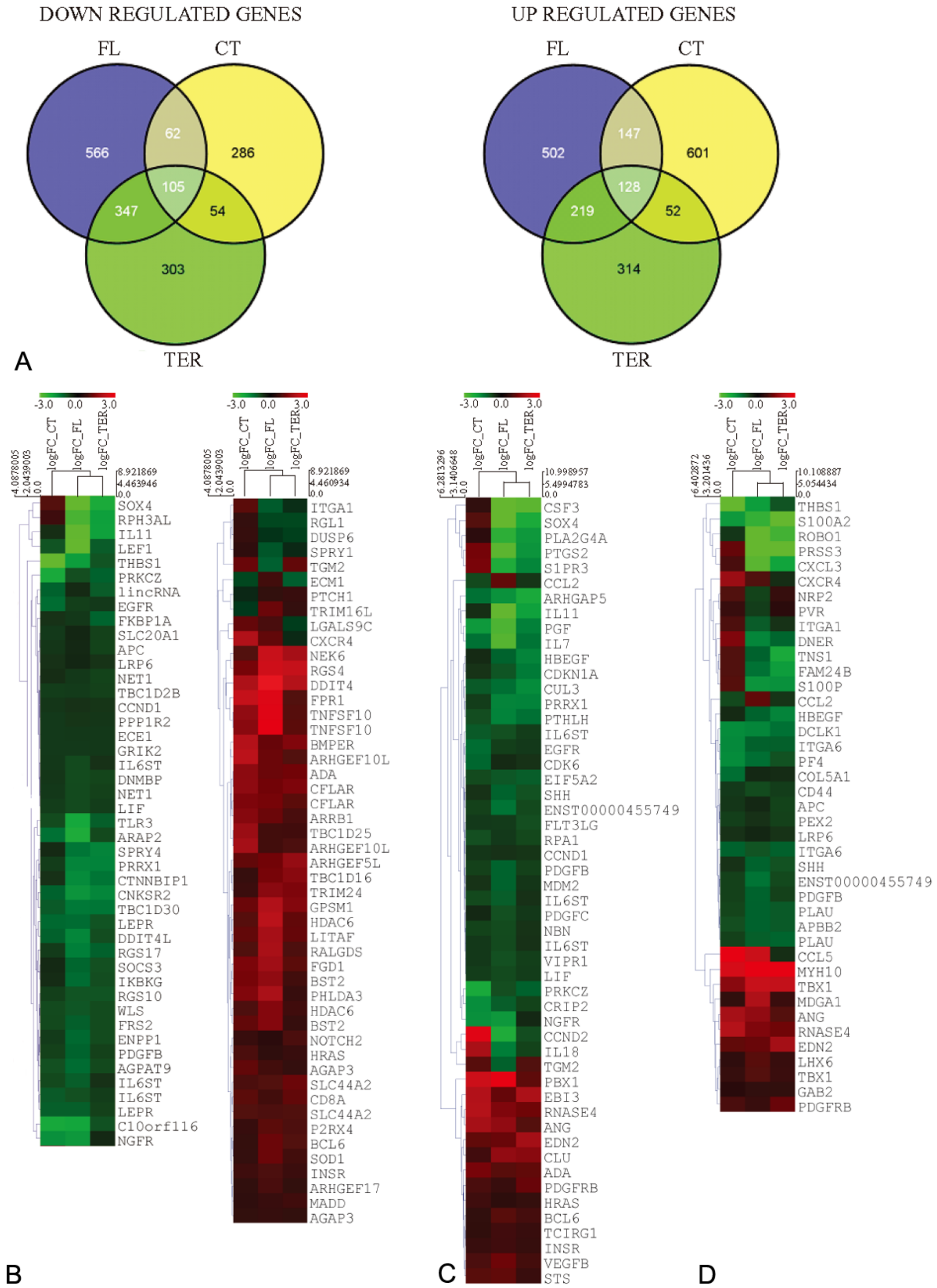

Figure 4. Gene expression and Gene Ontology enrichment analysis. Total RNA was extracted from MDA-MB-231 cells expressing TER, CT, FL or Mock cells and hybridized to Human Whole Genome Oligo Microarray 8 x 60 K (Agilent Technologies). A. Venn diagrams to depict the commonly regulated genes by expression of the three constructs (FL,

CT and TER) were created with the help of the on-line tool Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). B. The genes modulated in at least one condition in the three GO Biological Processes level 5 (BP5) (left panel: Regulation of Signal Transduction; medium panel: Positive Regulation of Cell Proliferation; right panel: Cell Migration) were selected to create expression matrices containing the log fold change of the expression of each gene in FL, TER and CT compared to Mock. The matrices were then loaded in Tigr MultiExperiment Viewer (TmeV version 4.6.1, http://www.tm4.org/mev/) to generate heatmaps by means of hierarchical clustering.

binding [5]. To assess whether these sequences have a key role in suppressing tumor properties in the context of the TER and CT modules, the TER cDNA was mutated in the EPLYA and EGLYA tyrosines (PG-TER) and the CT was deleted of the proline rich region (from amino acids 1000 to 1048) (DeltaPro-CT) (**Figure 3A**). Expression of the corresponding proteins in MDA-MB-231 cells was confirmed by western blot (**Figure 3B**, left panel). As shown in **Figure 3B**, right panel, the DeltaPro-CT and the PG-TER mutants did no longer immunoprecipitate Src and both Csk and Src, respectively, indicating that these specific mutations disrupt these binding capabilities.

For assessing cell proliferation, anchorage-dependent and independent assays were used. When an equal number of Mock, TER, PG-TER, CT, DeltaPro-CT and FL expressing cells was plated in a 12 well plate and counted every 48 hours for 10 days, the DeltaPro-CT mutant grew at the similar extent of Mock cells, rescuing cell proliferation compared to the CT expressing cells (**Figure 3C**, Right panel). Instead, the PG-TER mutant cells grew poorly as the TER cells and were not able to reach the Mock levels (**Figure 3C**, Left panel). Thus the proline rich domain is essential for inhibition of *in vitro* tumor cell proliferation, while the EPLYA and EGLYA motifs are not primarily involved in the control of this tumor feature. Interestingly, as shown in **Figure 3D**, when cells were tested for directional migration in transwell assays, the PG-TER expressing cells were capable to migrate like Mock cells, while the DeltaPro-CT cells migrate more than the CT cells, but significantly less than Mock cells. These data indicate that the tyrosines included in the EPLYA and EGLYA motifs, but not the proline-rich sequence, are key elements in the negative regulation of directional cell migration.

Cells were also plated in an anchorage-independent manner in a soft agar assay, to recapitulate the conditions where only aggressive tumor cells can survive and form colonies in the absence of adhesion. While consistently with previous data [5], FL expressing cells gave rise to a strong reduction in the number of colonies compared to Mock cells (**Figure 3E**), the expression of TER and CT proteins impaired colony formation, although to a lesser extent than FL. However this partial phenotype was rescued by expression of both PG-TER and DeltaPro-CT mutants (**Figure 3E**), suggesting that these sequences play a role in the ability of TER and CT domains of affecting anchorage-independent growth.

Overall, these data show that the tyrosines inserted in the EPLYA and EGLYA sequences in the TER module have an important role in the inhibition of tumor cell migration and of anchorage-independent growth. In contrast, the proline rich region in the CT domain is mainly relevant for inhibition of cell proliferation, while less involved in directional migration or anchorage-independent growth. Thus, using different assays, we were able to dissect the functions of tyrosine residues in the EPLYA and EGLYA sequences and of the second proline-rich stretch, ascribing to these three short peptides the ability of acting as negative regulators of cell migration and proliferation. These results suggest that these short peptides might potentially be used as tumor inhibitory molecules in the next future.

TER and CT regions share a common signature with 140Cap FL

The results shown here indicate that the TER and CT proteins are both equally effective in down-regulating tumor cell properties, at the same extent of the p140Cap FL. To investigate whether the three proteins can act through common pathways we performed a global gene profiling by microarray analysis. Comparison of genes modulated by expression of FL, TER and CT resulted in 105 commonly down-regulated and 128 commonly up-regulated genes (**Figure 4A**). As shown in the Venn diagrams, a stronger intersection was observed between FL and TER than between FL and CT, both for down and up regulated genes.

47 Gene Ontology Biological Processes level 5 (BP5) were commonly overrepresented in the lists of modulated genes in cells expressing FL, TER and CT. We further selected those genes modulated in at least one condition and involved in regulation of signal transduction (GO0009966) (**Figure 4B**), positive regulation of cell proliferation (GO0008284) (**Figure 4C**) and cell migration (GO0016477) (**Figure 4D**). The expression pattern of these genes were analyzed together to create expression matrices containing the log fold change of their expression in FL, TER and CT compared to Mock cells. This bioinformatic analysis shows that FL, TER and CT expression leads to down or up-regulation of a pattern of common genes, with FL and TER profiles more similar to one another, respect to CT.

We have already shown that p140Cap, TER and CT have similar ability in down-regulating biochemical activities inside the cells, such as Src kinase activation. Deeper analysis of the signatures specific for p140Cap, TER and CT domains will offer the possibility to explore the molecular mechanisms underlying the ability of p140Cap and of its regions to inhibit tumor properties at the level of gene expression, opening the perspective of further elucidating the pathways modulated by their expression.

Conclusions

In this work we have dissected the modular structure of p140Cap, providing evidence that the expression of the TER and CT regions of p140Cap is sufficient to impair proliferation, migration and *in vivo* growth of breast and colon tumor cells at a similar extent of the FL protein. Mutagenesis analysis has shown that in both TER and CT domains specific amino acid sequences can be involved in the control of cell migration and cell proliferation. Indeed, we have identified three short sequences that are relevant for the negative control of cell proliferation and migration (the EPLYA and EGLYA sequences and the second proline-rich stretch). Moreover gene expression profiling provides evidence of the existence of a common signature of down-regulated and up-regulated genes among FL, TER and CT expressing cells. In conclusion, this work represents the first attempt to identify short relevant regions that retain p140Cap inhibitory properties in tumor cells. In the next future it is conceivable to look for peptide sequences within these regions to produce small molecules with therapeutic potential.

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Abbreviations

Y, Tyrosine residues; Csk, C-terminal Src kinase; TER, Tyrosine Enriched Region; CT, Carboxy Terminus; FCS, Fetal calf serum.

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