PhD Thesis:

Analysis of the role of liprin proteins in breast cancer cell invasion

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Academic Year 2013/2014
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my Director of Studies Prof. Ivan de Curtis, for giving me the opportunity to carry out my PhD project in his laboratory and supervising all my work with extreme patience and motivation.

I would like to thank all the girls that shared with me these years in the lab. Valentina, Anna, Roberta, Elena, Romina, Kristyna, Martina, Vera, Diletta, Elisa, thank you for the discussions, the help, the funny moments and the support during hard moments.

I thank also my dad, Vale and Teresa for the moral support. And not only moral.

Finally, I want to thank Davide and Samuele that are my motivation for going on.
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ABSTRACT

The metastatic process requires the ability of cancer cells to break the basement membrane and migrate through a complex three-dimensional environment. The laboratory has recently identified the protein liprin-α1 as an important regulator of integrin-mediated focal adhesion dynamics and cell motility in non-neuronal cells (Asperti et al., 2009, Asperti et al., 2010). Liprins are a family of cytosolic scaffold proteins including the liprin-α and liprin-β subfamilies based on sequence similarities (Serra Pagés et al., 1998). The human genome encodes four liprin-α (liprin-α1-4) and two liprin-β proteins (liprin-β1 and liprin-β2). Interestingly, the gene PPFIA1 for liprin-α1 is frequently amplified in tumors. Moreover, the levels of expression of the liprin-α1 protein are often increased in human breast cancers (Astro et al., 2011). Functional analysis has revealed that liprin-α1 is specifically required for migration and invasion in vitro of highly invasive MDA-MB-231 human breast cancer cells. The analysis of lamellipodia dynamics has revealed a decrease of the stability of these protrusions in cells depleted of endogenous liprin-α1, which are defective in cell motility. Furthermore, liprin-α1 silencing causes a reduction of tumor cell invasion through Matrigel. The examination of the invasive potential has demonstrated that liprin-α1 is important also for the degradation of the extracellular matrix (ECM) (Astro et al., 2011). Starting from these observations, the first aim of my project has been to investigate the function of liprin-α1 in vivo. I have generated MDA-MB-231-derived cell lines with either stable overexpression or stable depletion of liprin-α1, and I have used these cells for injection or transplantation in mice, to determine their invasive potential. The characterization of these cell lines in vitro has confirmed that liprin-α1 overexpression causes an increase of both cell migration on FN and invasion through Matrigel, by promoting the stability of the lamellipodia. On the other hand, liprin-α1-depleted cells have reduced ability to both migrate and invade in vitro. However, all the cell lines with altered liprin-α1 levels have shown similar proliferation rates and viability compared to the control MDA-MB-231 cells. To investigate the involvement of liprin-α1 in invasion in vivo, experimental metastasis assays and spontaneous metastasis assays were performed. In both assays, the formation of lung metastases by the modified and control breast cancer cell lines has been evaluated. The results indicated that liprin-α1 overexpression did not affect lung colonization. Considering the high invasive ability of MDA-MB-231 wild type cells, increase in lung colonization by liprin-α1 overexpression may be irrelevant in vivo. On the contrary, injection of liprin-α1-depleted cells resulted
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in the reduction of the formation of lung metastases compared to control cells. This is the first evidence that liprin-α1 is not involved in primary tumor growth, while it is important for tumor cell invasion.

Being a scaffold protein, liprin-α1 is unlikely to act alone as a regulator of tumor cell invasion. Previous studies have described the interaction between liprin-α1 and liprin-β1 (Serra-Pages et al., 1998), and have suggested a possible role of liprin-β2 in migration and invasion (von Thun et al., 2012). However, the available data on the functions of liprin-β proteins and their relationship with liprin-α1 are not exhaustive. As the second aim of my PhD, I have addressed the biochemical interaction of liprin-α1 with either liprin-β1 or liprin-β2, and I have tried to elucidate the role of the two proteins in cell motility and invasion. While liprin-α1 interacts with liprin-β1, it does not interact with liprin-β2. This is the first evidence of the different ability of the two liprin-β proteins to interact with liprin−α1. The biochemical analysis has shown that the interaction between liprin-α1 and liprin-β1 occurs via the C-terminus of liprin-α1, and that two of the three SAM domains of liprin-α1 are sufficient to mediate this interaction.

The study of the subcellular localization has indicated that liprin-β1 colocalizes with liprin-α1 at the cell edge, whereas liprin-β2 partially colocalizes with cortactin-positive invadopodia. Functional analysis has shown that liprin-β1 silencing did not affect cell invasion through matrigel, whereas liprin-β2 silencing led to an increase of cell invasion, and enhanced ECM degradation, supporting the hypothesis of the different role of this protein in regulating the function of invadopodia with respect to liprin-α1 and liprin-β1. Analysis of the involvement of liprin-β1 and liprin-β2 in cell migration underlined the different effects of the two proteins. As previously observed for liprin-α1 (Astro et al, 2011), silencing of liprin-β1 led to a decrease of the speed of the cells in random migration assays. On the contrary, liprin-β2 silencing did not significantly affect cell motility. These data support the hypothesis of a cooperation between liprin-α1 and liprin-β1 in regulating cell motility, while they indicate that liprin-β2 does not have a relevant role in this process.

Altogether the work presented in my thesis sustains a key role of liprin-α1 as a positive regulator of the invasive apparatus of tumour cells in vivo, and has highlighted for the first time first distinct roles of liprin-β1 and liprin-β2 in tumor cell motility.
1 INTRODUCTION

1.1 Pathogenesis of Cancer disease

In the 70s, Stehelin and colleagues identified for the first time mutations in a set of regulatory genes that bestowed to the cells malignant properties (Stehelin et al 1976). Starting from this discovery, it has been proposed the theory of somatic mutation (SMT). This theory sustains that cancer is a disease that begins at cellular levels with a somatic mutation (M. Bizzarri et al., 2008). In the years, great efforts have been done to identify the genetic alterations that trigger carcinogenesis. Nowadays, tumorigenesis is widely considered a multistep process consisting in the progressive acquisition of molecular alterations that drive the transformation of a normal cell to a malignant cell. The acquisition of several alterations leads, in the time, to a considerable heterogeneity among cancers (Hanahan and Weinberg, 2000). Despite more than 100 types of cancers have been described, in the last decade scientists have identified a small number of circuits that control cell homeostasis and that are altered in almost the totality of cancers. Genetic alterations in these circuits confer to the cells specific capabilities, identified as the six hallmarks of cancer (Fig 1.1) (Hanahan and Weinberg, 2000). These hallmarks have been described as: self sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis ability, sustained angiogenesis, limitless replicative potential, tissue invasion and metastasis. None of these hallmarks can independently lead to a malignant transformation of a normal cell, but the combination among them allows cells to survive, proliferate and disseminate. After a decade, several studies have identified two new capabilities that are common to cancer cells and that have been added to the list of the initial six hallmarks: the metabolic reprogramming and the ability to evading immune response (Fig 1.2) (Hanahan and Weinberg, 2011). Moreover, two new enabling characteristics have been considered driving forces of transformation: genomic instability and tumor-promoting inflammation. In particular, it is evident that inflammation occurs in the early stages of tumorigenesis and can contribute to the acquisition of the hallmarks by supplying proliferative, survival and proangiogenic factors. Although the acquisition of the hallmarks is a concept widely accepted, recently, new theories and evidence about the bases of the cancer disease have added complexity to the scenario. An alternative view about the origin of the cancer disease sustains that cancer is a tissue-based disease rather than a somatic mutation-based disease. In contrast with the somatic mutation theory, it has been proposed the theory of the tissue organi-
zation field (TOFT) that identifies the reciprocal interaction between the stroma and the parenchymal cell as the target of alteration rather than the DNA. According to this theory, the chronical altered interactions between the stroma and the parenchima of a morpho-genetic field result in carcinogenesis. This theory suggests also that the acquisition of the hallmarks is a consequence of this altered link (Laconi 2007; Sonnenschien and Soto, 2013). Another emerging concept is the reversibility of cancer transformation. It has been proposed that the re-establishment of a physiologic interaction between cancer cells and microenvironment may reverse the neoplastic phenotype. In support of this hypothesis many studies indicated that “cancer can be epigenetically be reprogrammed into normal cell types” (Li et al., 2003). Supporting this idea, it has been shown that a neoplastic cell introduced into a blastocyst or implanted in a normal microenvironment, contributes to develop a normally structured organ (Lee at al., 2005; Cucina et al., 2006).

Although the SM and TOF theories are still under debate, it has become widely accepted that cancer is not only a mass of uncontrolled proliferating cells but a complex tissue composed by different types of cells interacting and influencing each other and with the microenvironment (Hanahan and Weinberg 2011). In particular, the role of the stroma as a promoter of cellular proliferation, genomic instability and cell motility is today object of increasing interest and attention (Laconi 2007).
Breast cancer: complexity and classification

Breast cancer is the most frequently diagnosed cancer among women ranking as second (10.9% of all cancers) and fifth as causes of death for cancer worldwide (Ferlay et al., 2010). Breast cancer is not a unique and simple disease, but it is an heterogeneous and complex pathology that includes different histopathological subtypes with genetic and genomic differences (Ellsworth et al., 2009). Traditional classification have regarded in particular the tissue components originating the tumor. Based on this criterion, breast cancers have been divided into two main groups: carcinomas and sarcomas. Sarcomas originate from the connective tissue of the mammary gland and are less frequent whereas carcinomas originate from the epithelial component of the organ (cells from the lobules or the ducts). The malignat lesion originating from the ducts is the ductal carcinoma in situ.
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(DCIS), that is the most common breast cancer, including the 80% of all breast cancer. This carcinoma can become invasive and develop an infiltrating ductal carcinoma (ICD). (Fig 1.2) This lesion presents cells that infiltrate the breast connective tissue, lymphatic vessels, intercostal vein and can interest also the vertebral plexus. The dissemination leads to a colonization of distant organs in particular lymph nodes, bones, lungs and nervous system (Vargo-Gogola and Rosen, 2007).

Tumor subtypes have been classified based on tumor size, lymph node involvement, histopathological grade, patient’s age and the histological evaluation of the expression of hormones receptor (oestrogen receptor ER, progesteron receptor PR) and epidermal growth factor receptor HER-2/ERBB2 (Yersal et al., 2014). However, recent studies have focused on the gene expression profile of breast cancer ductal carcinoma in order to provide a new classification of the different subtypes based on gene expression patterns (Eroles et al., 2012) (Fig 1.3). Perou and colleagues have identified two main classes of tumors described as ER-positive and ER-negative cancers (Perou et al., 2000). ER-positive tumors are characterized by a high expression of the gene specific of breast luminal cells such as ER-responsive genes and luminal cytokeratins (CK) but they do not express HER-2 at high levels. They constitute the group of the ER-positive luminal-like tumors. Other studies have identified subclasses of luminal cancers that have been divided in luminal A and

Figure 1.3 Schematic illustrating various breast cancer subtypes. The blue and pink rectangles group the subtypes based on the expression of ER/PR, positive in the blue (Luminal A and Luminal B) and negative (HER2+ and basal-like) in the pink. The central grey rectangle (with black outline) indicates the presence of HER2 amplification in Luminal B and HER2+ subtypes. Adapted from R. Sandhu et al., Labmedicine 2010.
luminal B (Sørlie et al., 2001). The ER-negative tumors are further divided into three subclasses: basal-like, HER-2 positive and normal-like tumors (Fig 1.3) (Yersal et al., 2014).

**Luminal A tumors:** they are the most common subtypes of cancers representing the 50-60% of all breast cancers. These cancers often present low histological grade and low proliferation rate (low expression of Ki67), so having a good prognosis and low rate of relapse. Relapse can frequently occur in bone (Kennecke et al., 2010). They are ER- and PR-positive and HER2-negative. Luminal A cancers are characterized by the expression of luminal epithelial cytokeratines 8 (CK8) and 18 (CK18) and other luminal markers such as hepatocyte nuclear factor 3 alpha (FOXA1), X-box binding protein1 (XBP1), GATA-binding protein 3 (GATA-3), B cell lymphoma 2 (BCL2), erb3 and erb4.

**Luminal B tumors:** these tumors constitute the 15-20% of breast cancers, they are also ER-positive but are more aggressive than the luminal A ones, presenting both higher proliferation rate and rate of relapse. In fact, they express higher levels of growth factor receptors such as fibroblast growth factor receptor 1 (FGFR1) and HER1, high levels of PI-3K, Src and proliferation-related gene such as cyclin E (CCNE1) and Ki67. Since there is not a clear distinction between luminal A and luminal B cancers, the evaluation of the levels of Ki67 has been proposed to be useful to discriminate the two subtypes of tumors. The overall survival of luminal B breast cancers is worse than the luminal A ones being similar to the basal-like and HER-2 positive cancers.

**HER-2 positive:** the HER-2 subtypes of cancer includes the 15-20% of breast cancer subtypes. These cancers are characterized by the high expression levels of HER-2 gene and genes correlated to the HER-2 pathway, and low expression of ER. They are more aggressive because of their higher rate of proliferation in comparison to the luminal cancers, higher histological grade and a higher rate of p53 mutations (Tsutsui et al., 2003). These cancers commonly give rise metastasis in the brain and visceral organs.

**Basal-like tumors:** this subtype represents the 8-37% of all breast cancers. They are also indicated as triple-negative cancers. However, the terms triple negative and basal-like are not completely synonymus. Triple-negative tumors lack the expression of ER, PR and HER-2 genes and constitute a subclass of basal-like cancers even though the term triple negative is more used in clinical settings. Basal-like cancers have altered expression levels of gene promoting cell proliferation, survival, migration and invasion. Basal-like cancers show a de-regulation of some signaling pathways such as MAPK, PI-3K, Akt, NF-kB and beta-catenin. Several myoepithelial markers have been identified for basal-like tumors, such as CK5, CK14, CK17 and laminin. It has been also reported that
basal-like tumors overexpress P-cadherin, fascin, caveolins-1 and 2 and EGFR. Finally they frequently harbor mutation in tumor protein 53 (TP53) gene and present inactivation of retinoblastoma (Rb) pathway (Hetiz et al., 2009). Basal-like subtype is often correlated with mutation in BCRA1 gene, constituting the 75% of the BCRA1 gene-related cancers.

Normal-breast like: they represent the 5-10% of all breast cancers. This subclass is poorly characterized and these tumors are often grouped with fibroadenoma and normal breast. Since they are negative for ER, PR and HER-2, sometimes they are classified as triple negative cancers, even thought they are not basal-like carcinomas because they lack the expression of CK5 and EGFR. They have an intermediate prognosis between luminal and basal-like cancers. Although molecular profiling has provided many information about gene expression of particular subtypes of breast cancers, some critics have been raised in particular about the validation of the new data and their clinical utility. The identification of some subclasses such as luminal A and B is not completely resolved and the difference between triple-negative and basal-like is disputed, yet. However, molecular characterization has contributed mainly in the modification of treatment protocol related to ER, PR, HER-2 and Ki67 status of breast cancer.

From the clinical point of view, ER-positive cancers are considered the more treatable ones since they can respond to endocrine therapies. HER-2 positive tumors can be treated with anti-ERBB2 therapies while triple negative cancers are not responsive to these treatment and can receive the classical chemotherapy. However, the main cause of death in breast cancer patients is the giving rise of long-term metastases. Because of this, the study of the mechanisms regulating the formation of metastasis constitutes a central issue to be clarified.

1.2 From tumor to metastasis: theories on metastatization

Metastasis derives from the evolution of a specific cancer cell sub-population whose expansion is promoted mainly by genomic instability and heterogeneity of tumor cells (Chiang et al., 2008). Metastasis formation is a multisteps process that can be synthetized in: loss of cellular adhesion, increased motility and invasiveness, entry and survival the circulation, exit into a new tissue and its colonization. The entire process is very inefficient: 10 000 cells can be shed in the circulation every day but only the 0.01% can survive and give rise to metastases (Chambers et al., 2002). Tumor cells die during the metastatic
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Fig. 1. 4 Linear and parallel models of metastasis. In the linear model of metastasis genetic modifications progressively accumulate in cancer cells of the primary tumor, and a more aggressive subpopulation is selected. Cells acquiring metastatic capacities are more effective in colonizing distant organs. In the parallel model of metastasis, cancer cells disseminate early during tumor progression, at a stage when the primary lesion is small or possibly even pre-malignant. In breast cancer both models are supported by clinical and experimental evidences. Metastatic cells can disseminate through lymphatic vessels and lymph nodes first or directly through the blood stream. Cancer cells recirculating from metastases to primary tumors might contribute to bad prognostic signatures. P, primary tumor; M, metastasis. Adapted from Lorusso and Ruegg, Seminars in cancer biology, 2012.

process mainly because of the hemodynamic forces of blood circulation and the induction of anoikis due to lack of cell-cell and cell-matrix adhesions (Lorusso and Ruegg, 2012). The basic events of metastatic process, such as the enhanced cell invasion and the entry in circulation system are common to different types of tumor, while others such as colonization of specific organs are peculiar for types of tumor and types of target organs. Two theories have been proposed to describe the metastatic dissemination process: the linear and the parallel theory (Fig 1.4). According to the linear theory, cells in the primary tumor acquire progressively genetic modifications and as the tumor growth, a subpopulation of
more-aggressive cells is selected. Because of this, it has been thought that dissemination occurs when the primary tumor has grown considerably. Furthermore, metastasis-driving mutations should be detected in metastatic foci and only rarely found in primary tumor (Chaffer and Weinberg, 2011). On the other hand, experimental observations of the breast cancer tumorigenesis have shown that dissemination occurs also during the early stages of tumorigenesis and also at pre-malignant stages. From these evidence it has been proposed the theory of the parallel metastatic process.

This theory sustains that dissemination is a process concomitant and independent from the primary tumor growth (Klein et al., 2009). Moreover, this theory hypothesizes that
metastatic cancer cells may recirculate from metastases to primary tumors, thus contributing to the onset of drug-resistance of the primary tumor and to a bad prognostic signature. This process is called reseeding of metastatic cells (Lorusso and Ruegg, 2012).

### 1.2.1 Early events in metastasis formation

At the bases of metastasis formation there is the ability of cancer cells to detach from the primary tumor and migrate through the extracellular matrix invading surrounding tissues. The invasiveness of cancer cells can occur mainly by three mechanisms: epithelial-mesenchymal transition (EMT) that drives individual cell migration, collective invasion and macrophage-tumor cell feed-back loop (Fig 1.5) (Cheung and Ewald, 2014).

#### 1.2.1.1 EMT in breast cancer

Initially, to detach from the primary tumor, cells need to break the cell-cell interactions, to remodel cell-matrix adhesion sites and follow a stimulus through the extracellular matrix. In this way, non-motile epithelial cells acquire a non-polarized, motile and invasive mesenchymal phenotype. This phenomenon is temporary and reversible and it is known as epithelial-mesenchymal transition (EMT) (Thiery et al., 2006). EMT also occurs in non-pathological conditions such as embryonic development. Similarly, in cancer-associated EMT, molecular pathways typical of embryonic development are re-activated (Polyak et al., 2009). EMT can be induced by cellular intrinsic signal such as genetic mutations, or external stimuli such as growth factor signaling, (TGFβ, HGF, EGF, IGF) (Zavadil et al., 2005), tumor-stroma interaction and hypoxia (Foroni et al., 2012). As mentioned, the first characteristic of EMT is the involvement of developmental transcription factors such as Snail1 and 2, Slug, ZEB1, ZEB2, Twist, Goosecoid, FOXC1, FOXC2 and E47 (Moody et al., 2005; Foroni et al., 2012). Moreover other pathways such as Wnt, Notch and integrin signals can regulate EMT. (Fig rev foroni) Moreover, emerging evidence underlines the role of microRNAs in inducing EMT-trascription factors and their regulation (Zhang et al., 2014). For example it has been demonstrated that Twist induces the expression of miR-10b. This microRNA targets and represses the expression of a homeobox gene relieving transcriptional inhibition of RhoC, a pro-metastatic Rho family GTPase. On the other hand, it has been shown that several other miRNAs have a metastasis suppressor effect (Fig 1.6) (Wang and Wang, 2011). A second characteristic of EMT,
is the loss of the E-cadherin expression. Loss of E-cadherin promotes actin-cytoskeleton rearrangement and changes in cell polarity promoting cell motility. Associated to the loss of E-cadherin it has been also observed a high expression of N-cadherin and vimentin in tumor circulating cells (Roussos et al., 2010). Another foundamental feature of EMT is its reversibility. In fact, upon the arrival in a new environment, cancer cells undergo mesenchymal-epithelial transition (MET), that restores the epithelial phenotype and allows cell proliferation and organization to give rise metastases. The role of hypoxia in EMT seems to be the support of long-lasting inflammation, caused by an increase of production of oxygen-reactive species which in turn activate HIF-1alpha and NF-kB signalling.

### 1.2.1.2 Individual tumor cell migration

Once loss of cell-cell junction has occurs, tumor cells can migrate and invade surrounding tissue individually, by adopting mesenchymal/elongated or amoeboid/rounded phenotype (Friedl and Wolf, 2010). The amoeboid/rounded migration occurs when low adhesion forces or high actomyosin contractility are involved in the cell. The amoeboid migration is characterized by the formation of Rac-dependent filopodia and small adhesion sites. Amoeboid movement can be alterantively induced by Rho-mediated blebbing of the membrane without the formation of defined adhesion sites (Friedl and Alexander, 2011).
An important characteristic of amoeboid migration is the lack of ECM degradation because cells migrate by adapting their shape to the tissue gaps and trails. Differently, mesenchymal migration occurs when cells develop prominent cytoskeletal protrusions and a great number of adhesion sites with clusters of integrins. In this case, proteases assemble at the cell membrane generating microtrack in the ECM allowing cell to penetrate the matrix. Different levels of stiffness of the matrix can influence the way of migration by promoting either an elongated or a rounded phenotype (Friedl and Wolf, 2009). Indeed, elongated or mesenchymal migration occurs when cells invade a highly rigid ECM. On the other hand, migration in a 3D environment that generates low tension force, leads cells to the acquisition of a rounded morphology. However, cells that migrate individually can switch from an elongated/mesenchymal migration to a rounded/amoeboid migration based on the 3D environment and the activation of different pathways inside the cell. In fact, the switch is mainly regulated by the balance between the activity of Rac and Rho GTPases. In particular, a decrease in Rac activity and a concomitant activation of Rho-mediate actomyosin contractility can induce a change from mesenchymal to amoeboid migration. The Rac/Rho balance can be regulated by different factors such as the therapeutic inhibition of metalloproteases (MMPs), the inhibition of chemokine-mediated Rac activation, the activation of Rho by inhibition of its GAP p190RhoGAP, or the engagement of indirect Rho activators like EphA2 (Gérard et al., 2007; Parri et al., 2009).

1.2.1.3 Macrophage-tumor cell interaction

In a physiological condition, for example during wound healing, macrophages can coordinate tissue repair by inducing cell migration, matrix remodelling and angiogenesis (Cousins and Werb, 2002). Clinical and experimental evidence has shown that tumor cells can recruit a specific population of macrophages, named tumor-associated macrophages (TAMs) that are able to facilitate tumor progression and metastasis formation. TAMs can promote tumor cell invasion through different mechanisms. It has been demonstrated that malignant cells can create a microenviroment that activates the macrophage function as in the physiological context by secreting chemoactrant molecules. Tumor-associated macrophages, in turn, secrete growth factors and other molecules supporting different processes that occur during tumor progression: chronic inflammation, matrix remodelling, tumor cell invasion, intravasation, angiogenesis and colonization of distant organs (Condeelis and Pollard, 2006). In particular, macrophages can promote invasion through
their ability to remodel extracellular matrix and to secrete growth and angiogenic factors (Condeelis and Pollard, 2006). For example, TAMs promote tumor cell extravasation and survival by producing vascular endothelium growth factor (Qian et al., 2009). The bidirectional paracrine signaling between macrophages and tumor cells is called macrophage-tumor cell feedback loop. Moreover, TAMs support tumor cell survival through the activation of the PI3K-Akt pathway. In fact, Cheng and colleagues showed that the integrin-α4 expressed on the surface of metastasis-associated macrophages binds vascular cell adhesion molecule-1 (VCAM1), which is highly expressed by metastatic cells in the lung. This interaction leads to VCAM1 clustering, which, in turn, activates PI3K-Akt, providing survival signals in tumor cells (Cheng et al., 2011). Moreover, a recent study has reported that a direct contact between macrophages and the tumor can induce RhoA-dependent formation of invadopodia in cancer cells (Roh-Johnson M et al, 2013).

1.2.1.4 Collective invasion

Several studies have shown that E-cadherin expression varies significantly among histological breast cancer subtypes. For example, only 10% of the ductal carcinoma presents the loss of E-cadherin (Gould-Rothberg et al., 2006). On the other hand E-cadherin is lost in the majority of lobular carcinomas. Since the ductal carcinoma is the most common breast tumor, while lobular carcinomas represent only the 10% of all the mammary carcinomas, it is reasonable to suggest that the majority of mammary tumors express E-cadherin. It has been also demonstrated that expression of E-cadherin induces tumor cells to migrate in a collective manner. In this direction, imaging studies demonstrate that collective invasion occurs in several solid tumors and many mechanisms have been proposed to explain this process (Nguyen-Ngoc et al., 2012). One mechanism that drives collective invasion is the presence of a cellular population that acts as leader for tumor cells. Two populations of cells have been identified as leaders of collective invasion: stromal fibroblasts and a subpopulation of breast cancer cells. Stromal fibroblasts are efficient in ECM remodelling and it has been demonstrated that they can create micro-tracks that drive cancer cells through the ECM. Interestingly, it has been observed that the ECM remodelling is regulated by Rho in fibroblasts, while tumor cells migration is under the control of Cdc42 pathway. Furthermore, the block of Rho signaling in fibroblasts can inhibit cancer cells invasion indicating that the targeting of the leaders is sufficient to impair the entire metastatic process (Gaggioli et al., 2007). Recently it has been proposed that fibro-
blasts can also support collective invasion of breast cancer cells in a subtype-dependent mechanism. Indeed, it has been shown that fibroblasts can lead invasion in basal subtypes but not in luminal cell lines. As mentioned, also breast cancer cells can promote collective invasion. Multiple studies indicate that within a tumor, cells with high migratory capability can act as leaders of collective invasion. For example, cells overexpressing MT1-MMP can create micro-tracks facilitating the migration of adjacent cells. A recent study has identified a specialized subpopulation of breast cancer cells that is able to trigger collective invasion (Cheung et al., 2013). Cheung and colleagues have observed that invasive leader cells express basal epithelial genes such as E-cadherin, cytokeratin-14 (K14) and the transcription factor p63. Leader cells interact with the follower cells via E-cadherin and do not undergo EMT. The expression of K14 has been described as a marker for the identification of the major population of leading cells. Consistently, knockdown of K14 blocks collective invasion in both 3D culture and in vivo. Although the presence of a population of leader cells has been demonstrated in different types of cancer even if the mechanisms that give rise to this population are not well understood. Probably, alterations in ECM composition and changing signals from the microenvironment, both gained and lost, may facilitate the emergence of an invasive cell subpopulation (Cheung and Ewald, 2014).

1.2.2 Late events in metastasis formation: extravasation and tissue colonization

Tumor circulating cells need to cross the vessel walls to colonize distant organs. In this step the permeability of the endothelium is fundamental. In some organs, as for liver and bone marrow, microvessels have high permeability, allowing tumor cells to easily extravasate. In other cases, as for the brain, blood brain barrier constitutes a wall not freely penetrable (Lorusso and Ruegg, 2012). After breaking the endothelial wall, tumor cells need to adapt to a new environment. Recently, several molecules have been identified that mediate tumor cells homing and colonization (Gupta and Massague, 2006; Fidler et al., 2003). For example, overexpression of selectin ligands on tumoral cell surface allows cancer cells to bind platelets and leukocytes, enhancing survival in the circulation. Another example is constituted by the amplified expression of CXCR4 and CCR7 receptor and ligands that allows breast cancer cells to arrest and migrate into a secondary organ. (Lorusso and Ruegg, 2012). The localization of metastasis does not occur randomly but is influenced by the expression of specific genes that confer a particular tropism.
and by the features of the target organ. These two aspects may explain why cells deriving from different types of cancer can colonize the same target organ. Recently, the genes that mediate the metastatization of breast cancer cells to the lung, bone and brain have been identified (Minn et al., 2009). In fact, microarray-based comparison of the parental cell lines to organotrophic metastasis has identified 43 genes overexpressed and 59 gene downregulated in bone metastasis induced by injection of triple negative breast cancer cell line MDA-MB-231 in mice. Genes such as MMP1, MMP2, LOX, CXCL12 have been shown to favour lung colonization by breast cancer cell, the expression of COX2 has been associated with brain metastasis and the expression of Src, NF-kB and VCAM1 has been observed in bone metastatic cells (Kang et al., 2003). After homing, metastatic cells can quickly produce a detectable metastasis. More frequently, the manifestation of a secondary lesion occurs after several years. This is indicated as a phenomenon called “metastatic dormancy”. This observation suggests that metastatic cells have a low rate of proliferation and are not able to give rise to a secondary tumor at the moment of the colonization. This ability is acquired with time, probably through the addition of new genetic and epigenetic alterations and in response to the signals from the new environment (Lorusso and Ruegg, 2008). However, there is a great variability in the rise of metastases by different types of cancer cells. The mechanisms that regulate the kinetic of this process are not well understood and their identification represents an important aim to try to prevent long-term cancer progression.

1.3 Dissecting cell motility: Molecular mechanisms of cell migration

Cell motility is a fundamental requisite of cancer cells to give rise to metastasis at distant organs. Motility involves dynamic cytoskeletal modifications, cell-matrix interaction, proteolysis, actin-myosin contractions and focal adhesion turn-over. These processes are spatially and temporally coordinated (Friedl and Wolf 2003). The migratory cycle starts in a cell that senses and responds to a stimulus activating actin polymerization and the extension of actin-driven protrusions (filopodia and lamellipodia). At the edge of the protrusion new adhesive site are generated and provide the traction force required to move the body cell forward. At the back of the cell, disassembly of focal adhesions and cell-surface detachment occur, thus allowing retraction of the cell rear and the translocation of the cell in the direction of the migration (Mattila and Lappalainen et al., 2008).
1.3.1 Actin cytoskeleton

The initial step of the migration cycle is the extension of a protrusion, driven by the dynamic assembly of actin filaments. Actin filaments grow below the plasma membrane and their fast polymerization produces the physical forces required for membrane extension. Actin filaments are characterized by an intrinsic polarity: they have a plus “barbed” fast-growing end and a minus “pointed” slow-growing end (Pellegrin and Mellor, 2007). This asymmetry allows barbed ends to drive membrane protrusion (Ridley et al., 2003). Three mechanisms have been described for the generation of barbed ends: de novo nucleation by Arp2/3 complex and formins, severing of pre-existing actin filaments by coflin, and uncapping of barbed ends on pre-existing filaments (Zigmond et al., 2004). In a cell, actin filaments are organized in networks that form different types of protrusions: lamellipodia, filopodia and podosomes (or invadopodia). Lamellipodia are flat, sheet-like membrane protrusions that drive migration through the attachment to the substrate, and generating the force to pull the cell body forward (Yamaguchi et al., 2006). Differently, filopodia are finger-like membrane extensions consisting of parallel bundles of actin filaments. Filopodia are considered to act as sensors that detect the local environment. Podosomes are actin-rich-structures generated by cells that physiologically cross the ECM, such as macrophages, and are required to remodel the ECM. Also cancer cells produce structures similar to podosomes in order to remodel the ECM and to allow cells to invade the surrounding tissues (Jiang et al., 2009). These structures are called invadopodia which are ventral protrusions composed by actin and actin regulatory proteins, such as cortactin, adhesion molecules, signal proteins and proteases. Actin can also organize with non-muscle myosin in complex and long structures called stress fibers. These structures provide the contractile force for cell movement. They can be divided into ventral stress fibers, dorsal stress fibers and arcs (Small et al., 1998). In particular, ventral stress fibers are linked on both ends to integrin-rich focal adhesions. Moreover, in motile cells ventral stress fibers are oriented parallel to the axis of locomotion thus providing the contractile force for tail retraction and disassembly of focal adhesions (Cramer et al., 1997). The organization and remodeling of the actin cytoskeleton is driven by a complex set of actin binding proteins (ABPs) (Pollard & Borisy, 2003). They include monomer-binding proteins (profilin), depolymerizing proteins (ADF/cofilin), nucleating proteins (Arp2/3, WASP, WAVE), barbed ends capping proteins (gelsolin), pointed ends capping proteins (tropomodulin), inhibitors of barbed end capping (DRFs, Ena/Vasp), F-actin stabilizing...
and crosslinking proteins (fascin, alpha-actinin) and F-actin anchoring proteins (cor-tactin, vinculin, spectrin). Furthermore, GTPases of the Rho family Rho Rac and Cdc42, play a central role in the regulation of the actin cytoskeleton and therefore in controlling cell migration.

1.3.2 The family of Rho GTPases

Rho GTPases are small proteins belonging to the Ras superfamily. They control acto-myosin cytoskeleton and cell adhesion (Wennenberg et al., 2005). The mammalian Rho GTPases comprise thirteen isoforms of Rho (Rho1-4 and Rho6-7, RhoA-E RhoG, Rho H), three isoforms of Rac (1 -3), Cdc42, and TC10 (Yoshimi et al., 2001). Small GTPases cycle between an active GTP-bound state and an inactive GDP-bound state (Fig 1.7). This cycle is regulated by guanine nucleotide exchange factors (GEFs) that activate GTPases, and GTPases activating proteins (GAPs) that inactivate Rho GTPases. About 80 GEF proteins have been identified in mammals. They present a Dbl homology domain (DH) important for GTPases activation, and a pleckstrin homology domain (PH) that is

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**Figure 1.7 The Rho GTPase cycle.** RhoGTPases act as molecular switches, cycling between two conformational states:GTP-bound active state and a GDP-bound inactive state. In the active state these GTPases bind to and activate downstream effectors to generate a biological response, until GTP hydrolysis switches the protein to the inactive state. The two classes of regulators that facilitate the switching on and off of Rho GTPases are the GEFs and the GAPs, respectively. Another class of proteins, the GDIs, keep the inactive GTPases cytosolic. Adapted from Etienne-Manneville, Nature, 2002.
important for their localization at the plasma membrane. The DH domain can bind the switch region of Rho GTPases and modify its conformation driving the release of GDP. Following that, GTP can bind the GTPase, activating it. The Rho GAP family consists of about 65 proteins. GAP proteins act by enhancing the intrinsic hydrolysis of GTP bound to Rho GTPases and by stabilizing their GDP-bound conformation (Rossman et al., 2005). In the cytoplasm, inactive Rho GTPases are complexed with guanosine nucleotide dissociation inhibitor (GDI). GDI binds Rho-GTPases through an immunoglobulin-like domain at the C-terminal region, and keep the GTPases in a GDP-bound form. The release of the GDP-bound GTPase from GDI occurs by a still unknown mechanism. Once GDI has released the GTPase, a GEF protein converts it in the GTP-bound form that can interact with downstream effectors. Rho, Rac and Cdc42 proteins localize in the cytosol and can reversible translocate to the plasma membrane (Yoshimi et al., 2001). Rho GTPases are involved in several cellular processes such as gene expression, cell adhesion, migration, cytokinesis, cell cycle progression, transformation and neuronal development (Etienne-Manneville & Hall, 2002). However, their main function is to regulate the assembly and organization of the actin cytoskeleton (Hall et al., 1996). In particular, Rho proteins regulate the formation of stress fiber and focal adhesion, while Rac and Cdc42 regulate the formation of lamellipodia and filopodia, respectively. The effects of Rho GTPases on the actin cytoskeleton occur through the action of downstream effectors. One of the identified Rho effectors is the serine/threonine kinase ROCK. This protein acts by interacting with many downstream targets such as the myosin binding subunit of myosin light chain phosphatase, myosin light chain, ERM, cofilin and mDia (Amano et al., 1996; Kimura et al., 1996; Maekawa, et al., 1999). It has been reported that ROCK and mDia cooperate to regulate the organization of actin cytoskeleton (Nakano et al., 1999). However, ROCK phosphorylates LIM kinase, which in turn activates cofilin that severs actin filaments (Maekawa et al., 1999).

1.3.3 Role of Integrins in cell-ECM interaction

Integrins are a family of transmembrane receptors that mediate cell-ECM and cell-cell interaction. These proteins are involved in several cellular processes such as embryonic development, tumor cell growth and metastasis, apoptosis, hemostasis and response of the cells to mechanical stress (E.A. et al., 1995). Integrins are heterodimeric proteins each composed by one α- and one β-subunit (Fig 1.8). Nineteen different α- and eight
β-subunits have been identified originating about 25 combinations. Moreover, the genes encoding both α and β subunits undergo alternative splicing, thus adding complexity to the possible combinations generated (Hynes et al., 1992). The extracellular region of integrins can bind many components of the cellular matrix such as fibronectin, collagen, vitronectin. Each integrin binds a specific ECM protein, even if a single ECM protein can be bound by more than one integrin. Some integrins can also bind soluble ligands such as fibrinogen or molecules on adjacent cells, such as the adhesion molecule ICAM, leading to homo- or heterotypic aggregation (Cadelwood, 2007). The short cytoplasmic domain of integrins does not have intrinsic enzymatic activity but aggregates cytoplasmatic proteins, both cystoskeletal and catalytic proteins, giving rise to large protein complexes and transmitting outside signals inside the cell (outside-in signaling). On the other hand, the interaction with intracellular ligands can induce a conformational change of the extracellular portion of integrins. In this way integrins can regulate their affinity for extracellular

Figure 1.8 The structure of integrins Integrin transmembrane receptors are composed by an α and a β chain. They are the structural link between actin cytoskeleton with the ECM, but also the functional link for signal transduction. From Pearson Education, 2009.
ligands (inside-out signaling) (Calderwood 2004). Cytoskeleton-associated proteins that interact with integrins are for example talin, vinculin, paxillin and kindlin, while one of the most important catalytic protein associated with the integrins is the focal adhesion kinase (FAK). In order to transmit the signals in the bidirectional way, integrin receptors can switch from a bent-inactive state in which the ligand binding site is close to the insertion of the transmembrane domain, to an extended-active state in which integrins bind their ligands. The binding to an extracellular ligand seems to stabilize the active conformation of the protein (Pinon and Wehrle-Haller et al., 2010). Ligand-binding causes integrins activation and clustering at the cell membrane. Integrin clustering leads to the formation of focal adhesions, proteins assemblies that connect the actin-cytoskeleton to the ECM (Clark and Brugge, 1995). Moreover, integrin activation can trigger intracellular signals such as protein phosphorylation, tyrosine kinase phosphorylation, activation of small-GTPases like Rho, variation in the concentration of intracellular calcium, changes in the production of phospholipids, regulation of intracellular pH and regulation of programme cell death (Harburger and Cadelwood, 2009). Moreover, integrins mediate the cross talk between different cellular pathways., The focal adhesion kinase FAK, following its activation by the β-subunit of integrin, seems to play a central role in this process. For example, FAK can interact with both the adaptor protein Crk and PI3K. Crk mediates integrin-mediated-Ras-signaling, while PI3K interacts with FAK after stimulation by platelet-derived growth factor. This results in a coordination of proliferation with actin cytoskeleton rearrangement (Ridley and Hall et al., 1994).

1.3.3.1 Integrin trafficking in cell migration

Integrins are internalized from the cell surface by both clathrin-dependent and clathrin-independent mechanisms. Integrins are recruited to clathrin-coated structures through the interaction between a conserved motif (NXXY motif) localized on the cytoplasmic beta-subunit and the adaptor protein AP2. In addition, it has been recently demonstrated that integrins recruitment for clathrin-mediated endocytosis occurs also through the interaction between a different integrin motif (NPXY) with other two adaptor proteins: DAB2 and Numb (Calderwood et al., 2003). On the other hand, integrins can be internalized in a clathrin-independent manner either through RAB21 by a cholesterol-sensitive caveolar mechanism (Pellinen et al., 2008), or through a direct interaction between the cytoplasmatic domain of β1 integrin and PKCα (Ng et al., 1999). After the internalization, integrins
are transported to the early endosomes, and then they are either sent to late endosomes and lysosomes for the degradation, or can be recycled to the plasma membrane. The recycling can occur either through Rab4-mediated mechanism or a mechanism involving Rab11 and Arf6 proteins. This process is regulated by several kinases and GTPases such as Rac pro-

Fig 1.9 Structure of a focal adhesion: A Transmembrane integrins that localize at focal adhesion bind both ECM and a complex of cytoskeletal proteins including alpha-actinin, vinculin, talin, paxillin and tensin that in turn bind actin filaments. B Signaling molecules that interact with FAK. FAK associates with the kinase Src, Csk, the adapter protein Crk and Grb2, the GEF Sos and C3G. A model for integrin signaling suggests that integrin clustering induces the phosphorylation of FAK Tyr 397. Once activated, FAK binds Src that in turn induces Ras activation at focal adhesion through Grb2-Sos complex Adapted from E. Clark and S. Brugge, Science, 1995.
tein (Caswell and Norman, 2008). The integrin traffic can influence cell migration through different mechanisms: by altering the recycling of growth factor receptors such as EGFR and VEGFR, and by regulating the actin polymerization and cell contractility through the Rho GTPases signaling (Caswell et al., 2009). In particular, Rho GTPases are closely linked to the endosomal transport. In fact, once inactivated, Rac protein recruited to the endosomal compartment. Endosomal membrane contains the Rac-GEF TIAM, that can re-activate Rac leading to its delivery to the plasma membrane. This allows the enhancement of actin polymerization that supports the extension of membrane protrusion for cell migration. Moreover, it has been demonstrated that different integrins can activate specific Rho-GTPases to regulate cell motility. For example, \( \alpha v \beta 3 \) activates Rac supporting the formation of lamellipodia, in this way promoting directed migration. On the other hand, the presence of integrin \( \alpha 5 \beta 1 \) promotes RhoA-dependent phosphorylation of cofilin, which causes the collapse of lamellipodia and increases random migration (Caswell, 2009).

### 1.3.4 The adhesion to ECM: focal adhesions assembly and disassembly

As mentioned, focal adhesions (FAs), or focal contacts, are adhesive sites that may be linked to actin-stress fibers, and contain actin binding proteins and adhesion molecules such as integrins. FAs have an elongated streak-like structure that are considered the mature form of integrin-mediated adhesive sites (Gardel et al., 2010). Other adhesive structures localized at the leading edge are the focal complexes, that represent the immature form of FAs (Nobes & Hall, 1995), and fibrillar adhesions, adhesive sites responsible of FN fibrillogenesis (Webb et al, 2003). The function of FAs is to stabilize the membrane protrusion and establish a structural and signaling link between the extracellular matrix and the actin cytoskeleton (Fig 1.9A) (Chan et al., 2010). Once actomyosin contraction has moved forward the cell, FAs disassembly allows further movement. The assembly of FAs occurs after the extension of a new membrane protrusion in order to stabilize cell-matrix contact. As the assembly, the disassembly of focal adhesions occurs at the cell edge, at the basis of lamellipodia, to allow the formation of new protrusions and the cell movement. The molecular mechanisms that regulate the assembly and disassembly of FAs are not well understood yet (Vicente-Manzanares et al, 2005). However, it is clear that Rho GTPases have a central role in these processes. Indeed, Rac and Cdc42 regulate the formation of nascent adhesions at the front, while Rho controls the assembly of mature focal adhesions and myosin-induced contractility at the cell rear. Rho proteins are in turn regulated by adhesion-related proteins like FAK, tyrosi-
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Introduction

ne kynases (Src), tyrosine phosphatases, and multiprotein complexes of G protein-coupled receptor kinase interactors (GITs). In particular, integrin-mediated adhesion induces the autophosphorylation of FAK in tyrosine 397. This residue is bound by the SH2 domain of Src kinase, which in turn can phosphorylate other residues of FAK. The FAK-Src complex activates the axis paxillin-GIT1-β-PIX-Rac1 by enhancing membrane protrusion, and inhibits cell contractility facilitating cell spreading (Huveneers & Danen, 2009). Moreover, the activated FAK-Src complex can promote Ras activation at focal adhesion through Grb2-Sos complex (Fig 1.9B) (Clark and Brugge, 1995). Focal adhesions disassembly is promoted by endocytosis of integrins in a microtubule (MT) dependent process (Ezratty et al., 2009), and by degradation by calpains of essential FA components such as FAK and talin (Chan et al., 2010). During cell migration, the formation of new adhesive sites stabilizes the membrane protrusions (Le Clainche & Carlier, 2008). When the cells move forward some of the focal complexes at the leading edge mature into FAs that are linked to the stress fibers, while FAs disassemble at the cell rear allowing the movement (Kaverina et al, 1998).
1.3.5 Lamellipodia formation: the driving force of migration

Lamellipodia drive migration through the attachment to the substrate and generating the force to pull the cell body forward (Yamaguchi et al., 2006). Formation of lamellipodia is regulated by PLC-PIP2-cofilin pathway and by GTPases proteins of the Rho family with the WAVE-ARP2/3 complex (Fig 1.10). Cofilin is a small protein of 19kDa that binds both monomeric actin and actin-filaments. Its main role is to severe actin filaments at the leading edge of a migrating cell, thus increasing the number of barbed ends, which in turn may result in the extension of a lamellipodia. This process is regulated by PLC and PI3K pathways that promote cofilin inactivation through its phosphorylation. (Yonwzawa et al., 1990). Either Rho through its effector ROCK, or Rac through PAK can also inactivate cofilin inducing its phosphorylation by the LIM kinase (LIMK) (Toshima et al., 2001). It has been observed that the precise balance between cofilin activation and inactivation by LIMK phosphorilation is crucial for tumor cell migration. Futhermore, several studies indicate that spatially and temporally localized activity of cofilin is determinant in chemo-taxis sensing during invasion (Mouneimne et al., 2004). The ARP2/3 complex localizes at lamellipodia to regulate it. ARP2/3 complex is activated by Cdc42 through WASP (Wi-skott-Aldrich syndrome protein family proteins) or by Rac via WAVE. Once activated, the complex ARP2/3 binds an actin filament and iniziates the extension of a new filament. In mammalian cells, five WASP-related proteins have been identified: WASP, neural WASP and WAVEs 1-3. It has been reported that WAVE2 has a specific role in the formation of lamellipodia while WAVE 1 stabilizes the protrusion (Yamaguchi et al., 2007).

1.3.5.1 Lamellipodia regulation: interplay between different GTPases

Several studies indicated that a fine balance between the signal of RhoA and Rac1 can regulate cell migration through the control of lamellipodia generation. Rac1 levels regulate the persistence of migration by influencing the number of peripheral lamellae and associated membrane protrusions. High activity of Rac1 promotes the extension of multiple lamellipodia, enhancing random migration with respect to directed migration that is usually mediated by a single stable protrusion. On the other hand, a reduction in Rac1 activity favours directed migration using axial lamellipodia (Pankov et al., 2005). This occurs because moderate or low activity of Rac1 results in the suppression of the extension of new protrusions, thus favouring the presence of a single lamellipodium that
promotes the persistence of migration in one direction. Once activated, Rac traslocates at the leading edge of the cells where it interacts indirectly with WAVE to stimulate Arp2/3-mediated actin polymerization, producing lamellar extension and forward cell movement (Pollard et al., 2000). The loss of directionality also occurs when RhoA activity exceeds Rac activity. This impairs the stability of lamellipodia driving the collapse of the protrusion (Caswell et al., 2009). The key concept, is that tight changes in Rac 1 and RhoA activity finely regulates random versus directed migration through the control of the number of lamellapodia generated at the cell periphery.

1.3.6 The degradation of ECM in cancer cell: invadopodia

Invadopodia and podosomes are ventral actin-rich protrusions originally indentified in v-src-trasformed fibroblasts (David-Pfeuty and Singer, 1980). Initially, both the terms have been used for to the same actin-rich invasive structures in the same cells. Nowadays, the term podosome is used to indicate these structures in normal cells that are able to remodel the ECM such as macrophages and osteoclasts and in Src-trasformed cells. Differently, the term of invadopodia is used for structures that mediate pericellular proteolysis of ECM formed in cancer cells and promote tumor cells invasion and metastasis (Murphy and Courtneidge, 2011; Artym et al., 2013). Invadopodia are structures that have a width between 0.5 and 2 \( \mu \text{m} \) and a length of about 2 \( \mu \text{m} \). They are composed by an actin-rich core colocalizing with several proteins. These structures have peculiar morphological features and a specific spatial localization that allow to distinguish them from filopodia and lamellipodia. Specifically, invadopodia can be identified by the colocalization between ventral actin puncta with focal degradation of the ECM. The proteins involved in invadopodia structure and function can be grouped in four functional classes: proteins of motility machine, adhesion proteins, signalling proteins and proteases. The first group includes regulators of F-actin polymerization and branching such as Arp2/3, N-WASP, Cdc42, Nck, coflin, capping proteins dynamin and cortactin. The second group includes integrins that mediate the interaction of invadopodia with the ECM. In the third group there are the signalling proteins regulating the actin cytoskeleton, and membrane remodelling such as Rho GTPases and kinases, while the fourth group comprises metalloproteases such as MMP14, MMP2, MMP9 and the urokinase-type plasminogen activator (uPA)/uPa receptor proteolytic system (Artym et al., 2013; Gimona et al., 2008). Key regulators of invadopodia formation have been identified: the N-WASP-Arp2/3-cortactin complex,
Tyr kinase substrate with four SH3 domains (TKS4), Tyr kinase substrate with five SH3 domains (TKS5), the Tyr kinase SRC and the transmembrane membrane type 1 matrix metalloprotease (MT1-MMP) (Clark and Weaver, 2008). Also phosphatases play a central role in the regulation of invadopodia. The most important phosphatases identified are the 5’ inositol phosphatase synaptojanin and the 3’ inositol phosphatase and tumor suppressor phosphatase PTEN (Murphy and Courtneidge, 2011). The main function of invadopodia is the degradation of ECM in order to allow cell migration through tissues, in particular during collective invasion. Recently it has been demonstrated that invadopodia can act also as mechanosensors (Albigez-Rizo et al., 2009). In fact, the formation of invadopodia seems to be promoted by a decrease in local cellular contractility (Burgstaller and Giona, 2004). For example the assembly of invadopodia occurs mainly at the ventral surface of cancer cells, where traction forces are weaker than at the periphery.

1.3.7 Mechanisms of invadopodia formation

Several studies indicate that the process of invadopodia formation includes four steps (Fig 1.11) (Artym et al., 2013). The first step is the formation of the core structure associated with actin puncta. This is induced in response to a great number of factors, which can be grouped in four main classes: growth factors (EGF, TGF-β, PDGF, HGF), oncogenic transformation, EMT induction and hypoxia (Beaty et al., 2014). It has been suggested that these factors may converge in the activation of the Rho GTPase Cdc42. This has been supported by the observation that Src, a key player in the invadopodia formation, phosphorylates and activates GEFs for Cdc42 such as Vav1. Therefore, it has been hypothesized that the axis EGFR-Src-GEF-Cdc42 may be the major actor in the invadopodia initiation (Beaty et al., 2013). Active Cdc42 induces the activation of cofilin-N-WASp-Arp2/3 complex, which in turn promotes actin assembly (Artym et al., 2013). Concomitant with this, cortactin accumulates at the ventral cell membrane adherent to matrix. Few seconds after that, also the adaptor protein Tks5 is recruited to the core and binds the PIP2 phosphoinositide in order to anchor the structure to the plasma membrane (Sharma et al., 2013). In the stage 2, it is formed a structure called adhesion ring that stabilizes the nascent invadopodia favouring the recruitment of β1integrins and other proteins such as the formin mDia that recruits microtubules to enhance vesicle transport to the invadopodia (Wickströme and Fässler, 2011). The role of integrins seems to be fundamental for invadopodia maturation since they can interact with the kinase Arg and the integrin-link kinase (ILK) (Branch et
Arg phosphorylates cortactin further promoting cofilin-Arp2/3-dependent actin polymerization, whereas ILK recruits the scaffold protein IQGAP that in turn induces the transport of MT1-MMP to the invadosome. The recruitment of MT1-MMP/MP14 constitutes the third stage of the invadopodia formation (Hoshino et al., 2013). At these step of invadopodia formation it occurs also the elongation of actin filaments through the action of cofilin. This is mediated by talin. Talin is a focal adhesion protein that is recruited to

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Fig 1.11 Model of invadopodia formation. (1) initiation: Growth factors (GF) interact with their receptor leading actin polymerization and invadopodia initiation. Signals from different pathways converge on N-WASP that activates Arp2/3 complex inducing actin polymerization. (2,3) During adhesion ring formation proteins such as integrins and ILK are recruited to the nascent invadopodia. The vesicle capture is mediated by molecules such as IQGAP, mDia, ILK. (4) at the stage four ECM degradation occurs. Moreover, proteinase activity may promote feedback signaling to enhance the formation of new invadopodia. Adapted from D. Hoshino et al., J. Cell Sci. 2013.
invadopodia by binding to the actin filament. Talin interact with the ERM family protein moesin. Moesin contributes to changes of the intracellular pH disrupting inhibitory interactions between cortactin and cofilin. This allows active cofilin to severe F-actin thus generating new actin barbed ends, for extension of the invadopodium (Beaty et al., 2014). At stage four, the matrix degradation occurs. Several studies indicate that, at this stage, mature invadopodia contain microtubules and intermediate filaments. Their recruitment promotes further elongation of the protrusion and the arrival of metalloproteases for ECM degradation. The number of invadopodia increases progressively at this stage due to the action of MT1-MMP (Artym et al., 2013). This metalloprotease can directly degrade ECM, but can also potentiate invadosome initiation by releasing growth factors through the proteolysis of their precursors. This occurs through different mechanisms. MT1-MMP cleaves MMP2 that disrupts the inhibitory complex of vascular endothelial growth factor (VEGF) releasing the VEGF and triggering invasion (Dean et al., 2007). Again, MT1-MMP cleaves the TGF-β binding protein LAP determining the release of TGF-β (Mu et al., 2002). In addition, MT1-MMP cleaves HB-EGF, which releases an EGF-like domain. This peptide strongly stimulates EGFR thus amplifying the invadosome assembly process.

1.3.8 The main actors of invadopodia formation: cortactin and MT1-MMP

Cortactin is an actin-binding protein and is one of the major substrate of Scr kinase. Cortactin is composed by different domains. Four are of particular interest: the N-terminal acidic (NTA), a tandem repeats domain, the C-terminal proline rich domain, and a Src-homology 3 (SH3) domain (Weaver et al., 2008). The N-terminal domain is the binding site for the Arp2/3 complex and actin filaments. Through this domain cortactin regulates branching of F-actin mediated by Arp2/3 complex (Weaver et al., 2001). The C-terminus proline rich domain has regulatory functions. It contains serine/threonine and tyrosine sites of phosphorylation, that are target of several kinases modulating cortactin function. The SH3 domain can bind signaling proteins such as N-WASP and WIP (Weaver, et al., 2008). Cortactin has different functions: it stabilizes the newly formed actin filaments network (Weaver et al., 2001), regulates the persistence of a protrusion, and promotes the formation of new adhesions site at the cell edge (Bryce et al., 2005). Moreover, cortactin plays a central role in invadopodia formation. Two mechanisms have been proposed to explain this function. First, cortactin may promote directly the actin assembly at invadopodial puncta. This hypothesis is supported by the observation that in Src-transformed cells,
cortactin silencing leads to a reduction of the number of invadopodia pucta (Arthym et al., 2013). Second, cortactin may influence invadopodia formation through its interaction with proteins N-WASP, WIP and Src kinase. Cortactin binds these proteins via its C-terminal domain. It has been observed that mutations in this region that prevent the binding affect invadopodia number and function (Webb et al., 2007).

MT1-MMP (or MMP14) protein belongs to the family of metalloprotease (MMP) that in humans includes 25 members (Poincluox et al., 2009). MMP are multifunctional zinc-dependent endopeptidases that can degrade a variety of ECM component such as collagen I, II and III, fibronectin. As the other metalloproteases, MT1-MMP is produced as a zymogen and it is cleaved in the Golgi giving rise the mature enzyme. The mature protein is anchored to the plasma membrane. MT1-MMP has a conserved structure and is composed by a signal peptide, a propeptide, and an extracellular catalytic domain (Poincluox et al., 2009). MT1-MMP is involved in the activation of MMP2 and MMP13, and in the regulation of cell migration through the proteolytic modification of CD44, the αv integrin and transglutaminase. Together with cortactin, MT1-MMP is required for the assembly of invadopodia. It has been demonstrated that its overexpression in cancer cells promotes migration, invasion and metastasis formation both in vitro and in vivo (Itoh et al., 2004). In particular, MT1-MMP mediates mesenchymal migration through the degradation of the collagen. Matrix degradation is also enhanced by the clustering of MT1-MMP with β1-integrin at sites of interaction between integrin and collagen fibers (Wolf et al., 2003). MT1-MMP can be regulated at different levels: gene transcription, intracellular trafficking and proteolytic activation. The intracellular trafficking is finely regulated both by clathrin-mediated and caveolar endocytosis, while the transport of MT1-MMP at invadopodia is regulated by exocytosis mediated by exocyst complex and IQGAP1 protein (Sakurai-Yageta et al., 2008). The internalization of MT1-MMP can result either in lysosomal degradation, or in the recycling of the protein to the plasma membrane. The cytoplasmic domain of MT1-MMP (20 aa) and in particular a Leucin motif (LeuLeuTyr573), is responsible for the clathrin-mediated endocytosis by its interaction with AP2-clathrin adaptor complex. On the other hand, several studies have demonstrated the association of MT1-MMP with caveolae, which are important for the correct localization and function of the protease during cell migration (Galvez et al., 2004). The endocytosis of MT1-MMP can be regulated by the binding of the protease with its principal inhibitor TIMP-2. The internalization of the complex results in a dissociation of MT1-MMP from TIMP-2 allowing the protease to be recycled to the plasma membrane. The internalization can be also affected by FAK that interferes with the fission of en-
docytic membranes and caveolae (Baldassarre et al., 2003). The transport of MT1-MMP to invadopodia is mediated by exocytosis regulated by Cdc42 and RhoA (Sakurai-Yageta et al., 2008). In addition, aggregation of both F-actin and cortactin enhances and controls the accumulation of the protease at invasive structures. MT1-MMP traffic is finely regulated by several mechanisms. This suggests that invadopodia are specific domain of the plasma membrane that are targets of MT1-MMP exocytosis and where MT1-MMP endocytosis is reduced with respect to the other regions of the membrane (Poincluox et al., 2009).

1.4 Liprin Family

1.4.1 Liprin subfamilies and isoforms

Liprins are cytosolic scaffold proteins involved in the regulation of synapse assembly and maturation, vesicular trafficking and cell motility (Zurner et al., 2011). These proteins localize mainly at the plasma membrane. Liprin proteins are classified into α- and β-types based on their sequence similarities (de Curtis, 2011). In vertebrates four liprin-α proteins have been identified, (Liprin-α1-4) and two liprin-β (liprin-β1 and β2) (Serra-Pages et al., 1998). Different liprin-α proteins have a 60-75% of sequence identity while liprin-β1 and liprin-β2 proteins are about 51% identical and about 28% identical to liprin-α family members.

The genomes of C.elegans and Drosophila encode a single gene homologous to human liprin-α1, termed synapse-defective-2 (syd-2) and Dliprin, respectively (Kaufmann et al., 2002; Zhen and Jin, 1999). Drosophila genome also encodes a single homolog of human liprin-β proteins, CG11206, (Serra-Pages et al., 1998), and a third protein called liprin-γ that has approximately equal homology to liprin-α and liprin-β (Astigarraga et al., 2010). Northern blot and quantitative RT-PCR analysis from different mammalian tissues revealed that liprin-α1 is ubiquitously transcribed with the low levels of gene expression in the brain (Zurner and Schoch, 2009), while liprin–α2 and liprin–α3 are mainly expressed in this organ. Liprin–α4 is mostly expressed in muscle and testis and at lower levels in brain, lung, heart and thymus (Serra-Pages et al., 1998; Zurner and Schoch, 2009). Liprin-β1 and Liprin-β2 are widely expressed in human tissues (Stafford et al., 2011). All the liprin genes are regulated post-transcriptionally by alternative splicing events (Zurner and Schoch, 2009; de Curtis, 2011). A recent comparative analysis between human and mouse liprin-α genes has shown a high homology in the sequence and the placement of
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1.4.2 Liprins structure

Both liprin-α and liprin-β proteins consist of an aminoterminal coiled coil region that mediates homo- and heterodimerization, and a carboxy-terminal region of 250 aminoacidic residues, named liprin homology (LH), that show high degree of conservation among isoforms and contains three steryl-alpha motifs (SAM domains) (Fig1.12) (Serra-Pages et al., 1998). SAM domains are placed in tandem and mediate the interaction with either proteins, RNA or lipid membranes (Qiao and Bowie, 2005). The most widely expressed liprin-α, liprin-α1, is composed by 1202 aminoacids and has a weight of 160 KDa, it is the only family member with a PDZ-binding domain at the carboxy-terminal and with a peptide (PEST sequence) placed between the N-terminal coiled coil region and C-terminal SAM region. The PEST sequence mediates the protein degradation by calcium/calmodulin-dependent protein kinase II (Hoogenraad et al., 2007; Sheng et al., 2007). Liprin-β1 and liprin-β2 are composed by 1011 and 876 amino acids respectively, and their N-ter-

Fig 1.12 Scheme of the structure of liprin-α and liprin-β. Liprin-α proteins include an amino-terminal coiled-coil region (CC) responsible for the formation of liprin-α dimers, and of a carboxy-terminal region including three SAM (steryl α motifs) and a carboxy-terminal PDZ-binding sequence (VRTYSC), which is missing in some liprin-α isoforms. Similarly, liprin-β include an amino-terminal coiled coil-rich region, shorter than in liprin-α1, and three carboxy-terminal SAM domains. Adapted from de Curtis, 2011.
minal coiled coil regions are significantly shorter than in liprin-α proteins (R.L Stafford et al, 2011). All liprin-α proteins coiled coil regions are able to interact with each other but not with liprin-β proteins. In particular, it has been reported that liprin-α1 mainly forms either homodimers or heterodimers with liprin-α2 through its amino-terminal portion (Fig 1.13A) (Serra-Pages et al., 1998). At the same time, all liprin-β proteins coiled coil regions are able to interact with each other. Liprin-βs can interact with liprin-α members through the C-terminal portion. Crystallographic studies on liprin-β2 coiled coil region have shown that the N-terminal portion of the protein mainly forms a dimer with parallel orientation. Moreover, this parallel dimer arrangement likely determine that all six C-terminal SAMs are positioned next to each other (R.L Stafford et al, 2011). Further crystallographic studies have indicated that SAMs domains are connected by H-bonding, hydrophobic and charge-charge interactions. The residues that connect SAM1 to SAM2/3 are highly conserved among liprin proteins, indicating that the head-to-tail assembly of the three SAMs is common to all the α and β molecules (Wei et al., 2011). Moreover, it has been demonstrated that the SAM domains form a supramodular structure responsible of the interaction properties of the proteins. In this respect, an important difference has been identified between the SAM repeat in α and β proteins. The β1 region lacks an alpha helix included in liprin-α1 SAM1, whereas liprin-β1 presents an additional helix at the
C-terminus of its SAM3 domain (Wei et al., 2001). This structural difference may determine the different target interaction properties of the two liprins subfamilies. Based on the orientation of the coiled coil region and the structure formed by the SAMs domains, two models for the interaction of liprin-α with liprin-β proteins have been proposed: the closed dimer and the open scaffold model. In the first model SAM domains of liprin-β's and liprin-α’s may come together by tail to tail interaction (Fig 1.13B). In the second model the the SAM domains of one α-dimer interact with SAM domains of two different β-dimers (Fig 1.13C), potentially creating large multiprotein complexes (Wei et al., 2011).

1.4.3 Liprins functions

Liprin-α1 has been identified for the first time in fibroblasts, as an interactor of LAR, a transmembrane tyrosin phosphatase with a role in axon growth (Dunah et al., 2005), cell adhesion and migration (Serra-Pages et al., 1998). Following studies have dissected the role of liprin-α proteins mainly in C.elegans and mammalian neuronal system, reporting their involvement in the assembly and regulation of the pre-synaptic active zone (Zhen and Jin, 2004), pre-synaptic vesicle trafficking (Zhen and Jin, 1999; Ko et al 2003b), but also in post-synaptic regulation with LAR (Dunah et al., 2005). More recently, it has been demonstrate the involvement of liprin-α proteins in the regulation of exocytosis in non-neuronal system, such as mast-cells (Nomura et al., 2009) and spermatozoa (Joshi et al., 2012), and in the regulation of cell motility in non-tumoral and tumoral cells (Asperti et al., 2009; Astro et al., 2011; Astro et al., 2014). Differently, only few data on the specific functional role of liprin-β proteins have been published. The specific roles of liprin-α and liprin-β proteins are discussed below.

1.4.3.1 Liprin-β proteins function

Liprin-β proteins have been identified as interactors of liprin-α1 protein by trap interaction screening (Serra-Pages et al., 1998). As for liprin-α proteins, some studies identified a possible role of liprin-β proteins in both neuronal and non-neuronal system, even if the data available are less than the data published about liprin-β proteins. Recently a single homologous of human liprin-β protein has been described in Drosophila. It forms homodimers via the N-terminus, and interacts with liprin-α through its C-terminal portion (Astigarra S., et al., J.Neurosci. 2010). These authors observed that liprin-α and liprin-β
have a synergistic function in Drosophila synaptogenesis although the mechanism and the contribution of each protein activity is different. Indeed, it has been demonstrated that both liprin proteins are needed to terminate the axon growth in response to target recognition even if in liprin-α mutants, the axon stops growing and remains in or retracts back to the target layer, while in liprin-β mutants a small percentage of axons maintain the capacity to extend projections but results in a less stable synapses formation. This indicates that liprinβ protein contributes to stabilize synapses. Moreover, it has been observed that liprin-β mutants cause reduced size of neuromuscular junction (NMJ) and affects bouton formation. This suggests an involvement of liprin-β in NMJ function (Asti-garra et al., 2010). More recently, it has been described that liprin-β1 may interact with S100A4 at plasma membrane of cellular protrusions in non-neuronal cells (Kriaevska et al 2002). S100A4 is a small calcium binding protein that participates in various cellular processes such as transducer of calcium signal. Since S100A4 is strongly expressed in undifferentiated types of cancer even at metastatic level, it was suggested that S100A4 acts as a modulator of liprin-α1/liprin-β1 complex formation, to influence cell adhesion, migration and invasion (Kriaevska et al, 2002). Another study has reported that liprin-β1 is highly expressed in lymphatic vasculature and that liprin-β1 silencing results in edema. This suggests an involvement of liprin-β1 in the development and integrity of lymphatic vasculature (Norrmén et al., 2009).

A more recent study has shown that liprin-β1 forms a protein complex with liprin-α1, KANK1 and KIF21A, which cooperates with LL5β, ELKS and CLASP proteins in cortical microtubules organization (van der Vaart et al, 2013). It has been also demonstrated that liprin-β1 immunoprecipitates with ELKS and liprin-α1, and immunofluorescent staining showed that endogenous liprin-β1 displays a significant colocalization with these proteins and LL5β (van der Vaart et al, 2013). Since it has been reported that the complex formed by liprin- α1, ELKS and LL5β plays an important role in the regulation of tumor cell migration and invasion (Astro et al., 2014), it may be hypothesised the involvement of liprin-β1 also in these same processes. However, it has not been demonstrated, yet. On the other hand, some authors have found that the silencing of liprin-β2 promotes tumoral cell invasion and increases the speed of cell migration. On the contrary, liprin-β2 overexpression causes a decrease in cell invasion (von Thun et al., 2011). Altogether, the data available on liprin-βs support the hypothesis of the involvement of these proteins in cell motility, possibly in the same processes in which liprin-α1 is involved. However, studies are needed to confirm these speculations.
1.4.3.2 Liprin-a function in neuronal cells

Liprin-α proteins have mainly been studied in the neuronal system, where they appear to be involved in several processes at both the pre-synaptic and post-synaptic level (Wentzel et al., 2013; Owald et al., 2012) (Fig. 1.14). Different studies have shed light on the different expression pattern of the four liprin-α in rat and mouse brain (Spangler et al., 2011; Zürner et al., 2011). It has been found that all four proteins are expressed in postnatal and adult rat brain, albeit the expression pattern and levels are different for each isoform. Liprin-α1 has been detected at very low level, except for the cerebellum and hippocampus where it is enriched in dentate gyrus. Liprin-α2 and liprin-α3 are mainly expressed in the postnatal brain in the outer layers of the cortex, in hippocampal cornu ammonis region (CA) and in the thalamus. The two proteins are detected at high levels also in the adult brain. The former protein is predominant in the olfactory bulb granules dentate gyrus, cerebellar granule cells, and amygdale nucleus. Liprin-α3 is expressed at higher level in hippocampus, cortex and cerebellar granule cells. Liprin-α4, is poorly expressed in the adult brain except for the cerebellum and the hippocampus. Furthermore, it has been

![Figure 1.14. Schematic representation of liprin-α1 recruitment at the presynaptic active zone in neurons.](image-url)

In synapses liprin-α1 is involved in the assembly and organization of a functional presynaptic active zone. Liprin-α1 interacts with other proteins, such as ELKS/ERC, RIM and Piccolo. (Grey lines) protein interaction; (Green circles) scaffolding proteins; prenylated Rab acceptor (PRA); Ras-related in brain (Rab); synaptosome associated protein (SNAP); Voltage-dependent Calcium Channel (VDCC). Adapted from Mittelstaedt et al., Biol. Chem., 2010.
observed that syd-2, the *C. elegans* homologous of liprin-α, is expressed in both neurons and muscles, where it is localized at the centre of pre-synaptic active zone. Syd-2 mutants show a significant increase in the area of the active zone but a decrease in its electron density. This effect could be rescued by the overexpression of syd-2 in neurons, but not in muscles, supporting a specific pre-synaptic role of this protein. Mutations in syd-2 result in altered presynaptic vesicle clustering (Zhen and Jin, 1999). Several studies have demonstrated that liprin-α could interact with different proteins involved in the formation and regulation of the active zone (Zhen and Jin, 2004). For example, the liprin-α N-terminal region interacts with Rab3 interacting molecules-α (RIM-α, Schoch et al., 2002), ERC/ELKS (ELKS-Rab6-interacting protein-CAST), MALS/Veli-Cask-Mint1 complex (Olsen et al., 2005), and Kif1a (Shin et al., 2003). In particular, RIM proteins regulate synaptic vesicle release. ERC family members influence the sub-localization of both liprin and RIM, and some data suggest that liprin-ERC/ELKS interaction may regulate the surface delivery of vesicles (Ko et al 2003b, Ohtsuka et al 2002). It has been proposed a model in which syd-2 recruits ERC-1/ELKS and other proteins to promote the assembly of a new presynaptic specialization. Subsequently the syd-2/Liprin-α ERC/ELKS-1 complex would bind RIM and other proteins allowing synaptic vesicle docking and priming (Dai et al., 2006). The MALS/Veli-Cask-Mint1 complex regulates the transport of presynaptic vesicles from the reserve pool to the releasable pool. Liprin-α can also bind KIF1A, a neuro-specific motor belonging to the kinesin superfamily (KIF) that is able to transport cargo vesicles along the microtubules (Hirokawa & Takemura, 2004). Liprin-α could link KIF1A to diverse kind of proteins either membrane-associated, cytoskeleton-associated or signalling proteins, to allow them to reach the presynaptic terminal (Shin et al., 2003). More recently, it has been identified a region of 107 amino-acids (aa 92-199) in the N-terminal portion of syd-2/Liprin-α named LH1 domain (Liprin homology domain 1) that is required for the function of this protein in presynaptic assembly and is sufficient to restore synaptic assembly in *syd-1* defective mutants (Taru and Jin, 2011). There is evidence supporting the importance of liprin-α2 as a regulator of presynaptic function and dynamics in mature hippocampal synapses: it controls synaptic output and synaptic protein composition, regulating the synaptic vesicle pool. Moreover, liprin-α1 undergoes an activity-dependent regulation: low neuronal activity reduces liprin-α2 levels, while high neuronal activity causes an increase of liprin-α2 expression (Spangler et al., 2013). In light of these results, it becomes evident a specific role of liprin-α proteins in synaptic vesicle transport along microtubules and in the control of vesicle trafficking, and exocyto-
sis of neuropeptides at synaptic sites, although the precise underlying mechanisms are not fully elucidated. Some studies have identified a postsynaptic role of syd-2/Liprin-α, showing that at this site liprin-α binds GRIP (Wyszynski et al., 2002), LAR (Dunah et al., 2005) and GIT/Cat/p95-APP/PKL (Ko et al., 2003a). LAR is important at postsynaptic sites since its depletion causes loss of synapses and dendritic spines (Dunah et al., 2005). The LAR/liprin-α/GRIP interactions are essential for the dendritic targeting of the cadherin–β-catenin complex and AMPA receptors. LAR complex promotes not only the synaptic accumulation of β-catenin, but also the adhesive function of the cadherin/β-catenin complex at synapses. GIT1 regulates endocytosis of various membrane proteins, like the β2-adrenergic receptor (Premont et al., 1998; Claing et al., 2000). It has been described that the interaction of GIT1 with liprin-α is essential for AMPA receptor targeting (Ko et al., 2003).

1.4.3.3 Liprin-a in exocytosis

Although Liprin-α proteins have been mainly studied in the neuronal system, they are widely expressed and there are increasing evidences showing their role in different processes.

1.4.3.3.1 Liprin-α in mast cell

The principal role of active zone is the release of neurotransmitter by exocytosis. Proteins involved in neuronal exocytosis are also expressed in other tissues, for example mast cells that are specialized in the release of inflammatory mediators. Nomura and colleagues found that ERC/ELKS is expressed in mast cells where it regulates positively the exocytic process (Nomura et al., 2009). More recently, the same authors have investigated the expression and the role of liprins in the same cells where liprin-a1, liprin-a2 and liprin-a3 are expressed. These authors have observed that liprin-a1 colocalizes with ERC/ELKS in the cytoplasm, and that liprin-a1 silencing impairs the exocytic release and a significant decrease of the cell area. These data indicate that liprin-a1 is a positive regulator of exocytosis and cell spreading of mast cells (Nomura et al., 2011).
1.4.3.3.2 Liprin-α in spermatozoa

The acrosomal reaction (AR) is the secretory event in the spermatozoa that is needed for the fertilization of the egg. Acrosome biogenesis is a multistep process in which the Golgi apparatus constantly secretes vesicles that fuse to form the acrosome. The acrosomal reaction is indispensable for a correct fertilization (Joshi et al., 2012). Several studies have compared acrosomal reaction to the vesicle exocytosis in synapses. For example, it has been demonstrated that diverse membrane-associate proteins like synaptotagmin, synaptobrevin, SNAP-25, RIM and Rab3A are involved in both the processes (Michau et al., 2011, Zitranski et al., 2010, Bello et al., 2012, Iida et al., 1999, Katafuchi et al., 2000).

Recently, it has been reported the expression of liprin-α3 and LAR in rat testis, epididymis, mouse and human spermatozoa (Joshi et al., 2013). Liprin-α3 colocalizes with LAR and RIM on the anterior part of the acrosome in mouse spermatozoa and depletion of liprin-α3 though liprin-α3 leads to a inhibition of the acrosomal reaction, thus affecting fertilization (Joshi et al., 2013). Moreover, it has been demonstrated that during the first phases of acrosome biogenesis liprin-α3 is associated to the Golgi, supporting the hypothesis that liprin-α3 is an acrosome-associated (Joshi et al., 2012; Joshi et al., 2013).

1.4.3.4 Liprin-a role in cell adhesion and motility: regulation cell spreading and FA dynamics regulation

Cell motility is a multistep process that requires cell spreading and the formation of new adhesions. Liprin-α1 is positive regulator of cell spreading: in 2007 Shen and colleagues demonstrated that liprin-α1 silencing in RKO cells suppressed cell spreading compared with controls (Shen et al., 2007). Previous work from our laboratory has confirmed these effects and has tried to elucidate the mechanisms by which liprin-α1 regulates cell spreading and motility. Initially, it has been shown that in COS7 cells liprin-α1 localizes at the periphery of the cells, and it is stably associated to the cytoplasmatic side of the ventral plasma membrane (VPM) derived from ECM-attached cells. The endogenous protein is enriched at the cell membrane together with talin, and partially co-localizes with more mature, central focal adhesions (Asperti et al., 2009). During cell spreading, it has been observed that liprin-α1 only partially co-localized with the newly formed focal adhesions at the edge of the cells. In fact, the costaining with paxillin and talin indicated that liprin-α1 localizes just behind the new adhesion sites. Talin is a protein that links integrins
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to the actin cytoskeleton and regulates integrin activation (Jiang et al., 2003). Indeed, the interaction between talin and the cytoplasmatic tail of integrins leads to a conformational change that increases the affinity of integrins for their extracellular ligands, mediating the cell spreading (Zhang et al., 2008).

Supporting the role of liprin-α1 in cell spreading, it has been observed that its overexpression strongly increases spreading by enhancing the formation of new actin lamellipodia. Furthermore, liprin-α1 overexpression leads to a redistribution of the newly formed focal adhesions that were enriched at the periphery of the cells. Finally, in liprin-α1 overexpressing cells, it has been observed an increase of Rac1 activity after 30 minutes of spreading on fibronectin (FN) while the co-expression of liprin-α1 with a Rac1 dominant negative, (N17Rac1 mutant) causes inhibition of spreading. These data indicate that the effects of liprin-α1 overexpression on cell spreading are mediated by the activity of the GTPase Rac1 (Asperti et al., 2009). Interestingly, liprin-α1 overexpression leads to an accumulation of activated β1-integrin at the cell edge whereas the overexpression of talin does not affect spreading but enhances β1 integrin activation. However, the co-transfection of liprin-α1 and talin1 prevents both liprin-α1 enhanced spreading and talin-induced integrin activation. Altogether, these data indicate that liprin-α1 and talin influence each other and cooperate in the regulation of cell motility (Asperti et al., 2009). The LAR protein is implicated in liprin-α1-dependent spreading: LAR depletion inhibited spreading also in cells overexpressing liprin-α1. In these conditions, the redistribution of β1-integrin in liprin-α1 overexpressing cells is not affected, indicating that liprin-α1 and LAR are involved in the same pathway with distinct roles: liprin-α1 and LAR are indispensable for cell spreading, but LAR is not required for liprin-α1 regulation of peripheral active β integrins. Further analysis on the role of liprin-α1 in controlling the FA dynamics has shown that in liprin-α1 overexpressing cells it occurs a re-localization of inactive integrin β1 but not of the active integrin pool, and that liprin-α1 colocalizes with inactive integrin β1 at the VPM of adherent cells (Asperti et al., 2010). Likewise, in chicken embryo fibroblasts (CEF) liprin-α1 overexpression induced the formation of a greater number of FAs, larger in size with respect to the control cells. Interestingly, liprin-α1 depletion leads to a faster internalization of integrin-β1 while liprin-α1 overexpression stabilizes integrin receptor at the cell surface, resulting in reduced internalization. Based on these finding it has been proposed a model in which liprin-α1 promotes cell motility, stabilizing the inactive integrins at the cell periphery and making them available for the formation of new FA (Asperti et al., 2010).
1.4.3.5 Liprin-a function in migration and invasion

Recently, it has been shown that liprin-a1 interacts with inhibitor of growth 4 (ING4) that is a candidate tumor suppressor playing a role in gene regulation, cell cycle control, apoptosis and angiogenesis (Shen et al., 2007). The ING protein family consists of five members (ING1-5) located on different chromosomes close to the telomeres (D. Ythier et al., 2008). The ING genes are conserved during the evolution supporting the hypothesis of the involvement of ING proteins in important cellular functions (G. H. He et al., 2005). In particular, it has been described that ING4 plays a role in cell migration. Indeed, ING4 overexpression impaires cell spreading and migration while its depletion promotes cell motility (M. Unoki et al., 2006).

Some studies have observed a loss of ING4 protein or a decrease of its mRNA levels in several tumors such as hepatocarcinoma, melanoma, gastric, ovarian, colon, brain and breast cancer (C. Guérillon et al., 2014). The observation that ING4 could affect cell migration through liprin-α1, has led to the hypothesis that liprin-α1 could be directly involved in tumor cell motility (JC Shen et al., 2007).

Interestingly, studies from our laboratory have found that liprin-α1 protein is frequently overexpressed in human breast cancer (Astro et al., 2011). Trying to dissect the role of liprin-α1 in tumor cell invasion and migration, it has been observed that this protein is essential for the stability of lamellipodia in a human breast cancer model (MDA-MB-231), affecting both cellular migration and invasion (Astro et al., 2011). Specifically, it has been shown that liprin-α1 depletion impairs lamellipodia dynamics, leading to an increase of the number of lamellipodia formed per cell but a decrease of the lamellipodia persistence. This data suggested that the defect of lamellipodia persistence may cause the defect in cell migration.

Further results have shown that liprin-α1 silencing reduces cell invasion through Matrigel and ECM degradation causing a decrease in the number of invadopodia formed per cell. In fact, time-lapse experiments on cell transfected with DS-Red-Cortactin and liprin-α1 siRNA have demonstrated a reduced lifespan of invadopodia in liprin-α1-depleted cells (Astro et al., 2011). Since liprin-α1 is a scaffold protein, it is reasonable to hypothesize that it does not act alone in controlling cell motility. Indeed, two recent studies have shown that liprin-α1 forms a complex with ERC1, LL5β, KANK1 and KIF1A (Van der Vaart et al., 2013, Astro et al., 2014). In particular, it has been demonstrated that ERC1 and LL5β colocalize with liprin-α1 at the front of migrating cells and cooperate with liprin-α1 in
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The regulation of tumor cell migration and invasion. Time lapses analysis reveals that the silencing of either liprin-α1, ERC1 or LL5β proteins decreases the stability of lamellipodia whereas their overexpression reduces the frequency but increases the stability of the protrusions. Interestingly, all the three proteins are required for the efficient endocytosis of β1 integrin, suggesting that this may affect cell motility (Astro et al., 2014).

1.4.5 Liprin binding proteins

As mentioned, liprin-α1 protein has been firstly identified by interaction trap assay in human fibroblasts studying potential partners of LAR (Serra-Pages et al., 1998). Moreover, several studies have shown that liprin-α1 interacts with other proteins (Fig 1.15) such as ERC1 (Ko et al., 2003), GITs (Kim et al., 2003), CASK (Wei et al., 2011), KIF1a (Shin et al., 2003) and RSY-1 (Patel and Shen, 2009). In particular, recent studies have demonstrated that LAR and GIT1 cooperate with liprin-α1 in the regulation of cell spreading (Asperti et al., 2011). On the other hand, Erc1a and its interactors LL5’s form a complex with liprin-α1 cooperating together in the regulation of tumoral cell migration and invasion (Astro et al., 2014).
1.4.5.1 LAR protein

Tyrosine phosphorylation of signalling molecules, adaptor proteins, and cytoskeleton-associated proteins is associated with different cellular processes such as activation, proliferation and migration (Clark and Brugge, 1995; Pulido et al., 1995) Early adhesion events depend on tyrosine phosphorylation and are consequently regulated by dephosphorylation from phosphatases (Guan et al., 1991). A family of phosphatases, the receptor protein-tyrosin-phosphatases (RPTPases), includes members with an extracellular region connected by a transmembrane portion to a cytoplasmatic part with a PTPase domain (Pulido et al., 1995). These proteins play a role in the transduction of signals from the extracellular environment. The extracellular region of several RPTPases includes Ig-like domains and FN-domains (Streuli et al., 1988). This architecture has been found also in adhesion molecules and in receptor for growth factors, and indicates that RPTPases regulate cell-cell and cell-matrix interactions (Pulido et al., 1995). LAR is a member of a subfamily of RPTPases (Serra-Pages et al., 1995) that includes three vertebrate homologue, LAR, RPTP-sigma, and RPTP-delta, as well as few invertebrates orthologue such as Dlarb (Chagnon et al., 2004). In the cytoplasm, the LAR pro-protein is cleaved into two subunits (P and E-subunit) that are then associated at the cell membrane by non-covalent bonds (Streuli et al., 1992). The P-subunit contains a small portion of extracellular region of the protein, the transmembrane region and the PTPase intracellular region that includes D1 and D2 domains. The D2 domain is responsible for liprin-α interaction with LAR, while the D1 domain is catalytic (Streuli et al., 1992). The P-subunit is shorter than the E-subunit. The E-subunit constitutes the extracellular portion of the protein, and includes a cell adhesion domain with three Ig-like domains and eight FN-III domains (Streuli et al., 1988). Together, Ig domains and FN-III domains form the CAM-like extracellular region of the receptor, commonly found in several other adhesion proteins (Streuli et al., 1992). Northern blot analysis has shown that LAR is expressed in human heart, brain, lung, kidney, pancreas, placenta, but not in skeletal muscle. In brain, it has been observed the expression of a splice variant of LAR indicating that this protein undergoes alternative splicing in a tissue-specific manner (Pulido et al., 1995). LAR protein has many functions: it transduces cell-cell adhesion signalling and cell-ECM signalling, can control actin organization (Beltran & Bixby, 2003), and it is responsible for the dephosphorylation of focal adhesion proteins, thus regulating focal adhesion turnover (Serra-Pages et al., 1998). Moreover, LAR regulates cell adhesion and migration through the desphosphorylation of
Trio, a GEF for Rac and Rho (Debant et al., 1996) and EphrinA2, a tyrosine kinase receptor involved in the axon (Wilkinson, 2000). Serra Pagés and colleagues have shown that the interaction between LAR and liprin-α1 is mediated by the D2 distal PTPase domain of LAR and involves the second SAM domain of liprin-α1 (Serra-Pages et al., 1995; Serra-Pages et al., 1998). It has been demonstrated that LAR cooperates with liprin-α1 in the regulation of cell spreading on FN, since LAR depletion causes a decrease of the cell area (Astro et al., 2011). The loss of the SAM2 domain of liprin-α1 prevents the enhancement of cell spreading observed after either liprin-α1 or LAR overexpression, indicating that the effect of liprin-α1 on spreading is mediated by the interaction with LAR. On the other hand, LAR silencing has no effects on tumor cell invasion, thus suggesting that liprin-α1 may regulate cell invasion independently from LAR (Astro et al., 2011).

1.4.5.2 GIT family proteins

Arf-GAP proteins form a heterogeneous family characterized by the presence of an ArfGAP domain. Arf-GAP GTPase-activating proteins stimulate the hydrolysis of GTP by Arf GTPases (R. J. Hoefen, 2006), small GTP-binding proteins involved in Golgi/ER perinuclear membrane trafficking and recycling of plasma membrane components at the cell periphery (H. Sabe et al., 2006; A. Spang et al., 2010). There are 31 genes in humans encoding proteins with an ArfGAP domain. (PA Randazzo and RA Kahn, 1994). Arf-GAP proteins have been divided in 10 subfamilies, including the family of GIT proteins. The human genome encodes two GIT proteins: GIT1/p95-APP1 and GIT2/p95-APP2/PKL (Premont et al., 1998). GIT1 is expressed in a unique form while GIT2 undergoes alternative splicing in a tissue-specific way (Premont et al., 2000). GIT1 presents at the N-terminus an ArfGAP domain, three ankyrin repeats (ANK) that mediate protein-protein interaction, and a Spa2-homology domain (SHD). The C-terminal region includes a coiled coil domain and a paxillin-binding site (PBS) (Paris et al., 2003). GIT proteins bind several partners such as PIX family of Rac1/Cdc42 GEF, that forms stable complexes with the GIT proteins (Z.S. Zhao et al., 2000), the focal adhesion adapter paxillin (R.T. Predemont et al., 2000), and the focal adhesion kinase (FAK) (Z.S. Zhao et al., 2000). These proteins have been observed in a complex localized at focal adhesions, and it has been suggested that they may be involved in the regulation of membrane trafficking, cytoskeleton dynamics and focal adhesion turnover (de Curtis, 2001). At the same time, evidence supports the role of GIT proteins in regulating cytoskeletal dynamics during...
cell spreading and migration (Manabe et al., 2002). For example, GIT proteins play an important role in maintaining the directionality of cell movement possibly by recruiting Rac1 at the cell edge (R. J. Hoefen, 2006). In fact, the overexpression of a constitutively active Rac leads to an increase of GIT1 localization at focal adhesions and at the leading edge in lamellipodia. (Manabe et al., 2002). Consistently, the overexpression of GIT1 enhances cell migration and causes a translocation of paxillin from focal adhesion to perinuclear vesicles (Zhao et al., 2000). Some studies indicate that GIT1 is a scaffold protein for MAP kinase activation, to promote the activation of ERK1/2 at focal adhesions (G. Yin et al., 2005). Other studies have hypothesized that GIT1 and GIT2 regulate focal adhesion and membrane trafficking in different ways. It has been observed that phosphorylation of GIT2 by Src or FAK is necessary for the translocation of the Nck-PAK-Pix-GIT2 complex to the adhesion sites and for the regulation of their turnover (MC Brown et al., 2005). Consistently, it has been observed that downregulation either of Src or FAK resulted in the inhibition of GIT2 recruitment at adhesion sites, whereas no effects have been observed for GIT1 (Bagrodia et al., 1999). Moreover, GIT1 localizes both at focal adhesion as well as along stress fibers, while GIT2 localizes only to paxillin-positive focal adhesion (Y. Shikata et al., 2003). GIT complexes play a role also in regulation of chemoattractant-induced cell motility and receptor trafficking although GIT1 and GIT2 seem to control different subsets of agonist-induced responses (M. Gavina et al., 2010). In 2003 Ko and colleagues have identified the interaction between both GIT1 and GIT2 with liprin-α1 by yeast two-hybrid screen. It has been shown that the minimal region of interaction between GIT1 and liprin-α1 is the portion of GIT1 including 523-770 amino acid residues and residues 513-673 for GIT2 (J. Ko et al., 2003). Furthermore, it has been observed that GITs proteins form complexes with both liprin-α1 and liprin-α2. Recently, work from our laboratory has demonstrated that liprin-α1 competes with paxillin for the binding to GIT1, while liprin-α1 does not interfere with the interaction between GIT1 and βPIX (C. Asperti et al., 2011). Moreover it has been shown that both GIT1 and liprin-α1 play a role during cell spreading in reorganizing the cell edge. The depletion of each protein in fact, leads to the inhibition of cell spreading. Finally, the silencing of GIT1 reverts the positive effect of liprin-α1 overexpression on cell spreading and migration. This indicates that the two proteins work in the same pathway during cell migration (C. Asperti et al., 2011). Conversely, in a study on the role of liprin-α1 in invasion, it has been observed that silencing of GIT/PIX complexes does not influence either cell invasion, or migration (Astro et al., 2011).
1.4.5.3 ERC/ELKS and ELKS-associated proteins

ERC/ELKS has been first identified as a gene fuse to RET, a tyrosin kinase receptor rearranged in thyroid papilloma (Nakata et al., 1999). ELKS is also known as Rab6IP2 (Monier et al., 2002), CAST (Ohtsuka et al., 2002) and ERC (Wang et al., 2002). The mammalian genome encodes two Erc proteins: Erc1 and Erc2 (Ko et al., 2003). ERC transcript undergoes alternative splicing generating a short isoform that is brain-specific, ERC1b, and five ERC1a proteins, with different C-termini: ELKSα, ELKSβ, ELKSγ, ELKSδ and ELKSε. ELKSα and ELKSβ are present in the brain, ELKSγ and ELKSδ are expressed in testis and thyroid while ELKSε is detected also in other tissue. ERC2 is brain specific and it is expressed in a unique form. Although both ERC1b and ERC2 are neuro-specific, they show a different subcellular localization in neurons (Ko et al., 2003). In fact, ERC1b is expressed as a cytosolic protein as well as an active zone component, while ERC2 is an active-zone specific protein (Ko et al., 2003; Nakata et al., 1999). Prediction studies on the secondary structure of the peptide indicate the presence of coiled coil regions throughout the protein, and a leucin zipper at the C-terminus (Ducut Sigala et al., 2004). The C-terminus ends with a PDZ-binding domain (Songyang et al., 1997). The interaction between liprin-α1 and ERC1 has been described by Ko and colleagues in a neuronal system (J. Ko et al., 2003). The active zone specific protein ERC2 promotes the synaptic accumulation of liprin-α, and the two proteins regulate together the trafficking of vesicle at the active zone.

ERC1a (ELKSε) interacts with the active form of Rab6A, Rab6A’, and Rab6B. Rab6 is a member of the Rab family, small GTPases that play a role in both retrograde vesicle transport from the endosomes to the ER and in anterograde transport, from the Golgi to the cell membrane (Monier et al., 2002; Grigoriev et al, 2007). ERC1a interacts with Rab6 through a portion of the C-terminus (aa 950-1015) (Grigoriev et al, 2011). Cells expressing the Rab6-binding domain of ERC1a (Rab6BD) show a partial inhibition of the transport between the Golgi and the plasma membrane, whereas the overexpression of the full length of Erc1a protein does not impair the exocytic process (Monier et al, 2002). Conversely, ERC1a depletion induces the accumulation of the exocytic Rab6-positive vesicles at the cell periphery. ERC1a silencing also affects the fusion of Rab6-positive vesicles at the cell membrane. On the other hand, ERC1a depletion impairs neither the formation of Rab6 vesicles from the Golgi, nor their movement mediated by microtubule. Finally, ERC1a localizes preferentially at regions of the cell membrane where the
docking and fusion of Rab6-positive vesicles occurs (Grigoriev et al, 2007).

Two other main functions of ERC1a have been described: the involvement in the exocytosis calcium-dependent in PC12 cells through the interaction with RIM (Inoue et al, 2006) and the regulation of exocytosis in mast cells and pancreatic β-cells. (Ohara-Imaizumi & Nagamatsu, 2005).

Furthermore, emerging evidence shows that ERC1a is involved in the regulation of cell migration and invasion through its interaction with liprin-α1 and LL5 proteins. LL5 proteins (LL5α and LL5β) have been identified for their interaction with PtdIns3P (PIP3) through their C-terminal PH domain (Dowler et al., 2000). It has been reported that LL5 can bind signalling proteins by recruiting them in PIP3 enriched membrane areas (Kishi et al., 2005). Moreover, it has been observed the interaction between LL5, ERC and cytoplasmic linker-associated proteins (CLASPs). This complex stabilizes microtubules and positively regulates the focal adhesion size (Akhmanova et al., 2001, Lansbergen et al., 2006). In view of these data, one hypothesis is that LL5β synchronizes actin filaments and microtubules, thus coordinating cytoskeletal components for efficient migration (Takabayashi et al., 2010).
1.4.5.3.1 The liprin-α1/Erc1a/LL5s complex in the regulation of tumor cell motility

Recent studies have demonstrated that liprin-α1, ERC1a and LL5 proteins cooperate together to regulate tumor cell migration and invasion affecting lamellipodia dynamics, β1-integrin internalization, focal adhesion turnover, and the organization of cortical microtubules (Lansbergen et al., 2006; Van der Vaart et al., 2013, Astro et al., 2014). Specifically, these proteins are co-expressed in both invasive breast cancer cell (MDA-MB-231) and HeLa cells, and it has been shown that they colocalize in cytoplasmatic structures concentrated near the active protrusions of migrating cells. The depletion of either ERC, LL5s or liprin-α1 inhibits cell spreading, migration on FN and Matrigel invasion. Moreover, their silencing results in increased lamellipodia dynamics, and in the inhibition of active β1 integrin internalization. Mutation in the PH domain of LL5 inhibits the accumulation of the proteins at the cell edge, and negatively regulates lamellipodia stability. Altogether these data indicate that the three proteins are recruited to the plasma membrane and may be a part of structures that regulate the stabilization of lamellipodia and cell adhesion turnover, two processes fundamental for an efficient migration (Fig 1.16) (Astro et al., 2014).
2 Aim

The study of the mechanisms that underlie the invasive and migratory processes of tumour cells represents a fundamental aim of the research in cancer biology. Cell migration is a cyclic process that requires remodeling of the cytoskeleton and cellular polarization. The migratory activity of cancer cells is frequently associated with ECM degradation that leads to neoplastic cell dissemination. Recent findings have revealed that liprin-α1 is a regulator of tumor cell migration and invasion in vitro. Interestingly, the gene PPFIA1 coding for liprin-α1 protein is frequently amplified in cancer, suggesting that liprin-α1 has a key role in malignant progression. Liprin-α1 is an ubiquitous scaffold protein that interacts with several partners, which in turn participate at events of membrane recycling, cytoskeletal reorganization, and adhesion assembly and/or disassembly.

The first aim of this thesis has been the study of the involvement of liprin-α1 in invasion in vivo, investigated by performing two different metastasis assays. These assays were performed with cell lines either overexpressing, or depleted of liprin-α1. I evaluated the ability of these cell lines to give rise to metastases in the lungs. The results of my work indicate that liprin-α1 is not a regulator of tumor cell growth, but it is specifically involved in tumor cell invasion in vivo.

In the second part of the thesis I focused on the investigation of the role of liprin-β proteins in the liprin-α1-mediated processes. For this purpose I first tested whether liprin-β1 and liprin-β2 interact with liprin-α1, and then whether their depletion had an effect on cell migration and invasion. I demonstrated that liprin-β1 and liprin-β2 have distinct roles in cell motility: the first interacts and cooperates with liprin-α1 to promote migration and invasion; the latter does not interact with liprin-α1, acting in an opposite way by inhibiting the migratory/invasive potential of the tumor cells.
3 RESULTS

3.1 Role of liprin-α1 for invasion in vivo

It has been shown that the PPFIA/liprin-α1 gene is amplified in about 20% of breast cancers (Al-Kuraya et al., 2004). In order to understand whether this amplification corresponded to an increased level of the protein, a previous study in our laboratory has included the immunohistochemical analysis of 116 human breast cancer samples (Astro et al., 2011). Immunohistochemistry for liprin-α1 has revealed an increase of liprin-α1 levels in about the 50% of the examined biopsies. Established data have demonstrated the involvement of liprin-α1 in breast tumor cell migration and invasion in vitro. In view of these results, I have addressed the involvement of liprin-α1 during invasion in vivo. In collaboration with the anatomo-pathology unit of the San Raffaele Hospital, I extended the analysis by adding new human breast cancer cases. This analysis confirms the increment of the liprin-α1 protein levels in the tumor tissue (Fig 3.1). Next, I explored the role of liprin-α1 in vivo by developing the following experimental plan: I generated stable cell lines with liprin-α1 overexpression or down-regulation, characterized them in vitro and then injected or transplanted these cells in vivo to test their tumorigenic and invasive potential. The high invasive human breast cancer cell line MDA-MB-231 was chosen to generate the stable clones. This lineage has been established from pleural effusion from metastatic adenocarcinoma (Brinkley et al., 1980). MDA-MB-231 cells are classified as basal-like subtype due to their lack of expression of the estrogen Receptor (ER), progesteron receptor (PR) and the transmembrane receptor HER2 and for the expression of the EGF receptor (EGFR) (Subik et al., 2010). Basal subtype tumors are often associated with visceral organs metastasis; they are more aggressive and poorly differentiated and have poor prognosis (Yersal et al., 2014). MDA-MB-231 cells are commonly used for invasion study in vitro and are regarded as a useful model for experimental metastasis in vivo (D.L. Holliday and V. Speirs 2011). Recent studies have also described the use of MDA-MB-231 in orthotopic transplantation NOD scid gamma (NGS) mice (E. Iorns et al., 2012).

3.1.1 Analysis of human breast cancer samples

Data from our laboratory have demonstrated an increase of the liprin-α1 protein levels in cancer tissues in comparison to healthy tissue (Astro et al., 2011). In collaboration with
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The Pathology Unit at San Raffaele Institute, I analyzed 142 new human breast samples by immunohistochemistry with two different antibodies specific for liprin-α1. We divided the samples in three categories based on the intensity of the signal for liprin-α1 (Fig 3.1) and analyzed the correlation between the levels of liprin-α1 protein and the stage of the tumor. The analysis of the new samples confirmed that the expression of endogenous liprin-α1 was increased in the majority of the cases (Fig 3.1A). Furthermore, in collaboration with professor Fesce of University of Insubria, we investigated by statistical analysis, the possibility of a correlation between liprin-α1 expression levels in biopsies and some parameters such as the stage of the tumor, the relapse of the tumor, the lymphonodal involvement and the expression of hormone receptors. This preliminary analysis did not show significant association between the amount of the protein and the diverse aspects of cancer probably due to the relative low number of cases analyzed. However, deeper analysis are required to define the relationship between liprin-α1 expression levels and the tumor phenotype.

3.1.2 Generation and characterization of cell lines stably overexpressing liprin-α1

The highly invasive human breast cancer cell line MDA-MB-231 has been chosen as a cellular model to generate stable clones of cells overexpressing liprin-α1. The monoclonal cell lines were tested for the rate of proliferation and the ability to migrate and invade in vitro. The data regarding the characterization of liprin-α1 overexpressing clones have been recently published in Journal of Cell Science (Astro et al., 2014).

Fig. 3.1 Immunohistochemical analysis of the expression of the liprin-α1 protein in human breast cancer. Samples from 142 human breast cancers were analyzed for Liprin-α1 expression in tumor cells. The signal for Liprin-α1 is very low in normal tissue within the same samples. Signal: 0-1, no/very low, as in sample (B); >1-2, clearly increased in tumor cells; >2-3, strongly increased in tumor cells, as in samples (C) and (D).
3.2.1A Overexpression of Liprin-α1 does not affect MDA-MB-231 cell proliferation and viability

MDA-MB-231 cells were transfected with either the pEGFPLiprin-α1 or the pEGFP construct (the latter was used as a control). After selection with G418, liprin-α1 protein levels were analyzed in three independent monoclonal cell lines overexpressing EGFP-Liprin-α1 (clones L4, L12 and L17) and in one control clone overexpressing EGFP (clone C9) (Fig.3.2A). Cell proliferation rate and viability were tested by growth curve and MTT assay, respectively. The results demonstrated that liprin-α1 overexpression did not affect either proliferation or cell viability (Fig 3.2 B-C).
3.1.2.B Liprin-α1 overexpression enhances cell invasion

In order to characterize the functional phenotype of the clones, I investigated the effects of liprin-α1 overexpression on invasion in vitro by using a transwell invasion assay on Matrigel. Liprin-α1 overexpression (Fig 3.3A, lane Luc) enhanced the invasive ability of the cells in comparison to the control cell line (C9) (Fig.3.3B). The increased invasion by these cell lines was prevented by liprin α-1 silencing (Fig 3.3A, lane Lip), confirming the specificity of the observed effects (Fig 3.3B). These results confirm the involvement of liprin α-1 in breast cancer cell invasion in vitro (Astro, et al., 2011). Moreover, these data show that the overexpression of liprin-α1 is able to potentiate the already high invasive potential of MDA-MB-2321 cells in vitro.
Fig 3.4 Liprin-\( \alpha \)1 is required for the efficient migration of human breast cancer cells. For random migration, 50,000 transfected cells were seeded for 2 h on 2.5 \( \mu \)g/ml FN-coated dishes before acquisition by video-imaging. (A) Analysis of random migration: velocity and total distance were evaluated during 5 h with ImageJ software. Bars represent normalized mean values ± s.e.m. (n= 42-78 cells per condition from two independent experiments). **P<0.005. (B) Cell tracking for 5 h of wild-type (WT), control (C9), and liprin-\( \alpha \)1 overexpressing cells (L4, L12, L17). Cells undergoing division and non-moving cells were excluded from the analysis.

Fig 3.5 Liprin-\( \alpha \)1 overexpression in 3D migration. For 3D fibrillar migration, 50,000 cells were seeded for 6 h on the top of fibroblast-derived 3D extracellular matrix before imaging. (A) Analysis of mean velocity, total distance and persistence of migration evaluated during 8 h with ImageJ software. Bars represent normalized mean values ± s.e.m. (n= 72-87 cells per condition, from two independent experiments). **P<0.005. (B) Cell tracking for 8 h of wild-type cell (WT), control (C9), and liprin-\( \alpha \)1 overexpressing clone (L12, L17). Cells undergoing division and non-moving cells were excluded from the analysis.
3.1.2.C Liprin-α1 overexpression enhances both 2D and 3D cell migration

Since liprin-α1 affects cell motility (Asperti et al., 2009), I tested the migratory ability of the clones overexpressing liprin-α1 in vitro. Two different approaches were used: 2D random migration and 3D fibrillar migration assays. For the 2D migration assay, cells were plated on FN coating (2.5 μg/ml) and were free to randomly move in absence of specific stimuli. For the 3D fibrillar migration, cells were plated on a fibroblast-derived 3D ECM composed mainly of FN fibers, and incubated for 6h to allow them to penetrate the matrix. The presence of the matrix gives some advantages: it creates a more physiological environment than 2D substrates. In addition, the fibronectin fibers act as guidelines for cell movement, thus allowing the study of the directionality of cell migration along the fibrillar meshwork (Cukierman et al, 2001). The results from these two different assays were similar: liprin-α1 overexpressing clones showed increased total distance covered by the cells both in 2D random migration assay (Fig 3.4 A) and in 3D fibrillar migration (Fig 3.5 A). Even though the liprin-α1 depletion led to a decrease in the persistence of migration, we did not observe any variation in the directionality in clones overexpressing liprin-α1(Fig 3.5).

3.1.2.D Liprin-α1 overexpression influences lamellipodia dynamics

Previous work in the laboratory demonstrated that liprin-α1 silencing causes an increase in the frequency of lamellipodia forming per cell and a decrease of the average lifespan of each lamellipodia (Astro et al., 2011). Consistent with the published data, the analysis of lamellipodia dynamics revealed that liprin-α1 overexpression caused a decrease of the frequency of lamellipodia formation (fig 3.6 A) but an increase of the average lifespan of these protrusions (Fig 3.6 B). These effects were abolished by silencing of liprin-α1 (Fig 3.6). These observations confirmed that liprin-α1 is a key regulator of lamellipodia dynamics. Liprin-α1 plays a role in the stabilization of these protrusive structure that are involved in cell migration and invasion.

3.1.3 Generation and characterization of liprin-α1 depleted cell lines.

In several organisms the introduction of double-stranded RNA has proven to be a powerful tool to suppress gene expression through a process known as RNA interference (Sharp
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**Fig. 3.6 Liprin-α1 regulates the stability of lamellipodia.** Cells overexpressing liprin-α1 (L4) and control cells (C9) were analyzed during random migration on FN. Images collected during 5h of acquisition were quantified using ImageJ. (A) Number of lamellipodia produced in 1h. Cells overexpressing liprin-α1 produce (A) less frequent and (B) more stable lamellipodia compared to control cells. The effects of liprin-α1 overexpression are abolished by silencing liprin-α1. Bars are means ± s.e.m. (n=10 cells per experimental condition). *P<0.05; **P<0.005.

**Fig 3.7 Liprin-α1 depletion does not affect cell viability.** (A) Levels of liprin-α1 residual expression detected by western blot. Lane 1: control cell line, lane 2: clone shRNA1-2; lane 3: clone shRNA1-5; lane 4: clone shRNA2-1; lane 5: shRNA2-3. (B) For the MTT assay, 2000 cells/well were seeded in 96 well plates and viability was measured every 24 h. No significant differences were detected by the unpaired Student’s t-test.
et al., 1999). However, the reduction of gene expression is transient. To overcome this limitation several mammalian vectors were developed to direct intracellular synthesis of siRNA-like transcripts. These vectors integrate in the genome to produce a small RNA transcript that specifically down-regulates gene expression. In order to obtain MDA-MB-231-derived cell lines that stably down-regulate liprin-α1, I took advantage of the pSuper RNAi system (Brummelkamp et al., 2002). This construct is bicistronic and encodes GFP and the neomycin resistance, allowing a double selection both by fluorescence and by the use of G418. In order to avoid off-target effects, I designed two independent shRNAs based on the sequence of validated siRNAs specific for liprin-α1. The empty pSuper vector expressing GFP was used to generate the control line. The shRNAs were cloned into the pSuper RNAi vectors and transfected into MDA-MB-231 cells. Monoclonal cell lines were obtained by limiting dilution in the selection medium. The clones were firstly tested for liprin-α1 silencing (Fig 3.7 A). Cell lines that showed the strongest silencing of liprin-α1 were examined for cell viability and invasion in vitro (Fig 3.7 B-3.8).

3.1.3.A Silencing of liprin-α1 does not affect MDA-MB-231 cell proliferation and cell viability

I analyzed the residual expression of liprin-α1 in the cell lines obtained. Several clones expressing either shRNA had a good level of silencing, indicating the high efficiency of the transfected silencing plasmid. On the other hand, as expected, transfection of the control vector did not affect liprin-α1 expression. In particular, western blots showed that clones shRNA1-5 (sh1-5) and shRNA2-1 (sh2-1) had a percentage of liprin-α1 depletion of 88% and 98% , respectively (Fig 3.6 A). The MTT assay revealed that liprin-α1 depletion did not affect cell viability compared to wild type (WT) and control cells (ctrl) (Fig 3.7 B).

3.1.3.B Silencing of Liprin-α1 affects MDA-MB-231 invasion

The liprin-α1-depleted clones were tested in order to assess their invasive ability in vitro. Invasion examined by transwell invasion assay on Matrigel was decreased for cells from clones with liprin-α1 silencing compared to cells from the clone transfected with the empty vector (Fig. 3.8).
3.1.4 **Analysis of the role of liprin-α1 in vivo**

To address the role of liprin-α1 in tumor cell invasion *in vivo*, I used the MDA-MB-231-derived cell lines described in the previous paragraph to perform metastasis assays *in vivo*. I have utilized for this purpose two different approaches: experimental metastasis assay and spontaneous metastasis assay (C. Khanna and K. Hunter 2005). The difference between the two methods resides in the way cells are delivered to the animals. In the first approach the tumor cells are injected directly in the systemic circulation, while in the spontaneous metastasis assay the tumor cells are transplanted in the anatomic location or tissue type from which they have been derived. The two methods give different information and in many cases they are complementary (C. Khanna and K. Hunter 2005).

The experimental metastasis assay focuses on the later steps of metastatization: the ability of the tumor cells to extravasate and on their ability to survive at distant metastatic sites. The site of injection determines the organs that tumor cells will colonize. For example, injection in the lateral tail vein results in pulmonary metastasis, intrasplenic and portal vein injection results in development of hepatic metastasis, while intracardiac injection causes metastasis formation in the bone (A.F. Chamber et al., 2000). The main advantages of this approach are the rapid clinical manifestation of the disease and the reproducibility of the results. I utilized athymic nude female mice as animal model for this kind of experiments according to published protocols (Fig 3.9 A) (M.M. Richert et al., 2005). The second approach, modelling the formation of spontaneous metastasis, mimics human cancers better, providing information more relevant for host-tumor interactions. Further-
more, this method gives the opportunity to study the early steps of the metastatic process (M.C. Bibby, 2003), and other characteristics of the pathology excluded by experimental metastasis assay such as primary tumor growth, vascularization, histology and gene expression (C. Khanna and K. Hunter, 2005).

The spontaneous metastasis assay consists in the inoculation of tumor cells either subcutaneously, intradermally or orthotopically. This results in the formation of a primary tumor that eventually could give rise to spontaneous metastasis (D. R. Welch, 1996). I performed orthotopic transplantation of MDA-MB-231-derived cell lines in NOD scid gamma (NSG) mice, as indicated in the literature (Fig 3.9 B) (E. Iorns et al., 2012).

3.1.4.A Liprin-α1 overexpression does not affect lung colonization in experimental metastasis assay

In order to study liprin-α1 involvement on invasion in vivo, I injected liprin-α1 overexpressing clones (L4, L12, L17), wild type cells and control cells (C9 GFP positive cells) in the

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Fig 3.10 Liprin-α1 overexpression does not affect lung colonization in experimental metastasis assay. (A) Analysis of metastatic area in 16 μm sections from left lungs of four mice injected with control cell line (C9 GFP+) as with cells of a clone overexpressing liprin-α1 (L17). Lung metastatic area was calculated as the ratio between the area occupied by the metastases and the total area of the section. Values are represented as percentages. The analysis was performed with ImageJ after setting a threshold to define for metastatic areas. Statistical analysis was performed with the unpaired t-test. (n=4 mice per experimental group). (B-C) hematoxillin and eosin staining of lung sections of mice transplanted with (B) control (C9GFP+) cells or with (C) liprin-α1 overexpressing clone L17. Arrows indicate examples of metastases.

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lateral tail vein of four-weeks athymic nude female mice (10 mice per experimental group) according to published protocols (M.M. Richert et al., 2005). After 4 weeks, mice were sacrificed, and lungs were explanted and processed. First, metastases on the surface of left lungs were counted at a dissecting microscope as described in the literature (Kwon et al., 2011). The analysis obtained was not sufficiently accurate because the metastases were different in size and too close to each other to be discriminated. Under these condition I could not observe significant differences in the number of metastatic foci between mice injected with liprin-overexpressing clones and control cell lines. To perform a more adequate analysis, the left lung of four mice injected with control cells (C9) and four mice injected with clone L17 were serially sectioned. Sections spanning the entire lung were stained with hematoxillin and eosin to identify the metastases (Fig. 3.10). For quantification, the ratio between the metastatic area and the total section area was measured. This preliminary analysis showed that liprin-α1 overexpression did not lead to differences in lung colonization in comparison with the control cells expressing endogenous levels of liprin-α1 (Fig 3.10).
3.1.4.B Liprin-α1 overexpression does not affect lung colonization in spontaneous metastasis assay

Since the results from the experimental metastasis assay did not show variations in lung colonization between mice injected with liprin-α1 overexpressing clones and control cell lines, I performed the spontaneous metastasis assay. Differently from the experimental metastasis approach, this experiment allows to understand whether liprin-α1 has a role in the formation of the primary tumor and its growth, and gives information on the ability of tumoral cells to degrade the matrix and to intravasate. Liprin-α1 overexpressing cell lines (L12 and L17), wild type cells (WT), and control cells (C9, GFP+) were inoculated into the fat pad of nine-weeks-old female NOD scid gamma (NSG) mice as previously
described (Mazzieri et al., 2011). Following transplantation, all the animals from each experimental group developed primary tumors. Tumor growth was measured four times during the experiments, and the growth curve derived from this analysis showed that liprin-α1 overexpression did not alter evidently the tumor growth by comparing with the tumors derived from control cells lines (Fig 3.11 A). Five weeks after the transplantation mice were euthanized, lungs collected, sectioned and analyzed as described for experimental metastasis assay (see methods). Once again, I did not observe difference in lung colonization among experimental groups (Fig 3.11 B). Considering the high invasive ability of MDA-MB-231 wild type cells, increase in lung colonization due to the liprin-α1 overexpression may be irrelevant in vivo. For this reason the study of the liprin-α1 silencing could be more informative and could highlight differences dependent on the expression of liprin-α1.

3.1.4. C Liprin-α1 silencing inhibits lung colonization in experimental metastasis assay

To study the effects of liprin-α1 silencing in lung colonization, I performed both experimental and spontaneous metastasis assays with liprin-α1-depleted cell lines. Wild type cells (WT), control cells (ctrl pSEV), and liprin-α1-depleted cell lines (sh1-5 and sh2-1) were injected into the lateral tail vein of four-weeks-old athymic nude female mice (4 mice per experimental group). After 4 weeks, mice were sacrificed and lungs were collected and processed as described (see methods). The analysis of lung metastatic areas revealed a reduction in lung colonization in mice injected with liprin-α1-depleted cells in comparison with mice injected with the control cell lines (Fig 3.12). The two liprin-α1-depleted cell lines showed a different efficacy to influence lung colonization. In particular, lungs from mice injected with clone sh2-1 presented a higher variability in the metastatic area even though the levels of liprin-α1 silencing were comparable between the two injected cell lines. This might be due to the intrinsic variability that characterizes in vivo experiments. Experimental metastasis assay was repeated and the reduction in metastatic area in mice injected with liprin-α1-depleted cells was confirmed (Fig 3.12 A). These are the first data supporting the requirement of liprin-α1 for efficient tumor cell invasion in vivo.
3.1.4.D Liprin-a1 silencing affects lung colonization in the spontaneous metastasis assay

The spontaneous metastasis assay was performed to confirm the results from the experimental metastasis assay, in a context that mimics better the metastatic process occurring in vivo. Control cells and liprin-α1-depleted cells were inoculated into the fat pad of nine-weeks old female NOD scid gamma (NSG) mice (4 mice per experimental group). Transplantated cells gave rise to primary tumors in all the mice from each experimental group. After transplantation tumor growth was measured four times and the growth curve showed that liprin-α1 depletion did not alter the tumor proliferation in comparison with the tumors originated by control cell lines (Fig 3.13 A). Five weeks after the transplantation, mice were sacrificed and primary tumors were analyzed by western blot and the immunofluorescence confirmed that the silencing of liprin-α1 was maintained until the end of the experiment (Fig 3.13 B-C). These data suggest that liprin-α1 is not involved in tumor cells proliferation. In support of this hypothesis. Then I analyzed lung colonization. For this purpose, sections spanning the entire lung

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were stained with hematoxillin and eosin to identify the metastases (Fig. 3.14). For quantification, the ratio between the metastatic area and the total section area was measured. Mice transplanted with liprin-α1-depleted cells presented a reduction in the formation of lung metastasis when compared mice transplanted with control cells expressing normal levels of liprin-α1. The results obtained in the spontaneous metastasis assays strengthened the results obtained with the experimental metastasis assay indicating that liprin-α1 plays a role in the process of metastasis formation (Fig 3.14). The reduction observed in spontaneous metastasis assay was stronger than in the experimental metastasis assay.

**Fig 3.13** Liprin-α1-depletion does not affect tumor growth. Orthotopic transplantation of liprin-α1-depleted cells (clones sh1-5 and sh2-1), wild-type cells (WT), and control cells (ctrl, pSEV) in NSG mice. (A) Primary tumor growth curve: tumor growth was measured at 20, 27, 34, 36 days after transplantation. No significative differences were detected by the unpaired Student's t test. Western blot analysis and immunofluorescence analysis on primary tumors showed that liprin-α1 silencing was maintained during the experiment. (B) Left panel: western blot performed on each mouse of the experimental group; right panel: quantification of liprin-α1 silencing; ** P<0.01, * P<0.05. (C) Immunofluorescence on a representative section of control (left) and clone sh1-5 (right) primary tumor. Staining with antibody for liprin-α1 (red), and DAPI (blue).
3.2 Study of liprin-α1 interactors: the liprin-β family members

Previous studies have described the interaction between liprin-α1 and liprin-β proteins (Serra-Pages et al., 1998; van der Vaart et al., 2013) and have suggested a possible role of liprin-β1 and liprin-β2 on migration and invasion (Kriajevska et al., 2002; von Thun et al., 2012). However, the available data regarding the liprin-β proteins and their relationship with liprin-α1 are not exhaustive. In order to understand better the mechanisms by which liprin-α1 regulates cell motility, I have investigated further the physical interaction between liprin-α1 and both liprin-β1 and liprin-β2 and to address their function in tumor cell motility.
3.2.1 **Biochemical characterization of the interaction between liprin-α1 and liprin-β proteins**

In 1998 Serra Pagès and colleagues identified liprin-β proteins as liprin–α1 partners by an interaction trap assay. They reported that liprin proteins interact through their C-terminal regions including the three SAM domains in both liprin-α and -β’s. They also performed immunoprecipitation experiments to confirm the data from the trap assay but they could not detect the interaction between liprin–α1 and liprin-β1, probably due to the strength of the detergent used (Serra-Pages et al., 1998). A recent study confirmed the interaction between liprin–α1 and liprin-β1 by co-immunoprecipitation from HeLa cells (van der Vaart et al., 2013) while the binding between liprin–α1 and liprin-β2 has been not investigated, yet. I performed immunoprecipitation experiments with either endogenous or overexpressed proteins in order to characterize the binding between alpha and beta liprins.

3.2.1.A Liprin-α1 interacts with liprin-β1 but not with liprin-β2

To observe the interaction between the endogenous proteins, I performed immunoprecipitation experiments from lysates of MDA-MB-231 cells. The lysates were immunoprecipitated with a monoclonal antibody anti-liprin–α1, then blots were incubated with the same antibody or with antibodies specific for either liprin-β1 or liprin-β2. The results indicated the good efficiency of the liprin–α1 antibody used to immunoprecipitate, since liprin–α1 was depleted from the unbound fraction of lysates (Fig 3.15, UB lane). I could observe the interaction between liprin–α1 and liprin-β1 confirming the published data (Fig 3.15). On the contrary, the binding between liprin–α1 and liprin-β2 was not detected, probably due to the low levels of liprin-β2 protein in MDA-MB-231 cells. In order to overcome the limitation of the low amount of liprin-β2 protein in MDA-MB-231 cells. In order to overcome the limitation of the low amount of liprin-β2, I performed immunoprecipitation with overexpressed proteins in COS7 cells. I used this cell line because transfection is more efficient in COS7 than in MDA-MB-231. FLAG-Liprin–α1 was co-transfected with either GFP-Liprin–β1 or GFP-Liprin–β2, and biochemical analysis was carried on by immunoblotting. The results pointed out that FLAG-Liprin–α1 interacted with GFP-Liprin–β1 but not with GFP-Liprin–β2 (Fig. 3.16). This is the first evidence of the different ability of the two liprin-β proteins to interact with liprin–α1 even though in literature was hypothesized a similar biological behavior for liprin–β1 liprin–β2, based on their sequence similarity. Van der Vaart and colleagues have been reported by that liprin–β1 is a member of the li-
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prin–α1 complex, (van der Vaart et al., 2013). I would speculate that liprin–β1 cooperates with liprin–α1 in regulating cell motility, whereas liprin–β2 may interact with different specific partners acting independently by the other liprin proteins.

3.2.1.B The interaction between FLAG-Liprin-α1 and GFP-Liprin-β1 is mediated by SAM domains

The interaction trap assay has shown that liprin–α1/liprin–β1 interaction is mediated by the C-terminal SAM domains of the two proteins (Serra-Pages et al., 1998). In order to confirm these data, I generated FLAG-Liprin-α1 mutants lacking either the C-terminal (ΔSAM mutant) or the N-terminal (ΔN–Term mutant) (Fig 3.17), and co-transfected each
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Results

**Fig 3.17 Liprin-α1 mutants used for the biochemical characterization of liprin-α1/liprin-β1 interaction**

Scheme of Liprin-α1 mutants generated to characterize liprin-α1/liprin-β1 interaction. In ΔSAM1-3 constructs, the red SAM is deleted.

**Fig 3.18 The C-terminal, but not the N-terminal part of liprin-α1 is required for liprin-α1/liprin-β1 interaction.** Co-immunoprecipitation with anti-GFP antibody from lysates of COS7 cotransfected with GFP-Liprin-β1 and either FLAG-Liprin-α1 full length (F.L.), FLAG-Liprin-α1 ΔSAM or FLAG-Liprin-α1ΔN-term mutants. Left panel, immunoprecipitation (IP): 300 μg lysates/immunoprecipitation were used. Right panel: lysates (LT); 30 μg of lysates were used. The membranes were blotted with anti-GFP antibody (upper panels) and anti-FLAG antibody (lower panels).

mutant with GFP-Liprin-β1 in COS7 cells. As expected, the ΔN–term mutant was able to interact with GFP-Liprin-β1, whereas the deletion of three SAM domains prevented the interaction between the two proteins confirming that the C-terminal region of liprin-α1 is necessary for the binding with liprin-β1 (Fig 3.18).
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**Fig 3.19 Deletion of a single SAM domain of liprin-α1 does not affect liprin-α1/liprin-β1 interaction.** Co-immunoprecipitation with antibody anti-GFP from lysates of COS7 cotransfected with either GFP (first lane, negative control) or GFP-liprin-β1 and either FLAG-liprin-α1 full length (F.L.), FLAG-liprin-α1ΔSAM1, FLAG-liprin-α1ΔSAM2, FLAG-liprin-α1ΔSAM3 or FLAG-liprin-α1F3F4. Left panel, immunoprecipitation (IP), 300 μg lysates/immunoprecipitation were used; Right panel: lysates (LT), 30 μg of lysates were used.

**Fig 3.20 liprin-α1 SAM3 domain alone does not interact with liprin-β1** Co-immunoprecipitation with antibody anti-GFP from lysates of COS7 cotransfected with GFPliprin-β1 and either FLAG-liprin-α1 full length (F.L.), FLAG-liprin-α1F2F3F4 or FLAG-liprin-α1F1. Left panel, immunoprecipitation (IP), 300 μg lysates/immunoprecipitation were used. Right panel: lysates (LT), 30 μg of lysates were used. The membranes were blotted with anti-GFP antibody (up) and anti-FLAG antibody (down).
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To understand the minimal region of liprin-α1 sufficient to mediate the interaction between liprin-α1 and liprin-β1, further biochemical analysis were performed. I used FLAG-Liprin–α1 mutants generated in the laboratory, lacking a single SAM domain, or the whole C-terminal region used as negative control (Fig 3.17, mutants ΔSAM1, ΔSAM2, ΔSAM3 and F3F4). Each mutant was co-trasfected with GFP-Liprin-β1 in COS7 cells. The results of the co-immunoprecipitation experiments showed that the deletion of a single SAM domain did not affect the interaction indicating that the binding between liprin–α1 and liprin-β1 does not required the three SAM domain together, since the presence of the two SAM domain (in all possible combination) is sufficient for binding to liprin-β1 (Fig 3.19).

To identify the minimal region of interaction between liprin–α1 and liprin-β1, I utilized mutants lacking different portions of the C-terminal. The data obtained from this analysis indicated that the deletion of SAM3 of liprin–α1 and a portion of SAM2 (mutant F2F3F4) partially inhibited the interaction (Fig 3.20). Furthermore, fragment including the only third SAM domain and a small portion of SAM2 of liprin-α1 (Fig. 3.17 mutant F1) was not sufficient to mediate the interaction with liprin-β1. Taken together, these data indicated that the three SAM domains form an organized super-structure that is redundant in some parts but that requires the presence of almost two SAMs to allow the interaction between liprin–α1 and liprin-β1.

3.2.2 Liprin-β1 but not liprin-β2 colocalizes with liprin-α1 at the cell edge.

Previous studies have shown that liprin-β1 co-localizes with liprin-α1 at the plasma membrane of COS7 and HeLa cells (Serra-Pages et al., 1998; van der Vaart et al., 2013). The subcellular localization of liprin-β2 has been never investigated. In COS7 cells, I could observe that endogenous liprin-β1 was enriched at the periphery of the cells and co-localized with Erc1α, a liprin-α1 interacting protein, at mature, central focal adhesions identified with paxillin (Fig 3.21). Liprin-β1 localized just behind the newly formed focal adhesions, as it has been described for liprin-α1 (Asperti et al., 2009) (Fig 3.21 B). Endogenous liprin-β2 could not be detected in COS7 cells probably due to the low amount of the protein in these cells. I also analyzed the localization of liprin-β1 and liprin-β2 in MDA-MB-231 cells, confirming the co-localization between liprin-α1 and liprin-β1 at cell periphery (Fig 3.22), while I could not detect endogenous liprin-β2 also in these cell line. Therefore, to verify the subcellular localization of liprin-β2, I overexpressed GFP-Liprin-β2 in MDA-MB-231 cells and I identified the protein by anti-GFP.
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Fig 3.21 Liprin-β1 colocalizes with Erc1a at the cell periphery. (A) Confocal images of COS7 cells plated on FN (10 μg/ml) for 1 h, fixed and immunostained for the endogenous liprin-β1 (green), Erc1a (blue) and paxillin (red). Scale, 20 μm. (B) Plot profile of the signal distribution two focal adhesions (FA) obtained with ImageJ plugin RGB profiler.

Fig 3.22 Endogenous liprin-α1 co-localized with liprin-β1 at cell periphery in MDA-MB-231 cells. (A) Confocal images of MDA-MB-231 cells plated on FN (10 μg/ml) for 1 h, fixed and immunostained for the endogenous liprin-α1 (red) and liprin-β1 (green). Scale, 20 μm. (B) Three-fold enlargement of the area indicated by the box in (A).
antibodies. I co-stained cells for GFP-Liprin-β2, endogenous liprin-α1 and endogenous liprin-β1. GFPliprin-β2 did not co-localized with the other two proteins (Fig 3.23A, 3.24). On the other hand, the GFP-Liprin-β2 signal revealed puncta in the perinuclear region of the cells, partially co-localizing with cortactin, a marker of invadopodia (Fig 3.24B) (Weaver, 2008). GFPliprin-β2 puncta seemed to form ring structures, next to cortactin positive puncta (Fig 3.24 B). The overexpression of GFPliprin-β1 did not lead to the same
pattern, thus excluding that the puncta formed by GFP liprin-β2 were artefacts due to the overexpression of the protein (Fig 3.23B).

These findings indicate that the two members of liprin-β subfamily have a different subcellular localization and suggest that could act differently in cell motility.

### 3.2.3 Liprin-β2 partially co-localizes with cortactin in NIH3T3 Src fibroblast and its overexpression affects podosomes formation.

Invadopodia are actin-rich protrusions of the plasma membrane into the ECM. The main function of these structures is to degrade ECM by the protease accumulation and secretion at these structures (Weaver, 2006). Invadopodia were first identified in Src kinase-tras-
formed cells, and are thought to constitute the invasive machinery of transformed cells (Weaver, 2006). Normal cells such as macrophages and osteoclasts physiologically form structures similar to invadopodia, called podosomes, in order to cross and remodel the tissues (Linder and Aepfelbacher, 2003). Podosomes and invadopodia are both characterized by branched actin assembly, the dependence on Src kinase and by the same molecular components including focal adhesion proteins, integrins and proteases (Buccione et al., 2004). Since active Src-transformed cells form large podosomes, also called rosettes, they are commonly used for the study of the invasive machinery of the cells (Murphy and Courtneidge).

My findings from the immunofluorescence analysis of MDA-MB-231 cells indicate that liprin-β2 formed ring-like structures localized next to cortactin. To visualize better the localization of liprin-β2 with respect to cortactin, I took advantage of NIH3T3 Src-transformed fibroblasts. I transfected GFP-Liprin-β2 in these cells to detect the subcellular localization of the protein (Fig 3.25 A-B). The images showed a partial colocalization between liprin-β2 and cortactin even though I did not observe the ring structures observed in MDA-MB-231 cells (Fig 3.25 B). The plot profile of one representative invadopodia showed a co-localization of phalloidin with cortactin as described in literature (Weaver, 2006) (Fig 3.25 B lower panel). On the other hand, the peaks of signal corresponding to liprin-β2 suggested that this protein localized around cortactin. This suggests that liprin-β2 may be part of the invasive machinery even though it seems not to be a structural protein of invadopodia. Furthermore, I noted a reduction in the number of cells forming podosomes after transfection with GFP-Liprin-β2 in comparison with the non-transfected cells or with cells overexpressing GFP only.

For quantification, I divided cells in three categories: cells forming podosomes (rosette +, Fig 3.25 C), cells without podosomes (rosette -, Fig 3.25 C), and cells containing no rosettes but only actin rich puncta, as described in a previous work (Fig 3.25 C) (Proszynski and Sanes, 2013). This analysis shows a reduction in the number of cells forming podosomes in NIH3T3 Src transfected with GFP-Liprin-β2 in comparison to cells transfected with GFP (Fig 3.24 C). These findings support the hypothesis on the role of liprin-β family members on invasion. However, functional analysis is required to demonstrate their involvement in this process.
3.2.4 Liprin-β2 silencing enhances cellular invasion

A recent work from the laboratory of JC Norman has shown that liprin-β2 is involved in tumor cell invasion (von Thun et al., 2012). On the contrary, the functional role of liprin-β1 on invasion has never been studied. Therefore, I investigated the invasion ability of MDA-MB-231 cells after liprin-β1 or liprin-β2 silencing and compared the results with the effect of liprin-α1 depletion. Specific siRNAs against liprin-α1, liprin-β1 and liprin-β2 were transfected in MDA-MB-231 cells (Fig 3.26 A), and then invasion assays in vitro were performed (Fig 3.26 B). The results show that liprin-β1 silencing did not influence invasion evidently, whereas liprin-β2 depletion led to a significant increase in the invasive ability of the cells. This observation is consistent with the published data on liprin-β2 (von Thun et al., 2012), and suggest that liprin-β2 has an opposite role on invasion in comparison with liprin-α1. Trying to understand whether the silencing of liprin-β1 could influence the inhibition of invasion caused by liprin-α1 depletion, I performed double silencing for liprin-α1 and liprin-β1. The results indicated that liprin-β1 silencing altered not significantly the inhibition of invasion caused by liprin-α1 silencing (Fig 3.27). I also performed double silencing of liprin-α1 and liprin-β2 together to test
whether liprin-α1 and liprin-β2 could cooperate in the regulation of invasion. Interestingly, the double silencing of liprin-α1 and liprin-β2 led to a further increase of cell invasion in comparison to the increase observed in liprin-β2-depleted cells, suggesting a relevant role of liprin-β2 in regulating this process by acting independently from liprin-α1 (Fig 3.27). Further studies are required to clarify the functional link between these two proteins.

3.2.5 Liprin-β2 silencing enhances ECM degradation

Published data from our laboratory indicate that liprin-α1 silencing inhibits ECM degradation, whereas liprin-α1 overexpression increases it (Astro et al., 2011). Following the observation that liprin-β1 and liprin-β2 silencing have different effects on cell invasion,
I investigated whether liprin-β1 and liprin-β2 depletion might influence the function of invadopodia by assessing ECM degradation. The silencing of liprin-α1 was used as positive control of the experiments. The results showed that liprin-β1 silencing caused a reduction of the area of the cells, as seen after liprin-α1 depletion, whereas silencing of
liprin-β2 did not (Fig 3.28 B). Total degrading area was decreased after either liprin-α1 or liprin-β1 silencing, indicating that liprin-β1 could cooperate with liprin-α1 in regulating ECM degradation (Fig 3.28 C). On the contrary, the depletion of liprin-β2 protein led to an increase of ECM degradation, measured both as total degraded area and relative degraded area (Fig. 3.28 C-D). The relative degraded area was calculated as the value of total degradation area normalized on the projected cell area (Fig 3.28 B,D). The effect of liprin-β2 silencing on ECM degradation strengthens the hypothesis of a different role of this protein in the regulation of the function of invadopodia in respect to liprin-α1 and liprin-β1.

3.2.6 Liprin-β1 and liprin-β2 silencing have opposite effects on the migration of MDA-MB-231 cells.

Liprin-β1 functions during migration are unknown, whereas previous work indicated that the silencing of liprin-β2 caused a marginal increase in the persistance of migration (von Thun et al., 2012). I tested the ability of MDA-MB-231 to migrate after liprin-β1 and liprin-β2 silencing. The analysis was performed also in cells transfected with siRNA for liprin-α1 as control for the experiment. The 2D random migration assay showed that the
silencing of liprin-β1 led to a decrease of the speed and the total distance covered by the cells (Fig 3.29). Similar negative effects on migration were observed after silencing of liprin-α1 (Astro et al, 2011). On the contrary, preliminary results indicate that liprin-β2 silencing did not affect cell motility (Fig 3.29). These data support the hypothesis of the cooperation between liprin-α1 and liprin-β1 in regulating cell motility, while they indicate that liprin-β2 acts as an antagonist in this process.
4 DISCUSSION AND CONCLUDING REMARKS

4.1 Discussion

Liprin-α1 is a cytosolic scaffold protein involved in the regulation of cell adhesion and migration (Asperti et al., 2009; Asperti et al., 2010). Acting as a scaffold, liprin-α1 interacts with several partners, that mediate its function in the regulation of lamellipodia stability, focal adhesion turnover, membrane recycling and remodelling the actin cytoskeleton. Moreover, the gene PPFIA1, coding for liprin-α1, has been found amplified in human cancers. Consistently with this observation, a study from our laboratory in collaboration with the anatomo-pathology unit at San Raffaele Scientific Institute, has revealed that liprin-α1 protein levels are increased in about the 50% of human breast cancer samples analyzed by immunohistochemistry (Astro et al., 2011). Another study from our laboratory has demonstrated that liprin-α1 cooperate with ERC1a and LL5 proteins to regulate tumor cell motility in vitro (Astro et al., 2014). Indeed, the silencing of liprin-α1 in the invasive breast cancer cell line MDA-MB-231 causes an increase of lamellipodia. This results in a reduction of cell migration on FN and invasion through Matrigel. Conversely, the overexpression of liprin-α1 leads to the formation of more stable lamellipodia in comparison with the lamellipodia formed by control cells, resulting in increased cell motility. Starting from these interesting results, during my PhD I have investigated the role of liprin-α1 in vivo. I have tried to understand whether the effects of liprin-α1 overexpression/silencing observed in vitro were mirrored by an altered ability of the tumor cells to invade tissues and give rise metastases in mice. Further studies on mechanisms of action of liprin-α1, have shown that liprin-α1 interactors can mediate its functions in cell motility. Indeed, LAR and GITs proteins, two direct interactors of liprin-α1, cooperate with liprin-α1 in the regulation on cell spreading (Astro et al., 2011). It has been also shown that the liprin-α1 binding protein ERC1a, and the ERC1a-associated proteins LL5, cooperate with liprin-α1 in the regulation of tumor cell migration and invasion. A model has been proposed, in which liprin-α1, ERC1a and LL5 proteins are recruited to the plasma membrane of migrating cells by localizing close to the adhesion sites of active protrusion. The results suggest that the complex may regulate the stability of lamellipodia and cell adhesion turnover by influencing integrin internalization and as a consequence, cell motility (Astro et al., 2014). In order to better understand the mechanisms by which liprin-α1 influences tumor cell motility, the secondary aim of my PhD work, I have addressed the
role of other liprin-α1-binding proteins: the members of the liprin β-subfamily, liprin-β1 and liprin-β2. Few data are available about the role of liprin-β1 and liprin-β2 in cell migration and invasion. It has been described that liprin-β1 belongs to a complex including liprin-α1, ERC1a and LL5 proteins, even if its function remains unclear (Van der Vaart et al., 2013). Liprin-β2 is considered a potential interactor of liprin-α1, based on its sequence similarity with liprin-β1, although a direct interaction has not been demonstrated yet. Some observations indicate that liprin-β2 affects cellular migration and invasion (von Thun et al., 2012). However, further studies are needed to clarify the physical and functional relationship between liprin-α1 and liprin-β2.

4.1.1 Role of liprin-α1 in vivo

Focusing on the first part of the project I used the highly invasive breast cancer MDA-MB-231 cells to generate cell lines with either stable overexpression or silencing of liprin-α1. As expected, the characterization in vitro confirmed what has been previously observed after transient transfection of either GFPLiprin-α1 or liprin-α1 siRNA, respectively. Cells overexpressing liprin-α1 showed a lower number of lamellipodia generated per time unit, but an increase of lamellipodia persistence with respect to control cells. This led to an increased speed of migration on in comparison with the control cells. Furthermore, liprin-α1 overexpression led to an increased invasion ability through Matrigel. Conversely, it did not affect cell viability and proliferation. Liprin-α1-depleted cell lines showed a significant reduction in the invasion ability through Matrigel, while viability and proliferation rate were similar to the control cells. In order to investigate the effect of liprin-α1 overexpression or silencing on the invasion in vivo, two different assays were performed: experimental metastasis and spontaneous metastasis assays. The first consists in the injection of tumor cells in the lateral tail vein of the animals. This assay gives information about the extravasation ability of the cells and their ability to colonize distant organs and give rise to metastases (M.M Richert et al., 2005). The spontaneous metastasis assay consists either in an orthotopic or subcutaneous transplantation of tumor cells that generate a primary tumor and eventually metastatize at distant sites. This assay mimics human cancer growth and the process of metastasis formation. It gives information not only about the later stages of metastasis formation, as in the experimental metastasis assay, but also about the tumorigenic capacity of the cells transplanted, and the ability of the cells to detach from the primary tumor and to invade the surrounding ECM. However,
the two techniques are considered complementary in the literature. At the end of both assays I analyzed the formation of pulmonary metastases, since the injection in the lateral tail vein is expected to give rise metastases in the lungs. Moreover, MDA-MB-231 cells are described to give rise pulmonary metastases after orthotopic transplantation (Xue B. et al., 2013). I used female athymic nude mice for experimental metastasis assay, and NSG (NOD scid IL2r\(^{null}\)) mice for spontaneous metastasis assay as described in literature (Iorns et al., 2012). After orthotopic transplantation, tumor cell growth was monitored. I could observe that tumors originating from liprin-\(\alpha\)1 overexpressing cells had a proliferation rate similar to the tumors originated by control cells. This suggests that liprin-\(\alpha\)1 does not have a role in tumor growth. Surprisingly, both the injection and the transplantation of liprin-\(\alpha\)1 overexpressing cells did not result in an increase of lung colonization in comparison with controls. The results obtained could be explained considering that MDA-MB-231 cells are highly invasive per se, thus the potentiated lung colonization due to the liprin-\(\alpha\)1 overexpression may be underscore under the experimental conditions used. The injection of liprin-\(\alpha\)1-depleted cells has been more informative. As hypothesized, the intravein injection of liprin-\(\alpha\)1-depleted cells resulted in a decrease of lung colonization in comparison with the lung colonization observed in mice injected with control cells. These data suggest that the silencing of liprin-\(\alpha\)1 affects cells extravasation, possibly because of the instability of lamellipodia and the consequent defect in cell motility. I excluded a defect in cell proliferation because proliferation and survival of the clones were comparable with the control ones. The transplantation of liprin-\(\alpha\)1-depleted cells gave rise to tumors comparable with the control ones for volume and weight. Because of this, it may be hypothesized that liprin-\(\alpha\)1 is not involved in the growth of primary tumor. Furthermore, I analysed liprin-\(\alpha\)1 protein levels in primary tumors. Both western blot analysis and immunofluorescence indicated that the silencing of the protein was maintained during the tumor growth and the formation of metastases. The analysis of lung colonization confirmed the result obtained in the experimental metastasis assay. However, in spontaneous metastasis assay the reduction in lung colonization by liprin-\(\alpha\)1-depleted cells was stronger than in experimental metastasis assay. This suggests that liprin-\(\alpha\)1 silencing may affect not only the extravasation, but also the detachment of cells from the primary tumor and the invasion of the surrounding ECM. The data obtained with the spontaneous metastasis assay indicate that the altered migration observed in the functional assays in vitro is reflected by defects in different steps of tumor cell invasiveness in vivo. This is the first observation that supports a role of liprin-\(\alpha\)1 in invasiveness in vivo.
In order to address the involvement of both liprin-β1 and liprin-β2 in liprin-α1-mediated cell motility, I used COS7 cells for biochemical analysis and MDA-MB-231 cells for functional assays. Initially I confirmed the physical interaction between liprin-α1 and liprin-β1. This binding has been previously detected by interaction trap assay in COS7 cells (Serra Pages et al., 1998) and by immunoprecipitation and GST-pull down in HeLa cells (van der Vaart et al., 2013). I detected the binding also in MDA-MB-231 cells, by immunoprecipitating either the endogenous or the overexpressed proteins. This is the first evidence of the interaction between liprin-α1 and liprin-β1 in a breast cancer cell line. I performed further immunoprecipitation experiments to identify the region that mediates the link between the two proteins. Interaction trap assays have shown that the binding is mediated by the SAM domains of the two proteins (Serra Pages et al., 1998). I co-expressed in COS7 cells GFP-Liprin-β1 and FLAG-Liprin-α1 mutants lacking either the C-terminal or the N-terminal portion. Immunoprecipitation confirmed that GFP-Liprin-β1 interacted with both FLAG-Liprin-α1 full length and the C-terminus of FLAG-Liprin-α1. As expected, the lack of the C-terminal region in FLAG-Liprin-α1 prevented the binding. Interestingly, the immunoprecipitation of FLAG-Liprin-α1 mutants lacking only one of the three SAM domains indicated that this deletion was not sufficient to prevent the binding with GFP-Liprin-β1. This indicates that the interaction between liprin-α1 and liprin-β1 does not require all three SAM domains. In fact, the presence of two SAM domains (in every possible combination) in liprin-α1 is sufficient for binding to liprin-β1. The deletion of SAM3 and a portion of SAM2 did not prevent the binding. On the other hand, the mutant including the isolated SAM3 and a small portion of SAM2 did not interact with GFP-Liprin-β1. These observations partially agree with crystallographic studies. Indeed, a crystallographic study indicates that the SAM domains are positioned in tandem with a head to tail interaction and that the head face of SAM1 in liprin-α1 mediates the interaction with the tail of SAM3 of liprin-β (Fig 4.1 C) (Wei et al., 2011). Another study proposes two different models for the interaction between liprin-α1 and liprin-β1. In the first model, liprin-α1 and liprin-β1 associate to create a “closed dimer”, that is mediated by the interaction between the SAM3 domains of a single dimer of liprin-α with the SAM3 domains of a single dimer of liprin-β proteins (Fig 4.1 A). A second model proposes the formation of an “open scaffold”, in which the SAM3 domain of both α and β molecules interacts with more than one dimer (Fig 4.1 B) (Stafford et al., 2011). These two studies
propose either SAM1 or SAM3 as mediator of interaction but do not clearly identify the SAM domain involved (Fig 4.1). However, they hypothesized that SAM domains are folded in a structure that acts as a structural and functional supramodule not described before for SAM domains. My findings are consistent with the hypothesis of a supramodule, even though I observed that SAM3 alone is not sufficient to mediate the interaction between liprin-α1 and liprin-β1 and also the deletion of SAM1 does not prevent the binding. I would like to speculate that the region connecting the SAM may have a role in mediating the interaction, since in the single SAM-deleted mutants the connecting regions were conserved. One possibility is that the SAM domains form a structure with some redundant regions that can compensate the deletion of other portions in vivo. According to this hypothesis two SAM domains and the connecting regions of liprin-α1 are sufficient to mediate the formation of liprin-α/β heterodimers. However, further analysis required to test this hypothesis. No interaction could be detected between FLAG-Liprin-α1 and GFP-Liprin-β2 by immunoprecipitation. This suggests that liprin-β1 and liprin-β2 may have different partners and possibly different subcellular localization. This hypothesis was confirmed by immunofluorescent experiments. Endogenous liprin-β1 colocalizes at the cell edge with liprin-α1 and ERC1a, a liprin-α1-binding partner, while endogenous
liprin-β2 was not detected in MDA-MB-231 cells, possibly due to the low levels of the protein in this cell line. In order to overcome this limitation, GFP-Liprin-β2 was over-expressed in MDA-MB-231 cells and then detected by an anti-GFP antibody. GFP-Liprin-β2 signal revealed puncta in the perinuclear region of MDA-MB-231 cells, where invadopodia localize (Weaver et al., 2008). Overexpression of GFP-Liprin-β1 did not show this type of distribution. Co-staining for cortactin, a marker of invadopodia, showed that GFP-Liprin-β2 and cortactin formed a ring-like structure. This findings suggests a potential involvement of liprin-β2 in the formation of invadopodia. I also observed that the overexpression of GFP-Liprin-β2 in Src-transformed NIH3T3 cells negatively affected the percentage in the number of cells forming podosomes. Src is a key regulator of podosomes formation and Src transformed cells are a cellular model commonly used for the study of invadopodia. In NIH3T3-Src cells, the overexpression of GFP-Liprin-β2 caused a reduction in the number of cells forming podosomes suggesting that liprin-β2 is implicated in their formation. To address the involvement of liprin-β1 and liprin-β2 in cell invasion and migration, I tested the effect of the silencing of either protein in specific assays. Random migration on FN was reduced after the depletion of liprin-β1, whereas silencing of liprin-β2 caused a weak increase of cell speed, although not statistically significant. From this analysis I speculate that liprinβ1 cooperates with liprin-α1 in the control of cell migration, whereas liprin-β2 may act by influencing cell motility by a different, liprin-α1-independent mechanisms. Analysis of invasion through Matrigel supports this hypothesis. Liprin-β1 silencing did not affect cell invasiveness, whereas liprin-β2 depletion led to an increase of invasion. Moreover, the silencing of both liprin-α1 and liprin-β2 together, led to a stronger increase of invasion. This suggests that liprin-α1 and liprin-β2 act in different pathways. Indeed, if they acted in the same pathway, the depletion of liprin-α1 are expected to revert the increase in invasion caused by liprin-β2 silencing. Finally, analysis on ECM degradation confirmed that liprin-β2 depletion promoted it whereas the silencing of liprin-β1 affected only the total degraded area. This strengthens the hypothesis of the involvement of liprin-β2 in a mechanism controlling tumor cell invasiveness.
4.2 Concluding remarks

My findings indicate that liprin-α1 has a functional role in tumor cell invasion in vivo. This is the first evidence of the involvement of liprin-α1 in the metastatic process in mice. I have hypothesized that this effect could be caused by the action of liprin-α1 on lamellipodia dynamics. However, future imaging studies are needed to shed light on the behaviour of liprin-α1-depleted cells during tumorigenesis and metastatization. Moreover, further analysis on human breast cancer samples are required, to understand whether liprin-α1 may be considered a predictive marker of particular subpopulations of human breast tumors. The findings of my work open also interesting questions about the involvement of liprin-β2 in the formation of invadopodia and about the unknown binding partners that could mediate the anti-invasive function of this protein.
5 MATERIAls AND METHODS

5.1 Antibodies and materials

Polyclonal antibody (pAb) anti-Liprin-β1 and anti-Liprin-β2 were from Sigma-Aldrich (St. Louis, MO, U.S.A.). The anti paxillin was from BD Biosciences (San Jose, CA, U.S.A.). The pAb against GFP was from Life Technologies (Paisley, Scotland, U.K.). The anti-liprin-α1 pAb was raised against the human glutathione-S-transferase (Knight et al)-liprin-α1 (818-1202) fusion protein prepared from bacteria transformed with the pGEX-Liprin-α1(818-1202) construct as described (Asperti et al, 2009). Rabbits were immunized with the GSTliprin-α1(818-1202) by Eurogentec (Seraing, Belgium). Affinity purification of antiliprin-α1 si1554 serum was performed by Simona Paris in the laboratory by using the p-MAL Protein Fusion and Purification System from New England Biolabs (Ipswich, MA, U.S.A.). Anti-liprin-α1 pAb commercially available from Protein Tech (Chicago, IL, U.S.A.). The monoclonal anti liprin-α1 was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) The polyclonal rabbit anti liprin-α1 and chicken anti-GFP antibodies were from Abcam (Cambridge, U.K.). Fluorescein isothiocyanate- (FITC) and cyanine 5 (CY5)-conjugated anti-rabbit, anti-mouse and anti-chicken secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.), FITC- and TRITC-conjugated phalloidin were from Sigma-Aldrich. Oregon 488-gelatin, Alexa-488, Alexa-568 and Alexa-546 anti-rabbit, anti-rat, anti-mouse and anti-hamster secondary antibodies were from Life Technologies. Fibronectin (FN) and matrigel were from BD Biosciences. EGF from Peprotech (Rocky Hill, NJ, U.S.A). Bovine serum albumin 138 (BSA) was from Roche. Peroxidase-conjugated anti-rabbit and anti-mouse antibodies, and the Enhanced Chemiluminescence (ECL) Detection System, were from Amersham Biosciences (Little Chalfont, U.K.). Peroxidase-conjugated anti-goat antibody was from Southern Biotech (Birmingham, AL, U.S.A); the anti-hamster was from Thermo Scientific (Waltham, MA, U.S.A). Bradford protein assay reagent was from BIO-RAD (Munich, Germany).

5.2 DNA constructs

The full length pFLAG-Liprin-α1 and pEGFP-Liprin-α1 (full length human liprin-α1) were described previously (Totaro et al, 2007). Fragments F1 including amino acids (aa)
1009-1202, F2 (aa 676-1009), F3 (aa 375-675) and F4 (aa 1-346) of human liprin-α1 were obtained by reverse transcriptase (RT)-PCR performed starting from mRNA isolated from SKNBE human neuroblastoma cells. The final constructs were assembled and subcloned to obtain the pFLAG-Liprin-F1, pFLAG-Liprin-F2F3 and pFLAG-Liprin-F2F3F4 plasmids. pFLAG-Liprin-ΔSAM1, pFLAG-Liprin-ΔSAM2 and pFLAG-Liprin-ΔSAM3 plasmids were produced by PCR performed starting from FLAG-Liprin-α1 plasmids and then were assembled and subcloned to obtain the final constructs. The construct pFLAG-Liprin-α1 ΔN-terminal and pFLAG-Liprin-α1 ΔC-terminal were produced by PCR performed starting from pFLAG-Liprin-α1 plasmids and then were assembled and subcloned to obtain the final constructs. pEGFP-C1 plasmids were from Clontech Laboratories (MountainView, CA, U.S.A.).

The constructs GFP-Liprinβ1 and GFP-Liprinβ2 were from Dr. Casper C. Hoogenraad (Spangler et al., 2011).

5.2.1 Small Interfering RNA (siRNA) ans Short hairpin RNA (shRNA)

Two siRNAs were used for human liprin-α1, liprin-1a and liprin1b, targeting the sequences 5’-CCAAGGTACAAACTCTTAA-3’ and 5’-CGAGGTGTGGTCATGAAAGA-3’ of liprin-α1 respectively. The siRNA were purchased from Life Technologies and Qiagen, respectively. Starting from the sequence of the two siRNAs, two shRNAs were designed according to the manufacture protocol of pSuper Vector (Oligoengine, Seattle, WA). The oligo obtained were cloned into the pSuperVector GFP.neo (Oligoengine, Seattle, WA), according to the manufacturer protocol. The plasmid obtained were named shRNA1, deriving from the sequence of siRNA liprin-α1a and shR-

<table>
<thead>
<tr>
<th>5’-3’</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>Hu-Liprin β2- (human) Invitrogen</td>
<td>[CAA CCA CAG GGU GAU GGA GUG GUU G] RNA</td>
<td>[UAA CCA CUC CAUCAC CCU GUG GUU G] RNA</td>
</tr>
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Table 5.1 Scheme of the siRNA sequence used in this work.
NA2, deriving from the sequence of siRNA liprin-α1b. The siRNAs used against liprin-β1 and liprin-β2 were stealth RNAi siRNA and target the following sequence: 5’-GATGAGTGATGCAAGTGACATGTGG-3’ for liprin-β1 and 5’-CAACCACAGGTTGATGGAGTG-3’ for liprin-β2. They were purchased from Life Technologies (Paisley, Scotland, U.K.). A siRNA specific for luciferase, used as negative, targeting the sequence 5’-CATTACGTACGCGGAATAC-3’ was purchased from Life Technologies. All siRNAs were transfected using Lipofectamine™ 2000 (Life Technologies) with 50 nM siRNA in serum-free medium. After transfection, cells were incubated in growth medium for 48 hours, and then used for biochemical and functional assays. All siRNAs could efficiently down-regulate the endogenous proteins in COS7 cells and/or human cancer cells MDA-MB-231.

5.3 Cell culture

5.3.1 Cell lines

COS7 cells were cultured in DMEM containing 10% FetalClone® III (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine.

MDA-MB-231, was growth in DMEM/F12 1:1, containing 10% FBS (Euroclone, Wetherby, U.K.), 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine.

NIH-3T3 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine. Cell lines up to the 9-10th passage after thawing were used for the experiments.

5.3.2 Generation of stable transfected cell lines

Independent cell lines stably expressing either GFP-Liprin-α1, GFP, ShRNA1, shRNA2, or pSuper vector GFP were obtained after transfection of MDA-MB-231 cells with 2 μg either of GFP-Liprin-α1, GFP, ShRNA1, ShRNA2, or pSuper vector GFP, respectively. Clones resistant to selection with 1 mg/ml of G418 (Merck Millipore) were expanded from single cells isolated by limiting dilution.

5.3.3 Viability and proliferation assay

Cell viability was assessed by a colorimetric assay based on the reduction of MTT
[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide]. Cells were treated at 37°C for 1 hour with 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, U.S.A.), added to the medium. After removal of the medium with MTT, DMSO was added and absorbance of the purple product was measured at 570 nm (Sunrise™, Tecan Group Limited). For cell proliferation, 2,000 cells were seeded in 24-well plates and their growth evaluated by counting in Burker chamber every 24 hours.

5.4 Trasfections

5.4.1 Lipofectamine

All the cell lines seeded in 3, 6 or 10 cm diameter plates or (6 to 24) well dishes were trasfected with Lipofectamine-2000® (Invitrogen Ltd) for biochemistry or immunofluorescence microscopy, respectively. For biochemistry 60-mm diameter plates, 2-4 μg of DNA and 10 μl of Lipofectamine-2000® were diluted separately in 300 μl of Optimem® (Gibco). In the case of 10 cm diameter plates 8 μg of DNA and 25μl of Lipofectamine-2000® were used, each diluted in 500 μl of Optimem®. For immunofluorescence, trasfection of siRNAs, DNA or co-trasfection of both, was performed in 6-well plates or 6 cm dishes with Lipofectamine-2000®. The procedure was similar to what previously described for DNA trasfection. SiRNA and/or DNA at the final concentration of 50-100 nM and 2-4 μg DNA respectively, were diluted in 250μl of Optimem®, and 5 μl of Lipofectamine-2000® were diluted separately in 250 μl of Optimem®. DNA solutions were added drop by drop to Lipofectamine-2000® solution, and incubated 20 min at RT before being added to the cells in Optimem®. After 4-5 hours Optimem® was replaced with complete medium. Cells were processed after 18-48 hours, as indicated.

5.5 Immunofluorescence

Cells grown on 10 μg/ml FN (BD Biosciences) coated glass coverslips (BDH Chemicals, Poole, UK), were fixed for 15 min at RT in 3% paraformaldehyde in PBS. After two washes with PBS, paraformaldehyde was blocked by 10 min incubation with 50 mM NH4Cl. After two washes with PBS, cells were permeabilized with 0.1-0.05% Triton X-100 in PBS for 4 min, washed twice with PBS, and incubated 10 min with 0.2%
gelatine from MERCK (Whitehouse Station, NJ, USA) in PBS. Coverslips were then incubated for 60-120 min at RT or overnight at 4°C with primary antibodies diluted in 0.2% gelatine in PBS. After two washes with 0.2% gelatine in PBS, the samples were incubated for 45 min with secondary antibodies. All coverslips were mounted with 1 drop of Gelvatol (20% polyvinyl alcohol, 2% propylgallate, in PBS), glycerol solution (glycerol 70% in PBS plus 0.01% phenylethilendiammine) or ProLong® Gold Antifade Reagent (Life Technologies) and observed either at epifluorescence or confocal microscopes.

5.6 Biochemical analysis

5.6.1 Preparation of lysates

Cells grown in 60 or 100 mm dishes were transferred on ice, washed twice with 10 ml of ice-cold TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and solubilised with 150-250 μl of lysis buffer [0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-Cl; pH 7.5, 2 mM MgCl2, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and anti-proteases 1x Complete® (Roche (Manheim, Germany)]. Lysates were transferred to tubes and rotated for 15 min by end-over-end mixing at 4°C. The insoluble material was removed by centrifugation at 12000g at 4°C for 15 min. Protein determination was done using Bradford protein assay reagent from BIO-RAD (Hercules, CA, U.S.A.).

5.6.2 Immunoprecipitation

Primary antibodies pre-adsorbed for 1 hour to 25 μl of protein A Sepharose beads (Amersham Biosciences) were added to lysates (2 mg protein/immunoprecipitation), and incubated for 3 h at 4°C with rotation. Immunoprecipitates were washed four times with 0.5 ml of lysis buffer with 0.1% Triton X-100.

5.6.3 SDS-PAGE and western blot analysis

Immunoprecipitates, lysates and unbound fractions were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Proteins from gels were electrophoretically transferred to 0.2 µm PROTRAN® nitrocellulose membranes (Schleicher & Schuell BioScience GmbH, Dassel, Germany), and stained with 0.2%
Ponceau in 3% TCA to visualise molecular weight (MW) standards and proteins. Filters were blocked for 1 hour at RT with 5% non-fat dry milk or 5% BSA (Durocher et al) in 50 mM Tris-HCl, 150 mM NaCl (pH 7.5), and then incubated for 2 h in the same buffer containing the diluted primary antibodies. For the detection of primary antibodies, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and revealed by ECL system (Amersham Biosciences).

5.6.4 Quantifications of protein levels

Densitometric analysis of blots was performed using the ImageQuant® software from Molecular Dynamics Inc. (Sunnyvale, CA) on images acquired with a Molecula Dynamics Personal SI Laser Densitometer (Amersham Biosciences). Each value was normalized to the protein levels of an internal standard in the same lysate (β-tubulin).

5.6.5 Stripping and reprobing

Filters were sometimes subjected to stripping and reprobing. Briefly, they were incubated in a large volume of stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) at 50° C for 30 min with gentle mixing. Filters were then washed twice for 10 min with 50 mM Tris-HCl, 150 mM NaCl (pH 7.5) supplemented with 0.5 % Tween-20. After stripping, filters were either quickly washed in the absence of detergent and stored at 4° C, or directly reprobed as described above. Alternatively, filters were subjected to ‘mild’ acid stripping. Briefly, filters were incubated in stripping solution (0.2 M glycine, 0.1% SDS, 1% Tween-20) set to pH 2.2, with gentle mixing for 10 minute at room temperature. Filters were then washed twice for 5 min in PBS, followed by 2 washes of 5 min each with 0.5 % Tween-20, 50 mM Tris-HCl, 150 mM NaCl (pH 7.5).

5.7 Microscope techniques

5.7.1 Wide-field and confocal analysis

Samples were observed using a Zeiss Axiovert 135 TV with QImaging Exi-Blue (Oberkochen, Germany) equipped with a Hamamatsu CCD Orca II camera from Hamamatsu Photonics K.K. (Hamamatsu, Japan). An Olympus IX70 microscope (Olympus, Tokyo, Japan)
Analysis of the role of liprin proteins in breast cancer cell invasion

was used to measure degradation, projected cell areas and number of cortactin-, filamentous actin-positive invadopodia. Confocal microscopy of biological samples was performed on the laser scanning confocal Ultraviewers from Perkinelmer (Waltham, MA, U.S.A.). For multichannel imaging, fluorescent dyes were imaged sequentially in frame or line-interlace mode to eliminate cross-talk between different channels. FITC and Alexa-488 were excited with a 488-nm ArKr laser line. TRITC and Alexa-568 were excited with a 543-nm HeNe laser line; CY5 was excited with a 633-nm HeNe laser line. Images were processed using Adobe Photoshop® CS4 from Adobe Systems Incorporated (Seattle, WA, U.S.A.), and analysed using the public domain NIH Image software (ImageJ).

5.7.2 Time-lapse wide-field microscopy

To observe movement of living cells in real time during 2D random migration, MDA-MB-231 cells were cultured for 1 day before trasfection with DNA and/or siRNAs. After 24-48 hrs cells were collected, washed with complete medium and plated on 6 well plates pre-coated with 2.5 μg/ml of FN. Images were acquired using a Zeiss Axiovert S100 TV2 microscope (Oberkochen, Germany) equipped with a Hamamatsu Orca II CCD digital camera from Hamamatsu Photonics K.K. (Hamamatsu, Japan), and a Cage Incubator designed to maintain all the required environmental conditions for cell culture, which was driven by an Oko-Vision software (OkoLab, Naples, Italy).

5.8 Morphological analysis

5.8.1 Measurement of the area of ECM degraded by MDA-231 cells on fluorescent gelatin

MDA-MB-231 cells were trasfected for 1 day with either Flag-βgalactosidase or Flag-Liprin-α1, or cotrasfected for 2 days with p-EGFP-C1 and small interfering RNAs (siRNAs). After 18 h on FN (10 μg/ml) cells were fixed with 3% PFA and subjected to immunofluorescence. For quantification, images were first subjected to adjustments using ImageJ software to remove diffuse background fluorescence. The area of degradation per cell was analyzed using ImageJ by performing a threshold of the green channel (488 nm) to create a binary image in which the total dark area corresponding to degraded gelatin was measured. (Clark et al, 2007). The measurement of the total cell area was evaluated on
thresholded images, by measuring the total area occupied by the whole cells. Data were pooled from 3 independent experiments in which a total of 60 cells were analyzed. Statistically significant differences were evaluated by the Student’s t-test. Value of P<0.05(∗) were considered statistically significant.

5.8.2 Quantification of migration and of lamellipodia protrusion

MDA-231 cells cotransfected with p-EGFP-C1 and siRNAs were plated on FN-coated 6-well plates (2D migration) or on the top of NIH-3T3 derived-matrix (3D migration) and subjected to time-lapse acquisition with an Axiovert microscope (Zeiss). Images were captured with 10x or 20x lens at 5-min intervals for 5 h, or at 6-min intervals for 8 h, respectively. Migration paths were calculated from the nuclear positions of cells obtained from 4 fields per well using two plugins available for ImageJ software (Manual tracking and Chemotaxis tool). The track of each cell was used to measure different parameters of migration: total (cell path) and Euclidean distances (length of the line segment, calculated between the start and the end point of the cell trajectory),
cell velocity and directionality. This parameter is an index of the persistence of the cell movement, given by the ratio between the Euclidean and the total distances. This value may change between 0 and 1, where 1 corresponds to the maximum linearity of the trajectory. Cells undergoing division and non-moving cells were ignored in this analysis (Rhoads & Guan, 2007). At least 50 cells from several experiments were recorded for each condition. To quantify lamellipodia dynamics, for each condition I analyzed 10 moving cells (90-100 lamellipodia) from two independent experiments. Images were quantified with ImageJ, by counting the number of lamellipodia forming during 1 or 5 h. The persistence of each protrusion was evaluated by considering the amount of time elapsed between the formation of lamellipodium and its disassembly. Statistically significant differences were analysed by the Student’s t-test.

5.8.3 Fluorescent-gelatin degradation assay

For gelatin degradation assay, MDA-231 cells trasfected for 24-48 h were replated for 5 h (5x10^4 cells) on Oregon-green 488-conjugated gelatin (Life Technologies) and 10 μg/ml FN pre-coated coverslips. Cells were then fixed with 3% PFA, stained with appropriate primary and secondary antibodies or fluorescent phalloidin, and observed with an
Olympus IX70 microscope (Olympus, Tokyo, Japan). ImageJ software was used to measure degradation (dark spots on fluorescent gelatin bright areas), projected cell areas, and number of active invadopodia (cortactin-, filamentous actin-positive dots that localized with areas of gelatin degradation.) (Clark et al., 2007).

5.9 Functional analysis

5.9.1 Migration assay

5.9.1.1 Two-dimensional (2D) random migration assay

For 2D random migration, MDA-231 trasfected cells for 48 h were seeded (5x10^4 cells/well) on 2.5 μg/ml FN pre-coated six well plates, and allowed to attach at 37°C for 3 h before time-lapse with an Axiovert microscope (Zeiss). Time lapse was performed for the following 5 hours. Images were captured at 5-min intervals using a CCD video camera (Orca II, Hamamatsu) with OKO Vision software. For each cell population, accumulated (cell path) and euclidean distances (cell displacement), mean velocity and directionality (persistence = path/displ) were calculated during 5 h assay with Manual tracking and Chemotaxis Tool of ImageJ (National Institute of Health, Bethesda, MD, USA). Cells undergoing division and non-moving cells were ignored.

5.9.1.2 Three-Dimensional (3D) fibrillar cell migration

For 3D cell migration, 5x10^5 MDA-231 trasfected cells were seeded on the top of NIH-3T3 fibroblast derived ECM for 6 h at 37°C, before live imaging acquisition with an Axiovert microscope (Zeiss). Images were captured at 6-min intervals for 8 h, using the CCD video cameras (Orca II, Hamamatsu). Different parameters were evaluated and quantified with ImageJ: accumulated (cell path) and euclidean distances (cell displacement), mean velocity and directionality (persistence = path/displ). The fibroblast-derived matrix was produced as previously described (Beacham et al., 2006). To evaluate the quality of FN fibers produced by fibroblast, ECM were fixed with 3% paraformaldehyde and stained with an anti-FN antibody. Z-stack projections were acquired at an Ultraview-ERS microscope.
5.9.2 **Matrigel invasion assay**

For invasion assay, MDA-231 trasfected cells (10 x 10^5 cells/100 μl) were seeded on Matrigel (BD Transduction, San Jose, CA, USA)-coated transwells in DMEM/F12 medium containing 0.1% BSA (Sigma-Aldrich). NIH-3T3-conditioned medium in the lower chamber was used as chemoattractant. The conditioned medium from NIH-3T3 mouse fibroblasts contains a variety of growth factors that could act as chemoattractants for tumor cells (Shaw, 2005). After 5 h of incubation at 37°C non-invading cells were removed from the upper side with a cotton swab, and cells invading and crossing the membranes were fixed with 100% cold methanol and stained with 1% crystal violet (0.5% crystal violet in 20% methanol). Cells invading to the lower side were counted from 6 representative 20x fields per condition, in at least 4 independent experiments, each performed in duplicate or in triplicate.

5.9.3 **Fluorescent-gelatin degradation assay**

For gelatin degradation assay, MDA-231 cells trasfected for 24-48 h were replated for 5 h (5x10^4 cells) on Oregon-green 488-conjugated gelatin (Life Technologies) and 10 μg/ml FN pre-coated coverslips. Cells were then fixed with 3% PFA, stained with appropriate primary and secondary antibodies or fluorescent phalloidin, and observed with an Olympus IX70 microscope (Olympus, Tokyo, Japan). ImageJ software was used to measure degradation (dark spots on fluorescent gelatin bright areas), projected cell areas, and number of active invadopodia (cortactin-, filamentous actin-positive dots that localized with areas of gelatin degradation.) (Clark et al., 2007).

5.10 **In vivo experiments**

5.10.1 **Experimental metastasis assay**

For experimental metastasis assay, cell lines stably expressing either GFP-Liprin-α1, GFP, shRNA1, shRNA2 or pSuperVectorGFP were seeded in 10 cm diameter plates and trypsinized after reached the 70% of confluence. MDA-MB-231 WT cells were used as positive control. Cells were counted with Tripan Blue and aliquots of 500 000 cells each in 100 μl of PBS were prepared. Four-weeks-old athymic *nu/nu* female mice were obtained from
Harlan. Animal care was in accordance with the guidelines of Italian Ethic Committee. Mice were injected in the left lateral tail vein. Eight-ten mice per experimental conditions were used. 5 weeks after the injection, mice were euthanized and lungs were washed twice by the injection of PBS into the thrachea and then explanted. Left lungs were fixed in 4% PFA for 2 hours, cryoprotected with sucrose in PBS (10-20 and 30%), embedded Tissue Tek® O.C.T.™ compound (Miles) and snap frozen by liquid nitrogen. In general, 5 -10 mice were used for each experimental condition.

5.10.2 Spontaneous metastasis assay

For spontaneous metastasis assay, cell lines stably expressing either GFP-Liprin-α1, GFP, shRNA1, shRNA2 or pSuperVectorGFP, were seeded in 10 cm diameter plates and trypsinized after reached the 70% of confluence. MDA-MB-231 WT cells were used as positive control. Cells were counted with Trypan Blue and aliquots of 4*10^6 cells each in 50 μl of PBS were prepared. NOD scid gamma (NSG) mice were obtained from TIGET colony at san Raffele Scientific Institute. NSG mice harbor mutation in Prkdc gene (DNA activated polypeptide gene) that causes lack of T- or B cells but still have high NK activity; SCID mutation into the NOD background resulting in decreased activity of NK cells, defects in immune system, deficiency in C5 and inability of macrophage to produce IL-1β in response to stimulation with LPS; and deletion of IL2 rγ, required for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signaling. Animal care was in accordance with the guidelines of Italian Ethic Committee. For mammary fat pad (MFP) tumor cell transplantation, mice were anesthetized with Avertin, (15mg/kg, Sigma-Aldrich) the right III MFP of 9-week–old NSG female mice was visualized through a small skin incision, and 2x10^6 tumor cells were injected in 50 μl of PBS with visual confirmation of MFP engorgement. The incision was closed with 3M™ Vetbound™ tissue adhesive (Alcyon, Italy). After transplantation, palpable tumors developed in 8-10 days and then the tumor size was determined every 3-4 days by caliper measurements. Tumor volume was calculated by a rational ellipse formula (m1 x m1 x m2 x 0.5236, where m1 is the shorter axis and m2 is the longer axis). In general, 4-10 mice were used for each experimental condition.
5.11 **Histological analysis**

5.11.1 **Immunofluorescence of cryosection**

From spontaneous metastasis assays, primary tumors were collected, fixed 2 hours in 4% PFA, cryoprotected with sucrose in PBS (10-20 and 30%), then embedded in Tissue Tek® O.C.T.™ compound (Miles) and snap frozen by liquid nitrogen. Twelve-μm frozen sections were taken by cryostat. For immunofluorescence, primary antibodies were revealed by incubation for 1.5–2 h with Alexa Fluor-conjugated secondary antibodies and 4′,6-Diamidino-2 Phenylindole (DAPI, Sigma-Aldrich) for nuclear staining. Confocal analysis was performed with a Leica TCS SP2 (Leica Microsystems).

5.11.2 **Quantification of metastatic area**

To quantify metastasis in experimental metastasis assay, I euthanized mice 5 weeks after injection. To quantify metastasis in spontaneous metastasis assay I euthanized mice 38 day after transplantation. In both cases left lungs were collected, fixed in 4% PFA for 2 hours and cryoprotected with sucrose in PBS (10-20 and 30%), then embedded in Tissue Tek® O.C.T.™ compound (Miles) and snap frozen by liquid nitrogen. Sixteen-μm frozen sections were taken at 160-μm intervals to obtain full coverage of the organ. Sections were then stained with H&E for histological examination. I photographed each lung section and quantified the relative metastatic area. To do this, the ratio between the metastatic area and the total section area was measured using ImageJ software by performing a threshold to create a binary image in which the total red area corresponding to the area covered by metastases. ImageJ software was used also to measure the area of the entire lung section. The ratio between metastatic area and total section area was measured and indicated as relative metastatic area per section. The average of all the metastatic areas per section of an entire lung was calculated.

5.11.3 **Hematoxillin & Eosin staining**

For H&E staining Hematoxillin solution and Eosin solution 2% were purchased from Sigma (Sigma Aldrich). Sections were stained for 30 seconds with Hematoxillin solution, washed three or four times in distilled water then stained with Eosin solution for 2 minutes.
and washed in distilled water again. Sections were dehydrated in 50-70-90-100% ethyl alcohol and then in xylene. Sections were mounted in DPX mounting medium (Sigma Aldrich) and let dry o/n at room temperature before the analysis.
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