

p130Cas and p140Cap as the Bad and Good Guys in Breast Cancer Cell Progression to an Invasive Phenotype

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1. Introduction

Breast cancer is an aggressive malignancy affecting a large woman population. Even though important progress have been made in providing new therapies to treat this neoplasia, our knowledge on the mechanisms underlying the transformation of breast epithelial cells in tumor cells is still superficial. The neoplastic phenotype results from the alteration of multiple cellular signaling mechanisms controlling proliferation, survival and invasiveness. Moreover, the prognosis of breast cancer patients is tightly correlated with the degree of spread beyond the primary tumor. However the mechanisms by which epithelial tumor cells escape from the primary tumor and colonize a distant site are not entirely understood. In this chapter we will discuss recent data on the relevance of p130Cas and p140Cap adaptor molecules in breast cancer signalling related to the acquirement on invasive properties. Due to the presence of adaptor modules, these proteins create signalling platforms proximal to plasma membrane cell surface receptors, such as integrins and growth factor receptors. p130Cas and p140Cap exert opposite regulation on cell signalling. Indeed p130Cas has been shown to increase survival, proliferation and migration of normal and transformed cells either in response to cell matrix adhesion or to hormones and growth factors. Moreover, p130Cas has been recently linked to resistance to breast cancer treatments, revealing its potential use as a novel therapeutic target. Instead, p140Cap behaves as a potent negative regulator of signalling pathways leading to cancer cell proliferation and migration. In this chapter, we will discuss the increasing evidence that highlight the importance of these adaptor proteins in breast cancer.

It is well established that to migrate and to invade a cell needs to detach from its neighbors, i.e. adjacent cells in an epithelium, to extend lamellipodia and filopodia from the leading edge and to create new dynamic adhesions, which form and rapidly disassemble at the base of protrusions (Mitra *et al.*, 2005; Ridley *et al.*, 2003). Cell invasion also requires the release or activation of proteases that degrade the extracellular matrix (ECM) and allows cells to sort out from the basal lamina invading surrounding tissues (Eliceiri *et al.*, 2002). Under physiological conditions cell motility and invasion are tightly controlled by a complex interplay among cell-cell, cell matrix and growth factors receptors resulting in the maintenance of the architectural integrity of human tissues. This subtle regulation is lost in

human tumours leading to uncontrolled dissemination of cancer cells into the body (Bex *et al.*, 2007; Cavallaro and Christofori, 2004; Giancotti, 2003; Guo and Giancotti, 2004),

At least three major classes of membrane proteins are involved in these events, namely, the E-cadherin, the Receptor tyrosine kinases (RPTKs), and the integrin receptors. The cell-cell adhesion receptor E-cadherin is the major membrane protein involved in binding between neighbouring cells in adherens junctions. As a practical consequence of its adhesive functions, E-cadherin has also been shown to prevent EGFR activation and downstream signalling, leading to negative regulation of proliferation (Bex and Van Roy, 2001; Gutkind, 2000; Perrais *et al.*, 2007; Qian *et al.*, 2004). E-cadherin is frequently down-regulated or lost in epithelial tumours, and its loss correlates with increased cancer cell invasiveness ((Peinado *et al.*, 2007; Reynolds and Carnahan, 2004);

Integrins are cell surface heterodimeric receptors for the ECM formed by the non covalent association of alpha and beta subunits (Hynes, 2004). Integrins specifically localize to focal adhesions, which are sites of close apposition with the ECM where actin filaments are anchored to the plasma membrane. Integrins are catalytically inactive and translate positional cues into biochemical signals by direct and/or functional association with intracellular adaptors or growth factor and cytokine receptors, thus regulating integrin ability to transduce signals inside the cells, the so called “outside-in signalling” (Cabodi *et al.*, 2010). A growing body of evidence shows that integrins, RPTKs and cytokine receptors 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 (Cabodi *et al.*, 2010b; Cabodi *et al.*, 2008; Desgrosellier *et al.*, 2009; Streuli, 2009; Uberti *et al.*). In the case of the EGF receptor (EGFR), beta1 integrin is both sufficient to partially activate the receptor itself and required for the full activation of the EGFR in response to EGF (Morello *et al.*; Moro *et al.*, 1998)). Integrin-dependent EGFR trans-activation accounts for a specific repertoire of mechanisms, namely cell survival and actin cytoskeleton organization involved in cell migration.

In this chapter we will focus on p140Cap and p130Cas adaptors as major regulators of cell migration and invasion (Cabodi *et al.*). Owing to their modular structure, both proteins can undergo tyrosine phosphorylation and association with effector proteins, leading to the assembly of molecular platforms that regulate the variety of signalling events originating from the complex cross-talk among integrins, E-cadherin and RPTKs.

2. p130Cas adaptor protein

2.1 p130Cas adaptor features

p130Cas is coded in human by the BCAR-1 (Breast Cancer Anti-oestrogen Resistance 1) gene. This gene is conserved through many species and in humans is localized on Chromosome 16q23.1. Knock-out of the mouse gene results in embryonic lethality at 9.5 days, indicating that any other protein cannot fill in for its role during development. p130Cas is an ubiquitously expressed multi-site docking protein that consists of i) a N-terminal Src homology 3 (SH3) domain, ii) a substrate domain, which contains 15 repeats of a YXXP sequence (tyrosine-any two aminoacids-proline), iii) a serine rich region, and iv) a C-terminal domain (Figure 1A). The presence of these multiple conserved sequence motifs and extensive post-translational modification, mainly consisting of tyrosine and serine phosphorylation, allow the assembly of specific multi-protein complexes. In particular, the SH3 domain interacts with polyproline-rich sequences present in several proteins including

Fak, PYK2/RAFTK, phosphatases like PTP-PEST, PTP1B, and effectors as C3G and CIZ (Sakai *et al.*, 1994; Tikhmyanova *et al.*). The substrate domain, upon Src family kinases activation, is tyrosine phosphorylated and exposes additional binding sites for SH2 containing proteins such as the Crk adaptors (Salgia *et al.*, 1996), while the serine rich region represents a docking site for other partners such as 14-3-3 and Grb2. Lastly, the C-terminus contains a polyproline-rich region responsible for the binding of the Src family kinase, PI3K, Bcar3/AND-34, Chat-H and ubiquitin ligases such as AIP4, APC/C and CDH1, as well as a binding site for the adaptor protein p140Cap (Bouton *et al.*, 2001; Cabodi *et al.*, 2004; O'Neill *et al.*, 2000).

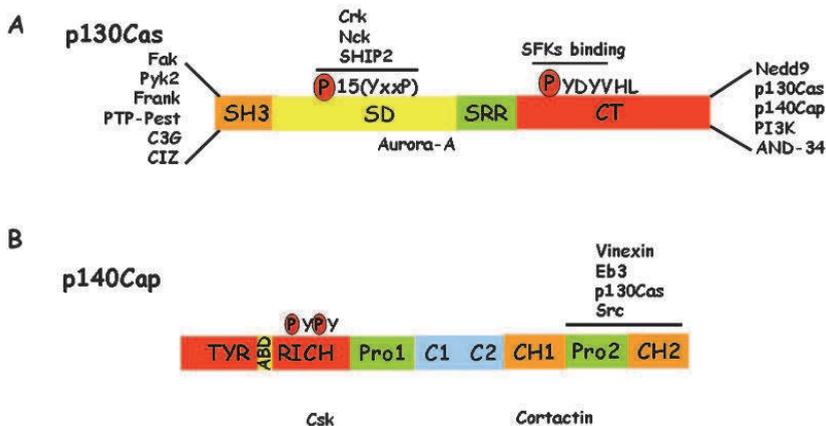


Fig. 1. p130Cas and p140Cap structure.

A) p130Cas consists of an N-terminal SH3 domain, a substrate domain (SD), a serine rich region (SRR), and a C-terminal domain (CT). The main interactors are indicated. In particular, many proteins associate to the N-terminal domain and the Src family kinases (SFKs) bind the CT domain. The 15 YxxP motifs are phosphorylated by Src family kinases to mediate Crk binding.

B) p140Cap consists of an N-terminal tyrosine-rich region (Tyr-rich), an actin binding domain (ABD), a proline rich domain (Pro1), a coil-coiled region (C1-C2), two domains rich in charged amino acids (CH1, CH2) and a C-terminal proline rich domain (Pro2). Src, p130Cas, EB3 and Vinexin bind to the Pro2 domain of p140Cap. The binding regions of Cortactin and Csk have yet to be defined.

2.2 p130Cas in human breast cancer

Although several reports highlight the relevance of p130Cas in tumour cell lines and animal models, investigation of its expression in biopsies of different human malignancies using immunohistochemistry, is still limited. However, it is noteworthy that a significant subset of human breast cancers where both ErbB2 and p130Cas are over-expressed are associated with increased proliferation and low prognosis (Cabodi *et al.*, 2006). In estrogen receptor (ER)-positive human breast tumours, p130Cas over-expression correlates with intrinsic resistance to tamoxifen treatment, high risk of relapse and loss of oestrogen-receptor in a large subset of human breast cancer samples, indicating that elevated BCAR1 might be a prognostic marker for breast tumours (Dorssers *et al.*, 2001; van der Flier *et al.*, 2001).

Therefore, at least in two classes of breast cancer that account for more than 90% of breast tumors, p130Cas over-expression is revealing its potential as prognostic factor in terms of therapy and disease progression.

2.3 p130Cas tyrosine phosphorylation in cell migration and invasion

p130Cas represents a nodal signalling platform on which integrin and RPTKs signalling convey. Integrins, RPTKs and oestrogen receptor (ER) are major upstream regulators of p130Cas, mainly through the activation of Src and Fak kinases, leading to p130Cas tyrosine phosphorylation on the C-terminal binding site YDYVHL (Figure 1) (Cabodi *et al.*). Moreover, physical stretching of p130Cas induces a conformational change that enables Src-family kinase-dependent p130Cas tyrosine phosphorylation. These findings point out a function for p130Cas as a sensor that integrates mechanical forces coming from the extracellular environment into intracellular signals leading to actin cytoskeleton reorganization (Kostic and Sheetz, 2006; Sawada *et al.*, 2006). The role of p130Cas in cell migration was initially inferred by studies performed on mouse embryo fibroblasts (MEFs) derived from p130Cas knock-out mice. p130Cas null MEFs show defects in stress fibre formation and cell spreading, impaired actin bundling and cell migration (Honda *et al.*, 1998), that were restored by full-length p130Cas expression. The tyrosine phosphorylation of the substrate domain of p130Cas provides binding sites for Crk proteins that in turn associates with DOCK180, a guanine nucleotide exchange factor that switches the small GTPase Rac1 from a GDP-bound inactive to a GTP-bound active state at lamellipodia and filopodia adhesion sites (Figure 2) (Kiyokawa *et al.*, 1998; Klemke *et al.*, 1998). This drives localized Rac activation, membrane ruffling and actin cytoskeleton remodelling, focal adhesion turnover, pseudopodia formation and extension. In addition, ARP2/3 and PAK kinase activation enhance cell migration (Heasman and Ridley, 2008). Uncoupling of p130Cas/Crk negatively regulates cell migration. Indeed, the non-receptor tyrosine kinase Abl phosphorylates Crk-II on tyrosine 221, inducing intramolecular folding that prevents binding of the C-terminal Crk-II SH2 domain to the phosphorylated p130Cas substrate domain, leading to decreased cell movement (Holcomb *et al.*, 2006; Kobashigawa *et al.*, 2007). Additional molecules that play important roles in modulating tyrosine phosphorylation of p130Cas leading to cell migration are the zyxin/Ajuba family of LIM proteins. These proteins bind to actin cytoskeleton and are implicated in cell motility. Ajuba allows p130Cas localization to nascent adhesive sites in migrating cells thereby leading to the activation of the small GTPase Rac, whereas Zyxin interacts with the SH3 domain of p130Cas and with a nucleocytoplasmic transcription factor, CIZ/NMP4/ZNF384 (Janssen and Marynen, 2006). Recent data also show that p130Cas activates several GTPases other than Rac. The association between p130Cas and And-34, an NSP family member, which acts as a GTP exchange factor for Ral, Rap1 and R-Ras enhances Src activation and cell migration, likely through a Rap1-dependent mechanism (Figure 2)(Riggins *et al.*, 2003). p130Cas tyrosine phosphorylation upon integrin or growth factor receptor activation has also been linked to cell invasion and it has been reported that the SH3 domain of p130Cas is also required for this process. Indeed, Focal adhesion kinase (Fak)-null cells are not invasive when transformed by v-Src, but they acquire invasive properties upon over-expression of p130Cas SH3 domain, indicating that this domain is required for rescue of v-Src cell invasion. In this context, the formation of Src/p130Cas/Crk/DOCK180 complex increases Rac1 and JNK activities and MMP-9 expression, leading to an invasive cell phenotype (Hsia *et al.*, 2003).

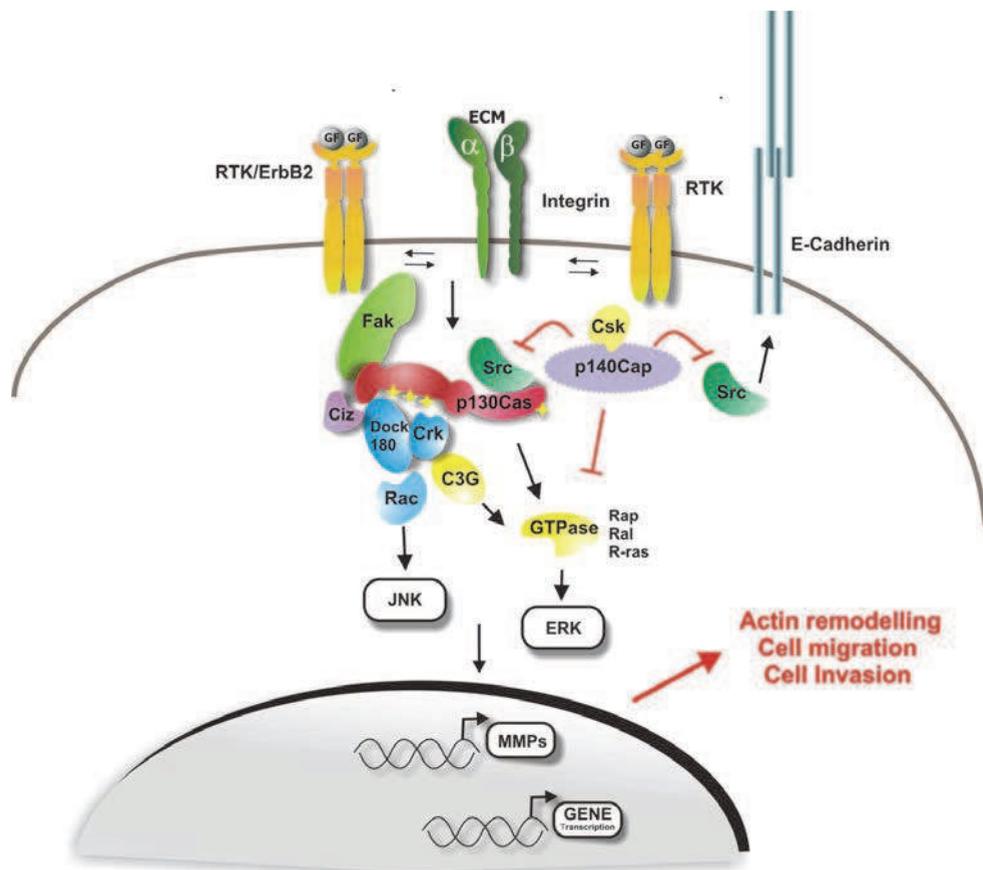


Fig. 2. p130Cas and p140Cap signalling involved in migration and invasion of breast cancer cells.

Upon extracellular matrix binding or growth factors stimulation, integrins and Receptor Protein Tyrosine Kinases (RPTK) represent the major upstream regulators of p130Cas and p140Cap, mainly through the regulation of Src kinase activity. Once tyrosine phosphorylated by Src, p130Cas recruits proteins that activate downstream pathways, resulting in actin cytoskeleton re-organization, increased cell motility and migration. p130Cas by acting on metalloproteinases (MMPs) promoter is also required for the invasive program. Upon cell matrix adhesion or mitogen stimulus, p140Cap inhibits Src kinase activity and p130Cas tyrosine phosphorylation and p130Cas/Crk complex formation. As a consequence, the effect of p130Cas on actin cytoskeleton re-organization is impaired and cell migration and invasion are inhibited. (Di Stefano et al., 2007) Moreover, by inactivating Src, p140Cap also regulates the epidermal growth factor receptor (EGFR) pathway through E-cadherin-dependent inactivation of EGFR signalling. p140Cap by interacting with E-cadherin and EGFR at the cell membrane, immobilizes E-Cadherin at the cell membrane thus preventing cell migration and invasion. (Damiano et al., 2010)

2.4 Role of p130Cas in c-Src dependent cell transformation

Hyper-phosphorylation or over-expression of p130Cas has been implicated in transformation induced by several oncogenes. For example, p130Cas involvement in c-Src-mediated tumorigenesis has been demonstrated by the inability of c-Src to transform p130Cas-null MEFs (Honda *et al.*, 1998). The C-terminal region of p130Cas containing the Src binding domain is sufficient to recover the ability of Src to promote anchorage-independent growth. In breast carcinoma cells p130Cas over-expression accelerates and up-regulates Src activity (Cabodi *et al.*, 2004) as well as increases tyrosine phosphorylation of multiple endogenous cellular proteins (Brabek *et al.*, 2004; Burnham *et al.*, 1996; Cabodi *et al.*, 2004). It was recently reported that bosutinib, a novel Src inhibitor, derived from breast cancer patients, inhibits cell spreading, migration, and invasion of human cancer cells, derived from breast cancer patients by stabilizing cell-to-cell adhesions and membrane localization of beta-catenin. These effects are dependent on the inhibition of the Src/Fak/p130Cas signaling pathway (Buettner *et al.*, 2008). It has been recently reported that Fak promotes mammary tumorigenesis by enabling Src-mediated phosphorylation of p130Cas. Consistently, knock-down of p130Cas causes proliferative arrest in breast cancer cell lines harbouring oncogenic mutations in K-Ras, B-Raf, PTEN and PIK3CA (Pylayeva *et al.*, 2009), underlying a role for p130Cas as a general regulator of breast cancer cell growth induced by different oncogenes.

2.5 Role of p130Cas in TGF-beta signalling in breast cancer cells

Transforming growth factor-beta (TGF-beta) is a powerful suppressor of mammary tumorigenesis because of its ability to repress mammary epithelial cell proliferation, as well as through its creation of cell microenvironments that inhibit mammary epithelial cells (MECs) motility, invasion, and metastasis. Yet, paradoxically, cancer cells elicit mechanisms that subvert the tumour suppressing functions of TGF-beta, and in doing so, confer oncogenic and metastatic activities upon this multifunctional cytokine (Massague, 2008). In epithelial cells, integrin beta1 suppresses apoptosis and growth inhibition induced by TGF-beta (Zhang *et al.*, 2003). In this context p130Cas has been shown to be a crucial player by binding to Smad3, and preventing its phosphorylation by TGF-beta receptor. As a consequence, the transcription of the cyclin-dependent kinase inhibitors p15 and p21 is inhibited, resulting in cell cycle progression (Kim *et al.*, 2008). Recently, it has been reported that p130Cas over-expression in MECs shifts TGF-beta signalling from Smad2/SMAD3 phosphorylation to p38 MAPK activation, rendering MECs resistant to TGF-beta -induced growth arrest and enhancing their metastatic potential (Wendt *et al.*, 2009). Overall, p130Cas can act as a molecular rheostat that switches the tumour suppressor function of TGF-beta to a pro-metastatic role during breast cancer progression.

3. The ErbB2 oncogene in breast cancer

The ErbB2 oncogene is a member of the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases (RTKs). This family comprises four related members: EGFR, ErbB2 (also known as Neu, HER-2), ErbB3 (HER-3), and ErbB4 (HER-4) (Holbro *et al.*, 2003). Over-expressed and mutated ErbB2 has been found in human tumors and cancer cell lines (Mukohara; Yarden *et al.*, 2004). In addition, several studies have shown a strong correlation of ErbB2 over-expression with a negative clinical prognosis in breast cancer (Choi *et al.*, 2009; Mukohara). Significantly, ErbB-2 may be useful not only as a prognostic marker but

also as a predictive marker, given that its elevated expression predicts tamoxifen resistance of the primary tumor and the response to anti-HER2 targeted therapy such as the monoclonal antibody Herceptin.

Further understanding of the mechanisms by which ErbB2 leads to tumorigenesis in the mammary gland comes from studies of ErbB2 mouse models. Expression of Neu mutation that promotes spontaneous receptor dimerization (NeuT), under the MMTV promoter, or more recently under the ErbB2 endogenous promoter (ErbB2/KI model), leads to the formation of mammary adenocarcinomas (Andrechek *et al.*, 2000; Muller *et al.*, 1998). Interestingly, the expression of the ErbB2 protooncogene in a MMTV-transgenic mice show late tumor latency with a low penetrance of lung metastasis, suggesting that gene amplification of the wild type receptor may be the main mechanism implicated in ErbB2-mediated tumorigenesis. Indeed, elevated protein and mRNA ErbB2 levels in the ErbB2/KI model also correlated with selective genomic amplification of the activated ErbB2 allele (Andrechek and Muller, 2000; Hodgson *et al.*, 2005; Montagna *et al.*, 2002). One of the most significant effects associated with ErbB2 activation is enhanced and sustained signal transduction cascades leading to the regulation a variety of cellular processes, including proliferation, apoptosis, cell polarity, migration and invasion (Feigin and Muthuswamy, 2009). Activation of specific ErbB homo- or heterodimer pairs leads to initiation of the mitogen activated protein kinase (MAPK) cascade, activation of phospholipase C gamma (PLC γ) and phosphatidylinositol 3 kinase (PI3K), as well as induction of the small GTPases Rho, Rac and Cdc42, among many other effectors (Hynes and MacDonald, 2009; Kurebayashi, 2001). Several reports have demonstrated a role for these pathways in ErbB-induced cell migration.

3.1 p130Cas in ErbB2 dependent transformation

In the context of ErbB2 positive breast cancer, previous studies generated by our group placed p130Cas as an important regulator of ErbB2-dependent tumorigenesis. To investigate the mechanisms through which p130Cas is linked to tumorigenesis, we generated mouse mammary tumor virus (MMTV)-p130Cas mice overexpressing p130Cas in the mammary gland. MMTVp130Cas transgenic mice are characterized by extensive mammary epithelial hyperplasia during development and pregnancy and by delayed involution at the end of lactation. These phenotypes are associated with activation of Src kinase, Erk1/2 MAPK, and Akt pathways, leading to an increased rate of proliferation and a decreased apoptosis. A double-transgenic line derived from crossing MMTV-p130Cas with MMTV-HER2-Neu mice expressing the activated form of the HER2-Neu oncogene develops multifocal mammary tumors with a significantly shorter latency than the HER2-Neu parental strain alone (Figure 3). MECs isolated from tumors of double-transgenic mice display increased tyrosine phosphorylation, c-Src, and Akt activation compared with cells derived from HER2-Neu tumors. In addition, p130Cas down-regulation by RNA interference increases apoptosis in HER2-Neu-expressing cells, indicating that p130Cas regulates cell survival. These findings provide evidences for a role of p130Cas as a positive regulator of both proliferation and survival in normal and transformed mammary epithelial cells. Its overexpression contributes to HER2-Neu-induced breast tumorigenesis, thus identifying this protein as a putative target for clinical therapy (Cabodi *et al.*, 2006).

More recent studies further assessed the functional role of p130Cas in ErbB2-dependent breast tumorigenesis by its silencing in breast cancer cells derived from mouse mammary tumours over-expressing ErbB2 (N202-1A cells), and by its re-expression in ErbB2-

transformed p130Cas-null mouse embryonic fibroblasts. We demonstrate that p130Cas is necessary for ErbB2-dependent foci formation, anchorage-independent growth and *in vivo* growth of orthotopic N202-1A tumours. Moreover intra-nipple injection of p130Cas-stabilized siRNAs in the mammary gland of MMTV-HER2-Neu mice decreases the growth of spontaneous tumours (Figure 4) (Cabodi *et al.*, 2010c).

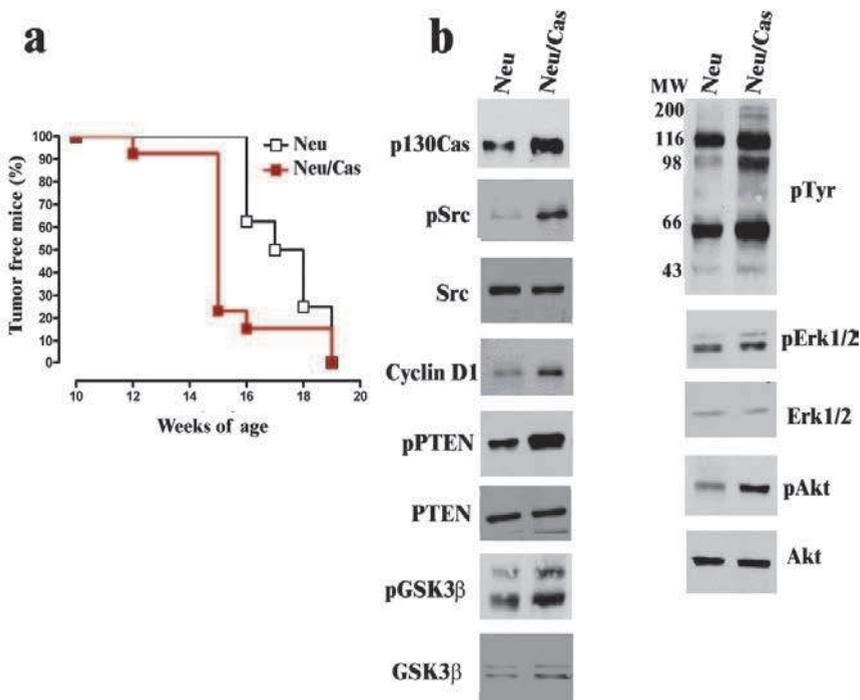


Fig. 3. Kinetics of tumor occurrence in p130Cas/HER2-Neu and HER2-Neu mice.

A) Tumor formation in p130Cas/HER2-Neu (gray line and black circles) and HER2-Neu (black line and empty squares) mice. Twenty mice were analyzed for each group. The difference of occurrence between the two groups is statistically significant, $P < 0.001$.

B) Independent epithelial cell cultures were derived from four distinct tumors excised from p130Cas/HER2-Neu and HER2-Neu mice. Western blot analysis of protein extracts was done with the indicated antibodies and representative results are shown. MW, molecular weight markers. The figure is modified from Cabodi *et al.*, 2006.

To precisely underline the mechanism implicated in p130Cas/ErbB2-mediated transformation, cultures of MECs grown on three dimensional matrix, that share several properties with breast epithelial acini were evaluated. These *in vitro* three-dimensional acini-like structures provide a developmental context and serve as an important tool to study the biological effects of oncogenic signals. Most oncogenic signals that promote proliferative signals have the ability disrupt acini organization with oncogene-specific features. For instance, activation of ErbB2 induces formation of abnormal non invasive structures consisting of individual units (Muthuswamy *et al.*, 2001). Interestingly, in human

mammary cells MCF10A.B2, the concomitant activation of ErbB2 and p130Cas over-expression provides invasive properties (Figure 5). Consistently, p130Cas drives N202-1A cells *in vivo* lung metastases formation. These results demonstrate that p130Cas is an essential transducer in ErbB2 transformation and highlight its potential use as a novel therapeutic target in ErbB2 positive human breast cancers (Cabodi *et al.*, 2010c).

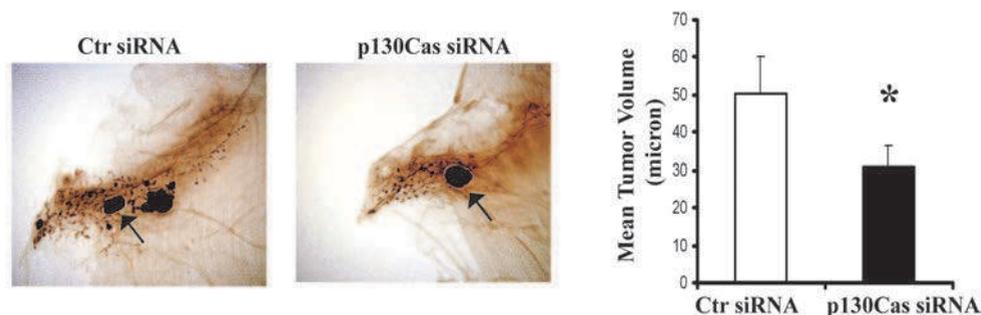


Fig. 4. p130Cas is required for *in vivo* ErbB2 tumorigenesis. Intra-nipple injection was performed in BalbC-NeuT female mice. Control (Ctr siRNA) or p130Cas stabilised siRNA (p130Cas siRNA) were injected once a week for 5 weeks starting from week 12. Left: Whole mount analyses of fixed mammary gland at week 18. The gland is composed of a tree-like structure of branching ducts. Small lesions that have histologic aspects of a solid carcinoma are visible. Black arrows indicate the lymph node. Ctr siRNA picture shows larger lesions on the right of the lymph node. Right: The histogram shows the mean tumour volume measured from two independent experiments with 8 mice per group. * $p < 0.0329$ (two-tailed P value). The figure is modified from Cabodi *et al.*, 2010c.

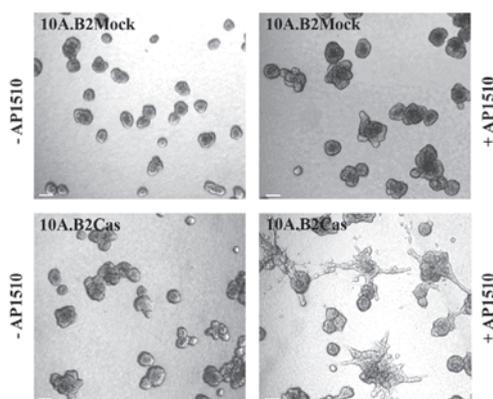


Fig. 5. p130Cas triggers acina invasion of ErbB2 transformed MCF10 cells. p130Cas over-expressing or Mock ErbB2 transformed MCF10 cells were plated on a Matrigel/collagen 1:1 matrix and left un-stimulated or activated for ErbB2 by treating with the small molecule AP1510. 3D invasive protrusions are present only in p130Cas over-expressing and ErbB2 activated acinar structures. The figure is modified from Tornillo *et al.*, 2010.

We further analysed the molecular mechanisms through which p130Cas controls ErbB2-dependent invasion in three-dimensional cultures of mammary epithelial cells. Concomitant p130Cas over-expression and ErbB2 activation enhance PI3K/Akt and Erk1/2 MAPK signalling pathways and promote invasion of mammary acini. By using pharmacological inhibitors, we demonstrate that both signaling cascades are required for the invasive behaviour of p130Cas over-expressing and ErbB2 activated acini. Erk1/2 MAPK and PI3K/Akt signaling triggers invasion involving mTOR/p70S6K and Rac1 activation, respectively (Figure 6). Moreover, *in silico* analyses indicate that p130Cas expression in ErbB2 positive human breast cancers significantly correlates with higher risk to develop distant metastasis, thus underlying the value of the p130Cas/ErbB2 synergism in regulating breast cancer invasion. In conclusion, high levels of p130Cas favour progression of ErbB2-transformed cells towards an invasive phenotype (Tornillo *et al.*, 2010).

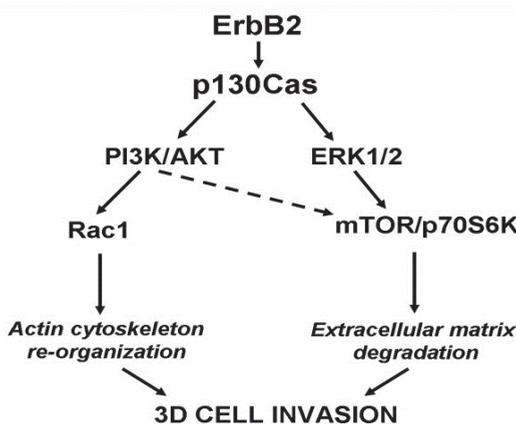


Fig. 6. Scheme illustrating the signaling pathways leading to 3D invasion of ErbB2 transformed MCF10 over-expressing p130Cas.

Both PI3K/Akt and Erk1/2 pathways are activated during invasion triggered by ErbB2 transformation of p130Cas over-expressing MEC. ErbB2/p130Cas/Erk1/2 MAPK signalling pathway preferentially targets mTOR/p70S6K, whereas the ErbB2/p130Cas/PI3K/Akt cascade triggers Rac1 activation. Both signaling pathways are required for mammary epithelia invasion in 3D suggesting that they cooperate in the regulation of different processes that ultimately lead to cell invasion. The figure is modified from Tornillo *et al.*, 2010.

4. p140Cap adaptor protein

4.1 p140Cap structure and phosphorylation

The human p140Cap (Cas associated protein) is codified by the gene *Srcin1*, previously known as SNIP, P140 or p140Cap. The *Srcin1* gene is conserved in human, mouse, rat, dog, cow, and zebrafish and in human is localized on Chromosome 17 q21.1.

The p140Cap protein was originally identified in rat brain as SNIP, a Synaptosome-associated protein SNAP-25b-interacting protein implicated in regulated exocytosis (Chin *et al.*, 2000). The name p140Cap derives from its identification as a protein associated to

p130Cas by affinity chromatography and MALDI-Mass spectrometry in epithelial cells (Di Stefano, 2004). p140Cap is a multisite docking protein, composed by a putative N-terminal myristylation site, a tyrosine-rich domain, two proline-rich regions, a coil-coiled domain, two regions rich in charged amino acids and a putative actin binding site (Figure 1)(Chin *et al.*, 2000; Di Stefano *et al.*, 2004).

p140Cap is mainly expressed in brain, testis and epithelial rich tissues such as mammary gland, lung, colon and kidney (Chin *et al.*, 2000; Di Stefano *et al.*, 2004; Ito *et al.*, 2008). The protein is present at least in two N-terminal alternative and two C-terminal different isoforms. The presence of many conserved sequence motifs that could undergo extensive post-translational modification, mostly tyrosine and serine phosphorylation, led to predict that p140Cap could promote protein-protein interactions, leading to the formation of multiprotein complexes. Indeed p140Cap is tyrosine phosphorylated in epithelial cells upon integrin-mediated adhesion and EGF receptor activation (Di Stefano *et al.*, 2004). In addition, global phospho-proteomic analysis of human brain extracts revealed that p140Cap is phosphorylated on serine 859 in the context of the sequence 857RGS*DELTIVPR866 (DeGiorgis *et al.*, 2005). The same sequence has also been found phosphorylated in mouse brain (Collins *et al.*, 2005).

4.2 p140Cap interacting proteins

Since its discovery, many proteins have been shown to bind directly or to associate in molecular complexes with p140Cap. In normal epithelial cells, p140Cap was found associated to the adaptor protein p130Cas. Although *in vitro* binding studies indicate that p140Cap and p130Cas are not directly linked, their association is mediated by the last 217 amino acids of the p140Cap C-terminal region and the p130Cas region encompassing amino acids 544-678. Through the same C-terminal region, p140Cap binds directly to the SH3 domain of the Src kinase. Moreover in MCF7 cells p140Cap has been shown by Far Western Blotting to bind directly the kinase C-terminal Src kinase (Csk), a potent negative regulator of Src (Di Stefano *et al.*, 2007). The physiological significance of p140Cap interaction with Src and Csk relates to p140Cap ability to regulate Src activation and downstream signaling (see below).

By two hybrid screen in human brain, the C-terminal motif of p140Cap has also been found to associate with the SH3 domain of Vinexin (Ito *et al.*, 2008), belonging to a family composed of vinexin, c-Cbl associated protein/ponsin, and Arg-binding protein 2 (Kioka *et al.*, 2002; Matsuyama *et al.*, 2005). In non-neuronal cells, Vinexin is localized at focal adhesions and shown to be involved in growth factor- and integrin-mediated signal transduction, actin cytoskeletal organization, cell spreading, motility, and growth (Kioka *et al.*, 2002). Always in brain, p140Cap directly associates with all the members of the microtubule plus-end tracking protein EB family through a short 92 amino acid C-terminal region, likely through a positively charged S/P-rich region (Jaworski *et al.*, 2009). The p140Cap interaction with Vinexin and EB family proteins in tumour cells remains to be established.

Finally, in breast cancer cells, p140Cap has also been shown to bind with Cortactin (Damiano *et al.*, 2011). 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 (Weed *et al.*, 2000; Wu and Parsons, 1993; Wu *et al.*, 1991). p140Cap/Cortactin association requires the second proline-rich domain of p140Cap and the Cortactin SH3 domain, suggesting a direct interaction between the two proteins. p140Cap binding to Cortactin controls invasion properties of breast cancer cells (Damiano *et al.*, 2011).

In conclusion, p140Cap is involved in direct interactions with several proteins (Figure 1). The p140Cap binding partners are mainly implicated in membrane fusion and actin cytoskeleton remodelling. p140Cap association to p130Cas, Src, Cortactin and the presence of a putative actin binding domain in the p140Cap sequence, suggest that p140Cap could be an actin binding protein. Indeed, p140Cap has been described to co-localize with actin stress fibers and cortical actin both in epithelial and in neuroectodermal cells (Chin *et al.*, 2000; Di Stefano *et al.*, 2004; Jaworski *et al.*, 2009).

4.3 140Cap in human breast cancer

So far, few data are available on p140Cap in human tumors. Immunohistochemistry analysis of normal mammary tissue show that p140Cap expression is confined to the luminal cells of alveoli, suggesting that in normal conditions p140Cap might play a role in mammary cell differentiation. In contrast, in human breast tumours p140Cap is not expressed in 70% of tumour specimens, showing an inverse correlation with the state of malignancy.

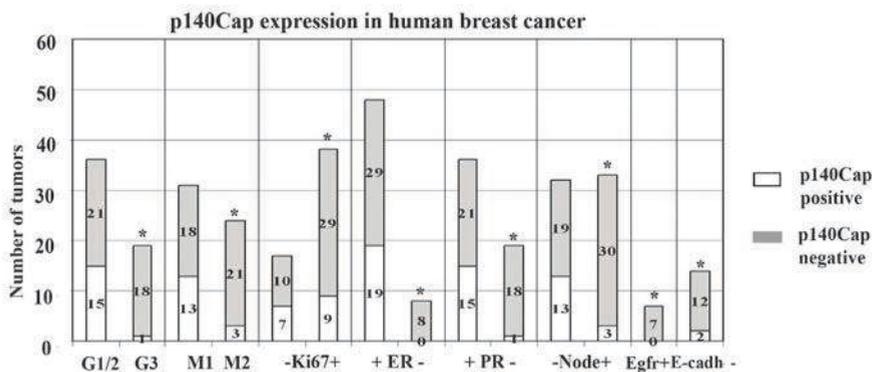


Fig. 7. p140Cap expression is lost in aggressive human breast cancers. In the histogram we reported the number of tumours positive (white) or negative (grey) for p140Cap expression according to tumour grade (low grade G1/G2, high grade G3), number of mitosis 10/10 HPS (M1 Mitosis < 10, M2 Mitosis > 10), Ki67 proliferation index (Ki67+ > 24%, Ki67- < 24%), Estrogen Receptor staining (ER-, ER+), Progesterone Receptor staining (PR-, PR+), infiltration in lymph nodes (Node+, Node-), EGFR staining (EGFR+), E-Cadherin staining (E-Cad-). The figure is modified from Damiano *et al.*, 2010.

Interestingly, 94.8% of aggressive G3 tumours, 87% of the Node +, 86.5% of tumours with a mitosis major number of 10/10HPF, and 76% of highly proliferative tumours (revealed by Ki67 staining), lose p140Cap expression. Moreover, none of the E-cadherin negative and EGFR positive tumours express p140Cap, suggesting mutually exclusive correlation between EGFR and p140Cap expression (Figure 7) (Damiano *et al.*, 2010). Therefore, although limited, these data point out that only low grade breast tumors express p140Cap. Further analysis is required to draw a general picture of the relevance of p140Cap in human breast cancers, and to delineate a potential use of p140Cap as a diagnostic and prognostic factor.

4.4 p140Cap modulates Src activity and EGFR signalling in breast cancer cells

The major function of the p140Cap adaptor is its ability to regulate Src kinase activation. In particular, in breast cancer cells, upon cell-matrix adhesion or EGF stimulation, p140Cap activates the Csk kinase, that phosphorylates the negative regulatory tyrosine 530 on the C-terminal domain of Src (Latour and Veillette, 2001), resulting in inhibition of Src kinase. Consistently p140Cap silencing increases Src activation, leading to a fine tuning of integrin and growth factor receptor signalling (Figure 2) (Damiano *et al.*, 2010; Di Stefano *et al.*, 2007). As a consequence, in breast cancer cells expressing high levels of p140Cap, upon integrin-mediated adhesion, the association between Src and Fak is impaired as well as integrin-dependent p130Cas phosphorylation (Figure 2). As described above p130Cas phosphorylation leads to the assembly of a p130Cas-Crk signalling complex that drives for cell migration and invasion through activation of Rac. Therefore elevated levels of p140Cap severely impair integrin-dependent Rac activity, while its down-regulation induces a sustained Rac activation (Di Stefano *et al.*, 2007).

In MCF7 breast cancer cells, p140Cap functionally interacts with E-cadherin and EGFR at the cell membrane, behaving as a new player in E-cadherin-dependent down-regulation of EGFR signalling. Indeed p140Cap-dependent inhibition of Src kinase activity results in E-cadherin immobilization at the cell membrane (Damiano *et al.*, 2010). E-cadherin is known to inhibit EGFR, either by interaction through the extracellular domains or by a beta catenin-dependent mechanism (Perrais *et al.*, 2007; Qian *et al.*, 2004; Takahashi and Suzuki, 1996). Consistently, EGFR activation, association and phosphorylation of Grb2 and Shc and Ras/Erk1/2 MAPK activities are profoundly impaired by p140Cap over-expression and enhanced by its silencing (Damiano *et al.*, 2010). Interestingly, rescue of Src activity and of E-cadherin mobility is sufficient to recover EGFR phosphorylation, but not Ras and Erk1/2 activation, that require an active RasV12, suggesting that p140Cap might regulate the Ras pathway through an additional mechanism. Therefore, in MCF7 cancer cells, p140Cap regulates EGFR signalling with dual mechanisms, involving both an E-cadherin-dependent inactivation of EGFR and a Ras-dependent inhibition of Erk1/2 activity (Damiano *et al.*, 2010).

Moreover, p140Cap expression also inhibits EGFR, Src and Erk phosphorylation in the highly aggressive MTLn3-EGFR breast cancer cells. Interestingly, in these cells, p140Cap affects also Cortactin phosphorylation in response to EGF (Damiano *et al.*, 2011).

4.5 p140Cap affects cell proliferation and in vivo tumour growth of breast cancer cells

The ability of p140Cap to regulate Src and Ras pathways profoundly affects cell proliferation. Elevated expression of p140Cap in both breast and colon cancer cells inhibits *in vitro* proliferation, but does not affect cell survival (Damiano *et al.*; Di Stefano *et al.*, 2007). Interestingly, p140Cap over-expression impairs colony formation in soft agar, while its silencing leads to a significantly increased number of colonies, demonstrating that p140Cap, likely through the regulation of integrin signalling, controls anchorage-independent growth (Di Stefano *et al.*, 2007). *In vivo* xenografts of breast and colon cancer cells show that cells expressing high levels of p140Cap are impaired in tumour formation. Consistently, p140Cap silencing in carcinoma cells dramatically increases *in vivo* tumour formation. Strikingly, p140Cap knock-down is sufficient for *in vivo* growth of MCF7 cells even in the absence of estrogen pellets, a condition in which control cells are unable to grow. These last findings also rise the possibility that p140Cap may regulate estrogen receptor signalling, contributing

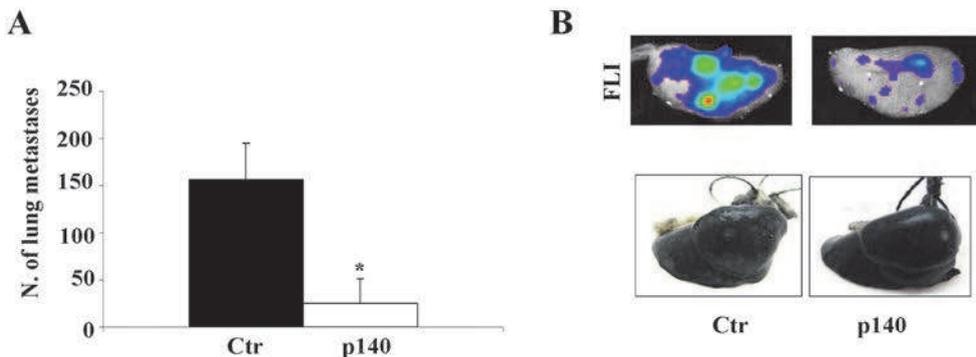
to breast cancer resistance to hormonal therapies. Thus these data provide evidence that p140Cap behaves mechanistically as a tumour suppressor molecule in breast and colon cancer cells, with a broad effect on cell proliferation and tumorigenesis.

4.6 p140Cap affects *in vitro* motility and invasion of breast cancer cells

As expected for the major role of Src in actin cytoskeleton dynamics and cell migration, high levels of p140Cap impair spreading and extension of lamellipodia and filopodia on extracellular matrix proteins of breast cancer cells. In addition, p140Cap over-expression also inhibits migration on fibronectin-coated transwells and invasion in Matrigel. Consistently, p140Cap silencing induces an increase in cell spreading in the early phases of cell adhesion, a fibroblastic-like shape and increased motility and invasion. Cells expressing a truncated form of p140Cap, lacking the Src-binding domain, restores integrin-dependent Src and Rac activation and are capable of migrating and invading properly (Di Stefano *et al.*, 2007).

In addition, p140Cap specifically interferes with invasive and migratory properties of cancer cells blocking E-cadherin/EGFR cross-talk in both breast and colon cancer cells. The ability of p140Cap to immobilize E-cadherin at the cell surface strengthens cell-cell adhesion and inhibition of cell scatter in response to EGF. Rescue of Src activity by the expression of a kinase-defective Csk mutant or by Csk silencing, recover E-cadherin mobility at the cell surface and the ability to scatter in response to EGF (Damiano *et al.*, 2010)

Moreover, we recently identified 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. Our data show that increasing p140Cap expression in the highly aggressive MTLn3-EGFR cells results in an 80% decrease in *in vivo* lung metastasis formation (Figure 8).



The figure is modified from Damiano *et al.*, 2011.

Fig. 8. p140Cap over-expression inhibits spontaneous lung metastasis formation.

A) 5×10^5 Ctr and p140 cells were injected subcutaneously in Rag2^{-/-}γc^{-/-} mice. Right panels: after sacrificing the mice, lungs were coloured 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).

B) 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 coloured with ink are shown.

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 tumour cells. 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. Remarkably, p140Cap over-expressing cells display an increased number and area of focal adhesions, which correlate with the presence of actin stress fibers consistent with a less dynamic turnover of adhesive structures. 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 in response to EGF is decreased. Indeed, the expression of the phosphomimetic cortactin mutant is sufficient to completely rescue the defects in migration and invasion of MTLn3-EGFR 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 (Damiano *et al.*, 2011).

5. Conclusions

As outlined in this chapter p130Cas and p140Cap adaptor proteins represent key elements in the control of cell migration and invasion in breast cancer cells. Interestingly, in breast cancers, p130Cas results frequently over-expressed, while p140Cap is not expressed in the more aggressive human breast cancers. Interestingly, Src kinase is a common target of these two proteins. However, even though both p130Cas and p140Cap have been described to bind to Src, they exert opposite roles on Src activity. Indeed p130Cas enhances and sustains Src activity, while p140Cap is a negative regulator of Src kinase. Therefore, it is likely that Src activity is finely tuned by p130Cas and p140Cap relative expression in cells in which they are co-expressed. As a consequence in breast tumors their reciprocal levels of expression might profoundly influence the ability of cancer cells to acquire invasive properties. Although still limited, the analysis of human breast tumors suggests that an overbalance towards p130Cas over-expression might represent a negative prognostic marker in human breast cancer specimens, indicating progression to a more aggressive phenotype.

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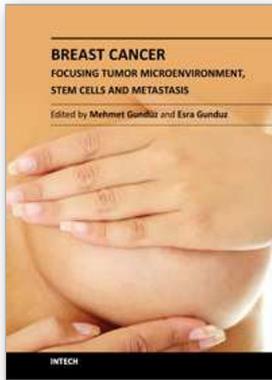
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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