Studies on Memo, an important ErbB2 receptor-mediated component of the cellular migratory machinery

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Maria Meira

aus Frankreich

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Prof. E. Parlow

Dekan

----------SUMMARY----------

The ErbB2 receptor tyrosine kinase has been shown to play an important role in cancer cell motility and metastases formation. This receptor is often overexpressed in human tumors of diverse origins, including breast and ovarian cancer. Individuals with ErbB2 over expressing tumors have shown poor clinical outcome.

Our studies are focused on signaling molecules that interact with autophosphorylated tyrosine residues of the cytoplasmic tail of the receptor. Two of the sites, Tyr 1201 (YC) and Tyr 1227 (YD) are fully able to restore the migratory phenotype of breast carcinoma cells. Studies of the functional role of ErbB2 phosphorylation sites identified PLCγ1 as an interacting partner of the YC autophosphorylation site, and Memo (Mediator of ErbB2-driven cell Motility) as a binding partner of the YD site that is required for ErbB2 induced cell motility.

Memo is encoded by a unique gene that is found in all branches of life, from bacteria to humans. Memo has no characterized domains, nor does it have obvious catalytic activity. Various approaches were used to position Memo in a signaling pathway and to uncover its biochemical function. Memo was initially detected based on its important role in ErbB2-induced cell motility. In fact, tumor cells with a specific knock-down (KD) of Memo failed to grow microtubules in response to Heregulin (HRG)-induced ErbB2 activation and were impaired in their migration.

Cell migration proceeds in distinct steps. In response to a chemotactic stimulus, cells extend protrusions at the front that help in attachment. This is followed by contraction of the cell body and tail detachment at the rear allowing movement in the direction of the ligand. The initial event in the process is sensing of the ligand in response to activation of cellular receptors like EGFR or ErbB2. Their activation initiates signaling pathways that lead to polymerization of new actin at the leading edge, which is necessary for generating the protrusive force allowing migration. An important goal of my thesis work has been to investigate the step(s) of the migratory process that require Memo.

In the first study, we explored migration using Dunn chambers and analyzed the chemotactic response of tumor cells in a shallow gradient of ligand. By tracing HRG-stimulated cell migration in time-lapse video microscopy, we found that Memo or PLCγ1 KD strongly impairs cell directionality, reflecting an important role for Memo and PLCγ1 in orchestrating directional cell migration. We also demonstrated that depletion of Memo or PLCγ1 resulted in very similar phenotypes, with a strong impairment of HRG-induced cytoskeletal organization.

To gain more insight into Memo's function, we carried out a Yeast-2-Hybrid (YTH) analysis and found a number of interesting new partners of interaction for Memo. Of particular interest

is the small protein cofilin, one of the major cellular actin severing and depolymerizing factors that is known to have an essential role in directional sensing during chemotaxis. This interaction was confirmed in vitro using recombinant proteins and in vivo in co-immunoprecipitation experiments where Memo was detected in complexes with cofilin, ErbB2 and PLCγ1. Interestingly, we also found that HRG-induced PLCγ1 phosphorylation was decreased in Memo KD cells, suggesting that Memo regulates PLCγ1 activation. Furthermore, by introducing GFP-tagged cofilin into control, Memo or PLCγ1 siRNA transfected breast tumor cells, we showed that HRG-induced recruitment of GFP-cofilin to lamellipodia is impaired in Memo- and in PLCγ1 KD cells, suggesting that both proteins lie upstream of cofilin in models of ErbB2-driven tumor cell migration. Finally, we examined the effect of Memo on cofilin binding and severing/depolymerizing properties. In vitro F-actin binding assays showed that Memo does not impair cofilin binding to F-actin, and revealed that Memo is a novel F-actin binding protein. In vitro F-actin depolymerization assays indicated that Memo promotes cofilin depolymerizing/severing activity. Altogether, these data suggest a novel role for Memo during the migratory process and its implication in the regulation of actin dynamics through cofilin binding.

In the second study, we used two different Memo-defective cellular models to examine Memo's function in more detail. We demonstrated that inhibition of Memo impairs activation of a number of signaling molecules including Src, Shc, ERK and PLCγ1. We also provide evidence that Memo interacts with the three Shc isoforms, $p46^{shc}$, $p52^{shc}$, and $p66^{shc}$, and showed that Shc is required for Memo binding to the ErbB2 receptor. Control and Memo-deficient cells were also scored for their migration and adhesion properties. These assays indicated that Memo is important in both cell migration and adhesion processes. Also, morphological and biochemical analyses of control and Memo-deficient cells suggested that Memo is involved in focal adhesion organization and rear cell deadhesion during the migratory process.
Altogether, these two studies revealed important roles for Memo at different steps of cell migration and metastasis, making it a potential interesting target for cancer therapy.

Genetic approaches in model organisms have been important for gaining insight into the function of evolutionarily conserved proteins. To position Memo within a genetic network, experiments in the model organism *S. cerevisae* that lends itself to rapid genetic screening were performed. We investigated cellular localization of Memo in yeast and found that Memo is located in the nucleus and cytoplasm of the cell. A *S. cerevisae memo* Δ strain has been generated and is viable. Considering the role of Memo in the microtubule and actin networks

that we described in mammalian cells, we examined the *memo* Δ strain for defects in different cytoskeletal dynamics. No significant effect was observed. We also performed a Synthetic Lethal Screen of genetic interactions between a *memo* Δ strain and an ordered array of 4700 Yeast strains containing non-essential gene deletions. This analysis revealed a limited number of synthetic interactions. Lethality was observed in combination with the plc1Δ strain. PLC1 encodes for the unique isoform of phosphatidylinositol-specific phospholipase C of *S. cerevisiae.* The results are intriguing and exciting considering the data obtained in the mammalian models; in fact, we demonstrated that Memo and PLCγ1 interact with ErbB2 autophosphorylation sites and are essential for directional migration. We also showed that Memo is found in a complex with PLCγ1 and ErbB2 and that Memo is likely contributing to PLCγ1 activation. We hypothesize that in Yeast, Memo and PLC1 act in the same or in distinct but related pathways, and suggest that the connection between PLC and Memo induced-pathways is also conserved through evolution.

----------INTRODUCTION----------

A. The Epidermal Growth Factor (EGF/ErbB) family of Receptor Tyrosine Kinases (RTK)

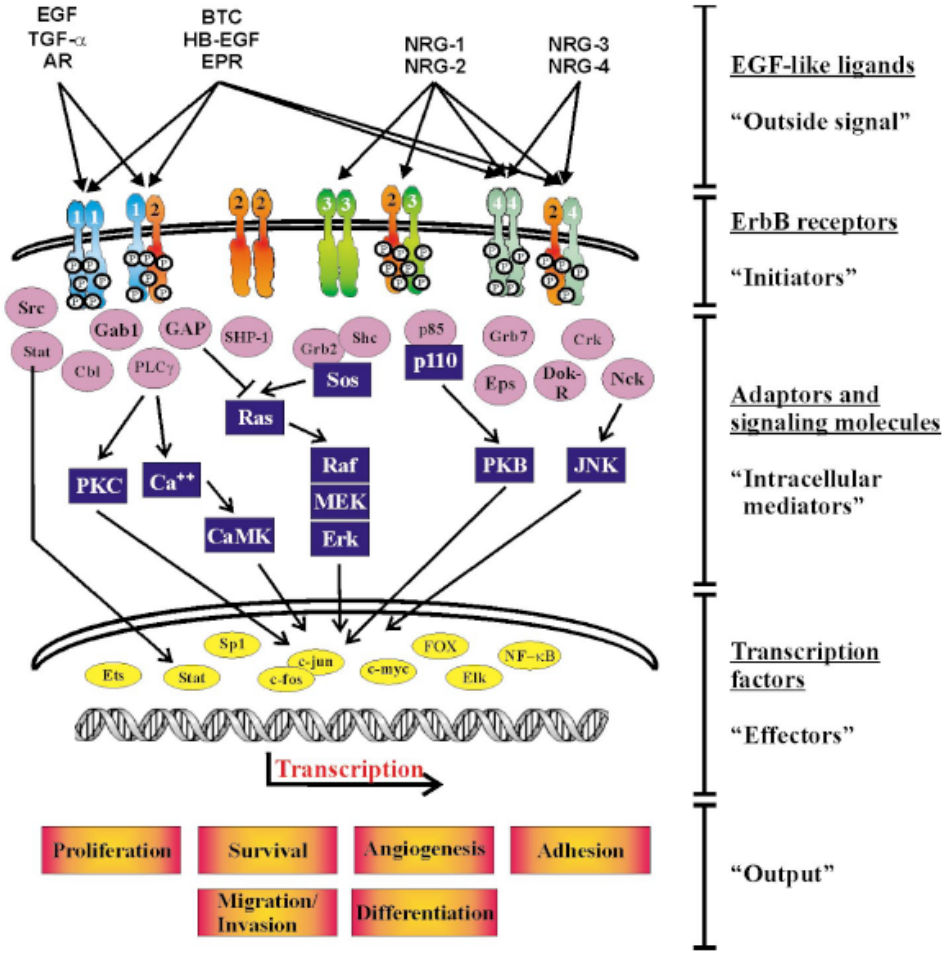
Cellular signaling processes are essential to the life cycle and for cellular biological functions. On the one hand, cells are constantly exposed to a variety of external stimuli, ranging from soluble factors to signaling molecules from neighboring cells. On the other hand, cells have to correctly interpret these extracellular signals in order to induce an appropriate response.

The family of Receptor Tyrosine Kinases (RTK) is part of a large number of systems that induce amplification from the external signal stimuli to the internal cell compartment. Indeed, RTKs are key regulator transmembrane proteins that are able to integrate a wide range of external stimuli with specific internal signals and responses. RTKs belong to the wider Tyrosine Kinase (TK) family and form a protein superfamily of 58 known members which is present in all metazoans and is divided into 20 different sub-families defined by their sub-domain composition.
The subfamily I, also called Epidermal Growth Factor (EGF/ErbB) family, originally named because of their homology to the erythroblastoma viral gene product, v-erbB (Downward et al., 1984), encompasses four structurally related type I growth factor receptors: EGF receptor (also called ErbB1/HER1), ErbB2 (Neu/HER2), ErbB3 (HER3) and ErbB4 (HER4), and a number of soluble ligands which bind to their cognate receptor (Yarden and Sliwkowski, 2001).

The ErbB receptors have a common cysteine-rich extracellular ligand binding domain, a single hydrophobic transmembrane spanning region and a cytoplasmic tyrosine kinase containing domain that is flanked by non-catalytic regulatory regions (Ullrich and Schlessinger, 1990). Ligand binding to the extracellular domain induces receptor activation with the formation of specific receptor homo or heterodimeric complexes, resulting in auto and trans-autophosphorylation of specific residues within the cytoplasmic kinase domain of the activated receptor (Schlessinger, 2000). These phosphorylated residues serve as docking sites for specific signaling SH2 (Src Homology 2) and phosphotyrosine-binding domain containing molecules (Yaffe, 2002; Olayioye et al., 2000). Additional effector proteins are

then recruited which turn on a plethora of cellular responses including cell growth, proliferation, differentiation, migration, survival, metabolism and ultimately, subsequent activation or repression of various subsets of genes (Holbro and Hynes, 2004).

The ErbB signaling network



Adapted from Holbro and Hynes, Annu Rev Pharmacol Toxicol 2004

Figure 1. Ligands of the EGF family bind to their receptors causing the formation of different ErbB dimers. ErbB2, which has no direct ligand inducing homodimerization, needs a partner to acquire signaling potential (indicated by the *encircled P*). Following receptor activation, various molecules (*violet*) with adaptor or enzymatic functions are directly recruited to the ErbBs. These then activate downstream signaling components (*dark blue*), which ultimately lead to changes in the activity of multiple nuclear transcription factors (*yellow*). For illustrative clarity, many components in each of the signaling layers are omitted.

1. The ErbB signaling network in evolution

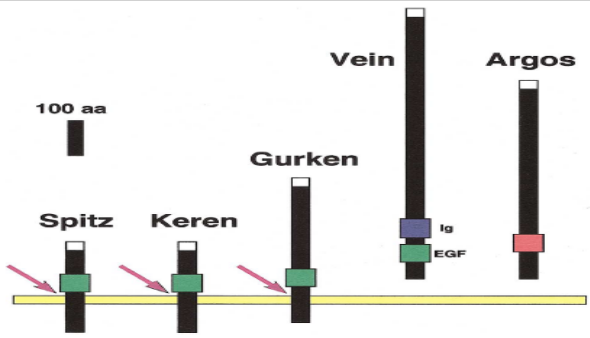
The components of the ErbB signaling pathway are evolutionarily conserved. In contrast with higher organisms where the ErbB pathway has developed an elaborated network, both *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* ErbB pathways display a linear version with a single ErbB homologue for each species.

In the *Caenorhabditis elegans* organism, the *let-23* and *lin-3* genes encode the single epidermal growth factor receptor homologue and an EGF-like/TGFα-like ligand, respectively (Aroian et al., 1990; Hill and Sternberg, 1992). LET-23 is differentially expressed at the beginning of the third larval stage of development (L3) in six vulval precursor cells (P3.p-P8.p also called VPCs) which are located along the ventral side of the hermaphrodite. Indeed, at this stage, LET-23 displays an increased expression on the surface of P6.p and a decreased expression on the surface of the other precursor cells. *Lin-3* is secreted by the anchor cell that is located in the somatic gonad, just above P6.p. The inductive signal results from the binding of *Lin-3* to the juxtaposed LET-23 receptor on P6.p, which in turn undergoes a determined number of cell divisions. This results in the formation of the primary fate, surrounded by the secondary fate presumably arising from the activation of LIN-12/NOTCH receptors and low levels of active LET-23 on the precursor cells flanking P6.p, namely P5.p and P7.p. The other three VPCs generate non-specialized hypodermis. Mutations in LET-23 and *lin-3* resulted in defective formation of vulval tissue (Aroian and Sternberg, 1991; Liu and al., 1999 respectively). Additionally to the role of the LET-23/*lin-3* pathway in vulval development and differentiation, this epidermal growth factor system is also involved in other processes such as viability (Aroian and Sternberg, 1991), spicule development (Chamberlin and Sternberg., 1994) and ovulation (Bui and Sternberg, 2002). Finally, epidermal growth factor signaling also has a neuronal function with an important role for EGF signaling in the regulation of behavioral quiescence (Van Buskirk and Sternberg, 2007).

The fruitfly *Drosophila Melanogaster* has a single receptor homologous to the four mammalian ErbB receptors also called DER (Wadsworth et al., 1985; Schejter et al., 1986), and a complex network of ligands composed by five activating ligands and one inhibitory ligand (Shilo, 2002). DER is repeatedly used at several stages during development. DER was shown to be involved during oogenesis in the establishment of both the anterior/posterior and dorsal/ventral body axes and in the pattern of the embryo (Gonzalez-reyes et al., 1995;

Wasserman and Freeman, 1998). A role for DER is also established during embryogenesis, where among other functions (Clifford and Schüpbach, 1992), DER is involved in the development of the central nervous system (Udolph et al., 1998). Wing vein formation and eye development in the *Drosophila melanogaster* larva are also controlled by DER (Sturtevant et al., 1993; Dominguez et al., 1998).

DER is activated in a versatile mode by ligand binding to the extracellular region. Four activating ligands are described: Spitz (Rutledge et al., 1992) is the primary activating ligand that similarly to Keren (Reich and Shilo, 2002) and Gurken (Neuman-Sillberberg and Schüpbach, 1993), is produced as a transmembrane precursor that is cleaved to generate the active secreted ligand. Vein (Schnepp et al., 1996) is the fourth activating ligand which is produced as a secreted protein. Argos is also a secreted protein which contains an atypical EGF-like motif and functions as an inhibitory ligand by competing with the activating ligands for the binding to the receptor (Schweitzer et al., 1995). Interestingly, a more recent study suggested that Argos sequesters the activating ligand Spitz without binding directly to the receptor (Klein et al., 2004). Moreover, Kekkon and Sprouty are two other proteins which interfere negatively with the DER signaling in some tissues (Ghiglione et al., 2003; Casci et al., 1999). These positive and negative feedback loops therefore regulate the different cell fate choices induced by DER activation



Adapted from Shilo, Exp Cell Res, 2003

Figure 2. Activating and inhibitory *Drosophilia* epidermal growth factor (EGF) receptor (DER) ligands. Five ligands are interacting with DER. Spitz, Keren, and Gurken are produced as transmembrane precursors and are cleaved (arrows) to generate the active secreted ligand. Vein and Argos are produced as secreted protein. The EGF domain (red) of Argos mediates binding to DER and inhibits binding of other ligands, as well as receptor dimerization.

The ErbB receptor family has evolved in higher vertebrates with the combination of a multitude of EGF-related ligands binding to four ErbB receptors which translate the information from the extracellular compartment to intracellular signaling pathways that modulate cellular biological responses. An important diversity in signaling is therefore possible with the numerous combinations between ligands and receptors. ErbB receptors are expressed in a variety of tissues throughout development where they play essential roles in cell proliferation and differentiation. Importantly, aberrant ErbB signaling has been correlated with the progression of human malignancies. Deregulated expression of the ErbB receptors, in particular ErbB1 and ErbB2, is associated with a more aggressive disease and with parameters predicting a poor clinical outcome (Hynes and Stern, 1994; Salomon and al., 1995). Accordingly, ErbB receptors are under intense scrutiny not only for their role in cancer biology, but also as therapeutic targets. Different approaches have been developed using ectodomain- binding antibodies or small-molecule tyrosine kinase inhibitors (TKIs) that compete with ATP in the tyrosine kinase domain (Mendelsohn and Baselga, 2000, de Bono and Rowinski, 2002, Rowinsky, 2004). Some of these therapies are either already in clinical use or in advanced clinical development.

2. The ErbB receptor/ligand network

The coordination of several processes such as cell morphology, proliferation, differentiation, motility, and survival is required for the formation and the maintenance of tissue organization during development. It is therefore vital that cells correctly interpret signals from their environment.

2.1. The first step: binding of EGF-related ligands to specific receptors

2.1.1. Classification of the ErbB receptors ligands

Growth factors that bind and activate the ErbB receptors belong to the EGF-family of growth factors (Harris et al., 2003; Falls, 2003). These specific agonists for ErbB receptors are each the product of a single gene. They are processed from large precursors into small soluble peptides.

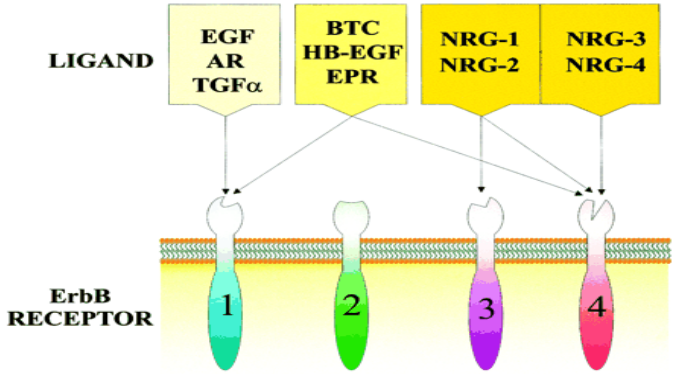
There are 11 soluble and small (6-10 kDa) growth factors, and according to their affinity for one or more ErbB receptors, the EGF-related growth factors can be divided into three distinct groups (Riese and Stern, 1998).

The first group includes ligands for the EGF receptor, namely EGF (Carpenter and Cohen, 1990), transforming growth factor α (TGF-α) (Massague, 1990), amphiregulin (AR) (Shoyab et al., 1988) and epigen (Strachan et al., 2001).

The second group encompasses growth factors that have dual binding specificity for EGFR and ErbB4, such as betacellulin (BTC) (Dunbar et al., 2000), heparin-binding growth factor (HB-EGF) (Higashiyama et al., 1992) and epiregulin (EPR) (Toyoda et al., 1995).

The third group comprises the neuregulins (NRGs also frequently referred to as Heregulins HRGs) (Falls, 2003) which can be divided into two subgroups: NRG-1 and NRG-2 which can bind to ErbB3 and ErbB4 receptors, and NRG-3 and NRG-4 which only bind to ErbB4. Of note is the fact that none of the EGF-family ligands directly bind ErbB2. However, despite having no soluble ligand, ErbB2 is activated via heterodimerization with the other ligand bound family members (Olayioye et al., 2000). In fact, ErbB2 appears to be the preferred heterodimerization partner of the other ligand-bound ErbB receptors (Graus-Porta et al., 1997).

This family of growth factors displays a similar structure with a characteristic consensus sequence containing six spatially conserved cysteine residues that form three intramolecular disulfide-bonds. This sequence composes the EGF-like domain or EGF motif which confers the specificity of binding (Jones et al., 1999). EGF ligands also contain additional structural motif such as immunoglobulin-like domains, heparin-binding sites and glycosylation sites (Harris et al., 2003).



Adapted from Olayioye et al., Embo J, 2000,

Figure 3. Binding specificities of the EGF-related peptide growth factors. There are four categories of ligands that bind ErbB receptors. EGF, AR and TGFα bind ErbB1; BTC, HB-EGF and EPR bind ErbB1 and ErbB4; NRG-1 and NRG-2 bind ErbB3 and ErbB4; and NRG-3 and NRG-4 bind ErbB4.

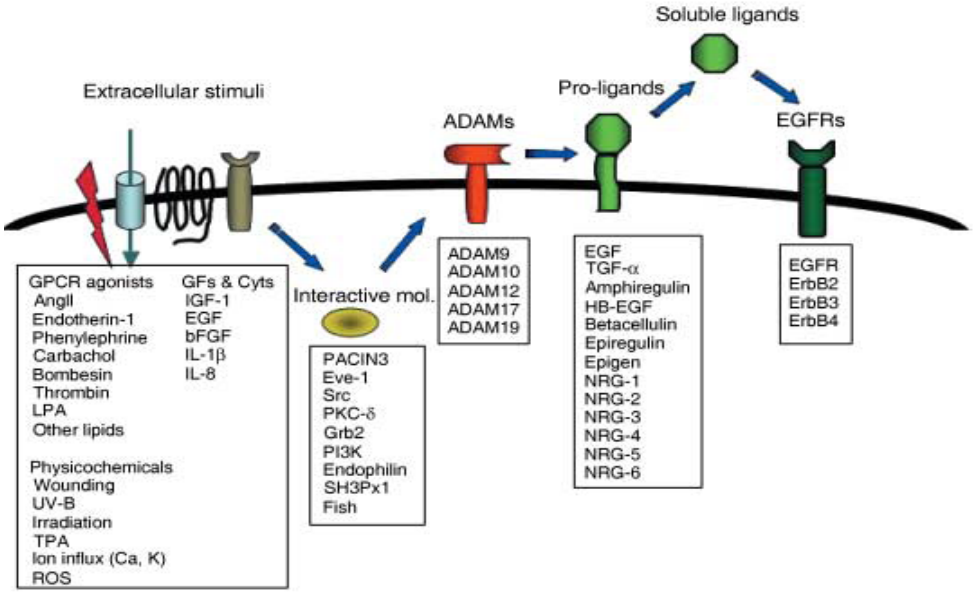
2.1.2. The production of soluble EGF family ligands

The mammalian ligands are regulated autocrine/paracrine and/or juxtacrine growth factors. Paracrine ErbB ligands are released from the stromal cells. Autocrine ligands are produced as transmembrane precursors that undergo proteolytic cleavage at the cell surface to release a mature soluble ectodomain which stimulate receptors on neighboring cells (Massague and Pandiella, 1993). The soluble rather than membrane-anchored forms of the ligands were shown to mediate most of the biological effects of EGFR ligands (Dong et al., 1999). This proteolytic cleavage is an important step in the process of receptor activation which is dependent on ligand availability. In fact, the presence of EGF ligands in tumors was correlated with constitutive activation of ErbB receptors (Baker, 2002). Therefore, this mechanism that controls ligand processing appears to be a potential therapeutic target (Borrell-Pagès et al., 2003; Higashiyama et al., 2008).

The proteases involved in the cleavage of the growth factors belong to the metalloproteinase family, in particular the zinc binding matrix metalloproteinases (MMPs) and ADAMs (a disintegrin-like and metalloproteinase-containing protein) family (Seals and Courtneidge, 2003; Sanderson et al., 2006). The first mechanism describing the production of soluble EGF family ligands through ectodomain shedding involve the activation of G-protein-coupled-receptors (GPCRs) (Prenzel et al., 1999; Daub et al., 1996). In this particular study, treatment of cells with different GPCRs agonists induces a rapid EGFR phosphorylation. This mechanism has been termed EGFR transactivation and resulted from metalloproteinase activation leading to the cleavage and release of HB-EGF, which in turn activated EGFR.

Importantly, these processes lead to the stimulation of intracellular signaling pathways such as MAPK (Luttrell et al., 1999). GPCR-induced transactivations of EGFR and ErbB2 have been extensively studied; however, it is important to mention that neuregulins, which are the ligands for ErbB3 and ErbB4, are processed by the same metalloproteinases (Wakatsukiet al., 2004).

This mechanism of proteolytic cleavage is also described in cancer cells, where members of the ADAM family were shown to be involved in the shedding of diverse EGF-like ligands (Fisher et al., 2003; Hynes and Schlange, 2006). Other physiological ligands inducing ErbB transactivation were recently identified. For instance, transactivation of EGFR by binding of WNT to the seven-pass membrane receptor Frizzled (FZD) was described (Civenni et al., 2003)



Adapted from Higashiyama et al., cancer Sci, 2008,

Figure 4. Ligand-shedding dependent epidermal growth factor receptor (EGFR) transactivation. Disintegrin and metalloprotease (ADAM) proteins are activated by various stimuli including wounding, ion influx, G-protein coupled receptor (GPCR) signaling, growth factor and cytokine signaling, protein kinase C (PKC) activation, and binding of cytoplasmic interactive proteins. EGFR ligand molecules are proteolytically cleaved by specific metalloprotease-activity of ADAMs, resulting in the production of soluble ligands and stimulation of EGFR in autocrine and paracrine manners. AngII, angiotensin II; Cyts, cytokines; GFs, growth factors; HB-EGF, Heparin-binding epidermal growth factor carboxy-terminal fragment; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; NRG, neuregulin; PI3K, phosphatidyl inositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; TGF-α, transforming growth factor-α; TPA, 12-O-tetradecanoylphorbol- 13-acetate.

In addition, juxtacrine stimulation of growth factor receptors has been described where the membrane-associated uncleaved form of HB-EGF could activate EGFR in adjacent cells (Higashiyama et al., 1995).

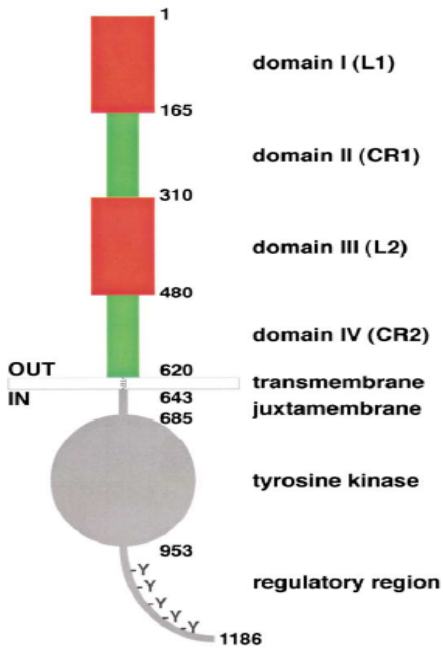
2.1.3. Architecture of the ErbB receptors

The ErbB receptors are type I transmembrane proteins containing a large extracellular ectodomain of 600-630 amino acids subdivided into four domains (I-II-III and IV). This extracellular domain is heavily N- glycosylated at 12 sites and characterized by two cysteine-rich domains (domain II and IV) that form 25 disulfide bonds. Domains I and III (also called L1 and L2) have a beta helical fold and constitute the ligand-binding site. A beta hairpin also called dimerization loop or dimerization arm (composed of a 10-reside sequence) in the subdomain II is involved in the process of dimerization between two receptors (Burgess et al., 2003).

The receptors also contain a single transmembrane-spanning α-helix that was usually assumed to play a passive role in ligand-induced activation of the receptor. However, mutations within some of these receptors, and studies with the EGFR and ErbB2 receptors have indicated that interactions between transmembrane domains do contribute to stabilization of ligand-independent and/or ligand-induced receptor dimerization and activation (Bell et al., 2000; Bennasroune et al., 2004).

The large cytoplasmic region (about 500 amino acids) encodes a juxtamembrane region, a highly conserved tyrosine kinase domain divided into two lobes, and a C-terminal region containing multiple autophosphorylation sites. The ErbB3 receptor is kinase inactive due to amino-acid substitutions in important residues (Kraus et al., 1989; Plowman et al., 1990; Guy et al., 1994). Therefore, ErbB3 has to dimerize with another ErbB receptor in order to be phosphorylated and signal.

These receptors are subjected to different co and post-translational modifications (N-glycosylation, phosphorylation and ubiquitination) (Blagoev et al., 2004; Wu et al., 2006; Guo et al., 2003; Huang et al., 2006). Many phosphotyrosine and some phosphoserine/threonine sites have been identified by conventional sequencing and more recently by the use of the mass spectrometry technique (Zhang et al., 2005). A multitude of signaling molecules associate with the ErbB receptor family through the phosphotyrosine residues (Schulze et al., 2005). Many of these associated proteins are tyrosine phosphorylated by the receptor. Others, like Grb-2, are not phosphorylated but act as adaptators to enhance downstream signaling pathways (Lim et al., 2000).



Adapted from Burgess et al., Mol Cell, 2003

Figure 5. Domain organization of the ErbB receptors (residue numbers for domain boundaries are for EGFR)

2.1.4. Alternative forms of ErbB receptors

Differential splicing or proteolytic processing results in the production of variant forms of the ErbB receptors. Oncogenic forms of the EGF receptor have been described in breast cancer (Moscatello et al., 1995). A spliced variant of ErbB2, deltaHER2 (with a deletion of an exon 16 amino acids long in the extracellular domain) has also been described to induce constitutive dimerization and transformation (Kwong and Hung, 1998). Also, soluble extracellular domains of EGF receptor and ErbB2 can be produced either through direct coding by spliced variant mRNAs or through proteolytic processing of full-length receptors (Petch et al., 1990; Doherty et al., 1999). An extracellularly truncated form of ErbB-2 which is produced through proteolytic cleavage of full-length molecules and which may be associated with lymph node metastasis is found in a subset of tumors that overexpress ErbB-2 (Christianson et al., 1998). ErbB4 is also cleaved to release a soluble 80 KDa intracellular domain termed s80, which translocates to the nucleus to promote cell differentiation (Linggi and Carpenter, 2006).

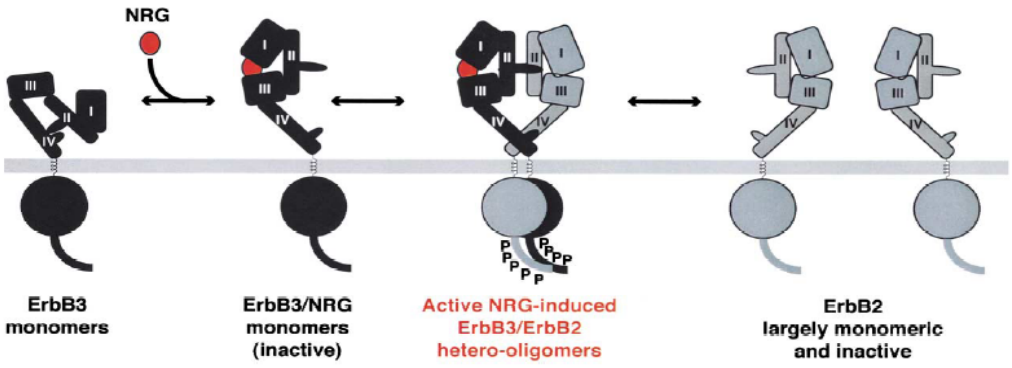
2.2. Mechanism of ErbB receptor activation upon ligand binding

2.2.1 Ligand induced dimerization of the ErbB receptors

The binding of the growth factors to the extracellular ectodomain results in homo- or hetero-dimerization of ErbB receptors, and activates the cytoplasmic tyrosine kinase, enhancing the stimulation of signaling pathways that direct cellular responses (Olayioye et al., 2000).

The mechanism of receptor activation by ErbB-ligand induced dimerization is well established since many years (Schlessinger, 2000), however, the exact mechanism of receptor-receptor interaction was unknown until recently. Significant improvement in the knowledge and the understanding of the molecular mechanism involved in ligand induced-receptor dimerization has emerged from high-resolution structure and crystal studies on the soluble ectodomains of EGFR (Garrett et al., 2002; Ogiso et al., 2002; Ferguson et al., 2003), ErbB2 (Garrett et al., 2003), ErbB3(Cho and Leahy, 2002) and ErbB4 (Bouyain et al., 2005). These studies indicated that in the absence of ligand, EGFR, ErbB3 and ErbB4 exist in a so called "tethered" intramolecular conformation due to an interaction between the dimerization loop in subdomain II and subdomain IV that leads to a closed conformation unable to mediate the interaction between monomers of receptors (Ferguson et al., 2003; Cho and Leahy, 2002). By contrast, a competent dimerization conformation is observed in ligand-bound EGFR with the exposition on the receptor surface of the dimerization arm. The contact between two monomers occurs mainly through this dimerization arm and leads to the formation of a dimer composed of two 1:1 receptor/ligand complex (Garrett et al., 2002; Ferguson et al., 2003).

The structure of ErbB2 differs significantly from that of the other receptors. Indeed, in the absence of ligand and, similarly to the structure of EGF-bound EGFR, ErbB2 is in a competent dimerization conformation which exhibits a protruding loop (Garrett et al., 2003). In this particular conformation, the proximity between the subdomains L1 and L2 prevents the binding of the ligand, which can explain the inability of ErbB2 to bind EGF-family ligands. More importantly, this particular conformation also explains the potential of ErbB2 to hetero-dimerize with other receptors, and highlights the position of ErbB2 as the preferred dimerization partner for all others ErbB receptors (Graus-Porta et al., 1997; Tzahar et al., 1996).



Adapted from Burgess et al., Mol Cell, 2003

Figure 6. Model for NRG-Induced Heterodimerization of ErbB2 and ErbB3. At left, a tethered ErbB3 monomer is depicted. Binding of NRG (red) is proposed to promote the extended configuration of ErbB3, with the dimerization arm exposed. Extended ErbB3 is thought to form homodimers only very inefficiently. On the right are shown ErbB2 molecules, constitutively in the extended configuration, that are thought to be primarily monomeric (although homodimerization can presumably be driven by substantial overexpression). When NRG-bound ErbB3 molecules are present in the cell membrane, ErbB2 preferentially forms hetero-oligomers with ErbB3, leading to receptor activation and mitogenic signaling. Additional interactions involving the transmembrane and kinase domains may also contribute to receptor oligomerization.

When associated with other ErbB receptors, ErbB2 induces amplification but also, diversification of the signal output. Evidence is provided that the EGF receptor homodimer signals differently from the EGFR-ErbB2 heterodimer (Muthuswamy and Muller, 1995). Interestingly, the ErbB2-containing heterodimeric receptor groups are the most potent complexes which induce cell proliferation and transformation (Citri et al., 2003). In fact, activation of this heterodimeric complex between a ligandless receptor (ErbB2) and a kinase-deficient receptor (ErbB3) enhances potent signaling pathways. (Harari and Yarden, 2000; Prigent and Gullick, 1994). Moreover, rapid internalization of the ErbB2/ErbB3 heterodimer is impaired, leading to prolonged signaling (Sorkin et al., 1993; Lenferink et al., 1998). Furthermore, the rate of ligand dissociation from the complex is decreased, leading to an increased affinity of the ErbB3 receptor to the ligands (Sliwkowski et al., 1994; Pinkas-Kramarski et al., 1997).

It is also important to highlight that autologous signaling by ErbB2 occurs when the receptor is mutated like in the rat Neu or over expressed, a situation that is observed in a variety of human cancers (Brennan et al., 2000; Sharpe et al., 2000; Penuel et al., 2002).

2.2.2 Ligand induced activation of the ErbB receptors

The kinase domain of the ErbB receptors is divided into two lobes, termed N and C domains that cooperate to form the active site. A recent study has brought new insights into the process of kinase activation following receptor dimerization (Zhang et al., 2006). The proposed model is the following: the C-lobe of one receptor kinase domain allosterically activates the kinase module of the other receptor by contacting its N-lobe. This interaction repositions the activation loop in a conformation which facilitates catalysis. The mechanism whereby ErbB2 gets phosphorylated in an ErbB2/ErbB3 heterodimer containing an enzymatically inactive ErbB3-kinase domain is poorly understood. However, this model proposed by Zhang and colleagues predicts that activation of ErbB2 is possible due to the interaction between the C-lobe of ErbB3 and the N-lobe of ErbB2. Moreover, a possible role of the juxtamembrane region and the C-terminal region in modulating receptor kinase activity has also been proposed (Aifa et al., 2005; Landau et al., 2004).

2.3. Activation of intracellular signaling pathways

Auto or trans-autophosphorylation of the ErbB receptors provides specific docking sites for a cascade of intracellular signaling SH2 or PTB domain- containing molecules leading to their recruitment and assembly at the receptor (Marmor and Yarden, 2004; Yarden and Sliwkowski, 2001).

In addition to their role in controlling the formation of defined ErbB partners through selective binding to the receptors, the ErbB ligands are also regulating the binding of specific adaptators proteins to the docking sites of the receptor, and consequently, influencing the downstream cellular responses (Olayioye et al., 1998; Sweeney et al., 2000).

Two major signaling pathways are activated downstream of all ErbB receptors: the Ras and Shc/Grb2-activated mitogen-activated protein-kinase (MAPK) pathway which is activated through the binding of Shc and/or Grb2 to the receptor, and the phosphatidylinositol-3-kinase (PI3K) pathway which is turned on following the recruitment of the p85 regulatory subunit to the activated receptor (Olayioye et al., 2000; Grant et al., 2002). Of note is the fact that the potency of the PI3K signaling differs among the homologous receptors. In fact, the p85 subunit is directly binding to the ErbB3 receptor, consequently positioning this receptor as the most efficient activator of this pathway (Prigent and Gullick, 1994; Fedi et al., 1994). ErbB4 has several isoforms that differ in their juxtamembrane and carboxyl terminal region and which therefore, contain or lack the PI3K binding sites (Elenius et al., 1999). PI3K activation is only possible if distinct isoforms are expressed. The E3 ubiquitin ligase Cbl is another

signaling molecule that binds preferentially to one member of the ErbB receptor family, in this particular case, EGFR, on a single tyrosine residue of the kinase domain. Cbl targets the EGFR to the lysosomal compartment, enhancing subsequent EGFR signaling downregulation by promoting receptor ubiquitination (Levkowitz et al., 1998).

The activated intracellular signaling pathways translate into the nucleus, where many cell cycle regulators and transcription factors determine the biological output of ErbB activation. Cyclin D1, which promotes the G1/S phase cell cycle progression, is a downstream regulator which appears to be up regulated and stabilized upon phosphorylation (Lee et al., 2000). A number of transcription factors are also activated downstream of ErbB receptors, including c-fos, c-myc, (Cutry et al, 1989) c-jun (Quantin et al., 1988), signal transducer and activators of transcription (STATs) (Olayioye et al., 1999), NF-kB (Biswas et al., 2000) and Ets family members (O'Hagan and Hassell, 1998).
Downstream effects on the expression of these targeted genes determine the biological response to receptor activation, ranging from cell division, migration, adhesion, differentiation and survival.

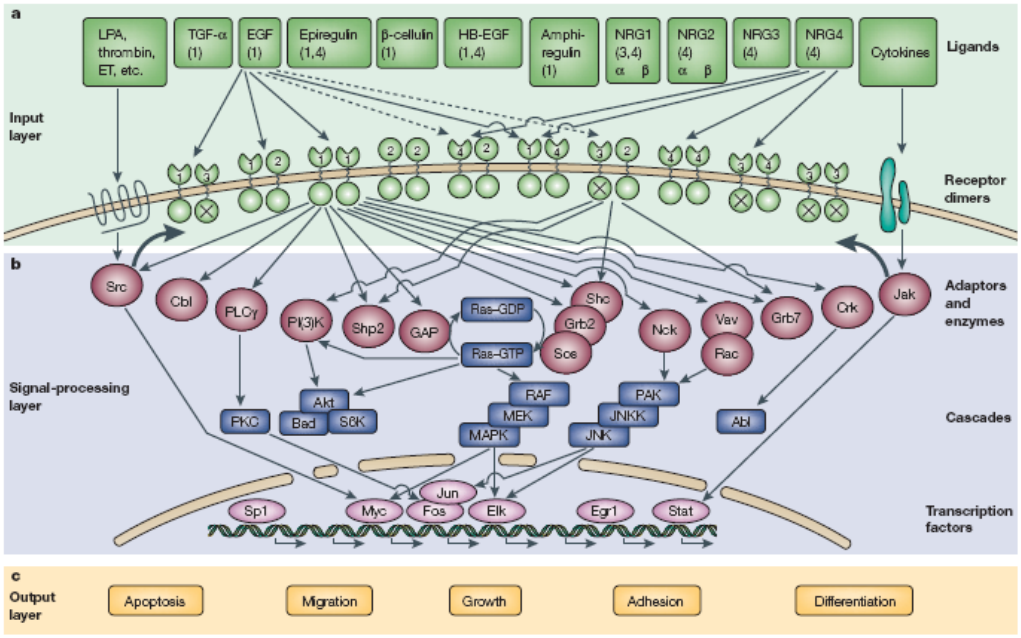


Figure 7. The ErbB signaling network. *From Yarden and Sliwkowski, Net Rev Mol Cell Biol, 2001*

2.4. Endocytosis and sorting of ErbB receptors

Endocytic removal of ligand-receptor complexes from the cell surface, and their subsequent sorting to degradation is a major process that regulates the amplitude of the signal induced by the activation of the ErbB receptors. Consistent with this paradigm, a mutant of EGFR whose endocytosis is impaired can deliver oncogenic signals (Wells et al., 1990). Receptor mediated endocytosis is a selective adsorptive uptake of specific ligand-bound membrane receptors. Caveolae and clathrin-coated pits are specialized in this internalization process (Waterman and Yarden, 2001). EGFR is rapidly internalized and degraded following activation, while internalization of ErbB2, ErbB3 and ErbB4 receptors is relatively slow (Baulida et al., 1996). Enzymatic tyrosine kinase activity has been shown to be essential for degradation of internalized receptors (Felder et al., 1990). Therefore, ErbB3, which is devoid of tyrosine kinase activity, recycles back to the plasma membrane presumably after unloading its ligand in an endosomal compartment (Waterman et al., 1998). Interestingly, more recent studies suggest a possible involvement of this endocytic pathway in the regulation of signal transduction through localized assembly of effector complexes, but also in the delivery of signaling molecules to the nucleus where these receptors enhance the induction of specific genes (Miaczynska et al., 2004; Lo et al., 2006; Giri et al., 2005).

2.5. The ErbB family: a broad and complex signaling network

The complexity of the ErbB signaling network is based on the diversity of interactions between ligands, receptors, downstream signaling effectors and transcription factors that translate into biological responses. The nature of the EGF-like ligand composes the first level of diversity. In fact, the activation of specific subsets of ErbB receptors and subsequent biological effects depend on the identity of the EGF ligand (Beerli and Hynes, 1996). Of note also is the fact that ligand affinity and pH stability of the ligand-receptor complex are important parameters that can influence signal response (French et al., 1995; Waterman et al., 1998). In this regard, it has been suggested that low-affinity ligands can induce a more potent signaling due to decreased receptor degradation and downregulation. Another level of diversity is achieved by the broad collection of intracellular signaling molecules which are recruited upon receptor stimulation and activate downstream signaling cascades, leading to a specific cellular response (Yarden and Sliwkowski, 2001).

2.6. Transactivation of ErbB receptors by other signaling pathways

Transactivation of ErbB receptors by different classes of membrane receptors leads to rapid receptor tyrosine phosphorylation and subsequent stimulation of downstream signaling pathways. This transactivation occurs through two different mechanisms: (i) phosphorylation of the ErbB receptors by other kinases, (ii) autophosphorylation in response to increased kinase activity. The representative example of the first mechanism is the phosphorylation of the cytoplasmic domains of EGFR and ErbB2 by activation of the Janus tyrosine kinase 2 (Jak2) following binding of the growth hormone and prolactin to their respective receptors (Yamauchi et al., 1997; Yamauchi et al., 2000). Src is another non receptor tyrosine kinase shown to induce phosphorylation of EGFR (Biscardi et al., 1999). Also, integrin-dependent EGFR activation has been described (Moro, 1998). The second mechanism involves G-protein coupled receptors (GPCRs) ligands, such as endothelin-1, bombesin, thrombin and lysophosphatidic acid which upon binding to GPCRs, induce rapid stimulation of metalloproteinases and cleavage of EGF-like precursors, therefore increasing ligand availability for ErbB receptors (Prenzel et al., 1999; Gschwind et al., 2002). Interestingly, ErbB receptor transactivation by GPCRs has been observed in both normal and cancer cells. For instance, deregulated expression of GPCRs and their ligands has been linked to tumor development in prostate models of cancer (Daaka, 2004). Interestingly, EGFR activation is well described in prostate tumors (Scher et al., 1995), indicating a possible link with GPCR altered expression. Another similar mechanism has been described where the binding of Wnt to frizzeled receptor enhanced stimulation of EGFR tyrosine kinase activity through the metalloproteinase-induced cleavage of EGF-like ligands in both normal and breast cancer cells (Civenni et al., 2003; Schlange et el., 2007). Also, another model of ErbB transactivation following the binding of oestradiol to the plasma-membrane-associated oestrogen receptor has been described (Razandi et al., 2003).

3. Role of ErbB receptors and their ligands in development

3.1. ErbB receptors in development

ErbB receptors are widely expressed in a number of organs and tissues. To examine their physiological role *in vivo* and gain more insight into the role of these receptors during development, mouse models carrying null mutations in the genes encoding these receptors have been created and resulted in embryonic or perinatal lethality.

EGFR null mice survive for up to 20 days after birth depending on the genetic background, and suffered from multiorgan failure and defects in the epithelial structures of different organs such as skin, lung, pancreas and gastrointestinal tract (Miettinen et al., 1995; Sibilia and Wagner., 1995, Threadgill and al., 1995). A post natal neurodegeneration characterized by increased apoptosis in the cortical and thalamic regions of the brain was also observed in EGFR null mice (Sibilia et al., 1998).

Embryos deficient in ErbB2 expression succumb during embryogenesis at day E10.5. These mutant embryos display an aberrant cardiac development associated with the absence of formation of the cardiac trabeculae (Lee and al., 1995). Interestingly, ventricular-restricted conditional deletion of ErbB2 resulted in a viable phenotype, however, these mice showed increased susceptibility for developing cardiomyopathy, highlighting a possible role of ErbB2 in the prevention of pathologic heart dilatation (Crone et al., 2002). ErbB2 has also been shown to be involved in the terminal differentiation of oligodendrocytes and myelin development (Park et al., 2001). Also, defects in the development of neural structures, such as neuromuscular synapses, show the importance of ErbB2 in neural development (Lee and al., 1995; Leu et al., 2003, Erickson et al., 1997).

ErbB3 null mutation resulted in embryonic lethality at day E13.5, with appearance of cardiac cushion abnormalities leading to blood reflux through defective valves. In addition to cranial ganglia defects similar to those observed in ErbB2 or ErbB4-deficient mice, a dramatic reduction in neural structures suggests a general effect of ErbB3 on the neural crest. Abnormal development of different organs such as stomach and pancreas has also been described (Erickson et al., 1997).

ErbB4 deficient mice also die during mid-embryogenesis at E10.5 due to aborted development of myocardial trabeculae in the heart ventricule. An important role for ErbB4 in the regulation of axon guidance in the central nervous system has been discussed (Gassmann et al., 1995). In addition, altered motor and behavioral activities were observed in ErbB4-

conditional deficient mice, suggesting a role for this receptor not only in the development but also in neuronal function (Golub et al., 2004).

3.2. ErbB ligands in development

Similarly to the studies performed for ErbB receptor characterization during development, animal models with null mutations for the expression of the ErbB ligands have been developed in order to investigate their role in different organs and tissues.

A functional role for EGF and TGF-α in prostate development has been suggested (Abbott et al., 2003). Also, expression of ErbB ligands such as EGF, HB-EGF and TGF-α has been detected in the central and peripheral nervous system where they regulate cellular functions such as proliferation, migration and differentiation (Xian and Zhou, 2004). Interestingly, the development and function of the nervous system is not significantly affected in knock-out TGF-α mice, however, a reduction in the number of neurons was observed in some brain areas (Blum, 1998; Tropepe et al., 1997). Moreover, abnormalities in the skin architecture, the hair and the cornea have been detected (Mann et al., 1993; Luetteke et al., 1993). A possible compensation for the defects in TGF-α function by other ErbB ligands has been suggested at different levels of development (Xian et al., 2001; Luetteke et al., 1999). In fact, in contrast to EGF receptor (EGFR) knockout mice, triple null mice lacking half of the EGFR ligand family (EGF, AR and TGF-α) were healthy and fertile, indicative of overlapping or compensatory functions among EGF family members.

HB-EGF-null mice display reduced lifespan and developed severe heart failure with dilated ventricular chambers and enlarged cardiac valves (Iwamoto et al., 2003). Betacellulin knock-out in mice did not affect survival and fertility, however, double null HB- EGF (-/-)/BTC (-/-) mice have a further reduced life span due to accelerating heart failure (Jackson et al., 2003).

Epiregulin-deficient mice develop chronic dermatitis and defective immune-related responses (Shirasawa et al., 2004).

As observed for mice defective in ErbB2 or ErbB4 expression, NRG-1 knock-out resulted in embryonic lethality at E10 due to defective cardiac trabeculae formation (Meyer and Birchmeier, 1995). In contrast, NRG-2 deficient mice survive embryogenesis without developing any apparent heart defect. However, an early growth retardation and reduced reproductive capacity were characterized (Britto et al., 2004).

3.3. ErbB family in mammary gland development

ErbB receptor signaling pathway is involved in normal mammary gland development. The mammary gland is a powerful model that helps to understand the potential role of the ErbB signaling in the adult organism. In fact, proliferation and differentiation mechanisms can be analyzed postnatally in this organ which undergoes extensive development from birth, where it consists in a rudimentary system of ducts, to puberty, where it develops under the influence of hormones and finally to pregnancy, when lobulo-alveolar proliferation occurs, followed by lobulo-alveolar-produced milk secretion (Hennighausen and Robinson, 1998).

ErbB receptors: All four ErbB family receptors are expressed in the mammary gland of adult females, but EGF receptor and ErbB-2 are preferentially expressed in young females (Sebastian et al., 1998) (Troyer and Lee, 2001).. Interestingly, the expression of the ErbB receptors in this organ is cell-type and developmental stage specific (Schroeder and Lee, 1998; Darcy et al., 2000). In fact, in the first postnatal episode of mammary development which occurs at puberty, EGF receptor and ErbB-2 are present and are tyrosine-phosphorylated, which is indicative of signaling activity (Sebastian et al., 1998; Schroeder and Lee, 1998). Hence, EGFR and ErbB2 seem to play a role at puberty, but also during late pregnancy and lactation, while ErbB3 and ErbB4 mostly act during pregnancy and lactation. The analysis of the role of ErbB receptors on the mammary gland development was not possible due to embryonic or early lethality. Consequently, other approaches such as dominant-negative (DN) receptor expression or reconstitution experiments were performed in order to elucidate the role of these receptors in this particular event. DN- ErbB2 or ErbB4 expressing transgenic animals display normal ductal outgrowth. However, defective lobulo-alveolar development and reduced milk secretion were observed in these strains (Jones and Stern, 1999; Jones et al., 1999). In contrast, transgenic experiments using DN EGFR (Xie and al., 1997) or reconstituted EGFR-/- neonatal mammary gland (Wiesen et al., 1999), resulted in abnormal ductal outgrowth.

ErbB ligands: numerous EGF ligands are expressed at various phases of mammary development, where they act on proliferation and differentiation (Sebastian et al., 1998; Schroeder and Lee, 1998). HRG-α has been show to be the only HRG isoform expressed in the mouse mammary gland. Severe defects in the development of the mammary gland lobulo-alveolae together with impaired lactogenesis were detected in HRG-α deficient mice (Li et al., 2002). In addition, mice with individual targeted disruption of EGF, AR, and TGF-α as well as triple null mice have been generated. Consistent with the abundant expression levels of AR in the mammary gland (Kenney et al., 1995), this ligand has been identified, among the

three of them, as a particularly important regulator of ductal outgrowth and lactation (Ciarloni et al., 2007). Indeed, AR knock out mice display an aberrant elongation of mammary ducts, pointing out the absence of functional redundancy of ErbB ligands in this particular process. Moreover, triple knock out mice lacking expression of AR, EGF and TGF-α showed aberrant mammary alveolar growth and reduced milk production. Furthermore, disruption of the AR gene also resulted in a significant impairment of activation of Stat5, which is an important mediator of mammary development that regulates production of milk proteins (Luetteke et al., 1999). Importantly, a possibly significant role for AR in driving human breast cancer progression has been suggested (McBryan et al., 2008).

4. ErbB family and cancer

Receptor tyrosine kinase activity is tightly controlled in normal cells. However, when they are mutated or structurally altered, RTKs become potent oncoproteins. In fact, the development and progression of many human cancers has been correlated with abnormal activation of these receptors that consequently, have become rational targets for therapeutic intervention.

4.1. Deregulation of ErbB signaling in human cancer

Different mechanisms leading to deregulation of the ErbB pathway have been reported; in fact, hyperactivation can be induced by either autocrine or paracrine overproduction of ligands, overexpression or constitutive activation of the receptors due to mutation. Also, impaired receptor downregulation is another described mechanism of RTK deregulation that could play a significant role in the pathogenesis of cancer (Peschard and Park, 2003).

EGFR in cancer: Overexpression and structural alterations of EGFR are frequent in human malignancies. In fact, amplification of the EGFR has been detected in a majority of carcinoma types (Normanno et al., 2003; Sibilia et al., 2007). In lung carcinomas, high levels of expression of EGFR have been associated with poor prognosis (Veale et al., 1993). However, a real correlation between EGFR expression and patient prognosis is still under debate. Interestingly, different studies suggest that EGFR overexpression requires ligand binding to induce receptor activation (Di Fiore et al., 1987). Accordingly, the relationship between co-expression of ErbB ligand/EGFR and tumorigenesis has been described (Salomon et al., 1995; Nicholson et al., 2001). Also, several deletions and point mutations resulting in increased catalytic tyrosine kinase activity of the receptors have been characterized. One frequent EGFR mutation is the EGFR vIII form (Voldborg et al., 1997) that is distinguished by a large deletion of the extracellular domain and results in a constitutive activation of the receptor. This mutation has been frequently found in gliomas, but also to a less extent in breast, ovarian, and lung carcinomas (Pedersen et al., 2001). Furthermore, in frame deletions or point mutations in the tyrosine kinase domain of the EGFR have been identified in non-small cell lung cancer (NSCLC) (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004).

ErbB3 in cancer: High expression of ErbB3 has been detected in certain human cancers, highlighting this ErbB member as a potential therapeutic target (Gullick, 1996). However, due to its impaired tyrosine kinase activity, ErbB3 is not a direct target of kinase inhibitors. Considering that ErbB3 is a positive regulator of the ErbB network (Citri and Yarden, 2006), impaired signaling downstream of this receptor is prone to lead to tumorigenesis. For instance, an increasing body of evidence shows that ErbB3 plays a critical role in EGFR- and

ErbB2-driven tumors (Hsieh and Moasser, 2007). In addition, the levels of expression of ErbB3 are generally comparable to EGFR. In ovarian and prostate cancer, ErbB3 is overexpressed and associated with poor prognosis (Tanner et al., 2006; Myers et al., 1994, respectively). Increased expression of ErbB3 has also been detected in primary human breast cancers (Lemoine et al., 1992), but also in diverse gastrointestinal cancers (Maurer et al., 1998; Slesak et al., 1998) and in human squamous cell carcinomas (Funayama et al., 1998). The prognostic role of this receptor in human carcinomas is still under debate.

ErbB4 in cancer: Neuregulins, betacellulin and heparin-binding EGF-like growth factor are binding to and activating ErbB4, leading to cellular proliferation, chemotaxis or differentiation (Carpenter, 2003). Although the structure and mechanism of action of ERBB4 are similar to those of other ERBB members, relatively little is known about the general or specific roles ERBB4 in the development of human tumors. However, high expression levels of ErbB4 have been reported in thyroid (Haugen et al., 1996), breast (Srinivisan et al., 2000), ovarian (Furger et al., 1998), endometrial (Srinivisan et al., 1999) and oral squamous cell cancer (Bei et al., 2001), as well as in medulloblastoma (Gilbertson et al., 1997), ependymoma (Gilbertson et al., 2002), and osteosarcoma (Hugues et al., 2004). Interestingly, ErbB4 expression has been shown to be down-regulated in prostate (Lyne et al., 1997), renal (Thomasson et al., 2004) and pancreatic (Graber et al., 1999) cancer. In addition, the significance of ErbB4 expression levels for clinical outcome is still unclear. Of note however is the prognostically-favourable ER phenotype associated with ErbB4 overexpression in breast cancer (Knowlden et al., 1998).

ErbB ligands in cancer: EGF-like ligands have been detected in a majority of human carcinomas (Normanno et al., 2001). For instance, overexpression of TGF-α has been observed in many tumors (Salomon et al., 1995) such as breast carcinomas or colon adenomas (Révillion et al., 2008; Habel et al., 2002), where a potential role for this growth factor in the early phases of tumorigenesis has been suggested. AR and HB-EGF have also been associated with several types of human cancer (Yotsumoto et al., 2008). In particular, a strong correlation between AR overexpression and rapidly growing keratinocytic tumors was purposed (Billings et al., 2003). Expression of AR was also shown to be up regulated in human hepatocellular carcinoma tissues and cell lines (Castillo et al., 2006). An important role for AR in breast cancer progression is also described (Willmarth and Ethier, 2008). Overexpression of HB-EGF has been observed in several tumors (eg, liver, pancreatic, colon, gastric, breast, and esophageal tumors) and HB-EGF has been implicated in tumor progression, increased proliferation, and metastasis (Raab and Klagsbrun, 1997; Ito et al., 2001; Miyamoto et al., 2004). Neuregulins may also play a role in human malignancies. Their

expression has been mainly investigated in breast cancer where NRG are detected in about 25-30% of human primary breast carcinomas (Normanno et al., 1995). NRG are also shown to be involved in other types of cancer such as ovarian, endometrial, colon, gastric, lung, thyroid, glioma, medulloblastoma, melanoma as well as head and neck squamous carcinoma (Breuleux, 2007).

4.2. Focus on the role of ErbB2 in cancer and metastasis

In 1985, the complete primary sequence of a putative RTK that showed a high level of homology to both the human EGFR product and the v-erbB oncogene, was described; this receptor was therefore named human EGFR-related 2 (HER2) (Coussens et al., 1985). Another independent study identified this new EGFR relative and named it ErbB2 (King et al., 1985). Interestingly, the chromosomal location of the gene for this protein was similar to the neu oncogene, an erb-B-related gene present in rat neuron/glioblastomas (Schechter et al., 1984). Importantly, in 1987, the HER2 gene was found to be amplified in 25 to 30 percent of invasive breast cancers. For the first time, a significant correlation between the overexpression of the HER2/neu oncogene in tumors and reduced patient relapse and survival was advanced (Slamon et al., 1987; Berger et al., 1988). As identified for breast cancers, HER2/neu was also shown to be involved in the pathogenesis of different human cancers such as ovarian cancer (Slamon et al., 1989), colon cancer (Cohen et al., 1989) and non-small cell carcinomas of the lung (Weiner et al. 1990). Since this discovery, ErbB2 has been intensely studied in order to understand not only its role in cancer biology, but also and very importantly, as a therapeutic target (Hynes and Lane, 2005).

4.2.1. ErbB2: the preferred heterodimerization partner

ErbB2 is widely expressed in normal human organs and tissues, including ovarian epithelium, endometrium, heart, lung, prostate, kidney, pancreas, and liver. ErbB2 encodes an 185kDa orphan receptor tyrosine kinase for which no direct ligand inducing homodimerization has been identified. Therefore, its role within the cellular network was largely unknown until 1988, when EGFR was shown for the first time to induce transactivation of ErbB2 through heterodimerization (Stern and Kamps, 1988). The ErbB2 receptor can mediate the lateral signal transduction of all ErbB receptors (Carraway et al., 1994; Graus-Porta et al., 1997; Plowman et al., 1993). Therefore, activation of ErbB2 is highly dependent on the expression of other family members, to which it is recruited as a preferred heterodimeric partner (Graus-Porta et al., 1997). Alternatively, overexpression and/or mutation of ErbB2 lead to

constitutive activation of ErbB2 and spontaneous dimerization and stabilization of the homo-dimeric complex in a ligand-independent manner (Hynes and Stern, 1994).

A major coordinating role in the ErbB network is proposed for ErbB2 (Graus-Porta et al., 1997); in fact, ErbB2 enhances and stabilizes the dimerization with another ErbB partner (Olayioye et al., 1998), leading to the formation of ErbB2-containing heterodimers characterized by extremely high signaling potency (Tzahar et al., 1996). Indeed, ErbB2 strongly reduces the rate of ligand dissociation, therefore allowing potent and prolonged activation of downstream signaling pathways (Sliwkowski et al., 1994).

Furthermore, the ErbB2 receptor can be involved in the regulation of a variety of vital functions controlled by the ErbB-receptor family members, such as cell growth, differentiation, and apoptosis (Schlessinger, 2000).

4.2.2. ErbB2 overexpression and cancer metastasis

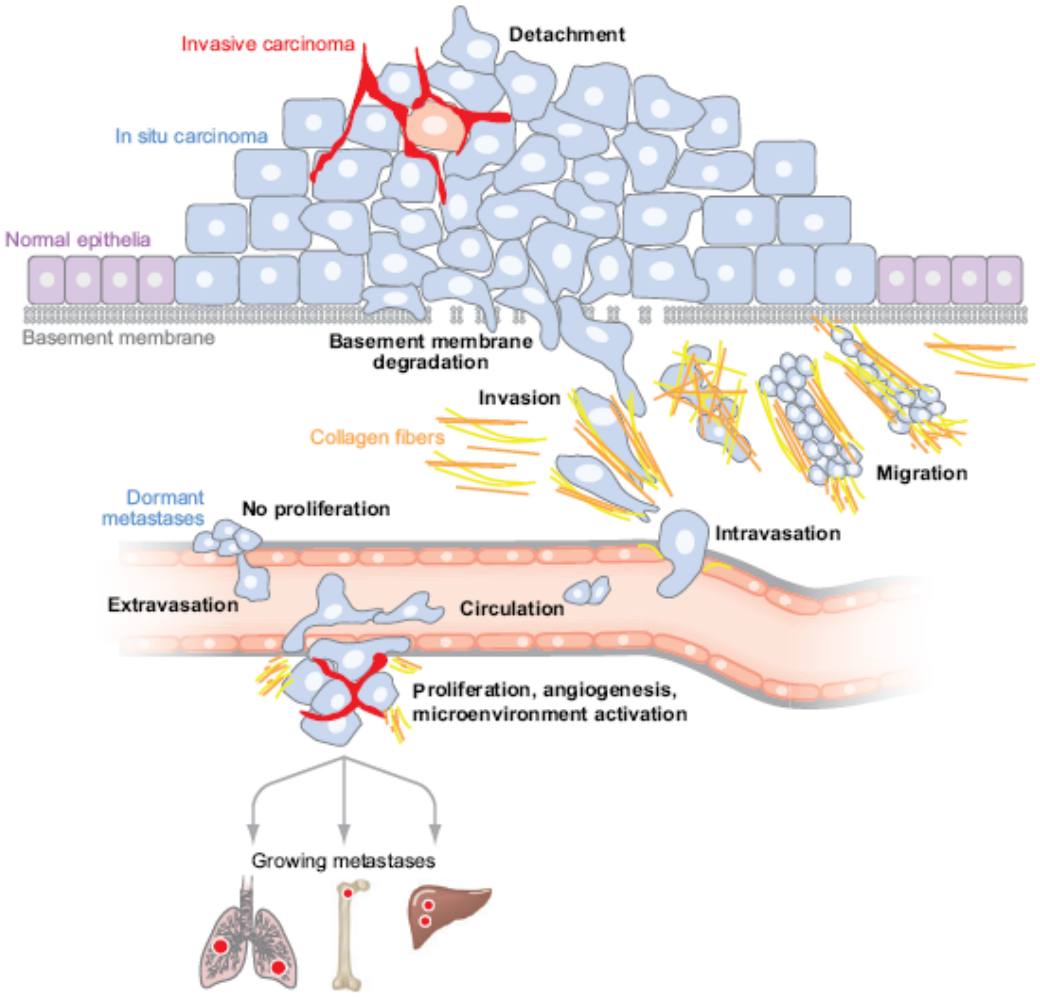
ErbB2 is overexpressed in breast, cervix, colon, endometrial, esophageal, lung, and pancreatic cancers (Blume-Jensen and Hunter, 2001; Salomon and al., 1995; Yarden and Sliwkowski, 2001). Studies of patients with ErbB2-overexpressing tumors have shown that they have a significantly poorer clinical outcome compared to patients whose tumors do not overexpress ErbB2 (Berchuck et al., 1990; Slamon et al., 1987; Slamon et al., 1989). Two major mechanisms underlie ErbB2-overexpression mediated poor clinical outcome. On the one hand, overexpression of ErbB2 enhances cellular properties such as invasion, angiogenesis, and increased survival of cancer cells, leading to increased cancer metastasis. On the other hand, overexpression of ErbB2 confers increased resistance of cancer cells to various cancer therapies (chemotherapeutic agents, hormones, γ-radiation and cytokines) that will result in poor response to cancer treatment.

The metastatic process: a therapeutic target for cancer.

Metastasis is the major driver of mortality in patients with cancer. Many cancers at the primary site are effectively controlled by surgery and radiation therapy. However, the development of metastatic disease correlates with a poor prognosis. Most metastatic lesions are not treated by surgery, but rather by chemotherapy, hormonal therapy, and radiation that serve palliative purposes in the metastatic development and sometimes lead to a significant extension of survival time.

Tumor metastasis consists of a series of biological processes that move tumor cells from the primary neoplasm to a distant location (Figure 8). Tumor cells invade the tissue surrounding the primary tumor, enter either the lymphatics or the bloodstream where they must survive

and eventually arrest in the circulation, then extravasate into a tissue and grow at the new site (Steeg, 2006).



Adapted from Bacac and Stamenkovic, Annu Rev Pathol, 2008

Figure 8. The tumor metastatic process. Transformation of normal epithelial cells leads to carcinoma in situ. At the primary tumor site, tumor cells invade into the lymphatics or directly into the circulation. Once in the bloodstream, tumor cells must survive and avoid immune attack to extravasate. Arrest is most often by size restriction in capillary beds but can involve specific adhesive interactions. The process by which tumor cells form micrometastases and then progressively growing, vascularized macrometastases in a distant organ is termed metastatic colonization. Metastatic colonization involves reciprocal interactions between tumor cells and cells in the microenvironment of the distant organ, and can pause for periods of dormancy.

Invasion

Invasion is the first step of the metastatic process. To invade the surrounding tissue, adhesion properties of cancer cells are modified. Cadherins mediate tumor cell-cell adhesion (Cavallaro

and Christofori, 2004), while tumor cell-extracellular matrix (ECM) adherence is mediated by integrins (Guo and Giancotti, 2004). Matrix metalloproteinases (MMPs), cathepsins and serine proteases at the cell surface induce proteolytic degradation of the surrounding tissue (Friedl and Wolf, 2003; Folgueras et al., 2004; Overall and Kleifeld, 2006). Tumor cell migration through the tissue is a dynamic mechanism involving the polarized extension of protrusions at the leading edge of the cell, translocation of the cell body, and cell contraction inducing migration through the tissue. In a tumor setting, chemokines and chemotactic cytokines induce cell migration (Balkwill, 2003; Wilson and Balkwill, 2002). Activation of cell migration may be also cell autonomous or may involve paracrine loops with cells in the environment (Goswami et al., 2005). Receptor tyrosine kinase interaction with integrins stimulates the formation of a focal adhesion kinase (FAK)-Src complex (McLean et al., 2005; Mclean et al., 2004; Mitra et al., 2005; Playford and Schaller, 2004). Downstream cellular changes in invasion are triggered by sequential binding of protein cascades to FAK (Steeg, 2006).

Survival and arrest in the bloodstream

Beyond survival in the bloodstream which is a harsh environment, metastasizing tumor cells must arrest in the circulatory system. The generally accepted model of arrest and extravasation is that a proportion of cells nonspecifically arrest by binding coagulation factors and by size restriction in the capillary beds (Weiss et al., 1986). Tumor cells extravasate by attaching to the subendothelial ECM, followed by reformation of the capillary (Al-Mehdi et al., 2000).

Metastatic colonization

Breast, prostate carcinomas and multiple myeloma metastasize to bone. Brain metastases are most common in individuals with lung and breast cancer, and are also frequent in individuals with melanoma. The majority of metastasis assays measure lung metastases. (Steeg, 2006).

Growth of metastases in a tissue requires blood supply, which provides oxygen, growth factors, nutrients and metabolites. Angiogenesis is the formation of a new blood supply from preexisting vasculature (Rak and Yu, 2004; Hicklin and Ellis, 2005). Multiple factors stimulate endothelial cells to induce angiogenesis including vascular endothelial growth factor (VEGF), angiopoietin, ephrin (Eph), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β and basic fibroblast growth factor (bFGF) families. VEGF is the best studied and has advanced as a molecular target through clinical approval of VEGF inhibitors (Leung et al., 1989; Yang et al., 2003; Cobleigh et al., 2003; Motzer et al., 2006; Weis et al., 2005).

Finally, many pathways involved in cell survival and resistance to cell death have been shown to promote metastasis and/or survival of cells after extravasation into the distant organ (Yu and Stamenkovic, 2004; Wong et al., 2001).

The contribution of ErbB2 overexpression to increased metastatic potential of cancer cells is evident from a number of studies. In fact, a correlation between the number of lymph node metastases and ErbB2 overexpression has been established in positive breast cancer patients (Slamon et al., 1987; Slamon et al., 1989). Furthermore, experiments using transgenic mice showed that introduction of the erbB2 gene into mice can induce mammary tumors and metastases (Guy et al.,1992; Suda et al., 1990). In addition, expression of activated rat Neu oncogene in 3T3 cells was shown to be sufficient to induce metastasis in nude mice (Yu and Hung, 1991). Also, overexpression of p185 ErbB2 enhanced the metastatic potential of human breast and lung cancer cells (Tan et al., 1997; Yu et al., 1994). Moreover, ErbB2 overexpression can up-regulate MMP-9 and MMP-2 protease activities and increase the invasiveness of breast cancer cells (Tan et al., 1997; Kossakowska et al., 1996). An increase in VEGF expression was also found in breast ErbB2 overexpressing cancer cells suggesting an enhanced angiogenic response (Petit et al., 1997). Additionally, ErbB2 overexpression confers resistance to apoptosis of breast cancer cells (Yu and al., 1998). Altogether, up regulation of matrix metalloproteinases, enhanced angiogenesis and resistance to apoptosis are factors that contribute to increased metastatic potential.

The molecular signaling mechanisms that are responsible for ErbB2-mediated cancer metastasis are under investigation. Multiple ErbB2 downstream signals (e.g., Shc/Grb-2-Ras, PI3K-Akt, MEK-ERK, JNK, p38MAPK, PKC, PLC etc…) may be involved in inducing a variety of metastasis-related properties that can contribute to higher metastatic potential of ErbB2-overexpressing cancer cells. Understanding and further manipulation of the signal transduction networks that are critical for ErbB2-mediated metastasis should facilitate the development of novel anti-metastatic therapies for ErbB2-overexpressing cancers.

5. The ErbB network as a target for cancer therapy

ErbB receptors are important mediators of growth factor signaling, cell proliferation, cell survival and cell migration, important processes that are involved in the development of malignancy. Aberrant activation of the ErbB receptors has been described in a wide number of human tumors, making the ErbB receptor family an important therapeutic target for selective anticancer therapies. Both academic and industrial researches are focusing on evaluating ErbBs as promising molecular targets for cancer treatment. Neutralizing antibodies, which block the bioactivity of RTK ligands, ErbB-targeted antibodies, which either target overexpressed receptors or receptor heterodimerization, and small molecule inhibitors of receptor tyrosine kinase activity have been developed to interfere with ErbB signaling. Owing to their increased expression in many tumor types, EGFR and ErbB2 have become very attractive targets for pharmacological intervention.

Two major therapeutic strategies are used in clinical or at advanced developmental stages to target the ErbB receptor family of RTK: inhibition of the tyrosine kinase (catalytic) domain with small molecules called TKIs, or targeting the extracellular domain of the receptors with monoclonal antibodies (Strome et al., 2007). Many of the intracellular pathways that are essential for cancer development and progression such as PI3K-AKT, MAPK, SRC and STAT signaling are affected by the treatment of tumor cells with these compounds (Hynes and Lane, 2005; Yu and Jove, 2004; Sliwkowski, 1999; Petit et al., 1997; Lane et al., 2000; Nagata et al., 2004). To note is the fact that toxicity of ErbB-directed therapeutics is related to their physiological roles (Hynes and Lane, 2005).

Antibody approach: EGFR and ErbB2 are currently considered as molecular targets for immunological cancer therapy (Friedlander et al., 2008).

- ErbB2 has been successfully targeted by the humanized monoclonal antibody Trastuzumab (Herceptin) in breast cancers where it is overexpressed (Yeon and Pegram, 2005) (Perez and Baweja, 2008). In addition to its binding to the extracellular domain of ErbB2 and antagonizing function, Herceptin induces the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the Rb-related protein p130, which in turn significantly reduce the number of cells undergoing S phase. Furthermore, interaction of Herceptin with the human immune system via its human immunoglobulin G1 Fc domain may potentiate its antitumor activities (Sliwkowski, et al., 1999). Herceptin has been approved for clinical use in metastatic breast cancer, both alone or in combination with chemotherapeutic agents. However, little or no effectiveness against cancers such as prostate (Ziada et al., 2004), pancreas (Safran et al., 2004), colon and rectum (Ramanathan et al., 2004), or lung epithelia (Clamon et al., 2005) has been observed.

Pertuzumab is a recombinant humanized monoclonal antibody (rhuMab 2C4) that binds to the extracellular domain II of the ErbB2 receptor and blocks its ability to dimerize with other ErbB receptors. It is one of the first in a new class of targeted therapeutics known as HER dimerization inhibitors (Agus et al., 2005) (Attard et al., 2007) that is in phase II/III of clinical trials for the treatment of ovarian, breast, prostate cancer, NSCLC and low ErbB2 expressing tumors.
MDX-H210, another targeting ErbB2 antibody, is in phase I of clinical trial for the treatment of advanced breast cancer. Ertumaxomab also targets ErbB2 and is in phase II against advanced metastatic breast cancer (Kiewe et al., 2006). The scFvFRP5 toxin fusion directed against ErbB2 is in phase I/II for the treatment of metastatic breast, prostate, head and neck, NLCSC and transitional cell carcinoma (Azemar et al., 2003; von Minckwitz et al., 2005)

- EGFR is being targeted with the monoclonal chimeric anti-EGFR IgG1 antibody Cetuximab which has been approved for the treatment of metastatic colorectal cancer as single agent or in combination with chemotherapy, in locally and regionally advanced head and neck squamous cell carcinoma in combination with radiation, and as monotherapy for recurrent and metastatic head and neck squamous cell carcinoma after failing platinum-based chemotherapy (Vincenzi et al., 2008). Other humanized mAb targeting EGFR are in clinical trials, such as Matuzumab (Schmiedel et al., 2008; Seiden et al., 2007; Kim, 2004) for the treatment of NSCLC, gynaecological, pancreatic and oesophageal cancers and Panitumumab for the treatment of metastatic colorectal cancer, renal cell cancer and NSCLC (Giusti et al., 2007). Nimotuzumab (Arteaga et al., 2007; Spicer, 2005) is another mAb targeting EGFR that has been approved for the treatment of head and neck cancer, and is in phase I of clinical trial for the treatment of pancreatic cancer. A number of other mAbs targeting EGFR are being studied in phase I clinical trial such as ch806 (Scott et al., 2007) and MDX-447 (Fury et al., 2008).

Combinations with radiopharmacons, peptide toxins, anticancer drugs, or drug-loaded liposomes are also tested to target the ErbB receptors (Friedlander et al., 2008).

Low molecular weight inhibitors approach: Inhibitors that block the activity of tyrosine kinases and the signaling pathways they activate provide a useful basis for drug development. (Levitzki and Gazit, 1995). Both reversible and irreversible inhibitors have been developed.
- EGFR is targeted with two low molecular weight ATP-competitive tyrosine kinase inhibitiors, Gefitinib and Erlotinib (Baselga and Arteaga, 2005). Several types of epithelial cancer with mutated or highly expressed EGFR have successfully been treated, including

head and neck, pancreatic, colorectal and a subset of NSCLC (Mendelsohn and Baselga, 2006).

Lapatinib is a dual (ErbB-1 and ErB-2) receptor tyrosine kinase inhibitor (TKI) that was recently approved for the treatment of advanced breast cancer (Mukherjee et al., 2007). AEE788 (Kuwai et al., 2008; Oehler-Jänne et al., 2007) and EXEL 7647/ EXEL 0999 (Trowe et al., 2008) are in clinical development and both target EGFR/ErbB2 and VEGFR. CI-1033 and EKB-569 are irreversible TKIs which target EGFR/ErbB2 and for which phase II trials are underway in NSCLC (Jänne et al., 2007; Erlichman et al., 2006).

In contrast to their receptors, the ligands of the EGF family of growth factors have not yet been considered as targets for cancer therapy. This can be explained because of the redundancy of EGFR ligands for each receptor, which has led to a general consensus that inhibiting receptor function is more effective than inhibiting multiple ligands for cancer therapy. However, EGFR ligands may be considered as valuable target molecules for cancer therapy (Yotsumoto et al 2008). For instance, HB-EGF has been proposed as a promising target for ovarian cancer (Miyamoto et al., 2004).

However, despite the broad development of ErbB-directed cancer therapy, multiple mechanisms appear to contribute to allow tumor cells to escape from the ErbB-targeted therapeutics. The concept of targeting several kinases using agents with dual activity, or using combination strategies has emerged (Hynes and Lane, 2005).

B. Cell migration: integrating signals from the front to the back

1. Cell migration: a central process in the development and maintenance of uni and multicellular organisms

Cell migration is a vital cellular response that plays a critical role during the life cycle of simple, uni-cellular organisms such as amoeba and of complex multi-cellular organisms such as mammals. In simple organisms, its main functions comprise mating and the search for food (Manahan et al., 2004). In multicellular organisms, specialization requires cell migration-mediated tissue organization, organogenesis and homeostasis (Ridley et al., 2003). Directed cell movement accompanies human life from conception to death. During embryogenesis, proper tissue formation requires complex patterns of cell migration. In adult life, wound healing which involves the migration of different cell types, including leucocytes, is required for the development of immune responses. Cell movement is also seen during remodeling of the vascular system in mature organisms. Finally, cell migration also occurs in diverse diseases, like in various chronic inflammatory and vascular diseases or in cancer where it leads to invasion and metastasis.

1.1 Cell migration in embryonic development

The cell migration process orchestrates morphogenesis throughout embryonic development. Gastrulation is the morphogenetic process in embryos of multicellular organisms by which the future mesoderm and endoderm move inside the ectoderm to form a three-layered embryo, consisting of the ectoderm on the outside, the endoderm on the inside, and the mesoderm between these two layers (Lu et al., 2001). During gastrulation, large groups of cells migrate individually or collectively as sheets to form this resulting three-layered embryo. The collective migration includes coordinated changes in cell shape and coordinated rearrangements (Keller, 2005). Following this three-layer formation, cells migrate to target locations where they differentiate to form the specialized cells that constitute the different tissues and organs (Locascio and Nieto, 2001). In fact, cell movement plays a critical role in the formation of the nervous system (Suzuki, 2007; Marín and Rubenstein, 2001) and organogenesis (Hogan and Kolodziej, 2002).

1.2 Cell migration during adult life

Cell migration is important in homeostatic processes such as immunity or wound healing. In fact, cell motility is a prominent component of immune surveillance that is mediated by leukocytes which migrate into the surrounding tissue to destroy invading microorganisms, debris and infected cells (Sànchez-Madrid and del Pozo, 1999). Leukocyte migration is controlled by a number of known attractants such as the extensive family of chemokines that are released by leucocytes and other cells in response to different stimuli, and mediate inflammation (Luster, 1998; Luster et al., 2005). Coordinated cellular movements also underlie response and repair of injured tissues during wound healing (Martin, 1997). Furthermore, cell migration is important during renewal of skin and intestine, where fresh keratinocytes or epithelial cells migrate up from the basal layer and the crypts, respectively (Fuchs and Raghavan, 2002; Heath, 1996).

1.3 Cell migration and disease

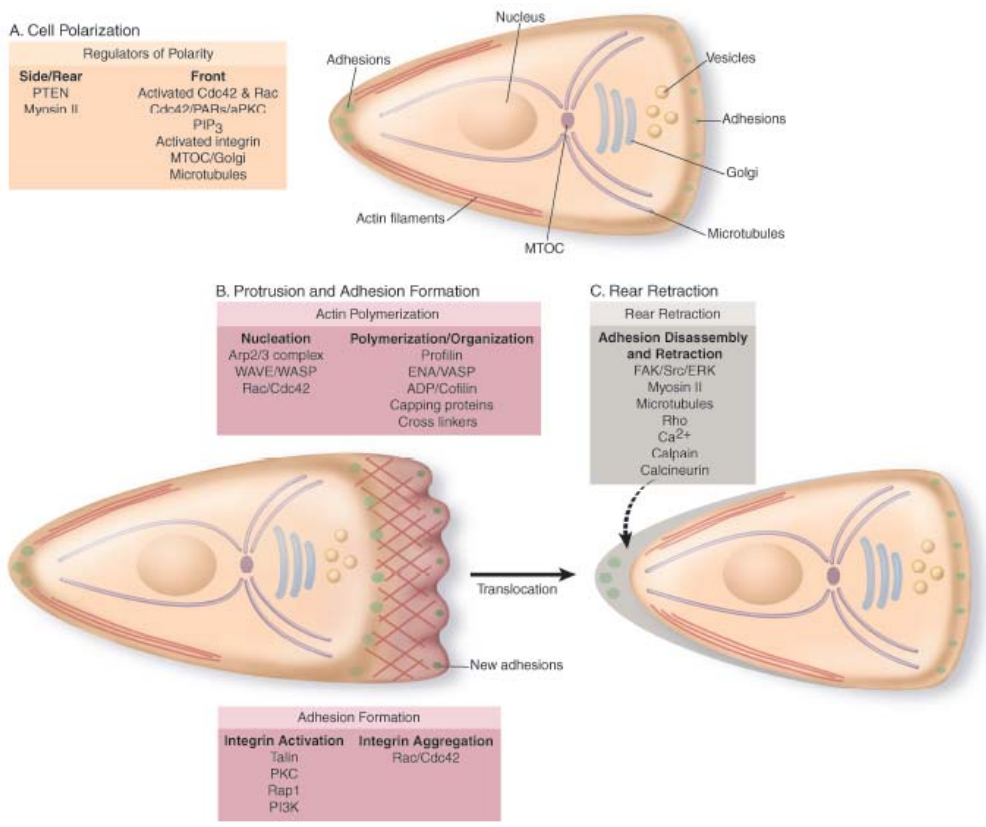
The cell migration process exhibits so many regulatory steps that many deficiency can impair or enhance cell migration, thus contributing to numerous pathological processes. At the initial steps of embryonic and fetal development, impairment of regulators of cell motility can lead to the failure of blastocyst implantation into the uterus or to malformed embryos with disorganized tissues (Stephens et al., 1995; Dominguez et al., 2005). A number of congenital abnormalities in brain development, such as epilepsy (Frotscher et al., 2003), neurological deficits (Givogri et al., 2006), mental retardation (Thelen et al., 2002), and also heart development defects (Davis and Katsanis, 2007), derive from deregulated cell migration processes. Chemotaxis has also been linked with pathological disorders such as osteoporosis (Susva et al., 2000), vascular diseases (Roque et al., 2002), or chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis (Hilden et al., 2006). Importantly, cell migration plays a critical role in cancer where it contributes to the formation of metastasis (Yamaguchi et al., 2005; Kedrin et al., 2007). In fact, the initial step in tumor metastasis requires chemotactic migration of cancer cells that invade the surrounding tissue and the lymphatic and blood vasculature which they subsequently leave and migrate into another site (Chambers et al., 2002; Friedl and Wolf, 2003; Steeg and Theodorescu, 2008).

Accordingly, the studies on the fundamental mechanisms underlying the migratory process should yield promising advances for the development of effective therapeutic approaches for the treatment of migration-related disorders.

2. Cell migration: a cyclic process

The current model of cell migration is derived from diverse studies performed on a wide number of migratory cell types and environments. As an example, cancer cells have a broad spectrum of migration and invasion mechanisms that include both individual and collective cell migrations strategies (Friedl and Wolf, 2003). As a general description, cell migration can be viewed as a physically integrated molecular system consisting in a sequence of coordinated steps which act in a cycle (Lauffenburger and Horwitz, 1996). The initial response of a cell to a migration-inducing agent is a morphological polarization and extension of a directed protrusion. These protrusions are stabilized by formation of cell adhesions to the extracellular matrix (ECM) or to adjacent cells. These cell-substratum adhesions serve as anchoring sites for cell body contraction and movement. In the last step of the migratory process, cell adhesions disassemble, resulting in a rear cell detachment.

Chemotaxis defines the ability of a cell to sense spatial heterogeneities in the concentration of extracellular chemoattractants and to respond by polarizing and migrating toward the source of stimulation (Iglesias and Devreotes, 2008).



Adatped from Ridley et al., 2003

Figure 9. Steps in cell migration

2.1 The first step: directional sensing and polarization

Chemotactic models, such as *Dictyostelium discoideum* that has the advantage of being a genetically and biochemically tractable organism, have been intensely studied to uncover the general principles by which cells sense asymmetric environmental stimuli (Chung et al., 2001; Iijima et al., 2002; Iijima et al., 2004). The molecular mechanisms of chemotaxis are highly conserved between mammalian cells and the *Dictyostelium discoideum* amoebae (Devreotes and Zigmond, 1998; Franca-Koh et al., 2006).

Directional sensing refers to the ability of a cell to detect an asymmetric extracellular signal and generate an internal amplified response (Parent and Devreotes, 1999; Iglesias and Levchenko, 2002). When cells are exposed to external gradients of chemoattractant, signaling molecules accumulate at the membrane and locally initiate the downstream signaling response. Polarization refers to the ability of a cell to assume an asymmetrical shape with a defined leading and trailing edge with distinct sensitivities for chemoattractants (Huttenlocher, 2005). Signals from the environment, including chemoattractants, growth factors and extracellular matrix, regulate cell polarization through the spatially controlled recruitment of signaling complexes that modulate the actin cytoskeleton and mediate the extension of membrane protrusions at the leading edge. Although directional sensing does not require actin polymerization, polarization depends critically on a signal input as well as on a reorganization of the cytoskeleton.

In both neutrophils and *Dictyostelium discoideum* models, PtdIns(3,4,5)P_3 has emerged as an important player of this first chemotactic step (Funamoto et al., 2001; Curnock et al., 2002; Charest and Firtel, 2006; Franka-Koh et al., 2007). In fact, upon gradient sensing in *Dictyostelium discoideum,* receptor-mediated activation of heterotrimeric G-proteins (Janetopoulos et al., 2001; Xu et al., 2005) enhances intracellular signaling activation with a graded activation of Ras-family small G proteins (Kae et al., 2004). PI3K that contain a Ras binding domain, is subsequently recruited to the plasma membrane (Sasaki et al., 2004), where the cell is exposed to a higher concentration of chemoattractant. This leads to the local activation and production of PtdIns(3,4,5)P_3 with localized accumulation of PtdIns(3,4,5)P_3 on the side of the cell facing the highest chemoattractant concentration. This polarized PtdIns(3,4,5)P_3 response is generated through the differential local regulation of PI3K, which produces PtdIns(3,4,5)P_3 by phosphorylating PtdIns(4,5)P_2, and the PtdIns 3-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten), which dephosphorylates PtdIns(3,4,5)P_3 back into PtdIns(4,5)P_2. PTEN is then located at the membrane at the lateral sides and at the trailing edge of the cell where it degrades any PtdIns(3,4,5)P_3 that might either diffuse laterally from the leading edge or be produced along

the lateral sides of the cell, thus restricting the polarized accumulation of PtdIns(3,4,5)P_3 (Huang et al., 2003; Iijima and Devreotes, 2002; Funamoto et al., 2002).
This role for local PI(3,4,5)P_3 in polarity and directional sensing appears to be conserved throughout phylogeny in cells such as *D. melanogaster* hemocytes, human neutrophils and fibroblasts, neurons, and a variety of embryonic cells (Stramer et al., 2005; Wang et al., 2002; Wu et al., 2000; Schneider and Haugh, 2005; Chadborn et al., 2006; Montero et al., 2003).
In multiple studies performed on different types of motile cells, including leucocytes, *Dictyostelium*, primary dendritic cells and fibroblasts, local polymerization of F-actin (filamentous actin) has been shown to be guided by chemoattractant-induced localized PtdIns(3,4,5)P_3 signaling (Affolter and Weiger, 2005).

In mammalian cells, directional sensing and polarization appear to be mediated by a set of interlinked positive feedback loops involving Rho family GTPases, PI3K, integrins, microtubules and vesicular transport (Ridley et al., 2003). At the rear of the cell, Myosin II and PTEN regulate polarity by restricting protrusions to the cell front (Vicente-Manzanares et al., 2007; Liliental et al., 2000). Cdc42 (cell division cycle 42) is an active regulator of cell polarity towards the front of a migrating cell (Itoh et al., 2002). Cdc42 defines the location and stability of the leading edge (Srinivasan et al., 2003), and along with Par proteins and aPKC, Cdc42 regulates the microtubule-organizing center (MTOC) and Golgi apparatus reorientation in front of the nucleus, facing the direction of the leading edge (Palazzo et al., 2001; Etienne-Manneville and Hall, 2003). Activation of Cdc42 is mediated by PAK1 (p21-activated kinase 1) (Li et al., 2003). Also, interaction of integrins with the extracellular matrix at the newly formed cell front was shown to induce activation and polarized recruitment of Cdc42 (Etienne-Manneville and Hall, 2001). In neutrophils, in addition to Rac activation at the leading edge of the cell (Gardiner et al., 2002), Gβγ-dependent activation of Cdc42 has been shown to be required for directional sensing (Li et al., 2003) Also, it has been proposed that neutrophil rear localization and activation of PTEN may involve phosphorylation by Rho kinase in a Rho-dependent manner (Li et al., 2005). Microtubules may also play a role in directed neutrophil migration via inhibition of the Rho GTPase (Xu et al., 2005). Also in other mammalian models, the Rho-mDia1 pathway is reported to regulate polarization by aligning microtubules and actin filaments and delivering Apc/Cdc42 to their respective sites of action (Yamana et al., 2006). In adenocarcinoma cells, Ras was also shown to be required for PtdIns(3,4,5)P_3 and lamellipodium production (Yip et al., 2007).

2.2 The second step: extension of the protrusion and adhesion formation

In the current paradigm for cell migration, formation and extension of the cell protrusion is controlled by actin polymerization (Pollard and Borisy, 2003; Ridley et al., 2003).

The core constituent of the actin cytoskeleton is a monomeric globular (G)-actin, a 43-kDa ATPase that can self-assemble into filamentous (F)-actin. Each asymmetric filament possesses a fast growing barbed end also called the + end, which is the more dynamic end of the actin filament, where growth and shrinkage are fast, and a slower less dynamic pointed end also called the – end. Hydrolysis of ATP in the filament is tightly coupled to polymerization and regulates the kinetics of assembly and disassembly, as well as the association of interacting proteins. The dynamic assembly and disassembly of filaments are under the control of at least hundred actin-binding proteins which bind directly to monomers or filaments and control the actin structure and dynamics and have specific coordinated actions: nucleating; capping; stabilizing; severing; depolymerizing; crosslinking; bundling; sequestering or delivering monomers; promoting monomer nucleotide exchange.

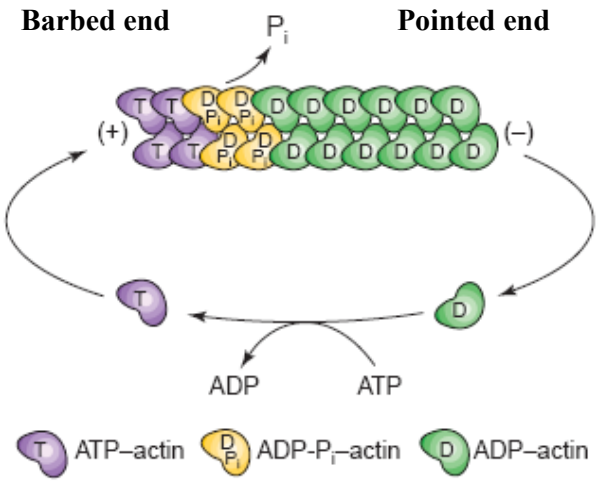


Figure 10. Turnover of an actin filament *Adapted from Chen et al., 2000*

An important set of actin regulators initiate formation of new actin filaments by a process that is called **nucleation**. Three classes of protein have been identified that initiate new filament polymerization: the Actin-related protein-2/3 (ARP2/3) complex, the Formins and Spire.

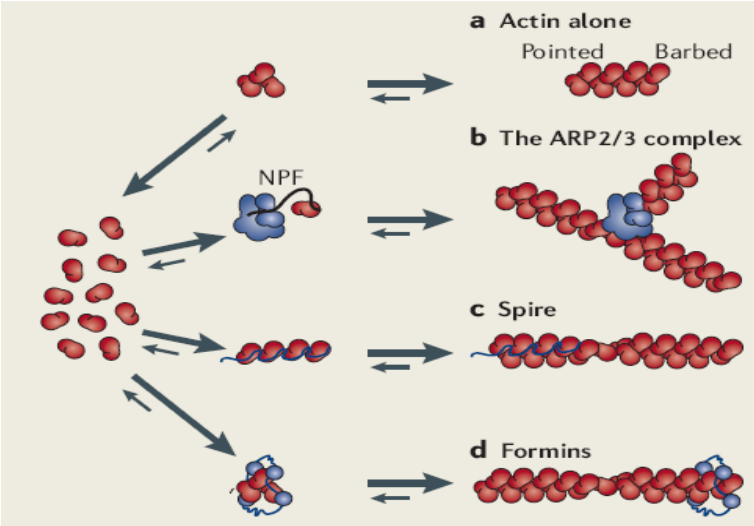
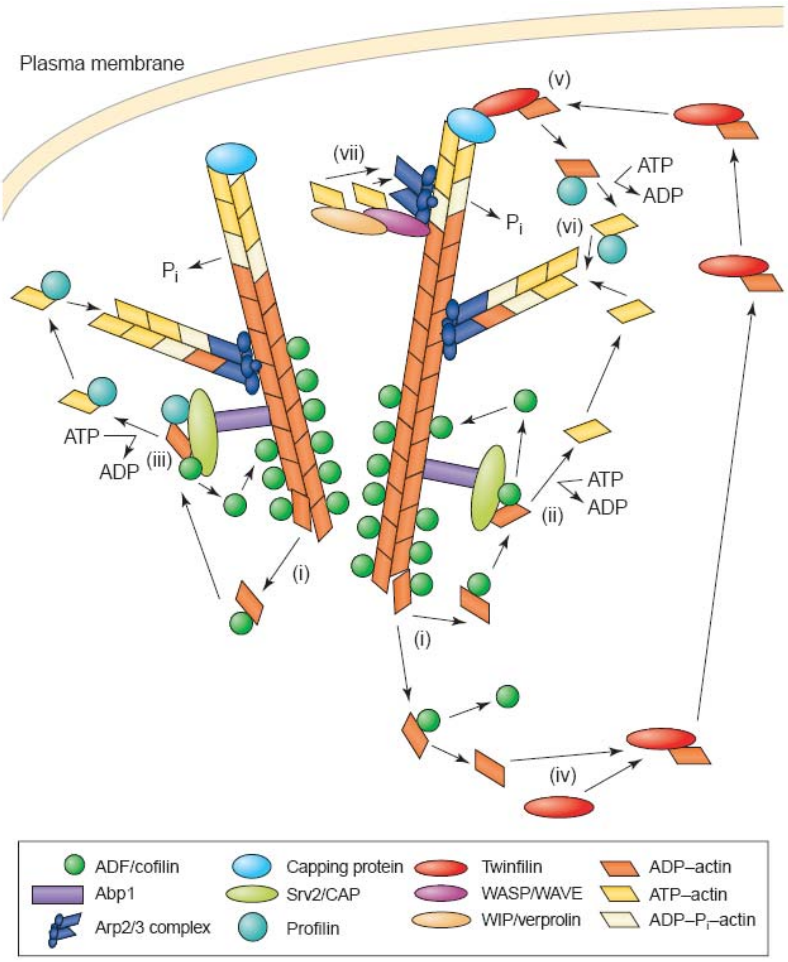


Figure 11. Paths to nucleation *Adapted from Goley and Welch, 2006*

- Arp2/3 complex is thought to mimic an actin dimer or trimer and binds to the sides of existing actin filaments and promotes the extension of a new actin filament from its pointed end, forming a 70° angle with the pre-existing filament (Weaver et al., 2003). Arp2/3 regulation is controlled by WAVE/Scar, WASP and N-WASP proteins. WAVE/Scar is part of a multimeric complex including Abi, Nap125, Sra-1 and HSPC-300. This complex is under the control of the small GTPase Rac, which induces the dissociation of Abi, Nap125 and Sra-1 from WAVE, thus mediating WAVE activation. WASP and N-WASP are regulated by Cdc42 (Vartiainen and Machesky, 2004; Goley and Welch, 2006).
- Spire proteins are conserved among metazoan species, and associate with four actin subunits through their four tandem G-actin-binding WASP-homology-2 (WH2) domains to function as a scaffold for polymerization into an unbranched filament (Quinlan et al., 2005; Kerkhoff, 2006).
- Formins are widely expressed in most eukaryotes and promote nucleation of unbranched filaments (Kovar, 2006). In contrast to Spire and Arp2/3, formins bind to the barbed end of actin filaments and promote actin growth in a linear movement. Mammalian Formins mDia1 and mDia2 are regulated by small GTPases (RhoA and Cdc42 for mDia1 and mDia2, respectively) and require interaction with G-actin bound profilin to promote actin polymerization (Watanabe and Higashida, 2004).

Polymerization/organization. Polymerization of the actin cytoskeletal network drives the initial extension of the plasma membrane at the cell front. This step is regulated by proteins that control the availability of activated monomers, capping proteins as well as depolymerization/severing proteins.



Taken from Paavilainen al., 2004

Figure 12. Regulation of actin-monomer turnover. Actin-depolymerizing factor (ADF)/cofilin (green circles) promotes the dissociation of actin monomers from the filament pointed end (i). Srv2/cyclase-associated protein (CAP) (light green ovals), which associates with actin filaments through interactions with the actin-filament-binding protein Abp1 (purple), recycles ADF/cofilin and actin monomers for new rounds of filament depolymerization and polymerization. Srv2/CAP might also promote nucleotide exchange on actin monomers, either on its own (ii) or with profilin (cyan) (iii). If the newly depolymerized ADP–actin monomer diffuses away from the actin-filament structure, it could associate with twinfilin (red), which inhibits nucleotide exchange and the spontaneous assembly of actin filaments from the ADP–actin monomer (iv). The twinfilin–ADP–G-actin complex is relocalized to the Arp2/3-nucleated actin structure through direct interactions with capping protein (light blue) (v). The ADP–actin monomer can then dissociate from the twinfilin–capping-protein complex, undergo profilin-induced nucleotide exchange and become attached to an uncapped filament barbed end nearby (vi). Wiskott–Aldrich syndrome protein (WASP)/WASP family verprolin-homologous (WAVE) protein (violet) and verprolin/WASP-interacting protein (WIP) (beige) regulate the activity of Arp2/3, and the actin-monomer-binding activity of these proteins might contribute to the actin-filament-nucleating activity of the Arp2/3 complex (dark blue) (vii). Abbreviation: Pi, inorganic phosphate.

Profilin is a small actin-monomer-binding protein that has a higher affinity for ATP–G-actin than for ADP-G-actin and catalyzes nucleotide exchange on actin monomers (Pollard et al.,

2000). When bound to an actin monomer, profilin inhibits spontaneous filament nucleation and, in the absence of free barbed ends, it functions as an actin-monomer-sequestering protein. However, when barbed ends are available and together with thymosin β-4, profilin promotes the assembly of actin filaments (Pollard et al., 2000; Pantaloni and Carlier, 1993). Profilin binds to formins, and is also required for Ena/VASP-mediated actin polymerization at the barbed end (Krause et al., 2003).

Ena/VASP proteins are a conserved family of actin regulatory proteins that associate with barbed ends of actin filaments and antagonize filament capping by the capping protein CapZ. They are also involved in the reduction of density of Arp2/3-dependent actin filament branches (Krause et al., 2003).

***Capping protein*s** are highly conserved proteins in eukaryotic cells that cap actin filament barbed ends with high affinity, thereby preventing the addition or loss of actin subunits (Wear and Cooper, 2004).

Twinfilin is present in eukaryotes from yeast to mammals and binds only to monomeric actin. This protein does not promote actin-filament depolymerization (Palmgren et al., 2002) but rather serves as a link between rapid actin filament depolymerization and assembly in cells.

Srv2/Cap is a multimeric protein complex (Balcer et al., 2003) that recycles ADF/cofilin and actin monomers for further filament depolymerization and polymerization, respectively. It stimulates nucleotide exchange on actin monomers and relieves the inhibitory effects of ADF/cofilin on this exchange (Paavilainen et al., 2004).

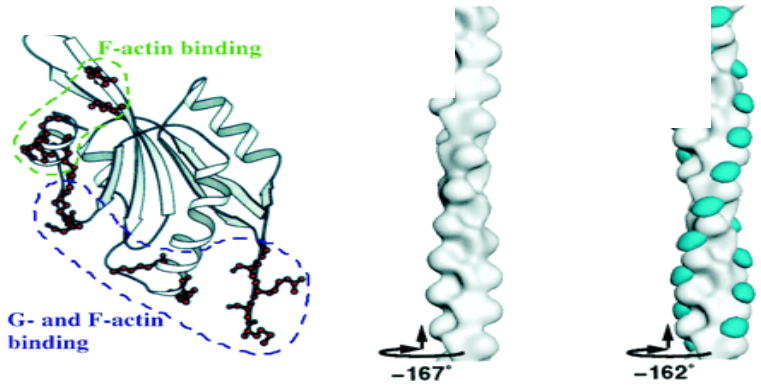
WASP/WAVE: The WASP family protein consists of two structurally distinct groups: WASPs and WAVEs (or SCAR). In mammals, this family comprises five members: WASP, N-WASP, and three WAVE proteins (Stradal et al., 2004). WASP family proteins interact with a large number of proteins such as Arp2/3, acting as intermediaries between signaling pathways and the actin cytoskeleton (Miki and Takenawa, 2003).

Verprolin/WIP are large proteins that interact with several regulators of the actin cytoskeleton and monomeric/filamentous actin. Through its C-terminal domain, Verprolin/WIP binds to WASP proteins. (Ramesh et al., 1997; Moreau et al., 2000; Martinez-Quiles et al., 2001; Kinley et al., 2003).

Actin depolymerizing factor ADF/Cofilin proteins belong to a family of abundant, related proteins that are found in all eukaryotes. Unicellular organisms such as yeast usually have only one ADF/cofilin-type protein, whereas multicellular organisms typically have several isoforms. In mammals, there are three different ADF/cofilins: cofilin-1, cofilin-2, and ADF. These proteins have distinct expression patterns: cofilin-1 is expressed in most embryonic and adult cells, cofilin-2 is expressed in muscle, and ADF is mainly found in epithelial and neuronal cells (Vartiainen et al., 2002). In some cultured mammalian cell lines and invasive mammary tumor cells, cofilin- 1 is the most abundant isoform, whereas ADF is expressed at much lower levels (5%) (Hotulainen et al., 2005; Wang et al., 2006). Furthermore, actin-depolymerizing factor and cofilin-1 were shown to play overlapping roles (Hotulainen et al., 2005).

ADF/cofilin are small ubiquitous proteins that bind to monomeric and filamentous actin in a pH-sensitive manner. Genetic studies in budding yeast, *Caenorhabditis elegans, Drosophila* and plants have shown that ADF/cofilins are essential for viability (Moon et al., 1993; McKim et al., 1994; Gunsalus et al., 1995).

Their most important physiological function is to enhance the rate of actin filament turnover by depolymerizing filaments from their pointed ends, thereby providing a pool of actin monomers for filament assembly (Carlier et al., 1997). ADF/Cofilin binds to ADP-F actin with higher affinity than they bind to ATP-F actin (Bamburg et al., 1999). Binding of ADF/cofilin to F-actin induces actin filaments to twist by ~5° per subunit, which promotes a modification of the thermodynamic stability of the filaments and leads to their depolymerization (McGough et al., 1997). Mutations in yeast cofilin result in an accumulation of abnormal actin-filament structures and decreased rates of actin-filament depolymerization (Lappalainen et al., 1997; Lappalainen and Drubin, 1997).



Adapted from Cell motility and the cytoskeleton, Prof. Peter K. Hepler

Figure 13. Structure and actin interaction of actin-depolymerizing factor (ADF)/cofilin. When bound to actin, ADF/cofilin increases the twist of the actin filaments by 5 degrees per subunit.

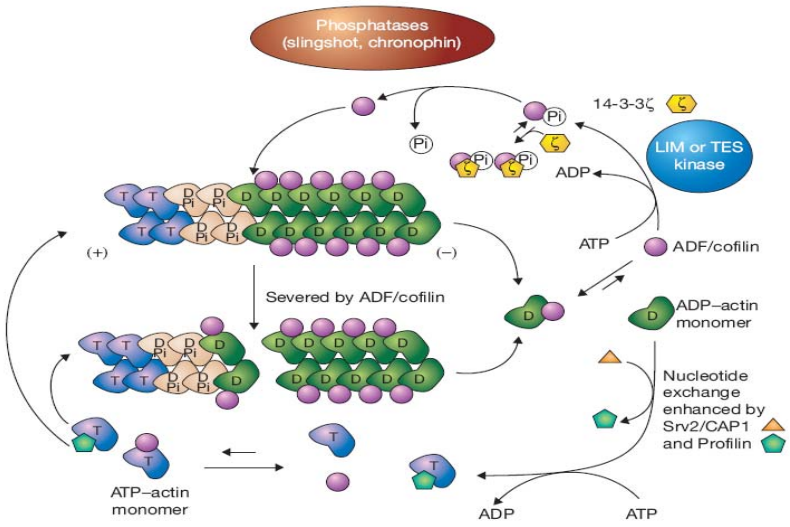
ADF/cofilins also stimulate the dissociation of γ-phosphate from ADP-Pi filaments (Blanchoin et al., 2000). In addition to their depolymerizing activity, ADF/cofilins also sever actin filaments and consequently increase the number of filaments barbed ends, promoting actin polymerization (Chan et al., 2000; Zebda et al., 2000; Ichetovkin et al., 2002; Ghosh et al., 2004). Whether ADF/cofilins contribute to cytoskeletal dynamics by depolymerizing actin filaments at their pointed ends or by creating new filament barbed ends for F-actin assembly through their severing activity, is still a matter of controversy. Finally, ADF and cofilin-1 bind preferentially ADP-G-actin (compared to ATP-G-actin) and inhibit spontaneous nucleotide exchange on monomeric actin (Carlier et al., 1997; Maciver et al., 1994; Blanchoin and Pollard, 1998; Vartiainen et al., 2002). ADF/cofilins are quantitatively different in their activities. ADF is the most efficient at turning over actin filaments and promotes a stronger pH-dependent actin filament disassembly than cofilin-1 or cofilin-2. The muscle cell-specific cofilin-2 has a weaker actin filament depolymerization activity than the other two and promotes filament assembly rather than disassembly (Vartiainen et al., 2002; Yeoh et al., 2002).

The regulation of ADF/cofilins activity by phosphorylation, phosphoinositides, acidity and interactions with other proteins:

Phosphorylation: mammalian ADF and cofilin are inhibited in their activity towards actin binding and dynamics by kinases that phosphorylate the proteins on a conserved serine residue (Ser-3) near the N-terminus which is predicted to be the actin-binding site (Wriggers et al., 1998)) (Agnew et al., 1995; Moriyama et al., 1996).

Several kinases that phosphorylate cofilin and mediate various signals for remodeling of the actin cytoskeleton have been identified. These include the LIM kinases (LIMK1 and LIMK2) (Meberg, 2000; Bernard, 2007; Wang et al., 2006) and the related testicular (TES) kinases (TESK1 and TESK2) (Rosok, 1999; Toshima et al., 2001). LIM kinases are regulated through phosphorylation by the Rho GTPase effectors Rho kinase (ROCK) and p21-activated kinase (PAK), whereas the TES kinases are downstream of integrin signals (Berbard, 2007; Toshima et al., 2001). In addition, 14-3-3ζ binds to phosphorylated cofilin and prevents dephosphorylation by a phosphatase (Gohla and Bokoch, 2002; Nagata-Ohashi et al., 2004).

Much less is known about the enzymes that activate cofilin by dephosphorylation. The general phosphatases PP1, PP2A and PP2B, are able to cause dephosphorylation of cofilin in some systems (Ambach et al., 2001). Another specific ADF/cofilin phosphatase, Slingshot, has been identified (Niwa et al., 2002; Ohta et al., 2003; Kousaka et al., 2008). Gohla et al. also identified another cofilin phosphatase, named chronophin (CIN) (Gohla et al., 2005).



Adapted from Wiggan et al., 2005

Figure 14. The activity of ADF/cofilin is regulated by a balance of phosphorylation and dephosphorylation.

Phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP2) also regulate cofilin activity. Indeed, PIP2 binds to ADF/cofilin and competes with actin (Yonezawa et al., 1990). A PIP2 binding site on ADF/cofilin is mapped to long helix α3 and several other positively charged residues which overlap with the actin-binding surface (Yonezawa et al., 1991; Van Troys et al., 2000; Ojala et al., 2001).

Acidity: the cofilin-mediated control of actin polymerization and depolymerization was shown to be pH-sensitive. In fact, when cofilin was reacted with F-actin at different pH, the depolymerized actin concentration was higher at elevated pH (pH>7.3) (Yonezawa et al., 1985; Pope et al., 2004).

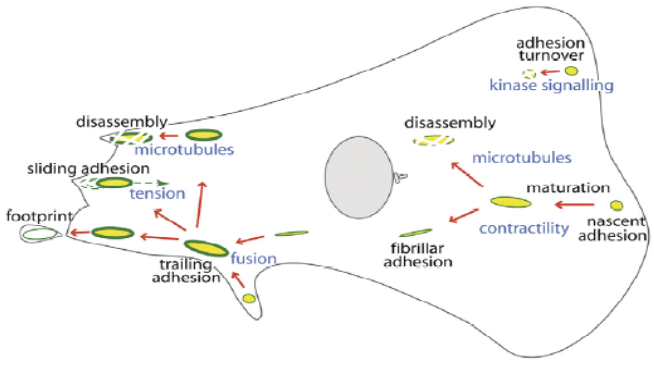
In addition to these regulatory mechanisms, ADF/cofilin activity is also regulated by its *interaction with other proteins.* Tropomyosin competes with ADF/cofilin for F-actin binding and inhibits depolymerization (Ono and Ono, 2002). Furthermore, several actin-binding proteins have recently been shown to collaborate with ADF/cofilin to promote actin filament dynamics. Profilin competes with ADF/cofilin for G-actin binding and enhances exchange of actin-bound nucleotide in the presence of ADF/cofilin, thereby increasing the rate of actin turnover synergistically with ADF/cofilin (Blanchoin and Pollard, 1998; Didry et al., 1998). Also, the N-terminal domain of Cap was found to bind to the actin-cofilin complex and to accelerate actin depolymerization from the pointed ends (Moriyama and Yahara, 2002). Finally, actin-interacting protein 1 (AIP1) was shown to enhances filament severing only in the presence of ADF/cofilin (Ono, 2003).

Actin polymerization is therefore regulated by a number of proteins, leading to the formation of a protrusion at the leading edge of the cell. These protrusions are stabilized to the surroundings by the formation of adhesions.

Formation of adhesions: The assembly of Focal adhesions (FAs) in response to adhesion to the ECM is gradual, usually occurring within 1 to 2 h after cell attachment. This process requires integrin activation followed by integrin aggregation and recruitment of signaling components to the nascent adhesions (Kiosses et al., 2001; Miyamoto et al., 1995). Integrin receptors support the adhesion to the extracellular matrix or to other cells and mediate the link with actin filaments on the inside of the cell (Geiger et al., 2001; Yamada and Miyamoto, 1995; Schoenwaelder and Burridge, 1999).

The integrins are heterodimeric adhesion glycoprotein receptors, consisting of α and β chains with a large ligand binding extracellular portion and short cytoplasmic domains. Ligand binding to the extracellular domain leads to conformational changes and integrin clustering (Emsley et al., 2000), inducing subsequent intracellular signaling such as protein tyrosine phosphorylation, activation of small GTPases and changes in phospholipid biosynthesis (Geiger et al., 2001). Proteins such as Talin, PKC, the GTPase Rap1 and PI3K regulate integrin activation (Kinbara et al., 2003; Hattori and Minato, 2003; Kolanus and Seed, 1997; Ratnikov et al., 2005; Katsumi et al., 2005; Arnaout et al., 2007). Integrin engagement and aggregation induces Rac activation (del Pozo et al., 2000).

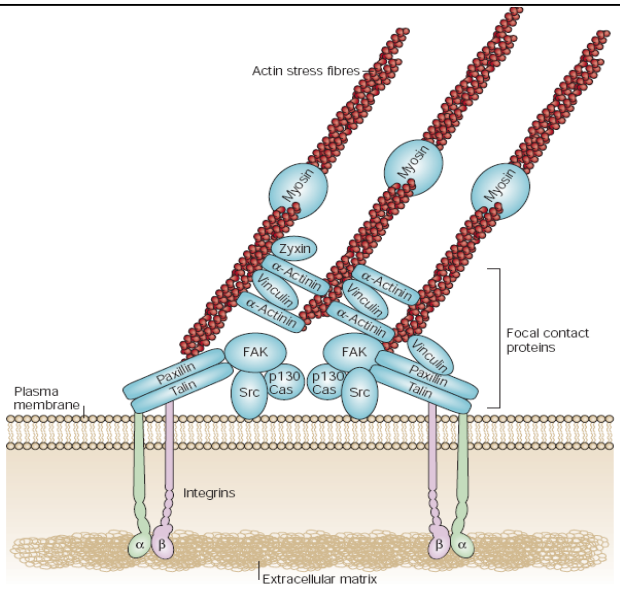
During migration, adhesions assemble at the leading edge and disassemble at the trailing edge. However, adhesions also disassemble at the front during protrusion and feed components to nascent adhesions at the leading edge in a process called adhesion turnover (Webb et al., 2004; Webb et al.,2002).



Taken from Broussard et al., 2008

Figure 15. Model of asymmetric adhesion dynamics. Nascent adhesions formed at the front either undergo turnover, which is predominantly controlled by kinase signaling, or mature in response to contractile forces. Mature adhesions can disassemble or be transformed into fibrillar adhesions. Trailing adhesions arise as a result of fusion of additional nascent adhesions and remaining fibrillar adhesions. Once formed, trailing adhesions slide because of tension from attached stress fibers and either eventually disassemble or detach in the form of membrane 'footprints'.

The classification of adhesions is based primarily on their morphology or on the method of their formation, and includes focal adhesions, fibrillar adhesions, and focal complexes. Fibrillar adhesions are central structures that contain $\alpha_5\beta_1$ integrins and tensin, whereas focal complexes (or nascent adhesions) are small adhesions induced by Rac activation at the periphery of the cell (Zamir et al., 2000; Nobes and Hall, 1995; Rottner et al., 1999). The small adhesions drive rapid cell migration (Beningo et al., 2001) and under tension, they mature into larger, relatively stable and more organized structures such as FAs (Chrzanowska-Wodnicka and Burridge, 1996; Sastry and Burridge, 2000; Bershadsky et al., 2003). Mature adhesions are, in turn, either disassembled underneath the approaching cell body, or diminished to form fibrillar adhesions (Rid et al., 2005; Zamir et al., 2000). The majority of nascent adhesions undergo rapid turnover such that their components can be incorporated into newly formed adhesion sites. The mechanisms by which adhesions turnover, and the mechanisms that regulate these processes are not well understood. The formation and disassembly of adhesions are complex processes that require a coordinated interaction of actin or actin-binding proteins, signaling molecules, structural proteins, integrins, adaptor molecules and microtubules. Over 50 proteins have been found in adhesions (Zamir and Geiger, 2001)



Adapted from Mitra et al., 2005

Figure 16. Molecular architecture of focal contacts

The current paradigm of cell migration favors a coupled formation of nascent-cell substratum contact sites at the leading edge and formation of a protrusion. Formation of these adhesions is dependent on Rac and Cdc42, and these adhesions stabilize the lamellipodium by mediating

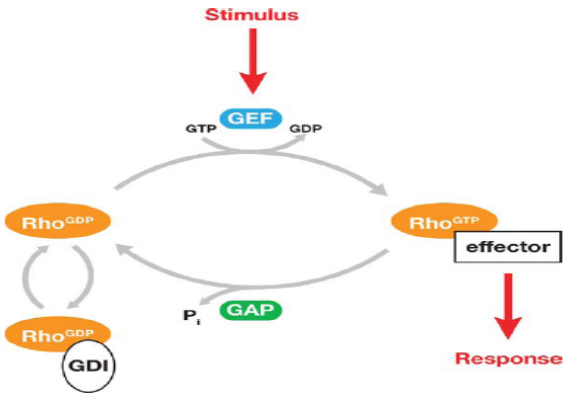
attachment to the ECM. A sequential recruitment of individual or classes of adhesion components around a nucleation center is proposed. In fact, integrin aggregation leads to the recruitment of structural and signaling proteins to FAs; the cytoplasmic components of FAs include cytoskeletal proteins, such as α-actinin, vinculin and talin, and signaling proteins such as the integrin-linked focal adhesion kinase, pp125FAK (FAK) (Miyamoto et al., 1995a; Miyamoto et al., 1995b; Yamada and Miyamoto, 1995). Subsequent accumulation of multiple signaling molecules is induced upon tyrosine phosphorylation. Indeed, extracellular matrix-dependent phosphorylation of FAK on Tyr-397 generates a high affinity binding site for SH2-domain containing proteins such as Src family kinases, PI3K, Grb7, and PLCγ1 (Chen and Guan, 1994; Schaller et al., 1994; Schaller et al., 1999; Schlaepfer et al., 1996; Shen and Guan, 2001; Xing et al., 1994; Zhang et al., 1999). $pp60^{c\text{-}Src}$ (Src) binding to FAK apparently contributes to Src activation, promoting further phosphorylation of FAK at additional tyrosine sites (Maa and Leu, 1998; Calalb et al., 1995; Calalb et al., 1996; Leu and Maa, 2002). There is evidence that Src phosphorylates FAK at Tyr^{925}, creating a binding site for the complex of the adapter Grb2 and Ras guanosine 5'-triphosphate exchange factor Sos (Schlaepfer et al., 1994). These interactions link FAK to signaling pathways that modify the cytoskeleton and activate mitogen-activated protein kinase (MAPK) cascades. Src family kinases and Src in particular, were shown to have a central role in regulating protein dynamics at cell-matrix interfaces, both during early stages of interaction and in mature focal adhesions (Volberg et al., 2001; Parsons and Parsons, 1997). In particular, the complex FAK-Src phosphorylates a number of focal adhesion components. The major targets include paxillin and tensin, two cytoskeletal proteins that may also have signaling functions, and $p130^{CAS}$, a docking protein that recruits the adapter proteins Crk and Nck (Panetti, 2002; Vuori et al., 1996; Schlaepfer et al., 1997; Schaller and Parsons, 1995; Lo, 2004).

Formation of FAs may also involve the coordination of several different events, including actin polymerization, actin stress fibers attachment to the membrane at sites of cell–ECM adhesion, and stress fibers contraction. The small GTP-binding protein, RhoA, was established to be involved in FA assembly through RhoA-induced actomyosin contractility that results in bundling of actin filaments to generate stress fibers and clustering of integrins and associated proteins (Ridley et al., 1992; Sastry and Burridge, 2000).

2.3 Tractional forces and cell translocation

Nascent adhesions at the cell front exert tractional forces on the substrate that lead to cell translocation (Beningo et al., 2001). In fact, integrins connect the ECM to the intracellular cytoskeleton, therefore serving as traction sites over which the cell moves, but also as

mechanosensors that transmit information about the physical state of the ECM into the cell (Galbraith et al., 2002). The strength of attachment is dependent of the density of ligands on the substrate, the density of receptors on the cell surface, and the affinity of these receptors for the ligands. The tractional force that is exerted on adhesions is induced by the interaction of myosin II with actin filaments that attach to these sites. Actomyosin-based contraction is controlled by the small Rho GTPases, Cdc42, Rac and RhoA (Jaffe and Hall, 2005).



Adapted from Jaffe and Hall, 2005

Figure 17. The GTPase cycle

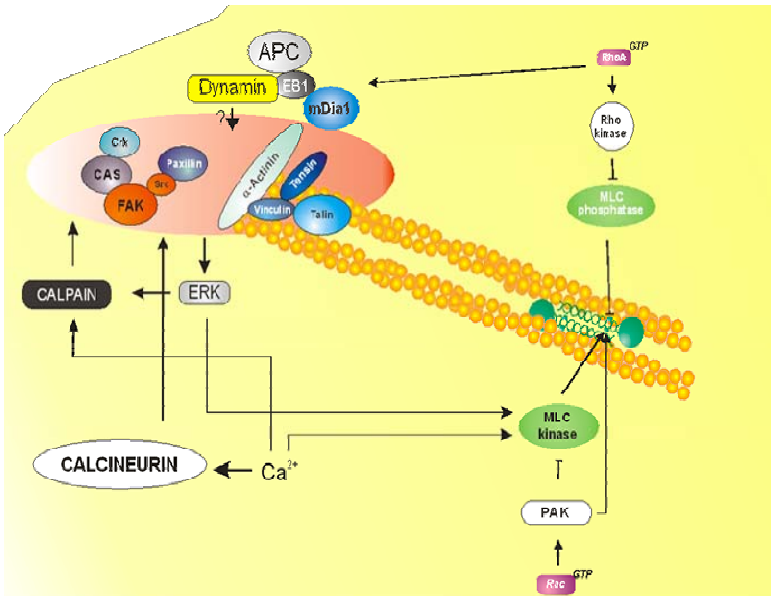
The regulation by these GTPases is antagonistic. Indeed, myosin II activation is regulated by Myosin Light Chain (MLC) phosphorylation, and results in increased contractility. MLC is phosphorylated by myosin light chain kinase (MLCK) or Rho kinase (ROCK) and dephosphorylated by MLC phosphatase, which is itself phosphorylated and inhibited by ROCK. MLCK is regulated by intracellular Ca^{2+} levels as well as phosphorylation by a number of kinases, whereas ROCK is activated by RhoA (Poperechnaya et al., 2000; Amano et al., 1996; Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996; Riento and Ridley, 2003; Kimura et al., 1996; Kaibuchi et al., 1999). RhoA is therefore promoting cell contractility. A similar mechanism has been shown for Cdc42, acting through the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (Wilkinson et al., 2005). Conversely, Rac activates PAK, which phosphorylates and inactivates MLCK, thus leading to decreased contractility and promoting spreading (Burridge, 1999). However, PAK may also phosphorylate MLC directly, thus promoting contractility (Brzeska et al., 2004).

2.4 The last step: retraction of the cell rear

Release of adhesions and retraction at the rear completes the migratory cycle allowing net translocation of the cell in the direction of movement (Kirfel et al., 2004). High tension exerted on the rear adhesions breaks the linkage between integrins and the actin cytoskeleton and contributes to detachment (Lauffenburger and Horwitz, 1996). This process of adhesion disassembly and rear retraction is mediated by several candidates and signaling pathways that include Rho, myosin II, Src/FAK/ERK, calcium, calcineurin, calpain and the delivery of components by microtubules (Ridley et al., 2003).

Figure 18. Trailing edge retraction

Adapted from Vicente-Manzanares et al., 2005



Rho is involved in disassembly of adhesions at the rear of migrating cells (Raftopoulou and Hall, 2004). In several cell types, inhibition of Rho leads to the formation of an extended tail, possibly because actomyosin-based contractility in the cell body is decreased. Rho may also act in the tail by stabilizing microtubules through the complex mDia-APC-EB1-Dynamin (Rodriguez et al., 2003; Palazzo et al., 2001; Small and Kaverina, 2003). Moreover, Rho and its effector, Rho kinase (ROCK), were shown to promote retraction of the tail of migrating monocytes (Worthylake and Burridge, 2003). Inhibition of ROCK or MLCK results in elongation of cell tails consistent with ROCK and/or MLCK being a positive effector of disassembly (Alblas et al., 2001; Wysolmerski and Lagunoff, 1990). PAK activation is also

implicated, through its effects on contraction and detachment, in retraction at the cell rear (Kiosses et al., 1999; Sanders et al., 1999; Sells et al., 1999; Zeng et al., 2000). Rac has also been implicated in the detachment at the rear of migrating cells (Gardiner et al., 2002). A role for myosin II in this rear deadhesion process has also been suggested (Koehl and McNally, 2002).

In addition, intracellular calcium levels are also implicated in this adhesion disassembly process. Indeed, the tension that is generated by strong adhesions at the cell rear can promote the opening of stretch activated calcium channels (Lee et al., 1999). As potential targets for calcium, the calcium regulated phosphatase calcineurin and the calcium activated protease calpain might be implicated in this process. Calpain, which is also activated by ERK, has the potential to cleave several focal adhesion proteins such as talin or FAK (Hendey et al., 1992; Glading et al., 2002; Carragher et al., 2003; Franco et al., 2004). Talin proteolysis by calpain has been shown to stimulate the dissociation of several major adhesion components including paxillin, vinculin and zyxin.

The involvement of microtubules in the rear deadhesion process has also been proposed (Wittmann and Watermann-Storer, 2001). In fact, Ballestrem and colleagues demonstrated that nocodazole-induced microtubule disruption resulted in cells that were unable to retract their tail (Ballestrem et al., 2000). Furthermore, microtubule-dependent targeting of dynamin and subsequent endocytosis of some adhesion components is another mechanism promoting focal adhesion disassembly (Ezratty et al., 2005). Also, by imaging microtubules and focal adhesions simultaneously in living cells, Kaverina and colleagues have revealed that focal adhesions are targeted by microtubule plus ends undergoing dynamic instability and that repeated targeting leads to focal adhesion disassembly (Kaverina et al., 1998; Kaverina et al., 1999; Broussard et al., 2008).

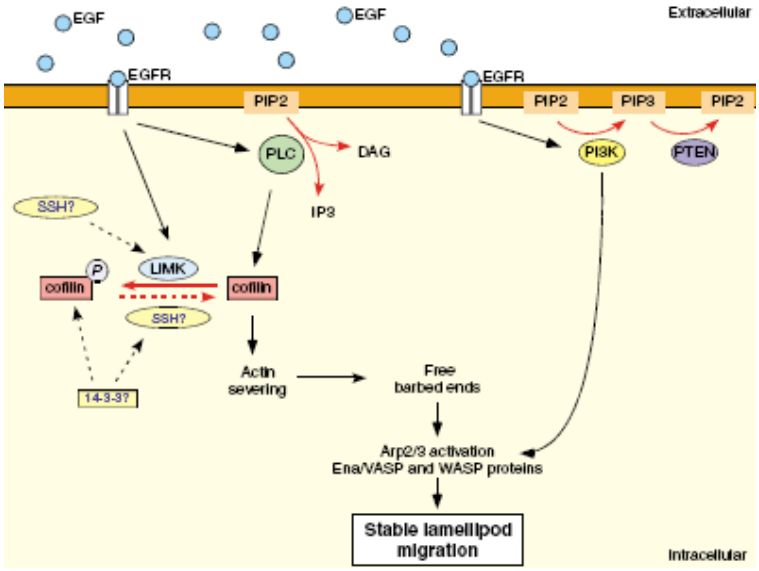
Finally, Src/FAK/ERK signaling is also involved in the focal adhesion disassembly process. Indeed, local rises in Ca2+ concentration can induce adhesion disassembly by increasing the residency of FAK at these sites (Giannone et al., 2004). Furthermore, FAK downstream pathways can cause a decrease in local myosin contractility. As an example, FAK-Src signaling stimulates adhesion disassembly through a signaling pathway that includes $p130^{CAS}$, paxillin, ERK and MLCK (Webb et al., 2004; Carragher et al., 2003; Westhoff et al., 2004). Specifically, treatment of cells with a Src inhibitor or expression of a kinase-defective mutant of Src decreased the rate constant of FA disassembly as observed using fluorescently tagged paxillin and zyxin (Webb et al., 2004). A similar effect was found after expression of Y397F-FAK mutant, consistent with Tyr-397 phosphorylation and subsequent recruitment of Src to FAs being necessary for FA disassembly.

3. Focus on PLCγ1 and cofilin, the gradient sensing machinery in adenocarcinoma cells

Phospholipase C (PLC) is involved in cellular proliferation and differentiation, and its enzymatic activity is up-regulated by a variety of growth factors and hormones (Rhee, 2001). PLC hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to generate inositol 1, 4, 5-trisphosphate (IP3) and 1, 2-diacylglycerol (DAG), which are implicated in the mobilization of intracellular Ca^{2+} and protein kinase C activation, respectively (Berridge and Irvine, 1989). Many growth factors such as platelet-derived-growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and nerve growth factor (NGF) elicit tyrosine phosphorylation of PLC-γ1 with stimulation of PIP_2 turnover in a wide variety of cells (Larose et al., 1993; Rotin et al., 1992; Peters et al., 1992). As a direct substrate for these receptors, PLC-γ1 has been shown to mediate chemotaxis toward these growth factors (Wells, 2000). PLC-γ1 is involved in cell adhesion and motility processes and its activation is therefore critical for tumor progression. The evidence for PLC-γ1 promoting tumor invasion has been reported in human cancer cells. Inhibiting PLC-γ1 signaling blocked human glioblastoma and prostate carcinoma cell invasion into normal tissue (Khoshyomn et al., 1999; Turner et al., 1997).

In the MTLn3 adenocarcinoma cells, EGF stimulation induces a biphasic F-actin polymerization response (Chan et al., 1998). The second peak of actin polymerization is dependent on PI3K activity, whereas the first peak is dependent on PLCγ1 and cofilin (Hill et al., 2000; Mouneimne et al., 2004). Additional studies strongly argue that PLC, together with cofilin, mediates gradient sensing in these cells (Ghosh et al., 2004; Mouneimne et al., 2006). Cofilin activity seems to be mainly dependent on PLCγ1-mediated PIP2 hydrolysis and does not involve an IP3-mediated Ca^{2+} release (Ma et al., 2000; Mouneimne et al.,2006; Yonezawa et al., 1991). Van Rheenen et al. recently showed that EGF induces a rapid loss of PIP(2) through PLC activity, resulting in a release and activation of a membrane-bound pool of cofilin. Upon release, cofilin was shown to bind to and sever F-actin, which is coincident with actin polymerization and lamellipod formation (Van Rheenen et al., 2007). Moreover, these data suggested an important role for PLC in the formation of protrusions in breast carcinoma cells during chemotaxis and metastasis towards EGF (Van Rheenen et al., 2007). In addition to activation by PLCγ1, cofilin activity in carcinoma cells is regulated by LIMK-mediated phosphorylation and SSH-induced dephosphorylation (Zebda et al., 2000; Nishita et al., 2005). Upon EGF stimulation, cofilin is phosphorylated and is essential for chemotactic sensing (Mouneimne et al., 2006), leading to the formation of F-actin rich lamellipodial protrusions in which SSH becomes locally activated and thereby, re-activates cofilin in the lamellipodium. This balance between inactivation by phosphorylation and reactivation by

dephosphorylation allows actin-filament turnover and ensures the dynamic nature of the lamellipodium (Soosairajah et al., 2005). However, recent studies in carcinoma cells have shown that the initial activation of cofilin does not involve ligand-induced dephosphorylation (Mouneimne et al., 2004; Song et al., 2006), but rather involves PLCγ1-mediated PIP2 hydrolysis and simultaneous inactivation by LIMK (Mouneimne et al., 2006; Hitchcock-DeGregori, 2006). When activated by PLCγ1-induced PIP2 hydrolysis, cofilin induces actin filament severing, thereby increasing the number of free actin barbed ends which become available for the binding of the Arp2/3 complex and Ena/VASP proteins, allowing initial protrusion and setting the direction of the movement. Activation of PI3K via EGF stimulation signals to Arp2/3 and promotes the formation of stable lamellipodia. Thus, the PLCγ1-cofilin and PI3K-Arp2/3 signalling pathways cooperate in chemotactic gradient sensing and lamellipodia generation in response to EGF stimulation.



Adapted from Kolsch et al., 2008

Figure 19. PLC and cofilin activities in adenocarcinoma cells

4. Memo: a novel component of the migratory machinery

Migration of breast tumor cells in response to EGF-related peptides is dependent upon ErbB2 activity (Spencer et al., 2000). In a screen for ErbB2 interacting proteins with roles in migration, a novel protein, Memo (Mediator for ErbB2-driven cell Motility) was identified as a binding partner of the ErbB2 autophosphorylation site Tyr1227 (Marone et al., 2004). Memo corresponds to the CGI-27/c21orf19-like hypothetical protein, which was identified by comparative genome identification using the *C-elegans* proteome as scaffold (Lai et al., 2000). A single Memo protein is encoded by the human genome, and Memo homologs are found in all branches of life. The sequence of Memo does not provide any relevant information about Memo's function since it does not contain any characterized domain. The 2.1Å crystal structure of Memo revealed that Memo is homologous to a bacterial class III nonheme iron-dependent dioxygenase (Qiu et al., 2008). Some of the key active site residues are conserved between dioxygenases and Memo, however, no evidence for a binding of Memo to metals or for an enzymatic activity was shown. Memo down-regulation in breast tumor cells resulted in a defect of cell migration, reflecting the requirement of this novel protein for ErbB2 dependent cell migration. Furthermore, following ErbB2 activation, Memo defective cells form actin fibers and grow lamellipodia, but fail to extend microtubules towards the cell cortex, suggesting that Memo controls cell migration by relaying extracellular chemotactic signals to the microtubule cytoskeleton (Marone et al., 2004). However, the mechanism whereby Memo mediates ErbB2-dependent cell motility is largely unknown and is under current investigation.

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----------AIMS OF THE WORK----------

Clinical studies have demonstrated that cancer patients with ErbB2 overexpressing tumors tend to have more aggressive, metastatic disease, which is associated with parameters predicting a poor outcome. However, the molecular basis underlying ErbB2-dependent cell motility and metastases formation still remains poorly understood. An initial study in our lab focused on the contribution of individual ErbB2 tyrosine autophosphorylation sites in cell migration. This study revealed that signaling downstream of Tyr 1201 and 1227 is important to induce efficient cell migration. Further investigation of the molecular interacting partners of these two tyrosine sites led to the identification of the novel protein Memo that specifically interacts with the phosphorylated Tyr 1227. This previous study also demonstrated that Memo is required for ErbB2-mediated breast carcinoma cell migration, and proposed that Memo controls cell motility by relaying extracellular chemotactic signals to the microtubule cytoskeleton. In the present study, we aimed at investigating in more detail Memo's function during ErbB2-induced cell migration, and determining the steps of the migratory process that involve Memo. We also aimed at positioning Memo within a genetic network by studying Memo in the model organism *S. cerevisiae*.

Our initial work on the role of Memo in mammalian cell migration was done with the Boyden chamber model that only allows quantification of migrated cells in response to a steep gradient of a chemotactic ligand. One important goal of the first study in the present thesis work was to explore the migratory process in more detail using the Dunn chamber model. This technique measures the chemotactic response of cells to a shallow gradient of ligand, and allows a direct visualization of the cell movement under video time-lapse microscopy. A quantification of different parameters such as track displacement, track speed and directional persistence is then possible. A second challenge in this first thesis project was to discover new partners of interaction for Memo in order to gain more insight into Memo's function. This was accomplished by the use of the Yeast-Two-Hybrid technique, which identified the cofilin protein among a number of interesting candidates. Considering the important role of cofilin on cell migration, particularly during the initial stages of the migratory process, we investigated the connection between Memo and cofilin, and examined the role of Memo on cofilin activation.

In the second part of the thesis, we aimed at positioning Memo in a downstream ErbB2 signaling pathway. In the original Memo study, the authors showed that there is a constitutive association of Memo and the adaptator Shc protein. Shc has three isoforms: $p46^{Shc}$, $p52^{Shc}$,

and $p66^{Shc}$ and each of them have different cellular functions. To orientate Memo in a specific cellular pathway, we investigated the ability of Memo to bind each of the three isoforms by using Shc-deficient mouse embryonic fibroblasts (MEFs) reconstituted with individual Shc isoforms. Furthermore, to uncover Memo's cellular and biochemical roles, we used two different models of Memo-deficient cells, and scored them for activation of different signaling cascades. Also, considering the previously described effect of Memo on HRG-induced breast carcinoma cell migration, we examined in more detail the morphology as well as cell motility and adhesion properties of Memo-null mouse fibroblasts, a good model for studying Memo function.

Memo orthologues are found in all species, from bacteria to human. To date, the function of Memo has only been studied in mammalian cells. In the third part of the present thesis work, we examined Memo in a model organism that is amenable to genetic manipulation in order to provide more information on Memo's role in other species. Genetic approaches in the model organism *S.cerevisiae* have been useful for gaining insight into the function of evolutionary conserved proteins. Thereby, to advance our knowledge of Memo's cellular and molecular role, we carried out a study of Memo in *S.cerevisiae*. We investigated Memo cellular localization, and generated a *memoΔ* strain that appeared to be viable. Considering the known role of mammalian Memo in the microtubule and actin cytoskeleton, we examined the *memoΔ* strain for defects in these networks. Also, given the viability of the *memoΔ* strain, we performed a Synthetic Lethal Screen (SLS) to characterize Memo's functions and dissect pathway structures.

----------RESULTS----------

I/ RESEARCH ARTICLE 1 in revision in *Journal of Cell Science.*

Memo is a novel cofilin interacting protein that influences PLCγ1 and cofilin activities, and is essential for maintaining directionality during ErbB2-induced tumor cell migration

Maria Meira, Régis Masson, Igor Stagljar[1], Susanne Lienhard, Francisca Maurer, Anne Boulay, Nancy E. Hynes*

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland; [1]Terrence Donnelly Centre for Cellular and Biomolecular Research (CCBR), Department of Biochemistry and Department of Molecular Genetics, University of Toronto, Toronto ON, Canada, M5S 3E1

*Corresponding author.

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Running Title – Memo and tumor cell migration

Summary

Heregulin (HRG) activates ErbB2/ErbB3 heterodimers thereby stimulating many cellular responses, including motility. Memo and PLCγ1 interact with ErbB2 autophosphorylation sites and are essential for HRG-induced chemotaxis. By tracing HRG-stimulated cell migration in Dunn chambers, we found that Memo- or PLCγ1 knock-down (KD) strongly impairs cell directionality. Memo has no obvious enzymatic activity and was discovered via its ability to complex with ErbB2. Using the yeast two-hybrid approach to gain insight into Memo function, an interaction between Memo and cofilin, a regulator of actin dynamics, was uncovered. The interaction was confirmed in vitro using recombinant proteins and in vivo in coimmunoprecipitation experiments where Memo was detected in complexes with cofilin, ErbB2 and PLCγ1. Interestingly, in Memo KD cells, HRG-induced PLCγ1 phosphorylation was decreased, suggesting that Memo regulates PLCγ1 activation. Furthermore, HRG-induced recruitment of GFP-cofilin to lamellipodia is impaired in Memo- and in PLCγ1 KD cells, suggesting that both proteins lie upstream of cofilin in models of ErbB2-driven tumor cell migration. Finally, in vitro F-actin binding and depolymerization assays showed that Memo binds F-actin and induces an enhancement of cofilin depolymerizing/severing activity. In summary, these data indicate that Memo also regulates actin dynamics by interacting with cofilin and enhancing its function.

Introduction

Cell migration is important in normal physiology and in disease. Acquisition of migratory ability by cancer cells is a characteristic that contributes to metastatic tumor cell spread to distant organs (Gupta and Massague, 2006). Proteins with essential roles in metastasis are under intense scrutiny in order to provide additional targets for cancer therapy. The ErbB2 receptor tyrosine kinase plays an important role in many human tumors (Hynes and Lane, 2005). In breast cancer, ErbB2 gene amplification and overexpression correlate with an aggressive metastatic phenotype (Hynes and Lane, 2005; Slamon et al., 1987). Accordingly, we have explored signaling pathways activated by ErbB2 in breast cancer, with the specific aim of identifying proteins recruited by the receptor that are essential for tumor cell migration.

ErbB2 is a member of the epidermal growth factor (EGF/ErbB) family that also includes EGFR, ErbB3 and ErbB4. ErbB receptor signaling is initiated by ligand binding to the extracellular region causing formation of receptor homo- and heterodimeric complexes, resulting in autophosphorylation at multiple Tyr residues in the cytoplasmic domain (Schlessinger, 2000; Yarden and Sliwkowski, 2001). ErbB2 has a central role in the family and is activated by heterodimerization with each of the other ErbB receptors (Graus-Porta et al., 1997). Functional inactivation of ErbB2 in T47D human breast cancer cells (Graus-Porta et al., 1995) impairs the ability of the EGF family ligands, EGF, betacellulin and heregulin (HRG), to stimulate cell migration in Boyden chamber assays (Spencer et al., 2000). Furthermore, introduction of Neu (rat ErbB2 homologue) mutants into the T47D cells showed that two Neu-Tyrosine add-back mutants, 1201/1196 and 1227/1222 (human/rat) are able to restore the ability of EGF and HRG to stimulate cell migration (Marone et al., 2004). In a screen for ErbB2 effector proteins with roles in migration, we identified PLCγ1 and Memo. These proteins are recruited to the ErbB2 autophosphorylation sites Tyr 1201 and Tyr 1227, respectively and both have essential roles in cell motility (Marone et al., 2004).

In contrast to the well-characterized PLCγ1, Memo is a novel protein. The 2.1 Å crystal structure of Memo revealed that it is structurally homologous to a class of non-heme iron dioxygenases that are mainly found in bacteria (Andujar and Santero, 2003), however, we were unable to detect metal binding or enzymatic activity (Qiu et al., 2008). To gain more insight into Memo function, we used the yeast two-hybrid (YTH) approach to identify Memo interacting proteins. We report here that Memo interacts with cofilin, a member of the conserved, ubiquitously expressed actin depolymerizing factor (ADF)/cofilin family, proteins

that control actin dynamics and regulate actin filament turnover (Moon and Drubin, 1995) (Ono, 2007).

ADF/cofilin proteins control actin dynamics at the cell membrane, a process which is essential for cell migration. In fact, depletion of cofilin impairs cell motility (Hotulainen et al., 2005). ADF/cofilin proteins bind actin and are involved in the actin filament assembly/disassembly process (dos Remedios et al., 2003). In vitro, it has been shown that cofilin stimulates F-actin disassembly by accelerating the rate of pointed end depolymerization, and by severing actin filaments (Carlier et al., 1997; DesMarais et al., 2005; Lappalainen and Drubin, 1997; Paavilainen et al., 2004). Although in vivo the predominant cofilin activity appears to be cell type dependent, cofilin has an essential role in promoting cytoskeletal dynamics by generating the pool of actin monomers needed for lamellipodium extension at the leading edge (Kiuchi et al., 2007). Cofilin is inactivated by LIM-kinase mediated phosphorylation at Ser-3 (Arber et al., 1998; Yang et al., 1998), and is reactivated by Slingshot-1L (SSH-1L) induced-dephosphorylation (Niwa et al., 2002). PLCγ1 is another regulator of cofilin; indeed, PLCγ1-mediated PIP2 hydrolysis releases PIP2-bound cofilin thereby increasing the pool of active cofilin available for interaction with F-actin (Ono, 2007; van Rheenen et al., 2007). Local activation of cofilin by PLCγ1 at the leading edge of migrating cells has been shown to be required for directed protrusion (Mouneimne et al., 2006; Mouneimne et al., 2004).

Using a KD strategy in this study, we observed that depletion of Memo or of PLCγ1 resulted in very similar phenotypes, with a strong impairment of HRG-induced cytoskeletal organization and directional migration of breast tumor cells. In contrast, cofilin KD, or simultaneous KD of Memo and PLCγ1, completely blocked cell movement. Furthermore, co-immunoprecipitation experiments revealed that complexes of active ErbB2, PLCγ1, Memo and cofilin are detected in HRG treated breast tumor cells. We also provide evidence that Memo positively regulates PLCγ1 phosphorylation and show that Memo and PLCγ1 are both involved in HRG-induced cofilin recruitment to the lamellipodia. We demonstrate that Memo also binds F-actin and promotes cofilin depolymerizing/severing activity. Considering these results, we propose that Memo and PLCγ1 lie upstream of cofilin in models of ErbB2 driven breast cancer cell migration.

Results

PLCγ1 and Memo are required for ErbB2-mediated cell motility

PLCγ1 and Memo bind to P-Tyr 1201/1196 and P-Tyr 1227/1222, respectively, two ErbB2 phosphorylation sites that we have shown to be important for cell migration (Marone et al., 2004). In the current study, we performed detailed studies on the role of Memo and PLCγ1 in HRG-induced tumor cell migration. As models, we used T47D and SKBr3 breast cancer cell lines that are representatives of, respectively, luminal, estrogen receptor-positive (ER+) cancer cells (Badache and Hynes, 2001; Marone et al., 2004) and ErbB2-overexpressing tumor cells (Lane et al., 2000). We have previously shown via siRNA mediated Memo knock-down (KD) and general PLC inhibition, that in T47D cells, both proteins have important roles in ErbB2-induced cell motility (Marone et al., 2004). In the following experiment, we decreased the expression of Memo and PLCγ1 in SKBr3 cells using specific siRNAs (Fig 1A *right panel*) and examined their motility. The directional (chemotactic) and random (chemokinetic) migration of control and KD cells was examined in Transwell assay chambers. HRG was added to the lower chamber to measure chemotaxis and to both chambers to measure chemokinesis. KD of Memo or PLCγ1 did not generally affect signaling in response to HRG (Marone et al., 2004) (Supp Fig S1 *left panel*, e.g. P-ERK). However, individual KD of Memo or PLCγ1 or simultaneous KD of both proteins strongly reduced chemotaxis in comparison to control LacZ cells (Fig 1A *left panel* and Supplementary Fig S1, *right panel*). Interestingly, the chemokinetic response of Memo or PLCγ1 KD cells was similar to that of control cells, whereas double KD of PLCγ1 and Memo resulted in a dramatic blockade of random cell motility (Fig 1A *left panel*), suggesting that Memo and PLCγ1 are cooperating during the migratory process.

Signal transduction pathways downstream of ErbB2 temporally and spatially regulate cytoskeleton remodeling during cell migration (Feldner and Brandt, 2002). Therefore, we examined the effect of Memo and PLCγ1 KD, or simultaneous KD of both proteins on HRG-dependent microtubule (MT) and actin organization. T47D cells were used for this experiment since following their exposure to HRG, they form large actin-rich lamellipodial protrusions with abundant MT extensions (Fig 1B LacZ control), however, results with SKBr3 cells are essentially the same ((Marone et al., 2004) and unpublished). Both Memo KD and PLCγ1 KD cells, as well as double KD cells, displayed similar actin-rich protrusions, however, the F-actin stress fibers appeared thicker and more predominant in the KD cells, suggesting a role for Memo and PLCγ1 in actin organization (Fig 1B). Furthermore, in comparison to control cells, the single and the double KD cells showed a strong reduction in MT extension to the cell periphery, a phenomenon previously reported for Memo KD cells (Marone et al., 2004). Thus, in response to ErbB2 activation, decreased expression of Memo

and of PLCγ1 have very similar consequences on the actin and MT network, raising the possibility that Memo and PLCγ1 act in concert during cytoskeleton organization.

PLCγ1 and Memo have important roles in directed tumor cell migration

Decreased expression of Memo or PLCγ1 reduces HRG-induced motility of tumor cells in Transwell assays (Fig 1A, Supplementary Fig S1 and (Marone et al., 2004)). We investigated these results in more detail using Dunn chambers, a system that allows real-time visualization of migratory cells (Wells and Ridley, 2005; Zicha et al., 1991)**.** The data generated in Dunn chambers provides additional information on the behavior of migratory cells compared to data obtained with Boyden chambers in which only the final distribution of cells can be monitored. For these assays, T47D cells were transiently transfected for 72 hrs with LacZ, Memo or PLCγ1 siRNA (Fig 2A *right panel*) and tracks of cells migrating on the bridge of Dunn chambers in a chemo-attractant gradient of HRG were traced using time-lapse video microscopy (Fig 2A *left panels*). Quantitative analyses of the tracks made by cells with Memo KD or PLCγ1 KD revealed that there was a 44% decrease in the net translocation distance (straight distance from the start to the end point) and a 30-35% decrease in their migration speed, compared to control cells (Fig 2B *left and middle panels*). Thus, as observed in the Boyden chamber assays (Fig 1A), the cells are still able to migrate although less potently than control cells. Importantly, there was a dramatic difference in their directional persistence. KD of Memo or PLCγ1 in T47D cells led to a 67% and 72% decrease, respectively, in the directional persistency index (Fig 2B *right panel*). The overall directionality of cell migration in control and KD cells is depicted in Fig 2C. Considering the final location of migrating cells positioned in the 180°C arc facing the HRG source, 86.6 % of control cells were located there, while only 37.5 % and 37.4 % of Memo KD and PLCγ1 KD cells, respectively were in this location. In summary, these results show that down-regulation of either Memo or PLCγ1 does not totally impair cell migration; however, the cells move in a more random manner compared to control cells that move up the HRG gradient. Thus, both Memo and PLCγ1 have important roles in orchestrating directional cell migration.

Complexes of ErbB2, PLCγ1, and Memo form upon HRG treatment of breast tumor cells.

Considering the similar role that Memo and PLCγ1 play in HRG induced cell migration, we explored the Memo-PLCγ1 connection in more detail. In Memo IPs from T47D cells (Fig 3A *left panel*) and from HEK293 cells (Supplementary Fig S2 *right panel*), complexed PLCγ1 could be detected. In the reverse immunoprecipitation, IPs of PLCγ1 revealed complexed Memo (Fig 3A *right panel* and Supplementary Fig S2 *right panel*). In each case, there is an

increase in the co-immunoprecipitating protein when lysates from HRG treated cells were used, probably reflecting stabilized complexes interacting with active ErbB2 (see Supplementary Fig S2 *middle panel*). Indeed, Memo and PLCγ1 were both detected in ErbB2 IPs from SKBr3 cells that have constitutive receptor activation (Supplementary Fig S2, *left panel*).

Memo has a role in HRG induced PLCγ1 activation

Phosphorylation of PLCγ1 on Tyr783 has been linked to its activation (Rhee, 2001). Upon HRG treatment of T47D breast tumor cells, there is a rapid increase in the P-Tyr content of immunoprecipitated PLCγ1 (Fig 3B *left panel*), showing that HRG activates PLCγ1. Interestingly, in Memo KD T47D cells, there was a strong decrease in the P-Tyr content of the immunoprecipitated PLCγ1 (Fig 3B *middle panel*), while the level of PLCγ1 remained the same (Fig 3B *right panel*). These results suggest that Memo has an important role in PLCγ1 phosphorylation in response to ErbB2 activation. Loss of Memo might directly or indirectly affect PLCγ1 activity, perhaps as a result of the alterations in the actin cytoskeleton following Memo KD.

Memo interacts directly with cofilin: results from yeast two-hybrid and GST-pull-downs

The molecular mechanisms underlying Memo's role in cell migration are not well understood. To gain more insight into Memo function, a YTH screen was performed (Pedrazzi and Stagljar, 2004). By screening a peripheral blood cDNA library, we identified cofilin-1, a ubiquitously expressed protein of ~19kDa that binds monomeric and filamentous (F)-actin (Ono, 2007), as a potential interacting partner for Memo. Mammalian cells express multiple ADF/cofilin-type proteins. Cofilin-1 is the dominant isoform expressed in many cells lines (Wang et al., 2004) (Hotulainen et al., 2005) and we will refer to cofilin-1 as cofilin throughout the text. The specificity of the YTH Memo-cofilin interaction was confirmed using control plasmids (Fig 4A). A bait dependency test also demonstrated a specific interaction between Memo and cofilin in the YTH system (Supplementary Fig S3).

To confirm the interaction between Memo and cofilin using purified proteins, Memo was expressed as a GST-fusion protein and its interaction with cofilin was tested in pull-down assays. Glutathione-sepharose bound GST-Memo was incubated with recombinant cofilin. Cofilin was recovered from GST-Memo beads but not from empty beads (Fig 4B lanes 4 and 6). The interaction is specific since preincubation of cofilin with an excess of soluble Memo decreased the amount of cofilin interacting with GST-Memo beads (Fig 4B lane 7). In summary, Memo is a novel cofilin interacting protein.

Memo and cofilin interact in mammalian cells and complex with active ErbB2.

Considering the importance of ADF/cofilin family members in cell migration (Ono, 2007) (Hitchcock-Degregori, 2006), we explored the Memo-cofilin interaction in mammalian cells. Memo IPs from lysates of T47D or MDA-MB435 cells, which express high cofilin levels (Wang et al., 2007) (Fig 4C *upper* and *middle panel*, respectively) were probed with a cofilin antibody (Song et al., 2006) revealing complexed cofilin. ErbB2 was also detected in Memo IPs from lysates of HRG-treated tumor cells, showing that Memo is recruited to the active receptor. The Memo interaction with cofilin and ErbB2 is specific since neither protein was detected in IPs carried out with an isotype matched control (Fig 4C). The cofilin specific antibody is not suitable for immunoprecipitation, which has precluded an examination of cofilin IPs for Memo and ErbB2.

The activity of cofilin is inhibited by LIMK mediated phosphorylation on Ser-3, a modification that prevents the interaction of cofilin with F-actin (Ono, 2007). We examined the ability of Memo to interact with P-cofilin using a specific antiserum. In Memo IPs from T47D cells, P-cofilin was detected (Fig 4C *lower panel*), suggesting that Memo binds both active and inactive cofilin. Interestingly, in comparison to control cells where low levels of cofilin (Fig 4C *upper* and *middle panel*) and P-cofilin (Fig 4C *lower panel*) were found in Memo IPs, higher levels of cofilin/P-cofilin were complexed with Memo in lysates from HRG-treated tumor cells (Figs 4C). Whether or not this result reflects stabilization of the Memo-cofilin interaction at the activated receptor remains to be explored.

Finally we examined the kinetics of formation of ErbB2, PLCγ1, cofilin and Memo containing complexes in response to HRG treated cells (Fig 4D). In lysates made from T47D cells treated 5 min with HRG, there was a strong increase in ErbB2 and cofilin coimmunoprecipitating with Memo and this complex was maintained throughout the 30 min time course. PLCγ1 was also evident in this complex after 5 minutes of HRG treatment and its level increased at 10 and 30 minutes (Fig 4D).

Cofilin is essential for cell movement in response to HRG in Dunn chambers

Cofilin has been shown to set the direction of tumor cell motility in response to EGF (Ghosh et al., 2004). We used Dunn chambers to examine the effect of cofilin KD on cell motility following ErbB2 activation. Cofilin levels were efficiently decreased in T47D cells (Fig 5 *insert*) and tracks of migrating cells in a gradient of HRG were traced. The cofilin KD cells showed a dramatic reduction in their migratory ability. The speed of migration was 16% that of control cells (Fig 5 *lower panel*), resulting in a net translocation distance that was 13% of that achieved by control cells (Fig 5 *upper panel*). Double KD of Memo and PLCγ1 in the cells resulted in a stronger block in movement compared to cells with individual KD (Fig 5

vs. Fig 2). These results are in accordance with those obtained in Transwell assays (Fig 1A), where double KD of Memo and PLCγ1 had the strongest effect on random migration/chemokinesis. Since the cofilin KD cells and the double Memo and PLCγ1 KD cells were essentially stationary, it was not informative to calculate a directional persistence index. These results provide additional evidence that cofilin has an essential role in the motility behavior of cells in response to ErbB2 stimulation. Furthermore, the results suggest that in the initial stages of migration, Memo and PLCγ1 cooperate to stimulate migration and that cofilin is likely mediating these effects.

PLCγ1 and Memo control GFP-cofilin localization in response to HRG.

To gain more insight into the effect of ErbB2 on cofilin, we introduced GFP-tagged cofilin into SKBr3 breast tumor cells and examined its cellular distribution in response to HRG. SKBr3 cells were used since T47D cells are not suitable for transient expression of proteins. In the absence of ligand, GFP-cofilin was diffusely distributed in the cytoplasm, while following HRG treatment, it was recruited to the actin-rich lamellipodium (Fig 6A). These results are similar to what has previously been reported for cofilin distribution in a variety of cell types following growth factor stimulation (Dawe et al., 2003; Nagata-Ohashi et al., 2004). GFP-tagged Memo also localizes to the lamellipodium in HRG-treated SKBr3 cells (Fig 6B).

Next, we monitored the localization of GFP-cofilin in Memo KD and PLCγ1 KD cells. In contrast to HRG-treated LacZ control cells in which GFP-cofilin localized to the lamellipodia, GFP-cofilin failed to be recruited to the F-actin rich lamellipodium and remained diffusely distributed in the cytoplasm of PLCγ1 KD cells (Fig 6C *right panel*) and Memo KD cells (Fig 6C *middle panel*). These results suggest that both PLCγ1 and Memo have a role in cofilin cellular distribution, perhaps reflecting the fact that Memo-depleted or PLCγ1-depleted cells have alterations in the actin cytoskeleton.

We also examined the effect of cofilin KD on the cellular distribution of Memo. Cofilin depletion in SKBr3 cells had a stronger effect on morphology than Memo KD or PLCγ1 KD, with the formation of multipolar lamellipodia. Individual cells (a typical one is shown in Fig 6D *middle panel*) displayed extensions of several protrusions in different directions, a phenotype that has been reported in other models (Nishita et al., 2005; Sidani et al., 2007). Similar results were seen with cofilin KD T47D cells (data not shown). In striking contrast to the effects of Memo or PLCγ1 KD on GFP-cofilin localization, neither cofilin KD nor PLCγ1 KD affected the ability of GFP-Memo to associate with the plasma membrane in response to HRG (Fig 6D, *middle* and *right panels*). In conclusion, these results suggest that PLCγ1 as

well as Memo are upstream of cofilin in response to ErbB2 activation. Cofilin activity is influenced by multiple mechanisms including PIP2 binding (Moon and Drubin, 1995). PLCγ1 mediated hydrolysis of PIP2, as reported downstream of EGFR (van Rheenen et al., 2007), likely contributes to the release of an active pool of cofilin that participates in actin dynamics in stimulated treated cells. Considering that Memo is involved in PLCγ1 activation, the effect of Memo KD on GFP-cofilin localization might be through PLCγ1, something that will be examined in the future.

Furthermore, phosphorylation of cofilin on Ser-3 also influences its activity. As a measure of cofilin activity in HRG-treated breast tumor cells, we examined its P-Ser3 status with a specific antiserum. The basal level of P-cofilin in T47D and SKBr3 tumor cells is high, and there were no obvious decrease in P-cofilin levels in response to HRG (Fig 4C, *lower panel*, Supplementary Fig S4 panel A). Furthermore, there were no changes in P-cofilin levels in Memo KD or in PLCγ1 KD cells (Supplementary Fig S4 panel B), suggesting that only a small pool of cofilin might participate in generating the dynamic actin structures observed following ErbB2 activation.

F-actin binding and depolymerization assays.

In the following experiments we examined the effects of Memo and cofilin on in vitro F-actin depolymerization. First, the ability of recombinant Memo and cofilin proteins to bind F-actin was examined. Actin was polymerized then mixed with Memo or cofilin alone, or in combination. Actin filaments were sedimented by centrifugation and the supernatant and F-actin containing pellet fractions were recovered. Scanning densitometry of the Coomassie Brilliant Blue-stained gels (Fig 7A *upper panel*) was performed to quantify the amount of proteins in each fraction (Fig 7A *lower panel*). Assays with α-actinin and BSA served as positive and negative binding controls, respectively. When incubated with F-actin, the majority of α-actinin, was found in the pellet (sample 3), while BSA remained in the supernatant (sample 4). Cofilin, a well established F-actin binding protein (Bamburg, 1999; Paavilainen et al., 2004) shifted from 35% in the pellet when alone (sample 5) to 58% in the pellet in the presence of F-actin (sample 6). Interestingly, there was also a shift for Memo from 35% in the pellet when alone (sample 7) to 45% when incubated with F-actin (sample 9), suggesting that Memo directly binds F-actin. When Memo and cofilin were both incubated with F-actin (sample 10), there were no differences in cofilin or Memo content in the F-actin pellet (58% and 47%, respectively). To confirm the Memo-actin interaction in vivo, Memo was immunoprecipitated from lysates of T47D breast tumor cells. In control IgG IPs, no actin

was evident, while actin was present in Memo IPs from cells treated or not with HRG (Fig 7B).

The ability of a protein to depolymerize/severe F-actin can be assessed by measuring the shift of actin from the pellet to the supernatant fraction. Memo addition to F-actin did not alter the % of actin in the supernatant (Fig 7A, 13.4% vs. 14% sample 1 vs. 9); while addition of cofilin, a known F-actin depolymerizing protein resulted in a shift of actin from 13.4% to 16% in the supernatant (Fig 7A, sample 1 vs. 6). Interestingly, there was a further increase of actin in the supernatant to 20% when cofilin and Memo were added together with F-actin (Fig 7A sample 10), suggesting that Memo might influence the activity of cofilin.

In Fig 8, additional experiments were performed to test Memo's effect on cofilin depolymerizing/severing activity. Increasing amounts of Memo were mixed with fixed amounts of G-actin and cofilin. Actin polymerization was initiated then the pellet and supernatant fractions were analyzed as above (Fig 8). When incubated alone, 9% of the actin was detected in the supernatant (sample 1), while addition of cofilin to actin resulted in an increase of the actin content in the supernatant to 12% (sample 3). In samples 4-6, increasing amounts of Memo were included in the assay. This resulted in an increase of cofilin (24%, 27% & 31%, resp.) and actin (17.3%, 19.8% & 17%, resp.) in the supernatant fractions. These results suggest that in the presence of Memo, the ability of cofilin to mediate F-actin depolymerization/severing is promoted.

Discussion

In breast cancer, ErbB2 overexpression correlates with an aggressive metastatic phenotype (Hynes and Lane, 2005). We have been exploring signaling pathways activated by ErbB2 with the aim of identifying proteins recruited by the receptor that are essential for tumor cell migration. Activated ErbB2 is complexed with multiple proteins that couple the receptor to a variety of intracellular signaling pathways (Olayioye et al., 2000). We show here that two proteins, Memo and PLCγ1, are required for HRG-induced tumor cell migration. Depletion of either Memo or PLCγ1 has similar effects on cell motility and on the actin and MT cytoskeleton. Furthermore, in Dunn chambers, we observed that in the absence of either Memo or PLCγ1, cells could migrate in response to HRG, albeit more slowly than control cells. However, loss of Memo or PLCγ1 had a dramatic effect on cell directionality. Indeed, both proteins are essential for the persistent directed migration of tumor cells up a gradient of HRG. Furthermore, we show that Memo binds cofilin and stimulates its F-actin depolymerization/severing activity. Finally, simultaneous KD of Memo and PLCγ1, like cofilin KD, almost completely blocked migration. Taken together, these results suggest that both Memo and PLCγ1 converge on cofilin, in an ErbB2 initiated migratory pathway.

PLCγ1 has a well-described role in PIP2 hydrolysis (Patterson et al., 2005) and is known to mediate chemotaxis towards a number of growth factor activated RTKs (Wells, 2000). We show here that transient KD of Memo in T47D cells causes an impairment of PLCγ1 activation in response to HRG. These results were confirmed using T47D cells with stable shRNA-mediated Memo down-regulation (provided by Gwen MacDonald) (data not shown). Although we cannot rule out a direct effect of Memo on the activity of PLCγ1 recruited to ErbB2, we consider it more likely that Memo's effects are indirect. There is accumulating evidence that PLCγ1 has an important role in integrin-mediated adhesion and migration. PLCγ1 has been detected in integrin complexes (Choi et al., 2007) where it is activated by Src (Jones et al., 2005). It is possible that alterations in the actin cytoskeleton resulting from Memo depletion impact on the ability of integrin complexes to activate PLCγ1.

Considering Memo's novel role in PLCγ1 activation, it is possible that the major effect of Memo depletion is via decreased PLCγ1 activity. Although it cannot be completely ruled out, we consider this unlikely for various reasons. Compared to cells with individual depletion of Memo or PLCγ1, simultaneous KD of both proteins has a more dramatic effect on the actin cytoskeleton of the cells (Fig 1B) and on their migration in Transwell assays (Fig 1A) and in Dunn Chambers (Fig 5). Interestingly, the double KD cells behave like add-back cells

expressing an ErbB2 mutant lacking all autophosphorylation sites (NYPD cells). These cells are impaired in their migratory ability in Transwell assays (Marone et al., 2004) and are totally blocked in the Dunn Chamber assay (supplemental data Fig S5). These results would not be expected if Memo's only cellular role was to ensure proper PLCγ1 activation. Furthermore, F-actin sedimentation assays showed that Memo positively influences cofilin activity, a role of Memo that is likely to be independent of PLCγ1.

We favor the hypothesis that Memo and PLCγ1 each have several roles in migration. Following acute ErbB2 activation, when both proteins are recruited to the receptor, they act in concert to stimulate the migratory process. PLCγ1 activation promotes the localized release of PIP2-bound cofilin, which stimulates alterations in actin dynamics. Indeed in the MTLn3 rat cancer cell model, rapid PLCγ1 activation following EGF treatment (van Rheenen et al., 2007) has been implicated in an increase in cofilin-mediated severing and an increase in actin barbed ends at the leading edge of the cell (Chan et al., 2000; DesMarais et al., 2005). However, PLCγ1 is not only activated by RTKs, but has also been detected in integrin complexes (Choi et al., 2007), where it is activated by Src (Jones et al., 2005). Indeed, in some cellular models it has been found that PLCγ1 has migratory functions independent of RTK activation (Jones et al., 2005).

Memo might also have multiple roles in the migratory process. Considering that Memo and cofilin were detected in complexes with activated ErbB2, and that Memo directly binds cofilin, it is possible that one role of Memo is to escort cofilin to the leading edge of the cell. Indeed, both GFP-Memo and GFP-cofilin rapidly associate with lamellipodia following HRG treatment (Fig 6). In the receptor complexes, Memo might also bind the pool of cofilin released by PLCγ1-mediated PIP2 hydrolysis. Both these activities would result in an enrichment of cofilin at the cell periphery where it could participate in regulating actin dynamics. In coimmunoprecipitation assays, Memo was also detected in complexes with inactive P-cofilin. Memo might also have a role in the dephosphorylation and ensuing activation of cofilin. Although it should be mentioned that in the breast cancer models used in our studies, we did not detect any changes in P-cofilin levels in response to HRG.

Memo appears to be a novel actin binding protein. A calponin homology (CH) domain has been identified in many actin binding proteins (Castresana and Saraste, 1995; Gimona et al., 2002). However, no CH domain has been identified in the crystal structure of Memo (Qiu et al., 2008). Future work will be aimed at testing if Memo binds both G-actin and F-actin, as well as determining the domain of Memo needed for binding. Moreover, by testing

recombinant Memo and cofilin in the F-actin sedimentation assay, we showed that Memo stimulates the F-actin depolymerizing/severing activity of cofilin. ADF/cofilin family proteins are regulated by multiple mechanisms, including interactions with other proteins, such as Aip1 and CAP (Paavilainen et al., 2004) that also influence actin dynamics. Future experiments will be aimed at determining the domains of Memo and cofilin that interact and the mechanism underlying Memo's role in cofilin regulation.

In summary, the results presented here show that Memo is a novel player in controlling actin cytoskeleton dynamics. The essential role of Memo in cell migration might make it an interesting target in metastatic cancer.

Materials and Methods

Reagents, recombinant proteins, antibodies and plasmids

HRG-β1, referred to as HRG, was purchased from R&D Systems (Inc., Minneapolis, MN). Human recombinant cofilin was from Cytoskeleton. For actin cosedimentation and depolymerization assays, the actin binding spin-down assay kit (BK001) from Cytoskeleton was used. Purification of Memo was described previously (Qiu et al., 2008). Polyclonal antibodies to a 19 amino acid peptide of Memo were produced in rabbits (aa 25-43 human Memo NM_015955: NAQLEGWLSQVQSTKRPAR); the same peptide was used for Memo mAb production in mice. Isotype matched IgG2a control serum for Memo and IgG3 control serum for PLCγ1 were purchased from Sigma. The affinity-purified chicken anti-cofilin antibody AE774 (Song et al., 2006) was provided by Dr. John Condeelis (Albert Einstein college of Medicine) and the rabbit anti-cofilin antibody was from Cell Signaling (≠3312). Phospho-cofilin (rabbit anti P-Ser3-cofilin ≠3311) and phospho-p44/42 MAPK (Thr 202/204) were from Cell Signaling. Rabbit and mouse anti-PLCγ1 antisera were from Santa Cruz (≠sc-81 and ≠sc-7290 respectively). β-Tubulin antiserum was provided by Dr. Brian Hemmings (FMI, Basel). Phospho-Tyrosine was detected with the 4G10 mAb (gift from Dr Juergen Mestan, NIBR Basel). For ErbB2, the 21N polyclonal antiserum was used in westerns (Lane et al., 2000) and mAb FRP5 was used in IPs (Harwerth et al., 1992). Memo was cloned into pCDNA3.1+ containing the GFP-tag. The GFP-Cofilin construct was provided by Dr. John. Condeelis.

Yeast Two-Hybrid (YTH) analysis

The analysis was carried out as described previously (Pedrazzi et al, 2004). Full-length human Memo cDNA was cloned into pLexA-Kan (Dualsystems Biotech AG) as a fusion with the LexA DNA binding domain, and was used as bait. The peripheral blood cDNA library was fused to the GAL4-activation domain in the pACT2 vector (Clontech Laboratories, Inc). The yeast reporter strain L40 was used. Transformants were selected in dropout plates and positive clones were identified. Growth in the absence of histidine and in the presence of βgalactosidase indicates an interaction. Plasmid DNA was isolated from the yeast clones and rescued into *E.coli* and a bait-dependency test was performed whereby each isolated plasmid was retransformed into yeast together with the control bait (pLexA-Kan without Memo), followed by growth selection and quantitative LacZ assay. Bait-dependent positive clones were sequenced and subjected to BLAST analysis.

Cell culture and transfections

T47D, NYPD, SKBr3, MDA-MB435 breast carcinoma cells and HEK-293 (Human Embryonic Kidney) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % Fetal Calf Serum (GIBCO Invitrogen AG, Basel, Switzerland). GFP-Memo and GFP-cofilin constructs were transfected into SKBr3 cells using FuGene -6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). T47D and SKBr3 cells were transfected with siRNA using HiPerFect (Qiagen) according to the manufacturer's instructions. For dual transfection of SKBr3 cells with siRNA and plasmid constructs, DharmaFECT DUO reagent (DHARMACON) was used. The following siRNAs obtained from Qiagen were used for transient KD. For control LacZ (target sequence: AAGCGGCTGCCGGAATTTACCTT), for PLCγ1 (target sequence: AAGGCCTGAAGACAGGATACA), for Memo, we used a previously validated siRNA (Marone et al., 2004) (target sequence: AAGACCTGCTAGAGCCATTAT), for cofilin (target sequence: AACCTATGAGACCAAGGAGAG). Knock-down was maximal after 72 hours; accordingly, cells were plated in Boyden chambers 3 days after siRNA transfections and allowed to migrate for 24 hours. For Dunn migration assays, cells were plated on coverslips 3 days after siRNA transfections and migration tracks were visualized over 4 to 12 hours.

Immunoprecipitations, GST-pull down and immunoblotting

Cells were stimulated or not with 1nM HRG, extracted in NP-40 buffer and protein lysates were immunoblotted as described previously (Marone et al, 2004). For immunoprecipitation experiments, cells (stimulated or not with 10nM HRG) were lysed in Triton X-100 buffer (50 mM Tris pH 7.5, 5 mM EGTA, 150 mM NaCl, 1.5 mM MgCl2, 25 mM β-glycerophosphate, 25 mM NaF, 1 % Triton X-100, 1 % Glycerol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 2 mM sodium orthovanadate, and 0.5 nM phenylmethylsulphonylfluoride). Equal amounts of cell lysates were incubated overnight at 4°C with antibody. During the course of our studies, we noted that pretreatment of cells with CoCl2 (500μM) enhanced the levels of Memo in the IPs. This was not due to a CoCl2-induced increase in Memo protein levels, but is likely a consequence of increasing epitope availability. Thus, in some experiments, Memo IPs were made with lysates from CoCl2 treated cells (Fig 4C), however, cofilin could also be detected in Memo IPs made with lysates from untreated cells (Fig 3A, *left panel* and 4D). Immunocomplexes were collected with protein-A or -G-sepharose beads (Sigma), and centrifuged. The supernatant was subjected to a second round of immunoprecipitation. The two pellets were combined and washed three times with Triton X-100 buffer. Proteins were then released by boiling in sample buffer, blotted onto polyvinylidene difluoride membranes (Millipore Corporation) and probed with the specific antibodies. Immunoprecipitations for

PLCγ1 was also performed on siRNA treated T47D cells. For GST pull-down experiments, purified GST-Memo was incubated with gluthatione sepharose beads (GE Healthcare) in TEN100 buffer (20mM Tris, pH 7.4, 0.1mM EDTA and 100mM NaCl), then beads were washed. Immobilized GST-Memo was incubated with 5 μg human recombinant cofilin (Cytoskeleton). Beads were washed four times with TEN300 buffer (20mM Tris, pH 7.4, 0.1mM EDTA and 300mM NaCl), and bound proteins were eluted in sample buffer and visualized using western blot analysis. In the same experiment, cofilin was also incubated with a five-fold molar excess of soluble Memo (40 μg) (Qiu et al., 2008) 1hour prior to incubation with GST-Memo immobilized beads.

Immunofluorescence microscopy.

Cells were grown on glass coverslips (BD Biosciences) coated with 25ug ml^{-1} rat tail collagen I (Roche Diagnostics GmbH), serum-starved overnight at 37°C and stimulated with 1nM HRG for different times. Cells were fixed with 4 % paraformaldehyde and 3 % sucrose in PBS, permeabilized in 0.2% Triton X-100 in PBS, and blocked with 1% bovine serum albumin in PBS before incubation with the primary anti-α Tubulin antibody. Alexa-Fluor 546 conjugated anti-rat antibody (Molecular Probes) was used as secondary antibody. F-actin was stained at RT with 2 U ml^{-1} FITC- labelled phalloidin (Sigma). Cells were washed with PBS-Tween 0.1% and mounted with a mounting solution (Calbiochem). Mounted samples were examined using an Olympus IX70 microscope linked to the DeltaVision workstation (Applied Precision). For GFP-transfected cells, F-actin was stained with 2 U ml^{-1} TRITC-labelled phalloidin (Sigma). Images were recorded with an Axioskop Zeiss Microscope coupled to a Sony 3 CDD camera.

Cell migration assays

For cell migration assays using Boyden chambers, serum starved transfected cells were seeded on a 8-μm-pore polycarbonate membrane (Corning Costar Products, Acton, MA) previously coated with rat tail collagen I (25μg ml^{-1}). For the chemotaxis analysis, the lower chamber was filled with 600μl of DMEM with or without 1nM HRG; for the chemokinesis analysis, both chambers contained 1nM HRG. After incubation at 37°C for 24 hours, non-migrated cells were washed and scraped from the membrane top. Migrated cells were fixed in 4% formaldehyde and stained with 0.1 % crystal violet. Cells were counted and migration was expressed as cell number per mm^2. Chemotaxis was also analyzed by direct visualization of cell migration in a gradient of HRG using Dunn chambers (DCC100; Hawksley). Serum-starved cells were seeded onto a glass coverslip previously coated with 25ug μl^{-1} rat tail

collagen I. Cells were inverted over the DCC chamber consisting of two concentric wells separated by an annular bridge. The outer well contained 10nM HRG in DMEM, whereas the inner well was filled with DMEM. A linear gradient of HRG was formed by diffusion across the bridge separating the two wells. Migration of the cells lying directly above the bridge was visualized using a Widefield TILL5, LONG RUN, Axiovert 200M (5 % CO2 and 37°C chamber). Time lapse images were digitally captured every 3 minutes with a CCD camera over a time frame of 4 to 12 hours. Migration paths were quantified using the Imaris software and the data were plotted with Microsoft Excel. The data are expressed as net translocation distance (straight distance between the start and the end point), migration speed (total length of cell migration during the capturing time) and directional persistency index (ratio between the straight distance and the total distance). Decagonal histograms were constructed to represent the directionality of cell migration. The percentage of cells whose final position was within the different sectors was indicated, the source of HRG was on the top.

F-actin cosedimentation and actin depolymerization assays

For these assays, the protocol provided by Cytoskeleton was used. For F-actin cosedimentation, 40µg of G-actin was polymerized in F-actin buffer (final concentration: 5mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 50mM KCl, 2mM MgCl2, and 1mM ATP). Polymerized F-actin was incubated for 30 minutes with: α-actinin (10µg), BSA (6.8µg), Cofilin (5µg) and/or Memo (3µg). Samples were ultracentrifuged at 150 000 x g. Equivalent amounts of supernatant and pellet fractions were separated on a 10% SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue (CBB) prior to quantification by densitometric analysis of the CBB-stained gel. In Fig 7A, the molar ratios of cofilin or Memo to actin were: cofilin:actin, 0.25:1, memo:actin, 0.05:1. The actin depolymerization assay was performed by incubating G-actin (40µg) for 30 minutes together with cofilin (20µg) and/or Memo (3, 6 or 9µg) in the same F-actin buffer. Samples were ultracentrifugated at 150 000 x g and the resulting precipitates and supernatants were analyzed as described above. Molar ratios of cofilin or Memo to actin were the following: cofilin:actin, 1:1, memo:actin, 0.05, 0.11 or 0.16:1.

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Figure legends

Figure 1. **PLCγ1 and Memo are required for ErbB2-mediated cell motility**. (A) *(Left panel)* SKBr3 cells were transfected with LacZ, Memo and/or PLCγ1 siRNAs. Control responses in the absence of ligand, as well as chemotactic responses towards 1nM HRG and chemokinetic responses in the presence of 1nM HRG were determined in Boyden chamber assays. The data are representative of several independent experiments. (B) (*Left panel*) Immunofluorescence microscopy of HRG-stimulated T47D cells treated with LacZ, Memo, or/and PLCγ1 siRNAs and labeled with Alexa-Fluor-488-phalloidin (green) or an anti-α-Tubulin (red) antibody. *(*A and B *Right panels)* Extracts were prepared and analyzed for Memo and/or PLCγ1 downregulation; α-Tubulin was used as a control. Bar, 10μm.

Figure 2. **Effect of Memo and PLCγ1 knock-down on HRG-induced chemotaxis in Dunn chambers.** T47D cells were transiently transfected with LacZ, Memo or PLCγ1 siRNAs and 3 days later were analyzed for their ability to migrate in a gradient of HRG. (A) Track displacement was followed during a period of 12 hours. The gradient follows the path from the bottom left to the top right. Gray arrows indicate the direction of individual cells. Cell displacement over the time course is indicated with the colored line (zero, intermediate and final time points are in blue, red and yellow, respectively). Down regulation of Memo or PLCγ1 was confirmed by western analysis, α-Tubulin was loaded as a control. (B) *(Left panel)* The net translocation distance is shown as the mean +/- SEM of the displacement of 61 cells. *(Middle panel)* The migration speed is shown as the mean +/- SEM of the paths of 61 cells. *(Right panel)* The directional persistency index of control LacZ, Memo or PLCγ1 KD cells is shown. (C) Decagonal histograms indicating the distribution of cells at their final location. The source of HRG is at the top.

Figure 3. **Memo complexes with PLCγ1 and ErbB2 and regulates PLCγ1 phosphorylation in tumor cells.**
(A) *(Left panel)* T47D cells were stimulated or not with 10 nM HRG and cell extracts were subjected to immunoprecipitation using a Memo mAb. IPs were probed for PLCγ1 and Memo; a mouse IgG2a mAb was used as a control. (*Right panel*). T47D cells were stimulated or not with 10 nM HRG. Cell extracts were subjected to immunoprecipitation using a PLCγ1 antibody and IPs were probed for PLCγ1 and Memo. Whole cell lysates (W) were loaded as controls. (B) (*Left panel*) T47D cells were stimulated or not with 10 nM HRG for 5 minutes.

Cell extracts were subjected to immunoprecipitation using a PLCγ1 mAb; IPs were probed for P-Tyr and for PLCγ1. (*Middle and right panels*) T47D cells were transiently transfected with LacZ or Memo siRNA, and stimulated or not for 5 minutes with 10 nM HRG. Cell extracts were subjected to immunoprecipitation using a PLCγ1 mAb (*Middle panel)* and PLCγ1 phosphorylation was analyzed by western blotting using a P-Tyr mAb. Blots were reprobed for PLCγ1. Memo and PLCγ1 levels in transfected cells were monitored in whole cell lysates (W) using the respective antibodies (*right panel*).

Figure 4. **Memo interacts with cofilin.**

(A) YTH analysis. The yeast reporter strain L40 was co-transformed with a plasmid encoding the LexA Binding Domain fused to Memo (pLexAKan-Memo) and a plasmid encoding GAL4 Activation Domain fused to the cofilin cDNA (pACT-Cofilin) (lower lane). The negative controls are: transformation with pLexAKan-Memo and the empty prey plasmid (2^{nd} row) or pACT-BLM encoding a nuclear helicase (3^{rd} row). βgalactosidase (βgal+) strain is used as a positive control (top row). (B) Pull down assay of GST-Memo/cofilin. Immobilized GST-Memo on gluthatione-sepharose beads was incubated with (lane 4) or without (lane 5) 0.24 nmoles of recombinant human cofilin. As a control, cofilin was incubated with the glutathione sepharose beads (lane 6). In lane 7, a five fold molar excess of soluble Memo was added to cofilin 1 hour prior incubation with the GST-Memo immobilized beads. After incubation the beads were pelleted, washed, and the bound proteins were eluted and visualized after western analysis with Memo and cofilin polyclonal antibodies. GST-Memo immobilized beads, glutathione-sepharose beads and cofilin were loaded on the same gel (lanes 1, 2 and 3, respectively). (C) T47D (*upper and lower panels*) and MDA-MB435 (*middle panel*) cells were stimulated or not with 10 nM HRG. Memo was immunoprecipitated using a mouse mAb; a mouse mAb IgG2a was used as a negative control. Co-immunoprecipitating proteins were analyzed by western blotting and probed with ErbB2, Memo, cofilin or P-cofilin polyclonal antibodies. Whole cell lysates (W) were loaded as controls. (D) T47D cells were treated with 10 nM HRG for the indicated times. Cell extracts were subjected to immunoprecipitation using a Memo mAb and IPs were probed for ErbB2, PLCγ1, Memo and cofilin. Whole cell lysates (W) were loaded as controls.

Figure 5. **PLCγ1, Memo and cofilin are required for ErbB2 mediated directional migration in Dunn chambers.** T47D cells were transiently transfected with either LacZ or cofilin siRNAs, or simultaneously transfected with Memo and PLCγ1 siRNAs. Cell migration

was analyzed in a gradient of HRG in Dunn chambers for 4.5 hours. Net translocation distance and migration speed were calculated for 78 cells. Cofilin, Memo and PLCγ1 levels in KD cells were checked by western blotting with the respective antibodies. α-tubulin levels were monitored as a control.

Figure 6. **HRG-induced recruitment of cofilin to the lamellipodia is dependent on Memo and PLCγ1.** (A) GFP-Cofilin-transfected and (B) GFP-Memo-transfected SKBr3 were stimulated or not with 1 nM HRG for 20 minutes. Cells were stained with a TRITC-labeled phalloidin antibody. Merged images are shown in the bottom panels. (C) SKBr3 cells were simultaneously transfected with GFP-Cofilin and LacZ, Memo or PLCγ1 siRNAs and HRG-dependent recruitment of GFP-cofilin to the lamellipodia was visualized. The actin cytoskeleton was examined with a TRITC-labeled phalloidin antibody. (D) SKBr3 cells were simultaneously transfected with GFP-Memo and siRNAs for LacZ, cofilin or PLCγ1 and HRG-dependent recruitment of GFP-Memo to the lamellipodia was visualized. The actin cytoskeleton was examined with a TRITC-labeled phalloidin antibody. Bar, 10μm.

Figure 7. **F-actin binding experiments.** For each reaction, samples of the supernatant (S) and pellet (P) fractions were collected and separated on a 10% SDS-gel. The gel was stained with 0.1% Coomassie Brilliant Blue and quantified for actin, Memo and cofilin levels. The molar ratios of cofilin or Memo to actin were: cofilin:actin, 0.25:1, memo:actin, 0.05:1. (A) (*Upper panel; left*) F-actin binding assay. Reactions: 1, F-actin; 2, α-actinin; 3, α-actinin and F-actin; 4, BSA and F-actin; 5, cofilin; 6, F-actin and cofilin (A+C); 7, Memo (M); 8, Memo and cofilin; 9, F-actin and Memo (A+M); 10, F-actin, Memo, and cofilin (A+M+C). (*Upper panel; right*) Percent of actin detected in the supernatant. (*Lower panel*) Quantification of cofilin *(left)* and Memo *(right)* levels in the supernatant and pellet fractions. (B) T47D cells were stimulated or not with 10 nM HRG for 5 minutes. Cell extracts were subjected to immunoprecipitation using a Memo mAb and an IgG mAb as a control ; IPs were probed for complexed actin and for Memo. Whole cell lysates (W) were loaded as controls.

Figure 8. **Effect of Memo on cofilin-induced actin depolymerization.** For each reaction, supernatant (S) and pellet (P) fractions were collected, separated on a 10% SDS-gel and quantified after staining of the gel with 0.1% Coomassie Brilliant Blue. Molar ratios of cofilin or Memo to actin were the following: cofilin:actin, 1:1, memo:actin, 0.05, 0.11 or 0.16:1. Reactions: 1, G-actin; 2, G-actin and Memo; 3, G-actin and cofilin; 4 to 6, G-actin and cofilin

plus increasing Memo amounts. Actin (*middle panel*) and cofilin (*lower panel*) levels were quantified.

Figure S1. **Memo and PLCγ1 are required for HRG-induced SKBr3 migration** (Left panel) SKBr3 cells were treated 10 minutes or not with HRG. Down-regulation of PLCγ1 and/or Memo in SKBr3 cells with specific siRNAs was confirmed by western blotting using specific antibodies. Phosphorylation of ERK in response to HRG was tested in control (LacZ) and KD cells. (Right panel) HRG-dependent chemotactic migration of SKBr3 cells was measured in Boyden chamber assays after Memo and/or PLCγ1 siRNA transfections. The results are representative of independent experiments.

Figure S2. **Memo is found in a complex with ErbB2 and PLCγ1** (Left panel) SKBr3 cells were stimulated with 10 nM HRG for 10 minutes. Cell extracts were subjected to immunoprecipitation using an ErbB2 mAb; IPs were probed for P-Tyr, PLCγ1, Memo and for ErbB2. (Middle panel) T47D cells were stimulated or not with 10 nM HRG for 10 minutes. Cell extracts were subjected to immuno-precipitation using a Memo mAb. IPs were probed for ErbB2, PLCγ1 and Memo. (Right panel) HEK293 cells were stimulated or not with 10 nM HRG for 10 minutes. Cell extracts were subjected to immunoprecipitation using either a Memo mAb and complexed PLCγ1 was detected, or using a PLCγ1 antibody and complexed Memo was detected; a mouse IgG3 mAb was used as a control for PLCγ1 IP. Whole cell lysates (W) are indicated in the three panels.

Figure S3. **Bait dependency assay**. Binding specificity was verified with a bait-dependency test whereby the reporter strain was transformed with the empty bait plasmid (pLexAKan) and the pACT-Cofilin plasmid. A false positive candidate is shown (pACT-TZF encoding for a testis zinc finger protein).

Figure S4. **Analysis of P-cofilin levels in breast tumor cells.** (A) T47D (upper panel) and SKBr3 (lower panel) breast tumor cells were stimulated with 1 nM HRG for the indicated periods of time. Western blot analysis was performed on extracts using a P-cofilin specific antibody. α-Tubulin was loaded as a control. (B) T47D cells were treated with LacZ, Memo

and PLCγ1 siRNA and stimulated with 1 nM HRG for the indicated periods of time. Extracts were prepared and a western analysis was performed to control for PLCγ1 and Memo down-regulation and to examine P-Ser3 Cofilin levels.

Figure S5. **HRG-induced chemotaxis of T47D and NYPD cells in a Dunn chamber assay.** T47D and NYPD were analyzed for their ability to migrate in a gradient of HRG. The migration of 23 cells was followed during 4.5 hours. Net translocation distance and migration speed were evaluated.

FIGURE 1.

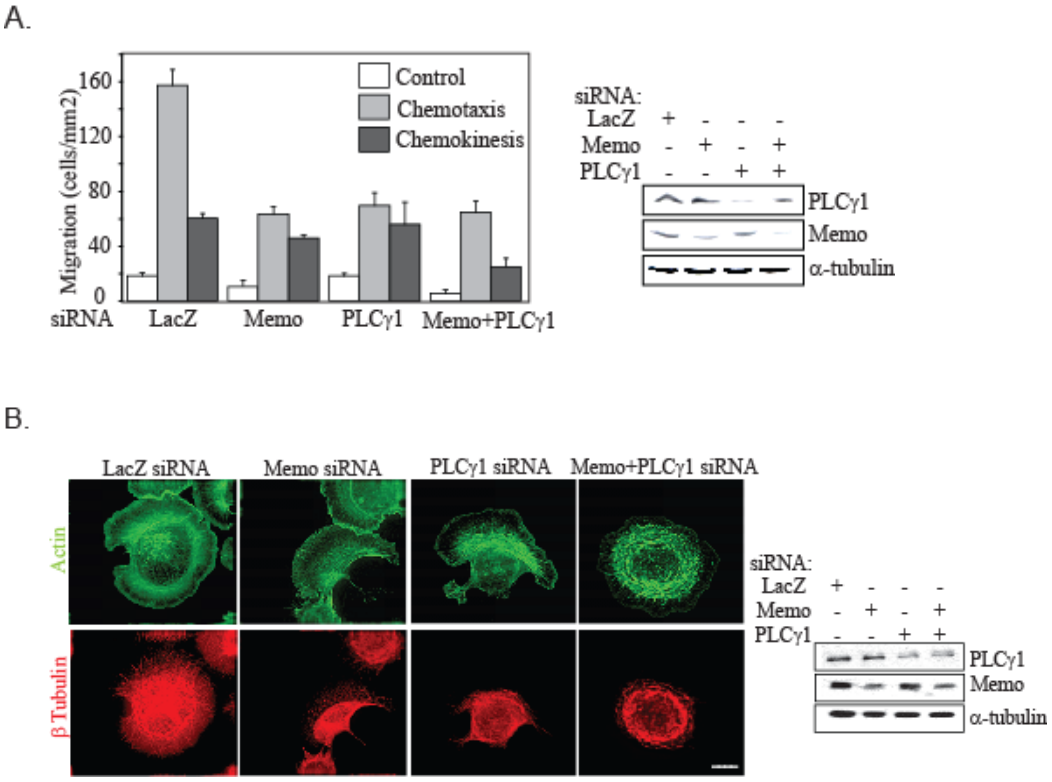

A.
Migration (cells/mm2)
160
120
80
40
0
Control
Chemotaxis
Chemokinesis
siRNA
LacZ
Memo
PLCγ1
Memo+PLCγ1
siRNA:
LacZ + - - -
Memo - + - +
PLCγ1 - - + +
PLCγ1
Memo
α-tubulin
B.
LacZ siRNA
Memo siRNA
PLCγ1 siRNA
Memo+PLCγ1 siRNA
Actin
β Tubulin
siRNA:
LacZ + - - -
Memo - + - +
PLCγ1 - - + +
PLCγ1
Memo
α-tubulin

FIGURE 2.

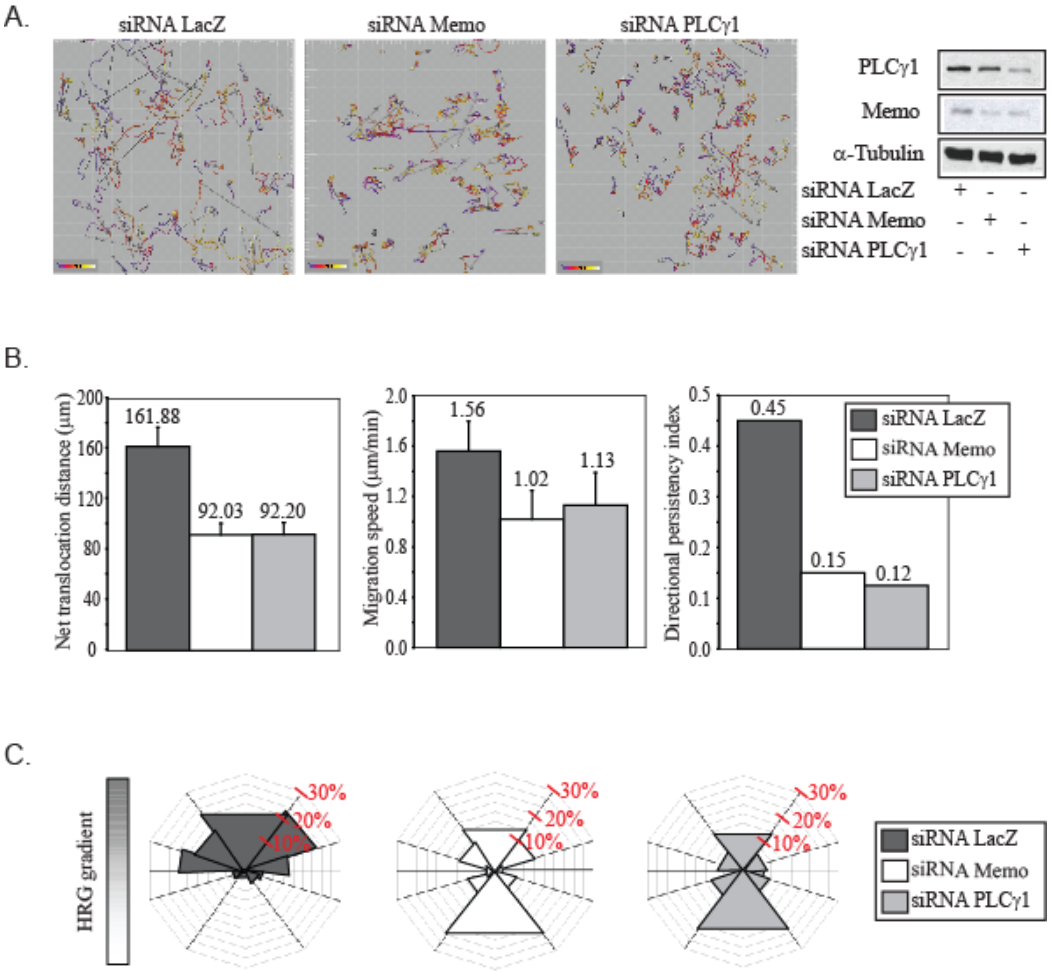

A.
siRNA LacZ
siRNA Memo
siRNA PLCγ1
PLCγ1
Memo
α-Tubulin
siRNA LacZ + - -
siRNA Memo - + -
siRNA PLCγ1 - - +
B.
Net translocation distance (μm)
161.88
92.03
92.20
Migration speed (μm/min)
1.56
1.02
1.13
Directional persistency index
0.45
0.15
0.12
siRNA LacZ
siRNA Memo
siRNA PLCγ1
C.
HRG gradient
30%
20%
10%
siRNA LacZ
siRNA Memo
siRNA PLCγ1

FIGURE 3.

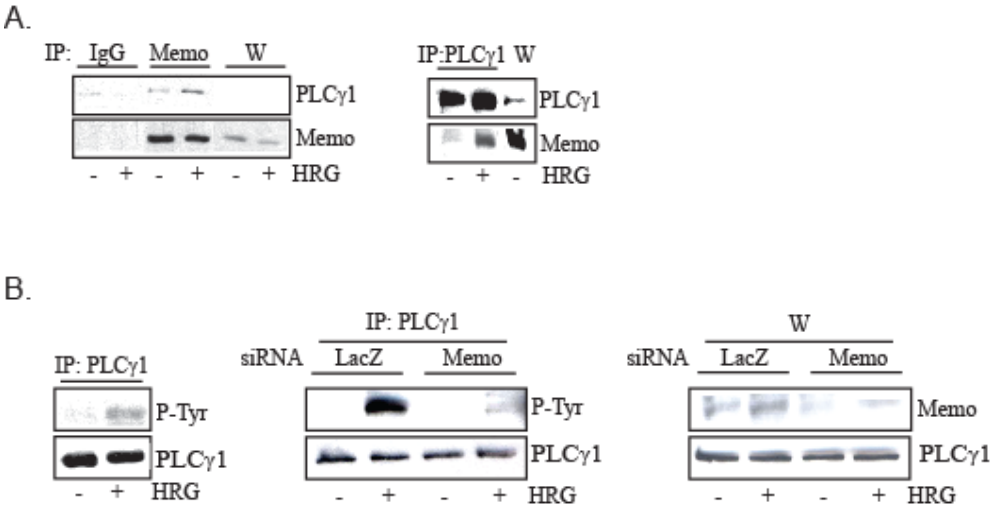

A.
IP: IgG Memo W
PLCγ1
Memo
- + - + - + HRG
IP:PLCγ1 W
PLCγ1
Memo
- + - HRG
B.
IP: PLCγ1
P-Tyr
PLCγ1
- + HRG
IP: PLCγ1
siRNA LacZ Memo
P-Tyr
PLCγ1
- + - + HRG
W
siRNA LacZ Memo
Memo
PLCγ1
- + - + HRG

FIGURE 4.

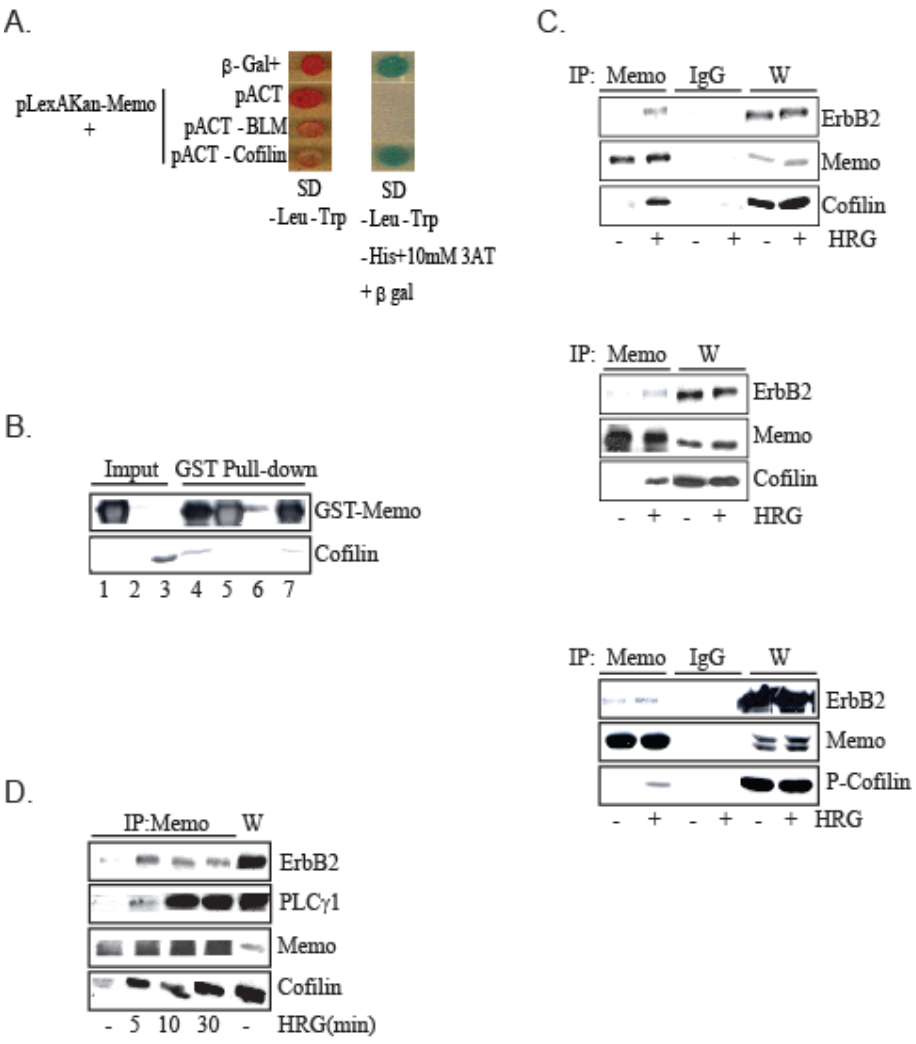

A.
pLexAKan-Memo
+
β-Gal+
pACT
pACT -BLM
pACT -Cofilin
SD
-Leu-Trp
SD
-Leu-Trp
-His+10mM 3AT
+β gal
B.
Imput
GST Pull-down
GST-Memo
Cofilin
1 2 3 4 5 6 7
C.
IP: Memo IgG W
ErbB2
Memo
Cofilin
- + - + - + HRG
IP: Memo W
ErbB2
Memo
Cofilin
- + - + HRG
IP: Memo IgG W
ErbB2
Memo
P-Cofilin
- + - + - + HRG
D.
IP:Memo W
ErbB2
PLCγ1
Memo
Cofilin
- 5 10 30 - HRG(min)

FIGURE 5.

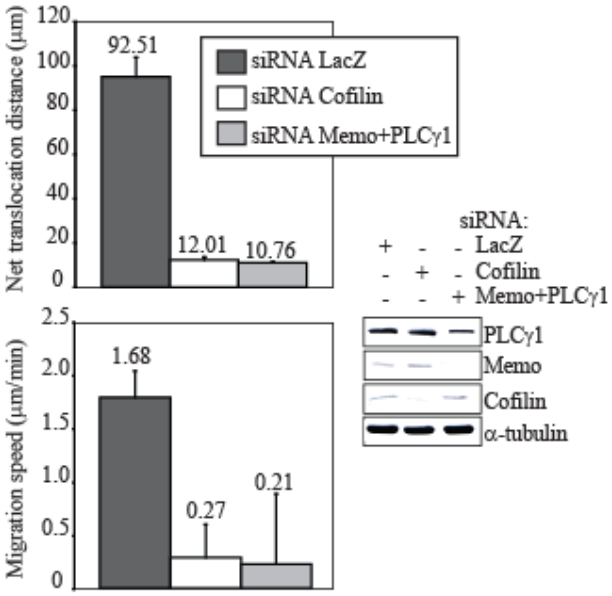

Net translocation distance (µm)
120
100
80
60
40
20
0
92.51
12.01
10.76
siRNA LacZ
siRNA Cofilin
siRNA Memo+PLCγ1
siRNA:
+ - - LacZ
- + - Cofilin
- - + Memo+PLCγ1
PLCγ1
Memo
Cofilin
α-tubulin
Migration speed (µm/min)
2.5
2.0
1.5
1.0
0.5
0
1.68
0.27
0.21

FIGURE 6.

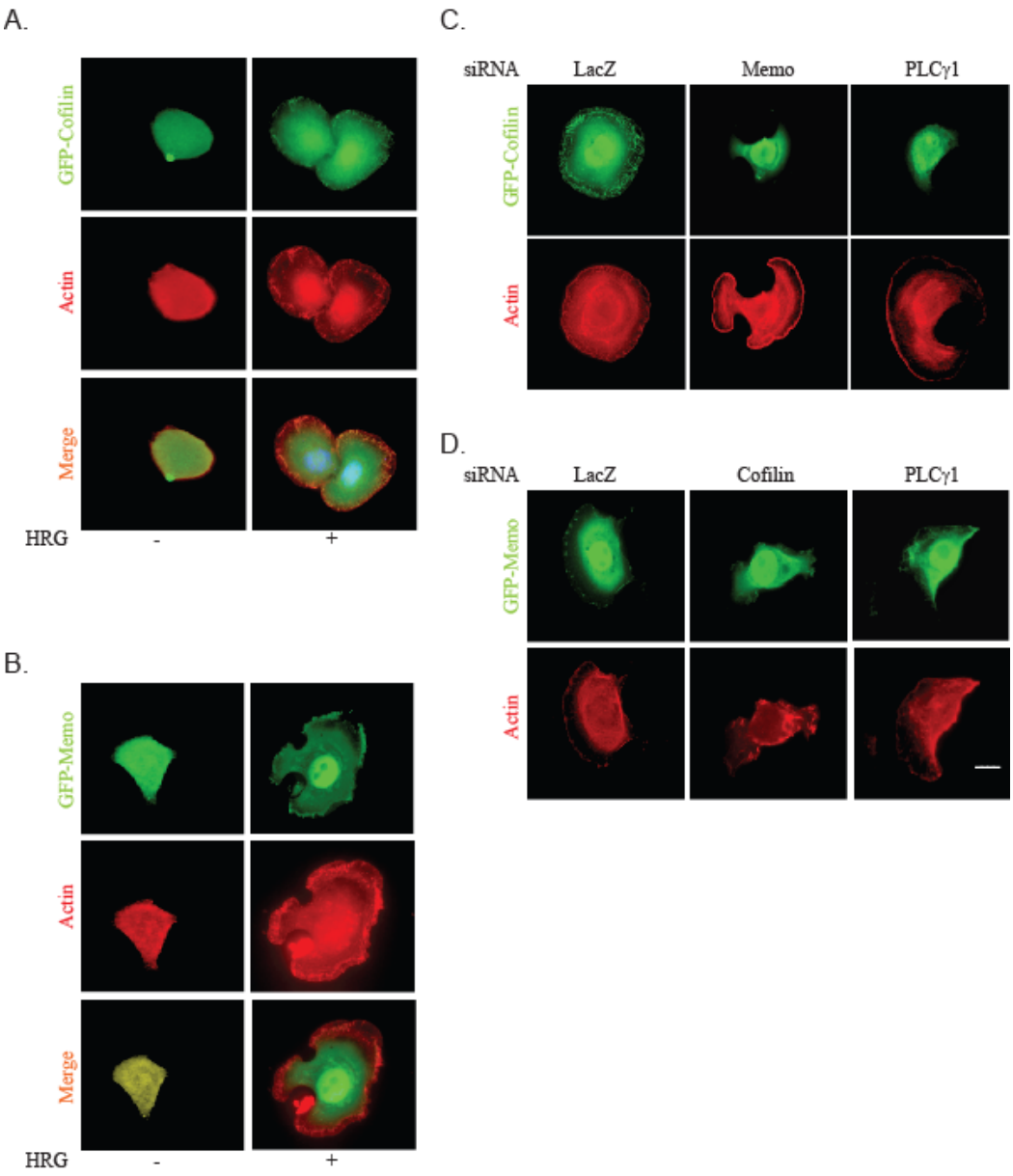
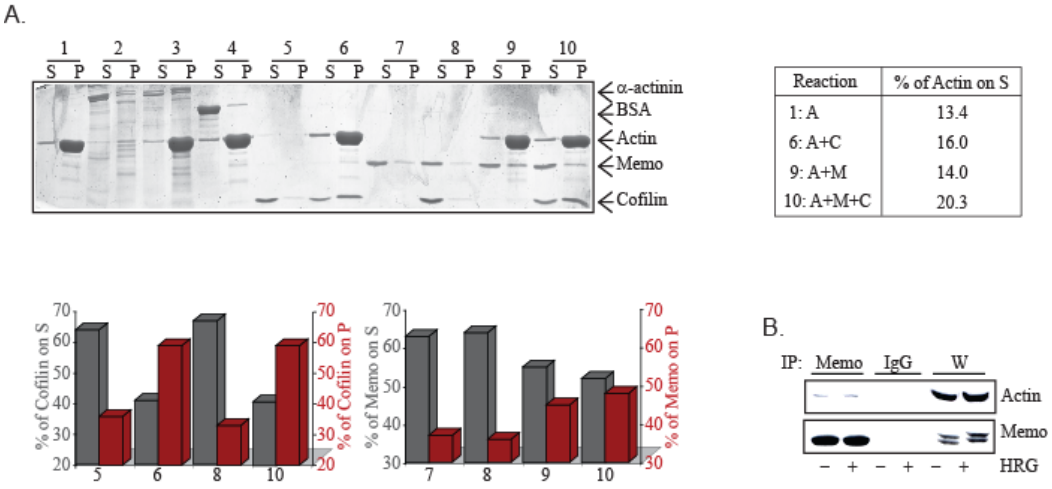

A.
GFP-Cofilin
Actin
Merge
HRG
-
+
B.
GFP-Memo
Actin
Merge
HRG
-
+
C.
siRNA
LacZ
Memo
PLCγ1
GFP-Cofilin
Actin
D.
siRNA
LacZ
Cofilin
PLCγ1
GFP-Memo
Actin

FIGURE 7.



Reaction	% of Actin on S
1: A	13.4
6: A+C	16.0
9: A+M	14.0
10: A+M+C	20.3

FIGURE 8.

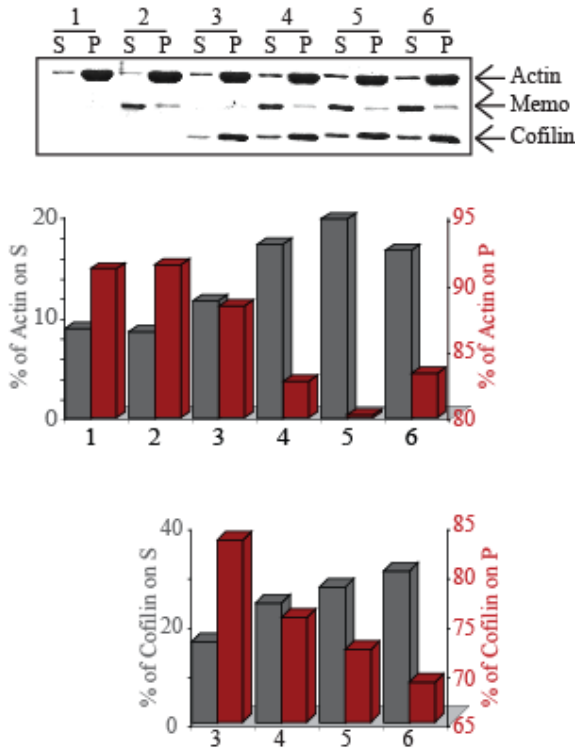

1 2 3 4 5 6
S P S P S P S P S P S P
Actin
Memo
Cofilin
% of Actin on S
% of Actin on P
% of Cofilin on S
% of Cofilin on P

FIGURE S1.

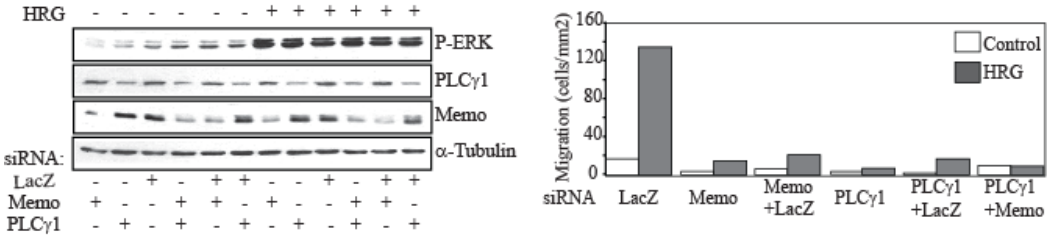


FIGURE S2.

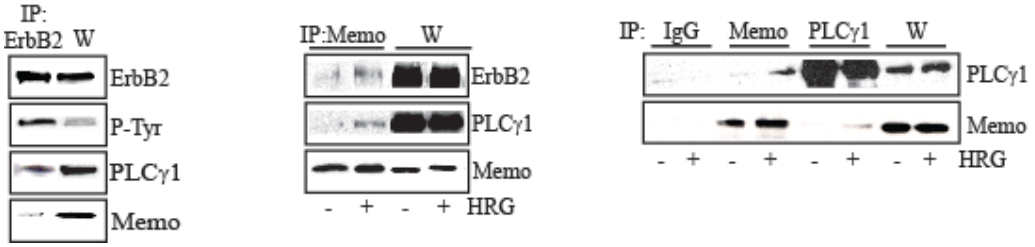


FIGURE S3.

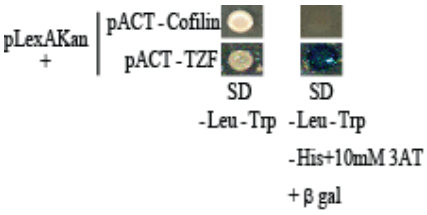


FIGURE S4.

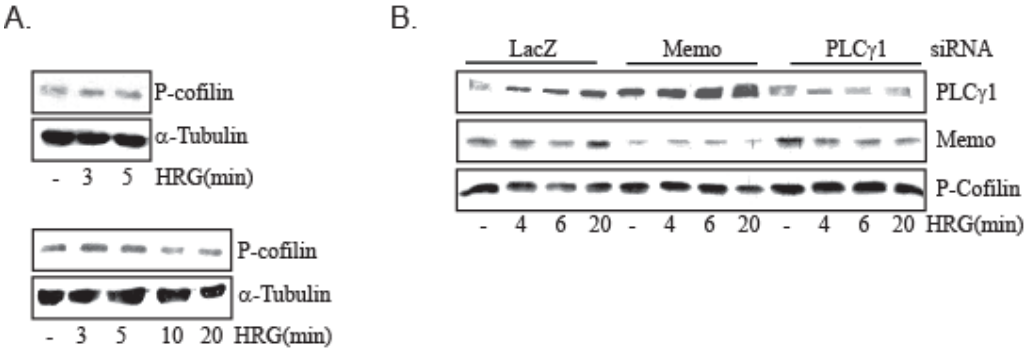
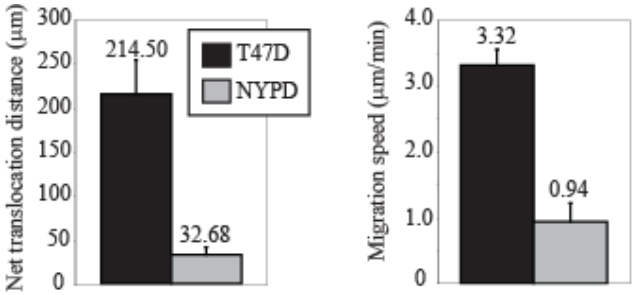


FIGURE S5.



----------RESULTS----------

II/ RESEARCH ARTICLE 2 in preparation for submission

Memo is required for ErbB-induced Src, Shc, ERK and PLCγ1 activations and is involved in the regulation of focal adhesion organization and deadhesion at the rear of the cell.

Maria Meira, Susanne Lienhard, Francisca Maurer, Gwen MacDonald, Barbara Haenzi, Régis Masson, Nancy E. Hynes*

Friedrich Miescher Institute for Biomedical Research, Maulbeertrasse 66, CH-4058 Basel, Switzerland

* Corresponding author

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Running Title- Memo in cell migration

Summary

ErbB2 controls key intracellular pathways that govern fundamental cellular processes including migration. Studies on the functional role of ErbB2 phosphorylation sites identified Memo (Mediator of ErbB2-driven Cell Motility), a 297 amino-acid protein that is required for ErbB2-induced cell motility. Memo has no characterized domains, nor does it have obvious catalytic activity, and its biochemical and cellular functions still remain poorly understood. In this study, we show that Memo interacts with the three Shc isoforms, p46^{shc}, p52^{shc}, and p66^{shc}, and we provide evidence that Shc is required for Memo binding to the ErbB2 receptor. To gain more insight into Memo's function, we used Memo-defective cells and showed that inhibition of Memo impairs activation of a number of signaling molecules including Src, Shc, ERK, and PLCγ1. Furthermore, Memo-deficient cells exhibited defective migration and adhesion properties. A morphological study revealed that inhibition of Memo results in a defect in rear cell deadhesion. Biochemical approaches indicated that Memo is involved in focal adhesion organization. Our data suggest a broad engagement of Memo in distinct steps of the metastatic process, making it an interesting target for cancer therapy.

Introduction

Cell migration is an essential and complex multistep process that plays an important role during development and adult life ((Kurosaka and Kashina, 2008); (Martin and Parkhurst, 2004); (Yang et al., 2002); (Moser and Loetscher, 2001); (Thelen, 2001)). Migration also contributes to several pathological disorders, such as cancer or vascular and inflammatory diseases ((Condeelis et al., 2005), (Smart and Riley, 2008); (Le et al., 2004); (Murphy, 2001); (Wardlaw et al., 2000); (Worthley et al., 2001); (Tarrant and Patel, 2006)). Increased motility and invasiveness are important properties that contribute to metastasis of cancer cells to distant organs (Kedrin et al., 2007). A growing number of proteins with roles in the metastatic process are being pursued as potential therapeutic targets. The ErbB2 receptor tyrosine kinase has been implicated in the development of many types of human cancer, including breast and ovarian cancer (Slamon et al., 1989). Clinical studies revealed that ErbB2 gene amplification and overexpression correlate with an aggressive metastatic phenotype ((Slamon et al., 1987); (Berger et al., 1988)). Accordingly, the role of ErbB2 in cancer biology and in the metastatic process has been under intense scrutiny (Hynes and Lane, 2005).

ErbB2 belongs to the epidermal growth factor (EGF/ErbB) family of receptor tyrosine kinases (RTK), which also includes EGF receptor (EGFR), ErbB3 and ErbB4 (Yarden and Sliwkowski, 2001). Ligand binding initiates signaling by inducing the formation of specific homo- or heterodimeric complexes, followed by activation of the cytoplasmic kinase domain which phosphorylates diverse Tyrosines (Tyr) in the tail region of the receptor (Burgess et al., 2003). Phosphorylation of these Tyr residues induces the binding of specific signaling molecules that initiates downstream signaling events (Schlessinger, 2000). ErbB2 is the preferred heterodimerization partner of the other ErbB members (Graus-Porta et al., 1997), and is exclusively activated via heterodimerization with another ligand bound ErbB receptor (Olayioye et al., 2000). In a screen for ErbB2 interacting proteins with roles in cell migration, a novel protein, Memo, and ShcA, were found to bind specifically the Tyr 1201 ErbB2 autophosphorylation site (Marone et al., 2004).

The adaptor protein ShcA, which is referred as Shc throughout the text, is involved in coupling receptor and non-receptor tyrosine kinases, to downstream signaling pathways (Ravichandran, 2001). ShcA encodes three widely expressed isoforms, $p46^{shc}$, $p52^{shc}$, or $p66^{shc}$ that derive from differential transcription and alternative splicing (Ravichandran, 2001) (Luzi et al., 2000). ShcA contains both phosphotyrosine-binding (PTB) and SH2 domains that bind to the activated receptors (Sakaguchi et al., 1998) and becomes phosphorylated on tyrosine residues in response to diverse stimuli ((van der Geer and Pawson, 1995); (Bonfini et al.,

1996)). All three isoforms contain three conserved tyrosine residues (Tyr 239/240 and Tyr 317) within the central proline rich CH1 domain that are phosphorylated, and serve as docking sites for downstream signaling molecules such as Grb2 ((van der Geer et al., 1996); (Salcini et al., 1994)). Activation of tyrosine kinases including Src, Fyn, and focal adhesion kinase (FAK) that phosphorylate Shc, lead to Grb2 recruitment , Ras and MAPK activation and entry into the cell cycle ((McGlade et al., 1992); (Mainiero et al., 1995); (Wary et al., 1996); (Wary et al., 1998); (Schlaepfer et al., 1998)).

Memo is encoded by a unique gene and orthologues are found in all branches of life, from bacteria to human. The 2.1 Å crystal structure of Memo revealed that it is homologous to LigB from *Sphingomonas paucimobilis,* a bacterial class III nonheme iron-dependent dioxygenase (Qiu et al., 2008). However to date, no enzymatic activity or metal binding have been detected, making it unlikely to be a novel dioxygenase. Memo was initially detected based on its important role in ErbB2-induced cell motility. In fact, tumor cells with a specific knock-down (KD) of Memo failed to grow microtubules in response to Heregulin (HRG) induced ErbB2 activation and were impaired in their migration (Marone et al., 2004). In a recent study, we explored in more detail the mechanism underlying Memo-mediated ErbB2-dependent cell motility, and showed that Memo regulates actin dynamics by interacting with cofilin, a known actin depolymerizing/severing factor, enhancing cofilin function (Meira et al., submitted). We also found that Memo is a novel F-actin binding protein that is required for HRG-induced cytoskeletal organization and directional migration of breast tumor cells (Meira et al., submitted), suggesting a novel role for Memo in the migratory process.

Cell migration proceeds in distinct coordinated steps which operate cyclically (Lauffenburger and Horwitz, 1996) and are controlled by different effector proteins (Raftopoulou and Hall, 2004). In response to a chemotactic stimulus, regulators of polarity at the front and the rear are activated ((Itoh et al., 2002); (Iijima et al., 2002); (Merlot and Firtel, 2003; Sakaguchi et al., 1998); (Yam et al., 2007); (Vicente-Manzanares et al., 2007)), leading to a polarized extension of protrusions and adhesion formation at the front ((Geiger and Bershadsky, 2001); (Rottner et al., 1999)). Actin polymerization and nucleation generates the protrusive force allowing cell translocation. This is followed by contraction of the cell body and tail detachment at the rear inducing movement in the direction of the ligand ((Kolega, 2003); (Kaverina et al., 1999); (Wehrle-Haller and Imhof, 2003)). Therefore, chemotaxis also depends upon focal adhesion disassembly at the cell rear and inhibition of this process results in a contractile or tail-retraction defect that impairs migration (Sturge et al., 2006).

Focal adhesions are specialized regions of tight interaction between cytoskeletal or membrane components of a cell and the extracellular matrix (Burridge and Chrzanowska-Wodnicka, 1996). These domains of integrin clustering are also sites of signal transmission into the intracellular compartment ((Juliano and Haskill, 1993); (Clark and Brugge, 1995)). A number of proteins with structural roles or with roles in signal transduction have been identified, mainly at the cytoplasmic face of focal adhesions (Sastry and Burridge, 2000).

Focal adhesion kinase (pp125FAK or FAK) is a non-receptor protein tyrosine kinase that acts both as a signaling and a scaffolding protein at sites of integrin adhesion ((Mitra et al., 2005); (Parsons et al., 2000); (Sieg et al., 2000)). Integrin clustering promotes FAK autophosphorylation at Tyr 397, which creates a binding site for the Src-homology (SH)2 domain of Src ((Schaller et al., 1994); (Calalb et al., 1995)). Src binding to FAK contributes to Src activation, promoting further phosphorylation of FAK at additional tyrosines (Brunton et al., 2005). The catalytic activity of Src has been shown to trigger focal adhesion disassembly (Fincham and Frame, 1998). Moreover, Src-dependent FAK phosphorylation has been shown to be required for focal adhesion turnover and cell migration (Westhoff et al., 2004). FAK null fibroblasts exhibit a decreased rate of migration and spreading, and an increase in the number and size of peripherally localized adhesions (Ilic et al., 1995). Similarly, inhibition of Src also results in a reduced motility and spreading, with the formation of large peripheral adhesions ((Klinghoffer et al., 1999); (Fincham and Frame, 1998)).

The FAK−Src complex mediates the phosphorylation of the well-studied scaffolding paxillin protein, which recruits other molecules to the adhesions ((Bellis et al., 1995); (Webb et al., 2004)). Another important target of FAK-Src signaling is the mitogen-activated protein kinase (MAP kinase)/ERK cascade (Schlaepfer et al., 1999). Activation of calpain-2 by ERK2 induces calpain-2 mediated cleavage of focal adhesion components, resulting in focal adhesion disassembly ((Carragher et al., 2003); (Franco et al., 2004); (Glading et al., 2004)). FAK was also shown to be involved in the recruitment of PLCγ1, a downstream effector of Src, to the plasma membrane at sites of cell-matrix adhesion, where it promotes PLCγ1 tyrosine phosphorylation and enzymatic activity (Zhang et al., 1999).

Remodeling of the actin cytoskeleton is a crucial step of the cell migration process that controls adhesion dynamics. Diverse key effectors of FAK, such as the members of the RhoGTPase family, are involved in actin remodeling ((Schlaepfer et al., 2004); (Zhai et al., 2003); (Chikumi et al., 2002)). Indeed, RhoA, a downstream FAK effector, regulates the assembly and disassembly of focal adhesions and their associated bundles of actin filaments ((Burridge et al., 1997); (Ridley, 1995)).

In this study, we show that Memo binds the three Shc isoforms, p46^{shc}, p52^{shc}, and p66^{shc}, and we provide evidence that Shc is necessary for Memo binding to the activated ErbB2 receptor. We also demonstrate that Memo is required for ErbB2-induced Src activation, and that loss of Memo impairs ErbB2-mediated phosphorylation of a number of signaling molecules, including ERK, PLCγ1 and Shc. Furthermore, in addition to their impaired migration and adhesion properties, Memo-defective cells exhibit a rear retraction defect, reflective of abnormal focal adhesion disassembly. The biochemical analysis of regulators of focal adhesions, such as FAK Paxillin, Rho, and Src, suggests that Memo is involved in focal adhesion organization.

Results

Shc interacts with Memo and is required for Memo binding to ErbB2

Shc and Memo bind to P-Tyr 1227/1222, one of the ErbB2 phosphorylation sites that have been previously shown to be important for cell migration ((Dankort et al., 1997); (Marone et al., 2004)). In our initial studies, various results suggested that memo's ability to interact with active ErbB2 might be dependent upon Shc. First, it was shown that immunodepletion of endogenous Shc from reticulocytes lysates expressing *in vitro* translated Myc-Memo resulted in a dramatic blockade of Memo binding to the phospho-mimetic Tyr 1227/1222 peptide. Secondly, Myc-Memo ectopically expressed in SKBr3 cells was shown to be constitutively complexed with Shc (Marone et al., 2004) and immunoprecipitations of ErbB2 revealed that it was complexed to Memo and Shc. Considering the importance of ShcA family members in the regulation of diverse cellular functions ((Ravichandran, 2001) (Migliaccio et al., 1997)), we explored the Memo-Shc interaction in more detail.

To directly test the role of Shc in Memo binding to active ErbB2, we used wild type (WT) mouse embryo fibroblasts (MEFs) and ShcA-deficient MEFs. Upon stimulation with EGF, there was a rapid increase in the phosphorylation of AKT and ERK, reflecting activation of these major cellular signaling pathways (Figure 1A). To note is the fact that those pathways are more activated in ShcA-deficient MEFs. Next, we examined the ability of Memo to bind to the activated receptor in control and Shc-null MEFs. Memo IPs from lysates of WT and ShcA-deficient MEFs were probed with ErbB2, Shc and Memo antibodies. Upon EGF treatment, there is a rapid increase in the P-Tyr content of ErbB2 (Figure 1B, *left panel*), reflecting receptor activation. Furthermore, Memo and Shc were constitutively complexed in WT control MEFs and there was an increase in co-immunoprecipitating Shc protein in Memo IPs from EGF-stimulated WT MEFs (Figure 1B, *right panel*). Importantly, ErbB2 was only detected in Memo IPs from lysates of EGF-induced WT MEFs, but not ShcA-deficient MEFs. These results clearly demonstrate that Shc is required for the binding of Memo to the active ErbB2 receptor (Figure 1B, *right panel*).

Next, we examined the ability of Memo to interact with the individual ShcA isoforms using Shc KO MEFs reconstituted with the individual HA tagged $p46^{shc}$, $p52^{shc}$, and $p66^{shc}$. Memo immunoprecipitations (IPs) from lysates of ShcA-deficient MEFs ectopically expressing the empty vector or one of the three Shc isoforms, $p46^{shc}$, $p52^{shc}$, or $p66^{shc}$, revealed complexed $p46^{shc}$, $p52^{shc}$ and $p66^{shc}$ molecules (Figure 1A). As seen in WT MEFs, Memo appears to bind slightly less to $p66^{shc}$ than to the $p46^{shc}$ and $p52^{shc}$ isoforms. The three isoforms were also detected in Memo IPs from T47D lysates (Supplementary Figure S1), where we observed an increase in the co-immunoprecipitating proteins in HRG-stimulated cells, probably reflecting

stabilized complexes interacting with ErbB2. The Memo interaction with p46^{shc}, p52^{shc}, and p66^{shc} is specific since neither protein was detected in IPs carried out with an isotype matched control (Supplementary Figure S1).

Memo has a role in EGF-induced Shc phosphorylation

Upon stimulation of most receptor tyrosine kinases, Shc is phosphorylated on Tyr 239/240 and Tyr 317, which serve as docking sites for the binding of molecules such as Grb2 and subsequent activation of the MAPK pathway (Ravichandran, 2001). Considering the Memo-Shc interaction, we asked if Memo has a role in EGF-induced Shc phosphorylation using WT and Memo-deficient MEFs. In WT MEFs, all three Shc isoforms were phosphorylated on Tyr 239/240 rapidly after EGF stimulation (3 minutes). Shc was also phosphorylated at 3 minutes after EGF treatment of Memo-deficient MEFs, however, lower p46 phosphorylated levels were observed. Phosphorylation was maximal at 5 minutes then decreased throughout the 60 minutes time course. By contrast, in the Memo KO MEFs, phospho-Tyr 239/240 levels were lower and decreased more rapidly than in WT MEFs. We also noted a pronounced decrease in the mobility of the p66 ^{shc} isoform in WT cells, but not in Memo KO MEFs, which likely reflects changes in its Ser-phosphorylation content. Altogether, these results suggest that Memo loss influences Shc phosphorylation in response to ErbB activation. Loss of Memo might directly or indirectly affect Shc activation, perhaps as a consequence of alterations in signaling molecules that act upstream of Shc.

Memo is required for proper ErbB-induced Src-phosphorylation

Src has been shown to phosphorylate Shc in vitro, mainly on Y239/240 (van der Geer et al., 1996). Furthermore, considering a report from our lab showing that Src inhibition reduces EGF and NRG-induced Shc phosphorylation (Olayioye et al., 2001), we examined Src activation in WT control and Memo-deficient cells. Upon EGF stimulation of WT MEFs, we observed a rapid increase in Src Y416 phosphorylated levels *(lower band)*, being maximal at 5 minutes, and progressively decreasing throughout the 60 minutes time course (Figure 3A, *upper panel*). Interestingly, EGF-induced Src Y416 phosphorylation was impaired in Memo-deficient MEFs, suggesting an important role for Memo in Src activation. To confirm these results, we used the T47D breast tumor cellular model where Memo expression was stably down-regulated following introduction of lentiviral shRNA expression vectors specific for Memo. A kinetic of Src activation following HRG stimulation was performed in shLacZ control or shMemo 5.2 expressing cells. HRG induced rapid Src phosphorylation in T47D LacZ control cells, being maximal at 1 to 3 minutes and rapidly decreasing after 5 minutes of stimulation (Figure 3A, *lower panel*). In accordance with the results obtained with Memo-deficient MEFs, in response to HRG, Src Y416 phosphorylation was impaired in T47D

shMemo 5.2 cells. These data suggest that Memo is required for efficient ErbB- induced Src activation.

ErbB receptor-induced Erk and PLCγ1 activation are impaired in cells lacking Memo

Src has been shown to be involved in ErbB-induced phosphorylation and activation of Erk1/2 (Olayioye et al., 2001). Therefore, we examined Erk1/2 phosphorylation status in WT control and Memo-deficient cells. Consistent with a previous report showing that EGF and HRG induced Erk1/2 phosphorylation (Graus-Porta et al., 1995), Erk1/2 (p42/p44) was activated rapidly (≤3 minutes) in WT control MEFs upon EGF stimulation, and in T47D LacZ control cells stimulated with HRG (Figure 4A *upper* and *lower panels*, respectively). Perhaps as a consequence of inhibition of Src activation, Erk phosphorylation was impaired in EGF-stimulated Memo-deficient MEFs and HRG-treated T47D shMemo5.2 cells. In fact, in Memo-deficient cells, there was a delay in full Erk1/2 activation (10 minutes in Memo-deficient MEFs vs 3 minutes in control cells). The level of phosphorylated Erk1/2 did not reach control levels, and decreased more rapidly. In T47D shMemo5.2 cells, HRG-induced Erk1/2 phosphorylated levels were also lower and delayed (10 minutes vs 5 minutes in control LacZ cells).

PLCγ1 is another downstream effector of Src. Indeed, pharmacological inactivation of Src has been shown to induce inhibition of PLCγ1 Tyr 783 phosphorylation (Tvorogov et al., 2005). Accordingly, we examined growth factor induced-PLCγ1 phosphorylation in WT and Memo-deficient cells. As shown in Figure 4B, EGF (*upper panel*) and HRG (*lower panel*) induce rapid phosphorylation of PLCγ1 in WT MEFs and T47D shLacZ cells, respectively. PLCγ1 phosphorylation on Tyr 783 in response to growth factor induction was impaired in Memo KO-deficient MEFs and T47D shMemo 5.2 cells. Altogether, these results indicate that loss of Memo is correlated with a major impairment of Src phosphorylation and downstream signaling components, more specifically Shc, Erk1/2 and PLCγ1. Whether or not these results reflect a direct or an indirect role of Memo on Src activation remains to be explored.

Memo has an important role in cell adhesion and migration processes

Memo is a novel protein that was discovered in a screen for ErbB2 effector proteins with roles in migration (Marone et al., 2004). Detailed studies on the role of Memo in HRG-induced tumor cell migration ((Marone et al., 2004); (Meira et al, *submitted)*) in T47D and SKBr3 models revealed an essential role for Memo in the migratory process. In the following experiments, we enlarged the study on the role of Memo in cell migration using WT control and Memo-deficient MEFs. The directional (chemotactic) and random (chemokinetic) migration of WT control and Memo-deficient cells was examined in Transwell assay

chambers. EGF was added to the lower chamber to measure chemotaxis and to both chambers to measure chemokinesis. In accordance with the results obtained using a knock-down strategy in the human breast carcinoma models, Memo-deficient MEFs displayed a dramatic blockade of cell motility (Figure 5A), confirming the importance of Memo in the cell migration. However, since the chemokinetic response of WT control cells was not significant, we could not assign a specific role for Memo in directional or random cell motility in this assay.

Cell adhesion also influences the migratory process. Thus, we investigated adhesion properties of WT control and Memo-deficient MEFs on different substrates. Cells were seeded onto collagen, laminin or fibronectin coated wells. After 45 minutes incubation at 37°C, cells were washed and adherent cells were fixed and stained. Representative images are depicted in Figure 5B. Compared to WT control cells, adhesion of Memo-deficient MEFs to collagen, laminin and fibronectin was impaired, suggesting that Memo is involved in the cell adhesion process.

Adhesion of a cell to the extra-cellular matrix (ECM) via transmembrane integrin molecules leads to the formation of focal adhesions complexes, which provide a link between the ECM and the actin cytoskeleton (Zamir and Geiger, 2001). We examined actin distribution in WT control and Memo-deficient MEFs seeded on collagen coated glass coverslips (Figure 5C). In contrast to WT control cells in which F-actin fibers were diffusely and strongly distributed in the cytoplasm, Memo-deficient MEFs exhibited an altered actin distribution, with a concentration of F-actin fibers in the center of the cell. Interestingly, Memo depletion in MEF cells resulted in a striking elongated phenotype which is indicative of a tail-retraction defect in some published models (Sturge et al., 2006). Defective cellular rear retraction resulting from the down-regulation of Memo was not restricted to MEF cells but was also confirmed in other cellular models, such as T47D (Figure 5D) and MDA-MB231 (Supplementary Figure S2) human breast carcinoma cells transiently transfected for 72 hours with LacZ control or Memo siRNA. The morphology of these cells migrating on the bridge of Dunn chambers in a chemo-attractant gradient of HRG was examined using time-lapse video microscopy. In both cellular models, Memo KD (Knock-Down) had a strong effect on cell morphology, with the formation of elongated tails (Figure 5D and Supplementary Figure S2 *left panels*). A quantification performed over 10 different time points revealed a significant increase in the percent of unretracted tails in T47D Memo KD cells (8-fold over the control cells) (Figure 5D *right panel*). We also examined the morphology of MDA-MB231 cells that were seeded on collagen coated glass coverslips and stimulated for 20 minutes with HRG before fixation and immunostaining (Supplementary Figure S2 *right panel*). In contrast to control cells in which no defect in rear cell deadhesion was apparent, Memo KD cells exhibit a collapsed morphology with the formation of visible unretracted tails.

Memo inhibition impairs focal adhesion organization

To understand the mechanism underlying Memo's effect on cellular adhesion disassembly and retraction, we performed a biochemical analysis of the pathways involved in this process. The exact mechanism involved in this migration step has not been completely described and might be cell specific since different studies have suggested the requirement of different candidates including the Rho-ROCK pathway, microtubules, calpains, FAK/Src and myosin II, for adhesion disassembly and retraction (Ridley et al., 2003). Considering our results showing that Memo inhibition impairs ErbB-induced Src-phosphorylation, and the described role of FAK/Src signaling in the regulation of adhesion disassembly ((Hamadi et al., 2005); (Webb et al., 2004)), we examined FAK activation in WT control and Memo deficient cells. A significant baseline level of phosphorylated FAK Y397 was observed in both WT control and Memo-deficient MEFs. Furthermore, in response to EGF, there was no change in FAK Y397 phosphorylation (Figure 6A) in either cell line. Phosphorylation of tyrosine Y397 is mainly due to autophosphorylation and it occurs in response to diverse stimuli, including integrin engagement (McLean et al., 2005). By contrast, focal adhesion kinase Tyr 861 has been described as a major Src phosphorylation site (Calalb et al., 1996). In accordance with inhibition of Src phosphorylation in EGF/HRG-stimulated Memo deficient MEFs and T47D cells, in both cell lines, there was a strong decrease of ligand-induced FAK Tyr 861 phosphorylation compared to control cells (Figure 6B, *upper and lower panels*).

To gain more insight into a possible role for Memo in the organization of focal adhesion complexes, and because many Src substrates, including FAK and Paxillin, are localized to focal adhesions, we examined focal adhesion distribution in EGF-stimulated WT control and Memo-deficient MEFs. Phospho-FAK Y397 (Figure 6C) and phospho-paxillin (Figure 6D) immunoreactivity were diffusely distributed on mature fibrillar adhesions in the cytoplasm as well as at sites of stress fibers attachment in WT MEFs. By contrast, phospho-FAK (Figure 6C) and phospho-paxillin (Figure 6D) labeled Memo-deficient cells did not exhibit a fibrillar staining distribution. Immunofluorescence remained concentrated in the cell periphery, perhaps at the sites of nascent adhesions (Figure 6C and 6D). Also in this experiment, Memo deficient MEFs exhibit an elongated phenotype with a number of visible unretracted tails (Figure 6C and 6D).

FAK-mediated phosphorylation of GTPases exchange factors has been shown to correlate with enhanced RhoA activation (Chikumi et al., 2002). Furthermore, considering another study showing that inhibition of ROCK, a downstream effector of RhoA, induces the formation of multiple and elongated tails (Sturge et al., 2006), we tested the hypothesis that Memo influences RHO-GTPase pathways. To examine the amount of active GTP bound-RhoA, the GST-C21 fusion protein was used in a pull-down assay (Malliri et al., 2002). Active RhoA was measured in lysates of WT control and Memo-deficient MEFs subjected to

a kinetic of EGF stimulation (Figure 6E). Lower levels of active RhoA levels were observed after 5 minutes of stimulation in Memo-deficient cells compared to WT control cells. The results suggest that the kinetics of RhoA activation are slower in the Memo null MEFs compared to WT cells.

Altogether, these results suggest that Memo is likely involved, directly or indirectly via Src inhibition, in the regulation of focal adhesion organization and in disassembly of focal adhesions.

Discussion

In the past decades, remarkable advances in the understanding of the role of ErbB2 in cancer and the development of targeted therapies for ErbB2-overexpressing tumors have emerged. In fact, ErbB2 activation turns on signaling pathways that enhance diverse metastasis-associated properties such as adhesion, migration and invasiveness, leading to an increase of cancer metastasis. Studies of individuals with ErbB2-overexpressing tumors have shown that they have a significantly poorer clinical outcome compared to patients whose tumors do not overexpress ErbB2 (Berchuck et al., 1990) (Slamon et al., 1987) (Slamon et al., 1989). Clinical trials using a recombinant monoclonal ErbB-2 antibody, Herceptin, have shown clear survival benefits when it is used in combination with chemotherapy for the treatment of patients in particular those with early-stage breast cancer (Hudis, 2007) (Romond et al., 2005) (Slamon and Pegram, 2001). Accordingly, ErbB2 and downstream signaling pathways have been intensely studied with the specific aim of identifying proteins that are essential for tumor development and progression. Once phosphorylated upon receptor dimerization, tyrosine residues that are located in the cytoplasmic tail of ErbB2 receptors serve as docking sites for diverse signaling molecules. These Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain-containing proteins are either enzymes that are tyrosine phosphorylated and activated, such as PLCγ1 and Src, or adaptator molecules that link receptor activation with downstream signaling pathways, such as Shc and Grb2 (Marmor et al., 2004). In a previous study, Marone and colleagues identified Memo as a novel ErbB2-interacting protein that binds Y-1227 of the receptor cytoplasmic tail (Marone et al., 2004). Shc was also found to bind the same residue, and was suggested to be an adaptator molecule for Memo binding to the receptor.

In the present study, we demonstrate that Memo interacts with the three Shc isoforms, p46^{shc}, p52^{shc}, and p66^{shc}, and we show that Shc is required for Memo binding to the ErbB2 receptor. Furthermore, when analyzed for the activation of ErbB2 downstream signaling molecules, Memo-deficient cells exhibited impaired phosphorylation of Src, Shc, ERK and PLCγ1. The adaptator protein Shc is a proto-oncogene product that has no intrinsic catalytic activity (Pelicci et al., 1992). Shc was assumed to work as an adaptator protein which, upon tyrosine phosphorylation, mediates the recruitment of other SH2-containing proteins such as Grb2, to the tyrosine residues. The activated receptor-Shc-Grb2 complex then activates the MAP (Mitogen-activated protein) kinase cascade through Sos (Bonfini et al., 1996). Interestingly, Sato and colleagues demonstrated that the Shc adaptor protein is a direct activator of c-Src in epidermal growth factor receptor signaling in A431 human epidermoid carcinoma cells (Sato et al., 2002). Furthermore, among the three Shc isoforms, p66^{sh} and p52^{shc}, but not p46^{shc}, were found to interact with and activate c-Src *in vitro* and *in vivo*. In addition, Shc-mediated activation of c-Src was enhanced by EGF signaling. Considering our results indicating that EGF or HRG-induced Shc and Src phosphorylation are impaired in Memo deficient MEFs, and given that Memo is found to bind all three isoforms, we propose a mechanism whereby Memo, through its binding to Shc, regulates Src activation. In cells lacking Memo, Src phosphorylation is decreased, leading to impaired phosphorylation of downstream components, such as Shc, Erk, and PLCγ1. To note also, is the fact that in our previous study, we did not detect any change in Erk phosphorylation in Memo down regulated breast tumor cell lines (Meira et al., submitted). In the present study however, stable shlacZ and shMemo transfections were performed in breast tumor cells, which were further subjected to a time course of HRG stimulation. We favour the model where Memo loss, rather than influencing the phosphorylation levels of Erk, and perhaps Shc, PLCγ1 and Src, delays the activation of these components. This could suggest the possible existence of a "compensatory" pathway for Memo.

Importantly, considering that Src and PLCγ1 activation are impaired in Memo deficient cells, Memo appears to be an important mediator of cellular processes such as migration and adhesion. In fact, in a previous study, we have shown that Memo KD in breast tumor cells resulted in a dramatic impairment of cell directionality during movement (Meira et al., submitted). In the present study, using another model of Memo deficient cells, we confirm the requirement of Memo for cell migration. Furthermore, we suggest an important role for Memo during the adhesion step, and propose a possible link for Memo, PLCγ1 and Src during this particular process. In fact, PLCγ1 has been shown to potentiate integrin-mediated cell spreading and motility (Choi et al., 2007). Furthermore, when scoring PLCγ1$^{-/-}$ fibroblasts

(Null) for their cell adhesion properties, Tvorogov and colleagues showed that Null cells displayed a significantly impaired rate of Src-mediated adhesion compared with PLCγ1 reconstituted fibroblasts (Tvorogov et al., 2005). Interestingly, Memo null MEFs also displayed a significant defect in cell adhesion, suggesting a possible connection between Memo, Src and PLCγ1 during the cell adhesion process.

Furthemore, by performing a morphological study of Memo deficient cells, we observed that inhibition of Memo results in a defect in rear cell deadhesion. Briefly, in the Dunn chamber assay, Memo KD in breast tumor cells resulted in a decrease of migration speed, translocation distance and directional persistency values (Meira et al., submitted). The directional persistency index reflects the ability of cells to migrate towards the ligand source and a decreased translocation distance could be directly due to decreased directional sensing. However, the moderate effect on migration speed could be related to impaired tail retraction during movement, therefore influencing general cell motility. Different candidates including the Rho-ROCK pathway, microtubules, calpains, FAK/Src and myosin II, have been suggested to be required for adhesion disassembly and retraction (Ridley et al., 2003). In the present study, we demonstrated that activation or distribution of components of the focal adhesion assembly/disassembly processes such as Src, FAK, paxillin, and RhoA, are affected. In addition, immunofluorescence labeling of focal adhesion components revealed an absence of fibrillar adhesions in Memo deficient cells, with the predominance of large, peripheral focal contacts. This could suggest that mature adhesions are not able to disassemble and be transformed into fibrillar adhesions, thus remaining at the periphery of the cell (Broussard et al., 2008).

Memo could also affect focal adhesion turnover. Indeed, in the initial assembly of focal adhesions, integrin-mediated phosphorylation of focal-adhesion kinase (FAK) leads to the recruitment a number of signaling and structural proteins. Once phosphorylated by Src, FAK recruits other signaling molecules, such as Erk2 and calpain-2 to focal adhesion sites, which subsequently mediate the cleavage of focal adhesion components, resulting in focal adhesion disassembly (McLean et al., 2005). Therefore, Src kinase activity is required for adhesion turnover. Src may also function as an adaptator to recruit other kinases that can phosphorylate key substrates including FAK (Brunton et al., 2005). Considering that Src phosphorylation is impaired in Memo deficient cells, and that phosphorylation of Y861, a Src site on FAK, is dramatically affected in Memo deficient cells we suggest that there is a direct effect of Src on subsequent FAK function, resulting in a defect of focal adhesion turnover. Interestingly, a significant correlation of FAK Tyr-861 activation and HER2 overexpression suggested that

HER2 is involved in tumor malignancy and metastatic ability of breast cancer through a novel signaling pathway in which FAK and Src participate (Schmitz et al., 2005). Considering the present results, we propose the involvement of Memo in this particular pathway.

Materials and methods

Reagents, antibodies and plasmids

HRG-β1, referred to as HRG, was purchased from R&D Systems (Inc., Minneapolis, MN). EGF was from Becton Dickinson. Polyclonal antibodies to a 19 amino acid peptide of Memo were produced in rabbits (aa 25-43 human Memo NM_015955: NAQLEGWLSQVQSTKRPAR); the same peptide was used for Memo mAb production in mice. Isotype matched IgG2a control serum for Memo was purchased from Sigma. Mouse anti-HA.11 antibody was from Covance. Phospho-AKT, AKT, phospho-Shc (Tyr 239/240), phospho-FAK (Tyr 861), phospho-Paxillin, p44/42 MAPK and phospho-p44/42 MAPK (Thr 202/204) were from Cell Signaling. Mouse anti-RhoA, rabbit anti-PLCγ1 and anti-FAK antisera were from Santa Cruz (≠sc-418, ≠sc-81 and ≠sc-932 respectively). Rabbit anti-Shc was from Transduction Labs. Phospho-Src (Tyr 418) and phospho-FAK (Tyr 397) were purchased from Biosource. Phospho-PLCγ1 (Tyr 783) was from Upstate. Phospho-Tyrosine was detected with the 4G10 mAb (gift from Dr Juergen Mestan, NIBR Basel). For ErbB2, the 21N polyclonal antiserum was used in westerns (Olayioye et al., 2000). Collagen I was purchased from Roche Diagnostics GmbH. Laminin and Fibronectin were provided by Dr. Ruth Chiquet-Ehrismann (FMI, Basel). pCDNA 3.HA vector, pCDNA 3 HAp46^{shc}, pCDNA 3 HAp52 ^{shc}, and pCDNA 3 HAp66 ^{shc} constructs were kindly provided by Dr. Yoshikuni Nagamine. The pBabe-puro vector was previously described (Morgenstern and Land, 1990).

Cell culture and transfections

Wild-type (WT) and ShcA-deficient MEFs were kindly provided by Dr. Yoshikuni Nagamine. Memo-deficient MEFs were provided by Dr. Régis Masson and Barbara Haenzi. WT MEFs, ShcA-deficient MEFs, Memo-deficient MEFs, T47D and MDA-MB231 human breast carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % Fetal Calf Serum (GIBCO Invitrogen AG, Basel, Switzerland).

Transient siRNA transfection. T47D and MDA-MB231 cells were transiently transfected with siRNA using HiPerFect (Qiagen) according to the manufacturer's instructions. The

following siRNAs obtained from Qiagen were used for transient knock-down. For control LacZ (target sequence: AAGCGGCTGCCGGAATTTACCTT), for Memo, we used a previously validated siRNA (Marone et al., 2004) (target sequence: AAGACCTGCTAGAGCCATTAT). Knock-down was maximal after 72 hours; accordingly, for Dunn chambers assays, T47D and MDA-MB231 cells were plated on coverslips 3 days after siRNA transfections and migration tracks were visualized during 12 hours. For immunofluorescence, MDA-MB 231 cells were seeded on coverslips 3 days after siRNA transfections and allowed to attach before stimulation with 1nM HRG and fixation.

Stable shRNA transfection. *shRNA sequences:* the shLacZ vector was produced by cloning the sequence CCGGGCGGCTGCCGGAATTTACCTTCTCGAGGGTAAAT TCCGGCAGCCGCTTTTTAATT into the pLKO.1-puro plasmid using the AgeI and EcoRI restriction sites. The glycerol stock for the shMemo1 vector was purchased from the Sigma (MISSION shRNA library, TRCN0000122898). *Production of retroviruses:* HEK 293 packaging cells were transiently transfected with polyethylenimine (PEI) (Polysciences Inc.) at a PEI:DNA ratio of 4:1. PEI was added to DNA diluted in serum-free media (8 µg pLKO.1-puro backbone with shLacZ or shMemo1, 0.4 µg HDM-tat16, 0.4 µg HDM-HgPM2, 0.4 µg pRC-CMV-RaII and 0.8 µg HDM-VSV-G), vortexed and incubated at room temperature for 15 minutes. The PEI/DNA mix was then added to the cells and incubated for 16 hours at 37 °C, at which point the media was changed. Media containing viruses was collected 72 hours post-transfection, filter sterilized, aliquoted and stored at -80 °C. *Infection of T47D cells:* T47D cells were infected by incubation in media containing 8 µg/mL polybrene (Sigma) and 600 µL of viral supernatant for 24 hours at 37 °C. The media was then changed and the cells were incubated another 24 hours at 37 °C. Successfully infected cells were then selected for using 0.5 µg/mL puromycin and knock-down of Memo1 was confirmed by western blot analysis.

Plasmid transfection. ShcA-deficient MEFs were co-transfected with 1 µg pBabe-puro and 6 µg pCDNA 3. HA vector control, pCDNA 3 HAp46^{shc}, pCDNA 3 HAp52^{shc}, or pCDNA 3 HAp66^{shc} constructs. Transfections were performed using polyethylenimine (PEI) (Polysciences Inc.) according to the manufacturer's instructions.

Immunoprecipitations and immunoblotting

For direct western blot analysis, cells were stimulated or not with 1nM HRG (for T47D and MDA-MB231 cells) or 100 ng ml^{-1} EGF (for MEFs), extracted in NP-40 buffer and protein lysates were immunoblotted as described previously (Marone et al, 2004). For immunoprecipitation experiments, cells (stimulated or not either with 10nM HRG in T47D cells, or 100 ng ml-^{1} EGF in WT and Shc or Memo-deficient cells) were lysed in Triton X-

100 buffer (50 mM Tris pH 7.5, 5 mM EGTA, 150 mM NaCl, 1.5 mM MgCl2, 25 mM β-glycerophosphate, 25 mM NaF, 1 % Triton X-100, 1 % Glycerol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 2 mM sodium orthovanadate, and 0.5 nM phenylmethylsulphonylfluoride (PMSF)). Equal amounts of cell lysates were incubated overnight at 4°C with antibody. Immunocomplexes were collected with protein G-sepharose beads (Sigma), and centrifuged. The supernatant was subjected to a second round of immunoprecipitation. The two pellets were combined and washed three times with Triton X-100 buffer. Proteins were then released by boiling in sample buffer, blotted onto polyvinylidene difluoride membranes (Millipore Corporation) and probed with the specific antibodies.

RhoA activity assay

To measure RhoA activation in WT and Memo-deficient cells, the GST-C21 fusion protein pull down assay was performed. Cells were stimulated with 100 ng ml^{-1} EGF for different time points, and extracted in GST-Fish buffer (10% Glycerol, 50mM Tris pH 7.4, 100 mM NaCl, 1% NP-40, 2mM MgCl2, 1 nM PMSF, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 2 mM sodium orthovanadate). Cell lysates were mixed with bacterially produced GST-C21 fusion protein bound to gluthatione-sepharose beads for 30 minutes at 4 °C. Beads were centrifuged, washed and captured active RhoA was eluted in sample-loading buffer. Protein was resolved by SDS-PAGE as described above, transferred to membrane and probed with a RhoA-specific antibody. Aliquots from the cell lysates were taken to analyse total amount of RhoA.

Immunofluorescence microscopy

Cells were grown on glass coverslips (BD Biosciences) coated with 25ug ml^{-1} rat tail collagen I (Roche Diagnostics GmbH), serum-starved overnight at 37°C and stimulated with 1nM HRG (for MDA-MB231 cells) or 100 ng ml^{-1} EGF (for WT and ShcA or Memo-deficient cells) for 20 minutes. Cells were fixed with 4 % paraformaldehyde and 3 % sucrose in PBS, permeabilized in 0.2% Triton X-100 in PBS, and blocked with 1% bovine serum albumin in PBS. Incubation with primary and secondary antibodies was done in the same buffer. Alexa-Fluor 488 goat anti-rabbit IgG (Invitrogen) was used for phospho-FAK and phospho-Paxillin stainings. F-actin was stained at RT with 2 U ml^{-1} TRITC-labelled phalloidin (Sigma). DNA was counterstained with 0.25 $mg.ml^{-1}$ Hoechst (Sigma). Cells were washed with PBS-Tween 0.1% and mounted with a mounting solution (Calbiochem). Mounted samples were examined using an Axioskop Zeiss Microscope coupled to a Sony 3 CDD camera.

Cell migration assays

For cell migration assays on MEFs using Boyden chambers, serum starved cells were seeded on a 8-μm-pore polycarbonate membrane (Corning Costar Products, Acton, MA) previously coated with rat tail collagen I (25μg ml^{-1}). For the chemotaxis analysis, the lower chamber was filled with 600μl of DMEM with or without 100 ng ml^{-1} EGF; for the chemokinesis analysis, both chambers contained 100 ng ml^{-1} EGF. After incubation at 37°C for 24 hours, non-migrated cells were washed and scraped from the membrane top. Migrated cells were fixed in 4% formaldehyde and stained with 0.1 % crystal violet. Cells were counted and migration was expressed as cell number per mm^2. Chemotaxis of T47D and MDA-MB231 cells were also analyzed by direct visualization of cell migration in a gradient of HRG using Dunn chambers (DCC100; Hawksley). Serum-starved cells were seeded onto a glass coverslip previously coated with 25ug μl^{-1} rat tail collagen I. Cells were inverted over the DCC chamber consisting of two concentric wells separated by an annular bridge. The outer well contained 10nM HRG in DMEM, whereas the inner well was filled with DMEM. A linear gradient of HRG was formed by diffusion across the bridge separating the two wells. Migration of the cells lying directly above the bridge was visualized using a Widefield TILL5, LONG RUN, Axiovert 200M (5 % CO2 and 37°C chamber). Time lapse images were digitally captured every 3 minutes with a CCD camera during 12 hours.

Cell adhesion assays

Cell adhesion assays were performed with WT and Memo-deficient MEFs. Microtitre plates (60wells; Nunc) were coated for 1 hour at room temperature with 5 μl per well PBS containing 0.01 % Tween and either 25μg ml^{-1} collagen I, fibronectin or 10 μg μl^{-1} laminin. Wells were then blocked for 30 minutes with PBS containing 0.025 % BSA. Cells were plated and allowed to attach for 45 minutes. Cells were then fixed with 4 % formaldehyde in PBS for 30 minutes at room temperature, and stained with 0.1 % crystal violet in H20 for 30 minutes. Photographs were taken with an Axioskop Zeiss Microscope equipped with a Sony 3 CDD camera.

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Figure legends

Figure 1. **Shc interacts with Memo and is required for Memo binding to ErbB2 receptor**. (A) WT and Shc-deficient MEFs were stimulated or not for the indicated periods of time with EGF. Western-blot was performed on extracts using P-AKT, AKT, P-ERK and ERK specific antibodies. (B) WT and Shc-deficient cells were stimulated or not with EGF for 10 minutes. (*Right panel*) Cell extracts were subjected to immunoprecipitation using a Memo mAb. IPs were probed for ErbB2, SHC and Memo. (*Left panel*) ErbB2 phosphorylated levels, as well as total ErbB2, SHC, and Memo levels in transfected cells were monitored in whole cell lysates (W) using the respective antibodies. (C) Shc-deficient MEFs were transfected with HA vector control, HAp46, HAp52 or HAp66 expressing constructs. Cell extracts were prepared and subjected to immunoprecipitation using a HA mAb or a Memo mAb. IPs were probed for Shc and Memo. Memo levels and Shc expression in WT control and Shc-deficient cells were monitored in whole cell lysates (W) using the respective antibodies.

Figure 2. **Effect of Memo on EGF-induced SHC phosphorylation.** WT and Memo-deficient MEFs were treated with EGF for the indicated periods of time. Western-blot was performed on extracts using P-SHC (Tyr 239/240) and SHC specific antibodies.

Figure 3. **Effect of Memo on EGF-induced Src phosphorylation.** (A) WT and Memo-deficient MEFs (*upper panel*) and T47D cells stably transfected with shLacZ control or shMemo 5.2 (*lower panel*) were stimulated or not with EGF or HRG, respectively, for the indicated periods of time. Western-blot was performed on extracts using P-Src (Tyr 416) and Memo specific antibodies.

Figure 4. **Effect of Memo on EGF-induced ERK and PLCγ1 phosphorylation.** (A and B) WT and Memo-deficient MEFs (*upper panel*) and T47D cells stably transfected with shLacZ control or shMemo 5.2 (*lower panel*) were stimulated or not with EGF or HRG, respectively, for the indicated periods of time. (A) Western-blot was performed on extracts using P-ERK (p42/44) and ERK (p42/44) specific antibodies. (B) (*Upper panel*) Western-blot was performed on WT and Memo-deficient MEF extracts using P-PLCγ1 (Tyr 783) and PLCγ1 specific antibodies. (*Lower panel*) Extracts from T47D cells transfected with shLacZ control or shMemo 5.2 cells were subjected to immunoprecipitation using a PLCγ1 mAb. PLCγ1 phosphorylated levels were monitored using P-Tyr and PLCγ1 antibodies.

Figure 5. **Effect of Memo on the cell adhesion and cell migration processes.** (A) Migration of WT and Memo-deficient MEFs was analyzed. Control responses in the absence of ligand, as well as chemotactic responses towards EGF and chemokinetic responses in the presence of EGF were determined in Boyden chamber assays. The data are representative of several independent experiments. Data are mean ± SEM. (B) WT and Memo-deficient cells were seeded onto coverslips coated with collagen type I, laminin or fibronectin and allowed to adhere and spread for 45 minutes before fixation. Adherent cells were stained and visualized. Images shown are representative of two separate experiments. (C) Immunofluorescence microscopy of EGF-stimulated WT and Memo-deficient MEFs labeled with a TRITC-labeled phalloidin antibody. (D) T47D cells were transiently transfected with LacZ or Memo siRNAs and 3 days later were analyzed for their ability to migrate in a gradient of HRG in Dunn chambers. Migration of the cells lying directly above the bridge was visualized during a period of 12 hours. The gradient follows the path from the top left to the bottom right. (*Upper panels*) Snapshot from representative time lapse videos taken at 8 hours are shown. Arrows indicate examples of the tails formed during the experiment. (*Lower panel*) Cells were scored for tail formation by counting >80 cells in each of the 10 time point acquisition. Data are mean ± SEM.

Figure 6. **Inhibition of Memo impairs regulation of focal adhesions.** (A) WT and Memo-deficient MEFs were stimulated with EGF for the indicated periods of time. Western-blot was performed on extracts using P-FAK (Tyr 397), FAK and Memo specific antibodies. (B) WT and Memo-deficient MEFs (*upper panel*) or T47D cells stably transfected with shLacZ control or shMemo 5.2 (*lower panel*) were stimulated for the indicated periods of time with

EGF or HRG, respectively. Extracts were collected and monitored for P-FAK (Tyr861) and FAK expression using the respective specific antibodies. (C and D) P-FAK (Tyr 397) (C) and P-Paxillin (D) stainings were examined in WT and Memo-deficient MEFs using the respective antibodies. (E) GST-RhoA pull down assay in WT and Memo-deficient MEFs stimulated or not for the indicated periods of time. Western-blot was performed using a specific RhoA antibody, and RhoA activation levels were quantified by densitometry. Data are representative of independent experiments.

Figure S1. **Memo interacts with the three Shc isoforms, p46^{shc}, p52 ^{shc}, or p66 ^{shc}.** T47D cells were stimulated or not with 10 nM HRG for 10 minutes. Memo was immunoprecipitated using a mouse mAb; a mouse mAb IgG2a was used as a negative control. Co-immunoprecipitating proteins were analyzed by western blotting and probed with Shc and Memo polyclonal antibodies. Whole cell lysates (W) were loaded as controls.

Figure S2. **Memo is required for rear cell deadhesion.** MDA-MB231 breast carcinoma cells were transiently transfected with LacZ or Memo siRNAs and 3 days later were analyzed for their ability to migrate in a gradient of HRG in Dunn chambers. Migration of the cells lying directly above the bridge was visualized during a period of 12 hours. (*Upper panels*) Snapshot from representative time lapse videos taken at 8 hours are shown. Arrows indicate examples of the tails formed during the experiment. (*Lower panels*) MDA-MB231 cells were transiently transfected with LacZ or Memo siRNAs and 3 days later were seeded on coverslips, and stimulated for 20 with HRG minutes before fixation. The actin cytoskeleton and cell morphology were visualized using a TRITC-labeled phalloidin antibody.

FIGURE 1.

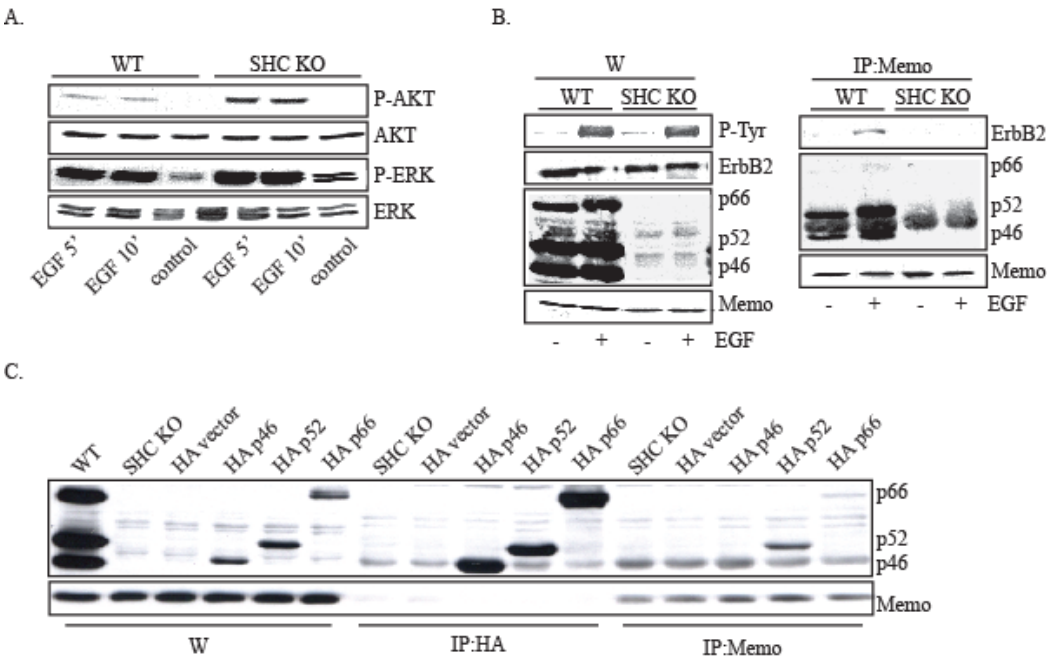

A.
WT
SHC KO
P-AKT
AKT
P-ERK
ERK
EGF 5'
EGF 10'
control
EGF 5'
EGF 10'
control
B.
W
WT
SHC KO
P-Tyr
ErbB2
p66
p52
p46
Memo
- + - + EGF
IP:Memo
WT
SHC KO
ErbB2
p66
p52
p46
Memo
- + - + EGF
C.
WT
SHC KO
HA vector
HA p46
HA p52
HA p66
SHC KO
HA vector
HA p46
HA p52
HA p66
SHC KO
HA vector
HA p46
HA p52
HA p66
p66
p52
p46
Memo
W
IP:HA
IP:Memo

FIGURE 2.

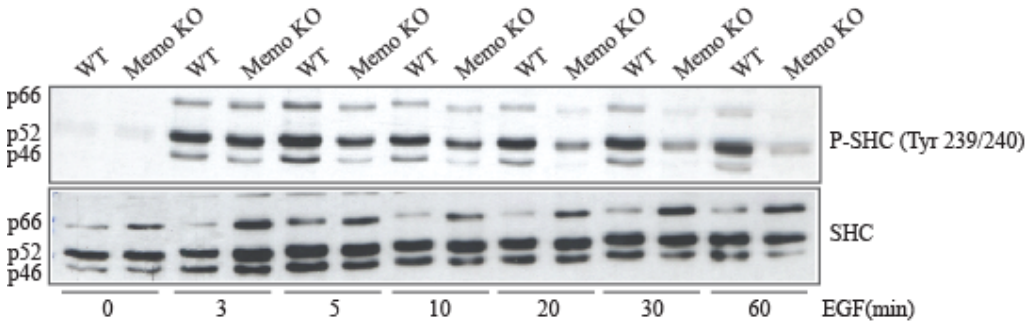

WT
Memo KO
WT
Memo KO
WT
Memo KO
WT
Memo KO
WT
Memo KO
WT
Memo KO
WT
Memo KO
p66
p52
p46
P-SHC (Tyr 239/240)
p66
p52
p46
SHC
0
3
5
10
20
30
60
EGF(min)

FIGURE 3.

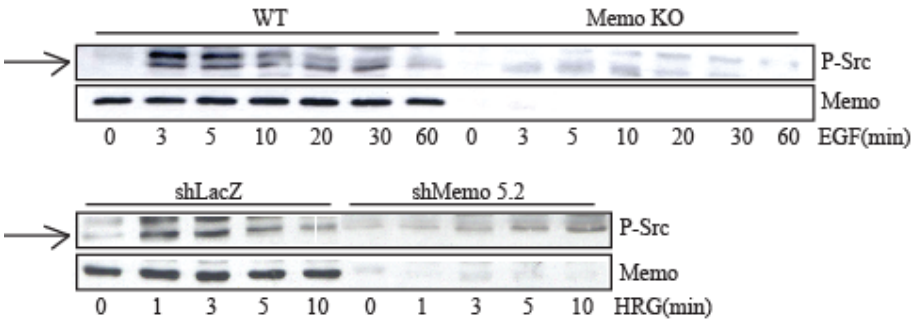

WT
Memo KO
P-Src
Memo
0 3 5 10 20 30 60 0 3 5 10 20 30 60 EGF(min)
shLacZ
shMemo 5.2
P-Src
Memo
0 1 3 5 10 0 1 3 5 10 HRG(min)

FIGURE 4.

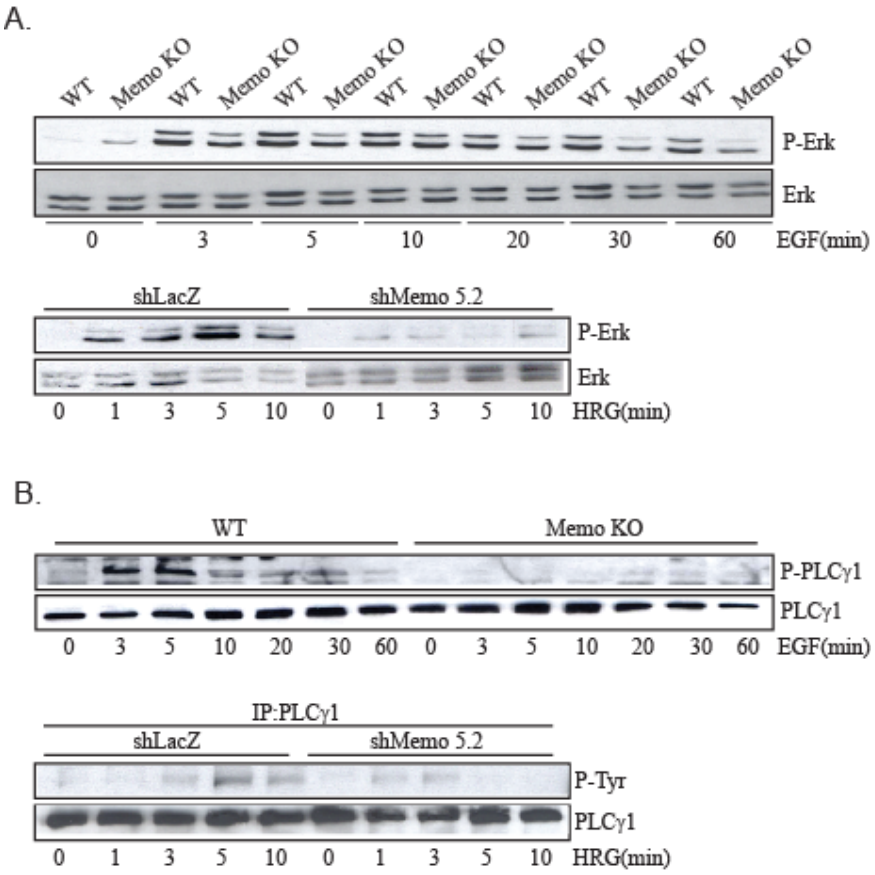

A.
WT
Memo KO
P-Erk
Erk
0 3 5 10 20 30 60 EGF(min)
shLacZ
shMemo 5.2
P-Erk
Erk
0 1 3 5 10 0 1 3 5 10 HRG(min)
B.
WT
Memo KO
P-PLCγ1
PLCγ1
0 3 5 10 20 30 60 0 3 5 10 20 30 60 EGF(min)
IP:PLCγ1
shLacZ
shMemo 5.2
P-Tyr
PLCγ1
0 1 3 5 10 0 1 3 5 10 HRG(min)

FIGURE 5.

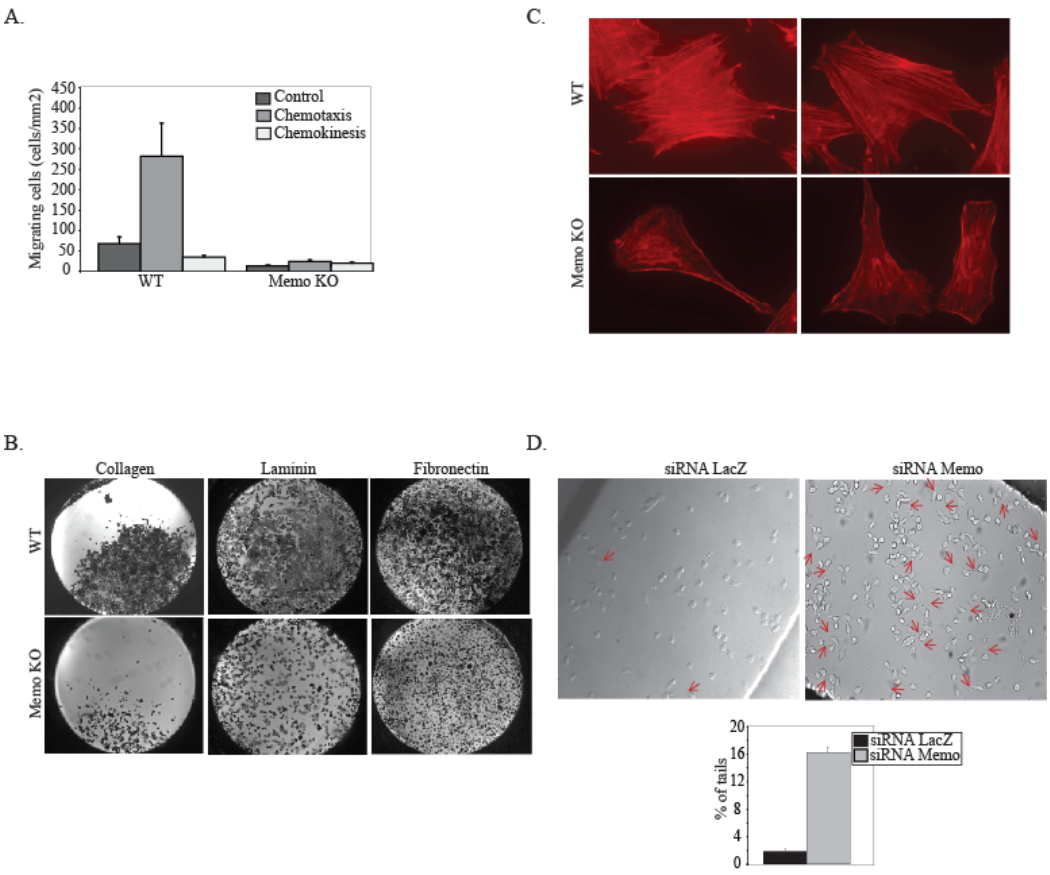

A.
Migrating cells (cells/mm2)
450
400
350
300
250
200
150
100
50
0
Control
Chemotaxis
Chemokinesis
WT
Memo KO
C.
WT
Memo KO
B.
Collagen
Laminin
Fibronectin
WT
Memo KO
D.
siRNA LacZ
siRNA Memo
% of tails
20
16
12
8
4
0
siRNA LacZ
siRNA Memo

FIGURE 6.

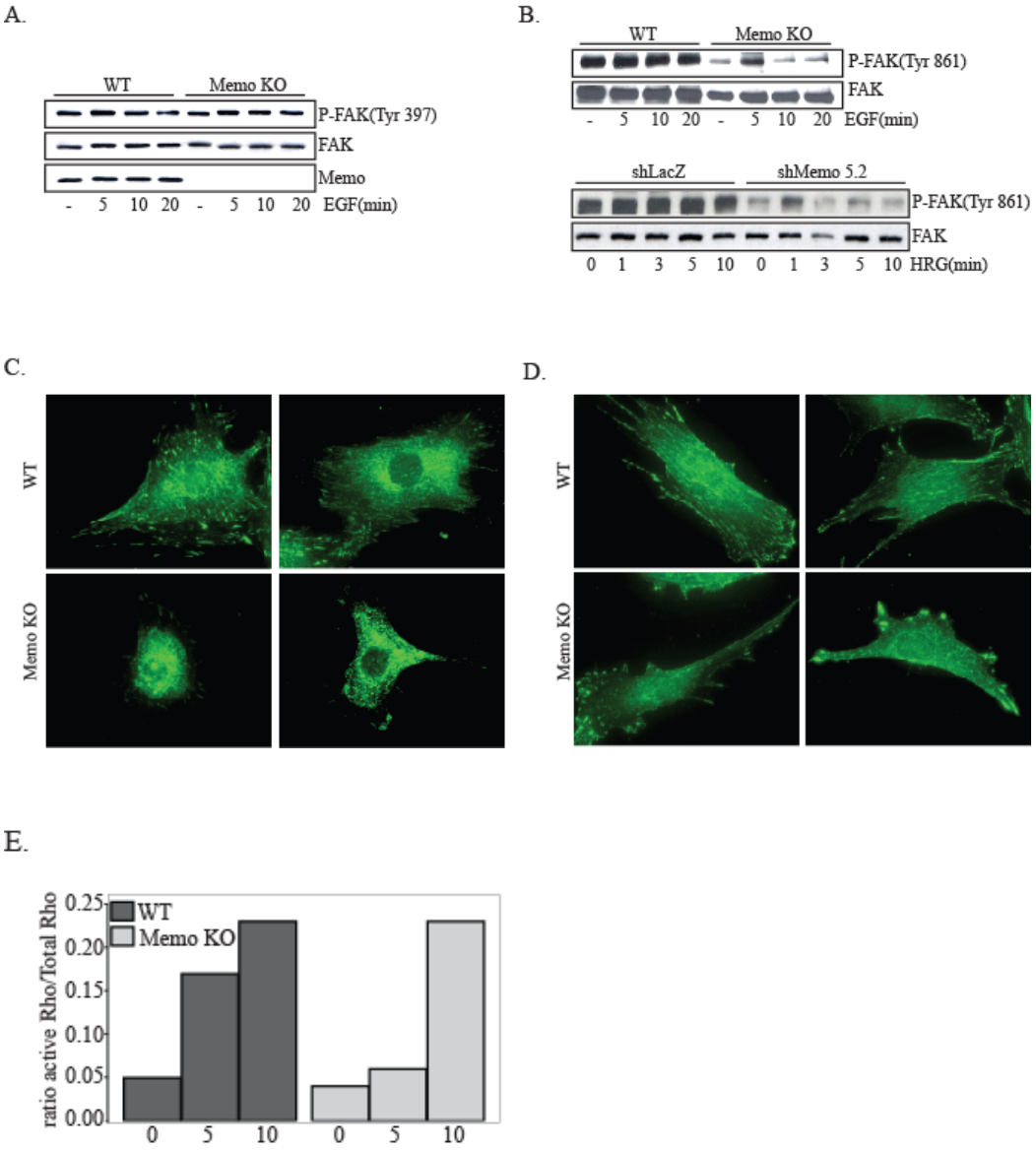

A.
WT
Memo KO
P-FAK(Tyr 397)
FAK
Memo
- 5 10 20 - 5 10 20 EGF(min)
B.
WT
Memo KO
P-FAK(Tyr 861)
FAK
- 5 10 20 - 5 10 20 EGF(min)
shLacZ
shMemo 5.2
P-FAK(Tyr 861)
FAK
0 1 3 5 10 0 1 3 5 10 HRG(min)
C.
WT
Memo KO
D.
WT
Memo KO
E.
ratio active Rho/Total Rho
0.25
0.20
0.15
0.10
0.05
0.00
WT
Memo KO
0 5 10 0 5 10

FIGURE S1.

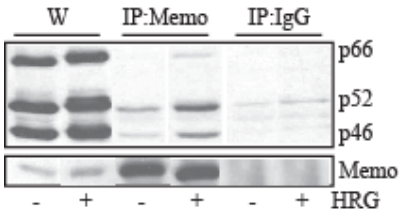
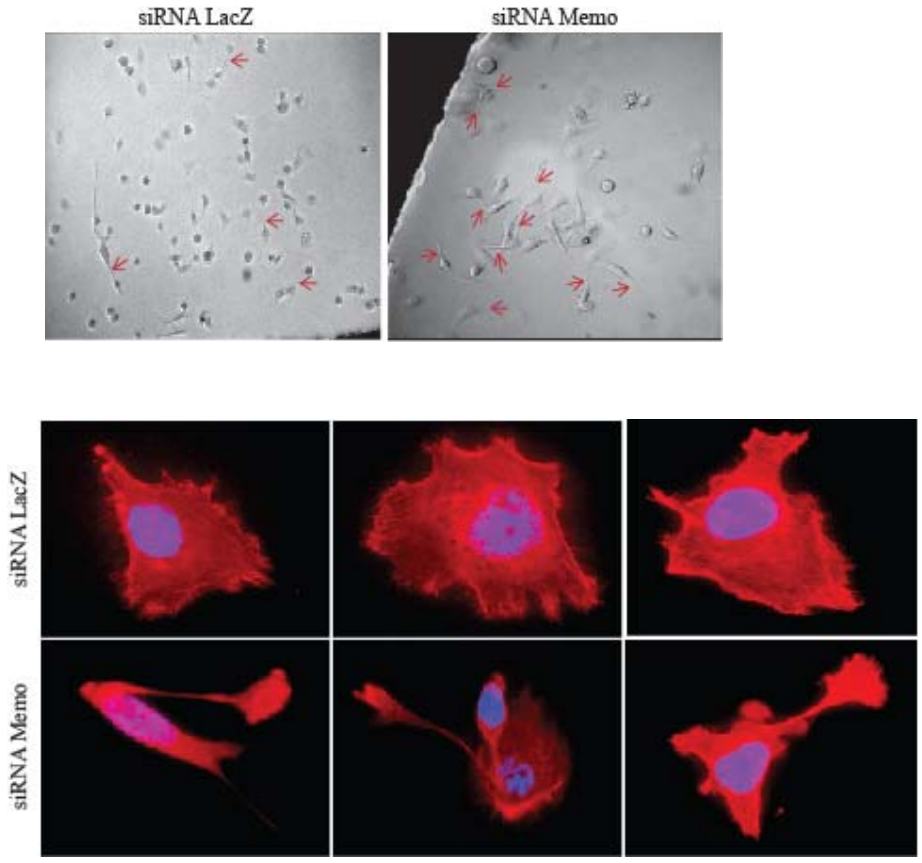


FIGURE S2.



----- III. Memo in a model organism- *S.cerevisiae* -----

1. INTRODUCTION

Genetic approaches in powerful molecular models such as *Saccharomyces cerevisiae* have been useful for gaining insight into the function of evolutionary conserved proteins. In fact, complex processes such as chromosome replication, transcription and translation, cell division, secretion, membrane trafficking, energy metabolism, cytoskeletal structure and mechanics, and intracellular signaling that occur in all eukaryotes can be explored in detail in this well-developed and simple-to-use genetic system which contains a nucleus and membrane bound organelles like mitochondria, peroxisomes, endoplasmic reticulum, and a Golgi complex.

To position Memo within a genetic network, experiments in the model organism *S. cerevisiae* that lends itself to rapid genetic screening were performed. *S. cerevisiae* has one homologue of the human *MEMO* gene, ***YJR008W.*** According to SGD (Saccharomyces Genome Database), *YJR008W* codes for the protein Yjr008wp, a putative protein of unknown function with 338 amino acids. We will refer to the gene and protein as *MEMO* (*ScMEMO*) and Memo (ScMemo), respectively. The yeast and the human proteins share over 50% sequence similarity and about 35% sequence identity.

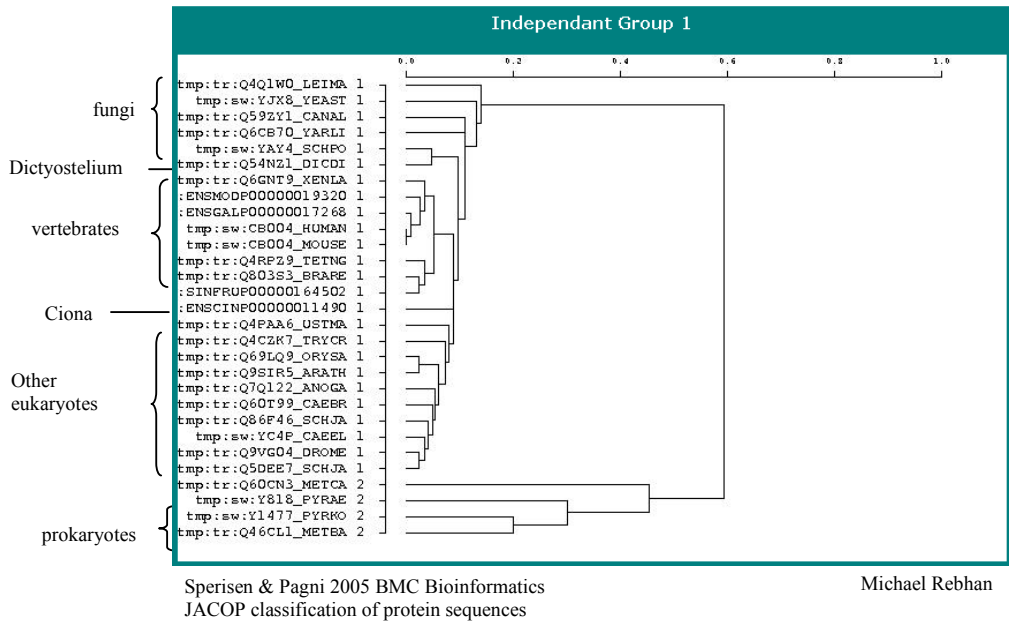


Figure 1. Memo is encoded by a single-copy gene present in all major taxonomic groups

2. RESULTS

2.1. Memo localization and function in S. cerevisiae

(In collaboration with Dr. Dominic Hoepfner and Dr. Peter Philippsen, Biozentrum, BASEL)

All experiments have been done according to the EUROFAN guidelines for gene deletions and modifications in *Saccharomyces cerevisiae*. The materials and methods used in this study are described in paragraph 2.1.4.

Auxotrophic Markers	GFP Labels	Deletions
his3Δ200 trp1Δ63 leu2Δ1 ura3-52Δ1	MEMO-GFP::kanMX4	
his3Δ200 trp1Δ63 leu2Δ1 ura3-52Δ1	kanMX4::GAL1/10- GFP-MEMO	
his3Δ200 trp1Δ63 leu2Δ1 ura3-52Δ1		memoΔ1::klTRP1
HIS3 trp1Δ63 LEU2 ura3-52Δ1	HHF2-GFP::His3MX6	memoΔ1::klTRP1
his3Δ200 trp1Δ63 leu2Δ1 ura3-52Δ1	promTUB1-GFP-TUB1::URA3int (pAFS125)	memoΔ1::klTRP1
his3Δ200 trp1Δ63 leu2Δ1 ura3-52Δ1	CAP2-YFP::His3MX6 promHIS3-CFP-PTS1::URA3 (pEW177)	memoΔ1::klTRP1
his3Δ200 trp1Δ63 leu2Δ1 ura3-52Δ1	ABP140-YFP::His3MX6	memoΔ1::klTRP1
his3Δ200 trp1Δ63 LEU2 ura3-52Δ1	MEMO-YFP::His3MX6	
his3Δ200 trp1Δ63 LEU2 ura3-52Δ1		memoΔ1::klTRP1
HIS3 trp1Δ63 leu2Δ1 ura3-52Δ1		memoΔ1::klTRP1
HIS3/his3Δ200 trp1Δ63/trp1Δ63 LEU2/ leu2Δ1 ura3-52Δ1/ ura3-52Δ1		memoΔ1::klTRP1/memoΔ1::klTRP1

Table 1. Yeast strains (all are FY1679 background).

2.1.1. Memo localization study

To examine the localization of Memo in yeast, in-frame fusion proteins of the green or yellow fluorescent protein (GFP/YFP) ORFs to that encoding Memo were constructed. Memo was either expressed under its own promoter and fused upstream of the GFP/YFP ORFs, or under the Galactose inducible Gal1/10 promoter and fused downstream of the GFP ORF (Figure 2). GFP/YFP fusions were introduced into FY1679 cells, and transformants (Table 1) were analyzed for Memo subcellular localization using fluorescence microscopy. When expressed under its own promoter, some cytoplasmic and strong nuclear Memo-GFP/YFP fluorescence were observed (Figure 2a). Overexpression of Memo from the inducible *GAL* promoter resulted in cells that don't exhibit GFP fluorescence when grown on Glucose, whereas growth on Galactose induced Memo-GFP expression in the cytoplasm and a particular intense nuclear fluorescence (Figure 2b). These results suggest possible nuclear and cytoplasmic functions of Memo in yeast.

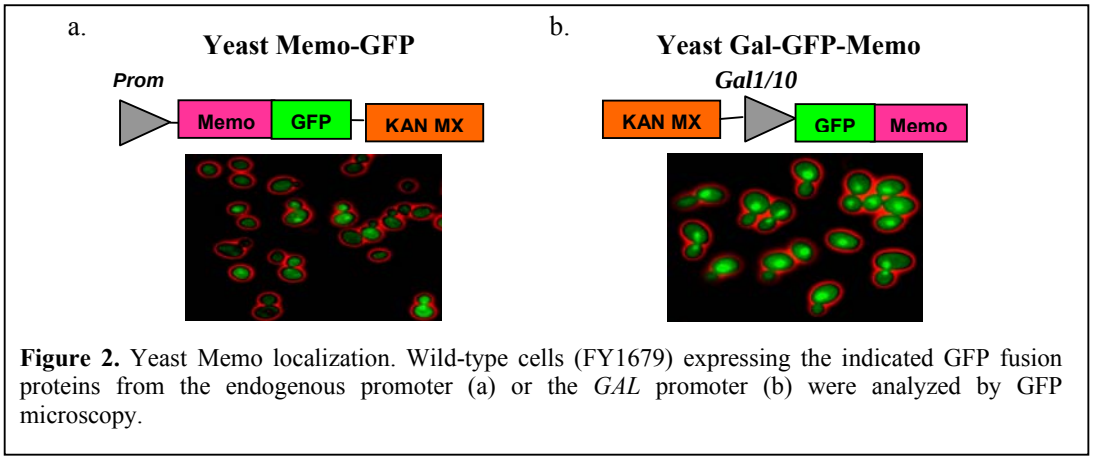


Figure 2. Yeast Memo localization. Wild-type cells (FY1679) expressing the indicated GFP fusion proteins from the endogenous promoter (a) or the *GAL* promoter (b) were analyzed by GFP microscopy.

2.1.2. Memo-GFP expression study

Only a certain percentage of GFP::kanMX-tagged cells showed detectable signal and only few accumulated Memo in the nucleus as observed in the overexpressing cells. Also, when checking the YFP tagged cells on the primary transformation plates, all cells showed clear cytoplasmic and nuclear Memo localization. But once replated on full YPD medium, Memo-YFP was no longer detectable. When the cells were plated back from the YPD to synthetic plates, Memo was again detected in all cells but only in some cells in the nucleus. These results suggest that i) Memo is only expressed under certain conditions, and that ii) Memo shuttles between cytoplasmic and nucleus and only accumulates in the nucleus upon a certain stimulus

In order to study in more detail Memo's function, and find out what induces the expression of Memo, particularly on the synthetic plates, we examined the expression of Memo-GFP transformants under different conditions of culture (pH, stress, N-source, temperature, osmolarity) (Figure 3).

Media and conditions	Memo-GFP expression
YPGal pH6.0 30°C	-
YPGluc pH6.0 30°C	-
YPGluc pH6.0 37°C	+
YPGluc pH6.0 42°C	+
YPGluc pH6.0 30°C stationary phase culture	++
YPGluc pH6.0 30°C 12mM Caffeine	+
YPGluc pH6.0 30°C 0.04% SDS	++++
YPGluc pH6.0 30°C 0.1M NaCl	-
YNB (NH4)2SO4 all AS, Glc	++++
YNB (NH4)2SO4 all AS, Gal	++++
YNB (NH4)2SO4 1/2x all AS, Glc	++++
YNB MSG all AS, Glc	++++
YNB CO(NH2)2 all AS, Glc	++++
YPGluc pH3.0 30°C	++++
YPGluc pH4.0 30°C	++
YPGluc pH5.0 30°C	-
YPGluc pH6.0 30°C	-
YPGluc pH7.0 30°C	+
YPGluc pH8.0 30°C	++
YPGluc pH9.0 30°C	++++
YPGluc pH10.0 30°C	++++

- Not expressed
+ Weakly expressed
++ Expressed
++++ Strongly expressed

Figure 3. Memo-GFP expression. FY1679 transformants were analyzed for Memo's expression levels under different culture conditions.

Memo appears to be strongly expressed in diverse stress conditions such as synthetic media, pH, and temperature. However, no individual triggering event for the induction of Memo's expression was uncovered. According to the expression data, Memo appears to be rather a general stress responder of unknown function.

2.1.3. memoΔ phenotype study

To gain more insight into the function of Memo in yeast, we carried out gene disruption experiments that replace Memo's sequence in the genome with the selectable marker gene *TRP1* (Figure 4), resulting in Memo's deletion. This process occurs by homologous recombination and uses the enzymes of the homologous recombination pathway.

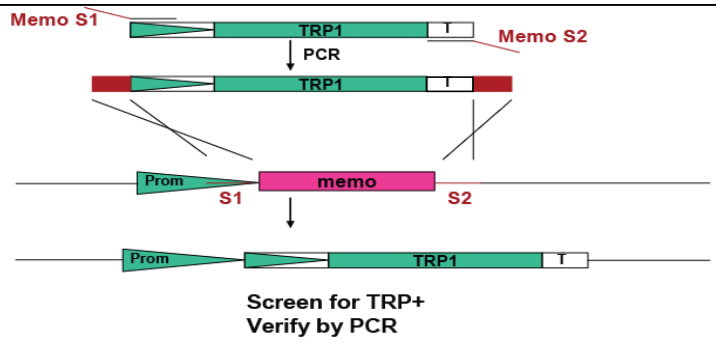


Figure 4. Memo disruption/deletion strategy.

*S. cerevisiae memo*Δ strains (haploïd a, α or diploïd a/α) grown at 30°C or 37°C were viable and showed no obvious phenotype compared to wild-type (WT) strains. We further scored *memo*Δ strains for their growth speed, pH sensitivity, sporulation efficiency, and sensitivity to several reagents such as Rapamycin, Caféine, SDS, MMS, Cobalt Chloride and Hydroxyurea (HU). No obvious differences were observed in *memo*Δ strains compared to WT strains in any condition. Cell polarity is important not only during growth and division, but also during the mating response, shmoo formation and cell fusion. *memoΔ* cells were tested in appropriate assays for each and were found to behave as WT strains.

To examine the role of Memo in cellular processes such as cell division, actin/microtubule dynamics and metabolite transport, we generated *memo*Δ strains in which nuclei (HHF2), microtubules (TUB1), actin patches (CAP2), actin cables (ABP140), and peroxisomes (PTS1) were GFP/YFP or CFP labeled (Table1). Since expression of Memo was found to be induced upon stress conditions (SD medium or alkalinized medium), yeast strains were cultured at 30°C in different media conditions: YPD, SD-medium or at pH4.0. Considering the known role of mammalian Memo in the microtubule and actin cytoskeleton, we carefully examined the *memo*Δ strain for defects in these networks. However, *memo*Δ microtubule and actin cable/patches morphology and distribution were indistinguishable from WT strains. Peroxisomes are ubiquitous subcellular organelles, which are highly dynamic and display large plasticity in response to cellular and environmental conditions (Visser et al., 2007). We examined peroxisome morphology and distribution in *memo*Δ and WT cells. No obvious difference was detected. Also, nuclear distribution was not affected in *memo*Δ cells in any of the culture conditions. Altogether, these results do not support a role for Memo in cytoskeletal organization in yeast, and indicate that Memo is rather involved in a general stress response.

2.1.4. Materials and methods used in this study

YPGlc liquid: 10g/l Yeast Extract (Oxoid), 20g/l Bacto Peptone (Becton Dickinson), 20g/l Glucose (Fluka); YPGal liquid: 10g/l Yeast Extract (Oxoid),20g/l Bacto Peptone (Becton Dickinson), 40g/l Galacotose (Fluka); Synthetic medium liquid: 6.7g/l Yeast Nitrogen Base w/o Amino Acids (Becton Dickinson), 0.69g/l CSM complete (Bio101), 20g/l Glucose (Fluka); Synthetic medium liquid, special Nitrogen Source: 1.7g/l Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Becton Dickinson), 1g/l Monosodium Glutamate or 1/gl, Urea, or 1g/l Glutamine, 0.69g/l CSM complete (Bio101), 20g/l Glucose (Fluka); YPGlc plates: 10g/l Yeast Extract (Oxoid), 20g/l Bacto Peptone (Becton Dickinson), 15g/l Bacto Agar (Becton Dickinson), 20g/l Glucose (Fluka); YPGal plates: 10g/l Yeast Extract (Oxoid), 20g/l Bacto Peptone (Becton Dickinson), 15g/l Bacto Agar (Becton Dickinson), 40g/l Galactose (Fluka).

To test for pH sensitivity the YPGlc and YPGal medium was acidified with 37% HCL. To make it alkaline, 10M NaOH was used. To test for SDS sensitivity, SDS in aqueous solution from a 10% stock was diluted and added to luke-warm YPGlc+Agar and YPGal+Agar medium prior to pouring the plates. To test for caffeine sensitivity, caffeine in aqueous solution from a 0.1M stock was diluted and added to luke-warm YPGlc+Agar and YPGal+Agar medium prior to pouring the plates. 800µM Cobalt Chloride, or 0.03% MMS, or 200mM HU were added to YPD and SD medium prior pouring the plates.

For sporulation the cells were grown in liquid presporulation medium (8g/l Yeast extract, 3g/l Peptone,100g/l Glucose) for 8 hours, washed twice and then plated on sporulation plates (10g/l Kac, 1g/l Yeast extract, 0.5g/l Glucose, 15g/l Agar) and incubated at 20° for 4-10 days.

Rapamycin sensitivity was tested using a stock solution (kindly provided by Mike Hall) of 1mg/ml Rapamycin in 90%EtOH/10%Tween-20. The drug was diluted 1/100 in 90%EtOH/10%Tween-20 and appropriate amounts were dissolved in luke-warm, sterile YPGlc+Agar or YPGal+Agar prior to pouring the plates. Sensitivity of the tester strain was compared to a WT control as well as Rapamycin hypersensitive and resistant control strains also provided by Mike Hall.

2.2. Synthetic lethal screen

(In collaboration with Dr. Marc Sohrmann and Dr. Matthias Peter,ETH, Zurich)

The study of genetic interactions is useful to characterize gene functions and to dissect pathway structures. Synthetic lethal mutants are often used to identify genetic interactions (Bender and Pringle, 1991; Guarente, 1993; Appling, 1999; Forsburg, 2001). The goal is to identify a double mutant combination that is inviable when both of the single mutants A and B alone are viable. A synthetic lethal screen can be performed with a deletion mutation in a nonessential gene or with a mutation in an essential gene that does not eliminate function. The interaction is assumed to reflect the compensatory relationship between the two genes. This strategy can be particularly useful for uncovering redundant pathways or direct protein interactions. In fact, synthetic lethality can indicate that A and B encode proteins that perform redundant functions in an essential pathway, such that mutation of both genes is necessary to eliminate the pathway. A second possibility is that the A and B gene products perform discrete steps in the same pathway. This would be more likely for non null mutations, where each protein retains partial function. The pathway can still function with reduced activity at one step, but ceases to function when two steps have reduced activity. Neither of these mechanisms implies a physical protein–protein interaction between A and B. In a third possible mechanism, a mutation in A weakens, but does not eliminate, its interaction with B. A mutation in B that further weakens the required interaction might then be synthetically lethal with the first mutation in A.

2.2.1 Synthetic lethal screen strategy

MEMO was identified in our *memo*Δ phenotype study as a nonessential gene since its deletion results in a viable mutant strain. We therefore performed a high-throughput synthetic lethal analysis using an assembled ordered array of ~4700 viable yeast gene-deletion mutants (Winzeler et al., 1999). Series of pinning procedures in which mating and meiotic recombination are used to generate haploid double mutants were performed. A query mutation, in our case *MEMO* deletion, was first generated in a haploid starting strain of mating type *MAT*α (Figure 5).

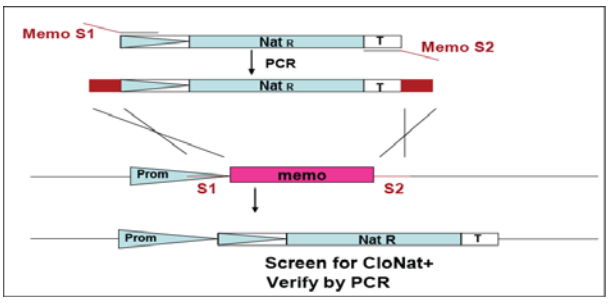
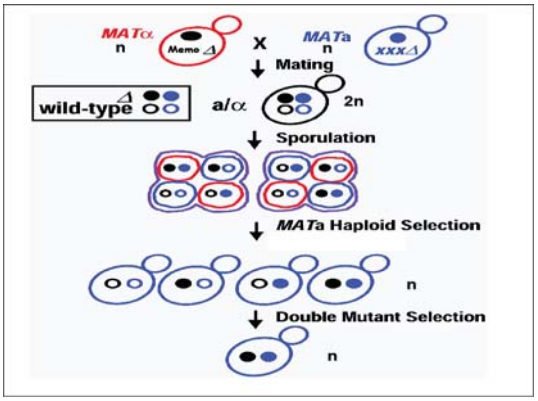


Figure 5. Memo disruption/deletion strategy for the synthetic lethal screen

The haploid *memo*Δ strain *MAT*α was then crossed to the array of gene-deletion mutants of the opposite mating type, *MAT***a** (Figure 6) using a robotic system for manipulation of high-density yeast arrays (Figure 7). Sporulation of resultant diploid cells leads to the formation of double-mutant meiotic progeny. The *MAT*α starting strain carries a reporter, *MFA1pr-HIS3*, that is only expressed in *MAT***a** cells and allows for germination of *MAT***a** meiotic progeny. Both the query mutation and the gene-deletion mutations were linked to dominant selectable markers (CloNAT or nourseothricin for MEMO, G418 for genes in the array) to allow for selection of double mutants. Final pinning results in an ordered array of double-mutant haploid strains whose growth rate is monitored by visual inspection or image analysis of colony size. To ensure reproducibility within a screen and to facilitate visual scoring, the deletion strains were arrayed in pairs. All materials and protocols were kindly provided by Dr. M. Peter, ETH Zurich.



Adapted from Tong et al., 2001

Figure 6. Synthetic lethal screen methodology. A *MAT*α strain carrying a query mutation (*memo*Δ) linked to a dominant selectable marker, such as the nourseothricin-resistance marker *natMX* that confers resistance to the antibiotic nourseothricin, and an *MFA1pr-HIS3* reporter is crossed to an ordered array of *MAT***a** viable yeast deletion mutants, each carrying a gene deletion mutation linked to a kanamycin-resistance marker (*kanMX*). Growth of resultant heterozygous diploids is selected for on medium containing nourseothricin and kanamycin. The heterozygous diploids are transferred to medium with reduced levels of carbon and nitrogen to induce sporulation and the formation of haploid meiotic spore progeny. Spores are transferred to synthetic medium lacking histidine, which allows for selective germination of *MAT***a** meiotic progeny because these cells express the *MFA1pr-HIS3* reporter specifically. The *MAT***a** meiotic progeny are transferred to medium that contains both nourseothricin and kanamycin, which then selects for growth of double-mutant meiotic progeny.

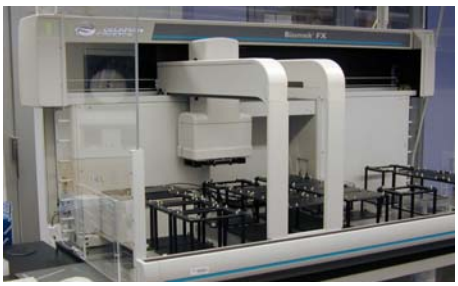


Figure 7. Robotic system for manipulation of high-density yeast arrays.

2.2.2. Synthetic lethal screen results

The analysis of genetic interactions between the *memo*Δ strain and ~ 4,700 viable gene deletion mutants revealed lethality with a *plc1*Δ strain (*PLC1:YPL268W*) (Figure 8). Indeed, the double-mutant cells *memo*Δplc Δ failed to grow, forming residual colonies that were relatively smaller than the equivalent colony on the wild-type control plate. Of note is the fact that the single plc Δ mutant strain exhibits a growth defect as reported previously (Yoko-o et al., 1993). Also, when the query mutation was identical to one of the gene deletions within the array, double mutants could not form because haploids carry a single copy of each allele; therefore, *memo*Δ appeared synthetic lethal with itself (Figure 8).

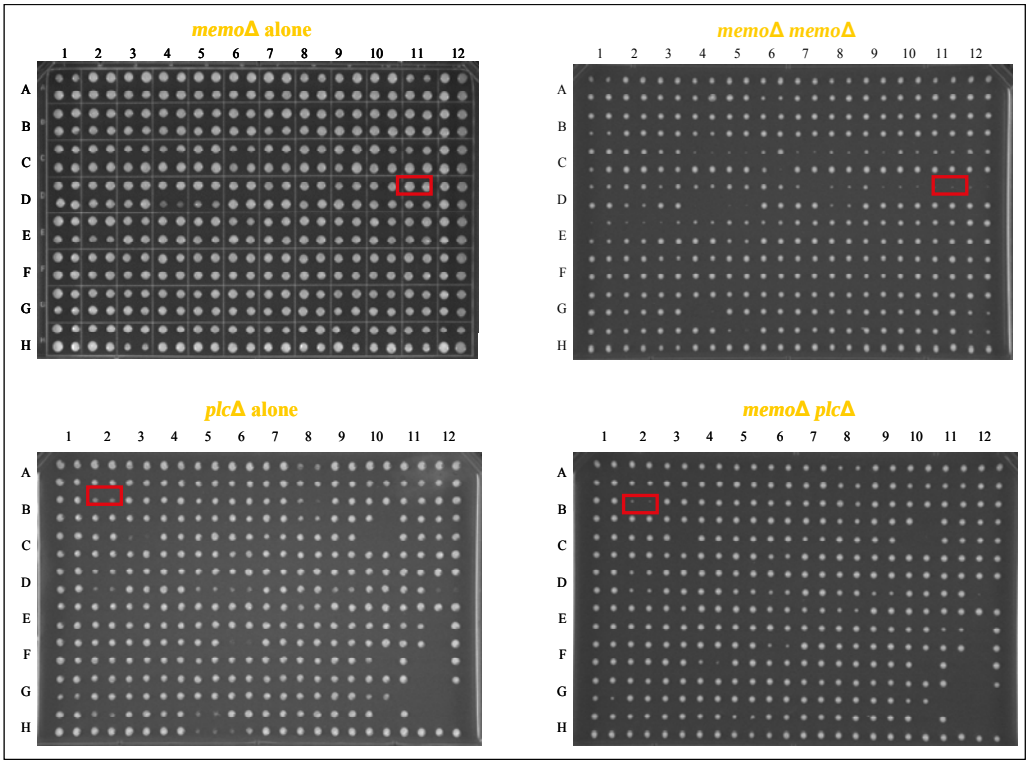


Figure 8. *MEMO* and *PLC1* are synthetic lethal. For each pinning, duplicates are shown. (Left panels, red square) *memo*Δ strain and *plc1*Δ strain are viable. (Right panels, red squares) *memo*Δ*memo*Δ and *memo*Δ *plc1*Δ strains are inviable.

PLC1 encodes the single phosphoinositides-specific phospholipase C of yeast. In mammalian cells there are multiple β-, γ-, and δ-PLC isoforms; the one found in yeast closely resembles the human δ- isoforms of PLC. PLC1p hyrdolyzes phosphatidylinositol 4-5-biphosphate (PIP2) to generate inositol 1, 4, 5-triphosphate (IP3) and 1, 2-diacylglycerol (DAG) (Yoko-o et al., 1993; Flick and Thorner, 1993). PLC1p is also involved in kinetochore function and pseudohyphal differentiation (DeLillo et al., 2003; Ansari et al., 1999). In most yeast strains,

the *PLC1* gene is not essential for viability at 25 °C, but Plc1p-deficient mutants arrest at temperatures above 35 °C as multi-budded enlarged cells unable to complete cytokinesis, they are sensitive against osmotic stress and nitrogen starvation, they do not sporulate as homozygous diploids, and they are defective in the utilization of non-fermentable carbon sources, suggesting that the hydrolysis of PIP_2 is required for a number of nutritional and stress-related responses (Flick and Thorner, 1993; Flick and Thorner, 1998).

These results are intriguing considering that mammalian PLCγ1 and Memo are found in a complex, and that Memo regulates PLCγ1 phosphorylation. Also, both PLCγ1 and Memo were shown to have essential roles in tumor cell motility (Meira et al., submitted). We hypothesize that in yeast, Memo and Plc1p act in the same or in distinct but compensatory pathways.

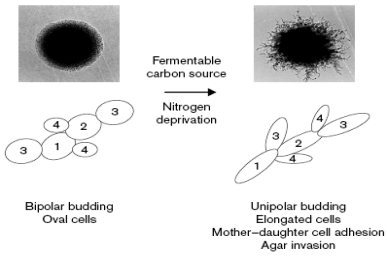
The synthetic lethal also identified a number of double mutants that were associated with a slower growth rate or "synthetic sick" phenotype, reflecting reduced fitness of the double mutant relative to the respective single mutants (Table 2).

GENE	**FUNCTION**
IMP2'/YIL154C	Transcriptional activator involved in maintenance of ion homeostasis and protection against DNA damage (Masson and Ramotar, 1996).
RAS2/YNL098C	GTP-binding protein that regulates the nitrogen starvation response, sporulation, and filamentous growth; localization to plasma membrane; homolog of mammalian Ras proto-oncogenes (Gimeno et al., 1992; Bhattacharya et al., 1995; Kataoka et al.,1984).
CAT5/YOR125C	Mitochondrial inner membrane protein directly involved in ubiquinone biosynthesis, essential for several other metabolic pathways including respiration (Jonassen et al., 1998; Marbois and Clarke, 1996).
MDG1/YNL173C	Plasma membrane protein involved in G-protein mediated pheromone signaling pathway (Leberer et al., 1996).
PPT2/YPL148C	Phosphopantetheine:protein transferase (PPTase), activates mitochondrial acyl carrier protein (Acp1p) (Stuible et al., 1998).
COQ5/YML110C	Putative mitochondrial C-methyltransferase, ubiquinone metabolism,mitochondrion (Dibrov et al., 1997; Barkovich et al., 1997).
PET8/YNL003C	Member of the mitochondrial carrier family (Marrobio et al., 2003).

Table 2. Synthetic sick mutants of Memo

2.3. Memo in filamentous growth

The polarity of cell division is critical in determining the size and shape of organisms. A cell that undergoes polarized cell division specifically orients its division axis or plane of division with respect to some reference point. The yeast *S. cerevisiae* divides mitotically by budding (Hartwell et al., 1974). A dimorphic transition in the life cycle of *S. cerevisiae* was described and called pseudohyphal growth (Gimeno et al., 1992). A pseudohypha is defined as a "fragile chain of cells which have arisen by budding and have elongated without detaching from adjacent cells, with morphological characteristics intermediate between a chain of yeast cells and a hypha" (Figure 9).



Adapted from Pan et al.,2000

Figure 9. Pseudohypahl growth Diploid cells of the yeast *Saccharomyces cerevisiae* undergo pseudohyphal differentiation in response to nitrogen limitation. In response to nitrogen-limiting conditions, diploid cells of *S. cerevisiae* change their growth pattern: the cells elongate and switch from bipolar to unipolar budding, the mother and daughter cells remain physically attached, and the cells invade the growth substrate. As a result, filamentous pseudohyphal colonies are formed.

This dimorphic transition is induced by starvation for a nitrogen source and is controlled by the RAS signal transduction pathway (Gimeno et al., 1992; Lorenz and Heitman, 1997). The enzyme phospholipaseC (Plc1p) was also shown to function with Gpr1 and Gpa2 to regulate yeast filamentous growth (Ansari et al., 1999). Given that genetic interactions of *MEMO* with *PLC1* and *RAS2* genes were identified in our synthetic lethal screen, we scored *memo*Δ strains for their ability to induce pseudohyphal differentiation under nitrogen starvation Figure 10). Nitrogen-limiting (SLAD) medium was used to allow diploid cells of the Σ1278b strain background to undergo pseudohyphal differentiation. MLY40 and MLY41 and MLY61 yeast strains were kindly provided by Dr. J. Heitman and described (Lorenz and al., 2000). *memo*Δ strains were generated and diploids were grown on SLAD medium containing only 0.05mM ammonium sulfate as sole nitrogen source. As shown in Figure 10, the transition from unpolarized colonial growth to pseudohyphal growth occurred on agar-based synthetic growth medium deficient in nitrogen in both Wild Type and and *memo*Δ strains, indicating that Memo is not involved in pseudohyphal differentiation in yeast.

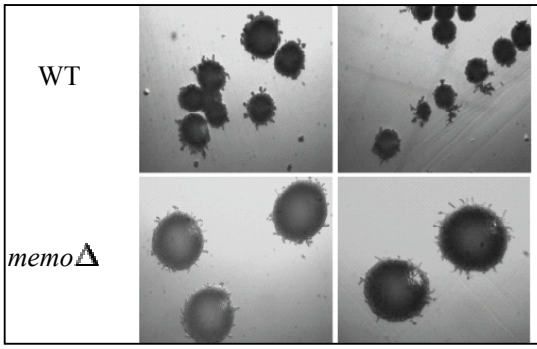


Figure 10. Memo does not control pseudohyphal growth. Wild-type cells (MLY61a/α) or *memo*Δ strains were grown on SLAD medium at 30°C for 5 days. Colonies were photographed.

3. CONCLUSIONS AND PERSPECTIVES

The expression data suggested that Memo is a general stress responder, however, no specific function was uncovered for Memo. These results showing that stress conditions lead to elevated levels of expression of Memo will be explored in more detail in different culture conditions (log-phase versus stationary phase) and using diverse drugs/reagents. By performing a synthetic lethal screen, we identified a genetic interaction between *MEMO* and *PLC1*. This result could reflect different possibilities; either Memo and Plc1p perform redundant functions in an essential pathway or Memo and Plc1p perform discrete steps in the same pathway. Interestingly, in mammalian cells, complexes of Memo and PLCγ1 were detected, and Memo was found to be required for efficient PLCγ1 phosphorylation, suggesting that Memo has a role in efficient PLCγ1 activation (Meira et al., in revision). Furthermore, Memo or PLCγ1 down regulation in breast tumor cells resulted in a strong impairment of cell directionality during cell migration; however, simultaneous down-regulation of Memo and PLCγ1 led to a stronger phenotype with a total blockade of cell movement and increased formation of actin stress fibers. These results indicate that, similarly to their homologues in yeast, mammalian Memo and PLCγ1 "cooperate" in essential steps in migration. To test a potential rescue of the memoΔplcΔ synthetic lethal interaction, yeast Memo will be replaced with the human Memo. Future experiments also include the use of the efficient inhibitor of the yeast Plc1p, 3-Nitrocoumarin (Tisi et al., 2001), to determine a potential effect of PLC1p activity on Memo localization. Furthermore, experiments in mammalian cells suggested that both Memo and PLCγ1 lie upstream of cofilin in models of ErbB2-driven cell motility, and Memo was found to bind directly to cofilin (Meira et al., submitted). Yeast *cofilin* is functionally similar to its human homolog, and binds to both monomeric and filamentous actin, promoting actin filament depolymerization/severing function (Theriot, 1997). *COF1* is an essential yeast gene (Iida et al., 1993; Moon et al., 1993). Its protein product, cofilin, is localized to the cortical patches, small, rapidly moving, actin-rich structures usually concentrated at sites of cell surface growth in budding yeast (Moon et al., 1993). Although no evidence was found for a role of Memo in actin patch distribution and actin cables formation/distribution, we can not exclude a link between Memo and cofilin in yeast. Currently, there is not much information on interacting proteins for yeast Memo. Only one candidate, Arp1, has been identified in a yeast high -throughput two hybrid screen (Ito et al., 2001). Arp1 is an actin-related protein of the dynactin complex that is required for spindle orientation and nuclear migration (Muhua et al., 1994). This interaction was confirmed in mammalian systems (data not shown). Interestingly, mammalian Memo was found to be required for microtubule cytoskeletal organization. However, *memoΔ* strains did

not exhibit any defect in microtubule distribution or nuclear distribution, suggesting that Memo does not influences Arp1 function. In order to confirm the Memo-Arp1 interaction and identify other Memo-interacting proteins, another technique will be used where Memo is expressed in the yeast strain with a C-terminus TAPtag (Tandem Affinity Purification tag). TAPtagged-Memo will be purified and complexed proteins will be processed by mass spectrometry analysis. Considering that expression of Memo is enhanced upon stress, Memo-complexed proteins content will be examined in different environmental conditions. Furthermore, to examine potential differences in binding partners, TAP-tagged-Plc1p will be expressed in the *memoΔ* strain or inversely, TAP-tagged-Memo will be expressed in *plc1Δ* strain. A TAP-tagged-cofilin construct will be also generated in order to test cofilin-Memo interaction in yeast. Finally, we will have a closer look at the "synthetic sick" candidates which have been identified in the yeast-two hybrid screen, in particular at the candidates associated with the mitochondria function and stress response in order to characterize a potential role of Memo in mitochondria.

Genetic approaches in models such as S. *cerevisiae* are powerful tools to study the function of evolutionary conserved proteins. Memo homologs are present in all species, from bacteria to human. We hope that the rapid genetic approaches that are possible in yeast will advance our knowledge of Memo's cellular and molecular role.

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----------APPENDIX----------

CANDIDATE	Accesion number
Acetyl coenzyme A acyltransferase	NM_006111
Casein kinase 1 epsilon	BC006490
Cofilin-1	BC012318
Crystallin lamda	BC008562
Developmentally regulated RNA-binding protein 1	AB036991
Ets-like protein	AC069287
Eukaryotic translation elongation factor 1α1	BC029343
FAD-synthetase	BC032323
FANCC-interacting protein	AF130255
Fc receptor related protein X	AF531423
Fc receptor.like and mucin-like	NM_032738
Ferritin	NM_002032
Glucosamine 6 phosphate deaminase	NM_005471
Gluthatione reductase	AY338490
Growth inducible transmembrane protein	NM_014394
Hemidesmosomal tetraspanin	NM_139030
Hexokinase 1	BC008730
Homo sapiens chaperonin containing TCP1, subunit 2 (beta)	NM_006431
HSPC041 protein	AF125102
H-unc-like protein	BC014794
Immunoglobulin lamda constant 1	BC073769
Iron inhibited ABC transporter	AF261092
KIAA0286 protein	NM_015257
KIAA2005	AB095926
MHC class II HLA DR beta1	BC071659
Nascent-polypeptide-associated complex alpha polypeptide	AY911673
Oxidored nitro domain containing protein	BC063380
PHD finger protein 20 like 1	BC036953
PRO 0992	AF116615
Programmed cell death 2	BC067759
Proliferation-inducing protein 10	AY239295
Proteasome 26 subunit	BC002589
Prothymosin alpha	BC034921
Ribosomal protein L4 variant	AB208977
Ribosomal protein S2	BC008329
Ribosomal protein S5	BC018828
Sin3 associated polypeptide	NM_005870
Solute carrier family 1, member 4	NM_003038
Solute carrier family 25	BC068199
TACC1	AY072876
TNF receptor associated protein 1	AJ890085
Tri iso-phosphate 1 (TPI1)	NM_000365
ZHX3 transcription factor	NM_015035
Zinc finger protein 317	BC009367
Zinc finger protein 394	BC062588

Table 1. List of potential Memo-interacting proteins identified in the YTH screen.

----------DISCUSSION----------

In multicellular organisms, the regulation and coordination of complex cellular processes such as growth, differentiation, migration and apoptosis, require efficient communication between individual cells. These biological processes are mediated by a plethora of signal transduction networks. Polypeptide growth factors induce signaling by activating cell surface receptors such as RTKs which are the primary mediators of such physiological cell responses (Yarden and Sliwkowski, 2001). In fact, these receptors couple ligand binding to downstream signaling cascades and gene transcription (Schlessinger, 2000). RTKs have been regarded as key regulators of normal cellular processes, but also appeared to be critically involved in the development and progression of human cancers and other hyper-proliferative diseases (Kolibaba and Druker, 1997). Therefore, signaling pathways controlled by tyrosine kinases offer unique opportunities for pharmacological intervention.

ErbB2, which belongs to the EGF/ErbB family of RTKs, has been implicated in the development of many types of human cancer. The *ERBB2* amplicon on chromosome 17q12-21 is found in approximately 20% of primary human breast tumors which show a dramatic overexpression of the receptor. The mechanism underlying the oncogenic potential of overexpressed HER2 may be related to the increased availability for heterodimer formation and heteromolecular interaction, and consequently, for high autophosphorylation levels and constitutive signaling (Lonardo et al., 1990) (Brennan et al., 2000). Clinical studies revealed that ErbB2 gene amplification and overexpression correlate with a more aggressive progression of disease and a reduced patient survival (Paik et al., 1990) (Slamon et al., 1987). Moreover, clinical and fundamental investigations revealed that overexpression of HER2 increases the metastatic potential of human breast and lung cancer cells and correlates with the number of lymph node metastases in node-positive breast cancer patients (Yu and Hung, 2000). The metastatic process is extremely complex and, unfortunately, targeting metastasis, the cause of 90% of deaths from solid tumors, is still a major clinical problem. The metastatic process, whose study suffers limitations since it can only be studied *in vivo*, consists of distinct steps, some important ones being loss of cellular adhesion and increased motility and invasiveness (Gupta and Massague, 2006). Data gleaned from *in vitro* cancer cell models demonstrate that ErbB2 controls key intracellular pathways that govern fundamental cellular processes including migration (Hynes, 2007). Accordingly, ErbB2 and downstream signaling pathways have been intensely studied with the specific aim of identifying ErbB2-regulated proteins that are essential for tumor cell migration. Marone and colleagues identified a novel 297 amino-acid protein as an interacting partner of an ErbB2 autophosphorylation site. This

protein, which was called Memo, was found to be important for ErbB2-induced cell migration, and knock-down of Memo in tumor cells resulted in dramatic alterations in microtubule outgrowth and actin stress fibers distribution (Marone et al., 2004).

In the present study, we dissected in more detail the diverse stages of the migratory process and uncovered novel functions for Memo at different steps of cell migration. By using the Dunn chamber model which allows direct visualization and quantification of cell movement, we showed that while the general movement (translocation distance and speed) of Memo-down-regulated human breast carcinoma cells was moderately affected, the directionality of these cells was strongly impaired, suggesting that Memo is involved in the very first steps of the migratory process. How does Memo regulate directed cell migration? In fact, directional movement requires the cell to be able to detect an asymmetric extracellular signal, in our case the HRG gradient, and to generate internal amplified responses, leading to cell polarization, protrusion formation and adhesion in the direction of the ligand. Thus, directional migration of cells towards chemoattractants depends upon the spatial and temporal regulation of the actin cytoskeleton by actin-binding proteins.

One mechanism by which Memo could affect cellular movement is by influencing the activity of regulator(s) of directional sensing and cell polarity. Indeed, in a screen for Memo binding partners, we uncovered a novel interaction between Memo and cofilin, a known F-actin depolymerizing/severing factor. While studying the in vivo biochemical function of cofilin and subsequent cellular response, Gosh and colleagues demonstrated that cofilin determines the direction of cell migration (Ghosh et al., 2004). Interestingly, by performing in vitro F-actin binding and depolymerization experiments with recombinant Memo and cofilin proteins, we found that Memo enhances cofilin depolymerizing/severing activity, suggesting a direct role for Memo on cofilin function, and subsequently, on directional migration. The same group also showed that inhibition of PLCγ1 inhibits cofilin activity in cells during the early transient of actin barbing, delays the initiation of protrusions, and inhibits the ability of cells to sense a gradient of EGF, suggesting that both PLCγ1 and cofilin are required for setting the direction of cell movement in response to EGF (Mouneimne et al., 2006) (Mouneimne et al., 2004). More recently, van Rheenen and colleagues demonstrated that EGF induces a rapid loss of PIP(2) through PLC hydrolysis activity, resulting in a release and activation of a membrane-bound pool of cofilin (van Rheenen et al., 2007). Importantly, we found that Memo is complexed with ErbB2 and PLCγ1 and is required for efficient ErbB2-induced PLCγ1 phosphorylation, suggesting another possible mechanism of action of Memo on cofilin. We also showed that ErbB2-induced recruitment of GFP-cofilin to the lamellipodia is

impaired in Memo and PLCγ1 knock-down cells, suggesting that both Memo and PLCγ1 are important for the lamellipodial localization of cofilin. Whether or not PLCγ1-mediated cofilin localization is dependent on Memo still remains unknown. Furthermore, Mouneimne and colleagues demonstrated that local activation of cofilin by PLCγ1 and its global inactivation by LIMK phosphorylation, combine to generate the local asymmetry of actin polymerization required for chemotaxis (Mouneimne et al., 2006). By performing co-immunoprecipitation experiments, we showed that Memo is able to bind both unphosphorylated and Ser3-phosphorylated cofilin, suggesting that Memo could influence the balance between the phosphorylated and the non-phosphorylated status of cofilin, although it should be mentioned that no change in cofilin phosphorylation status was observed in the breast carcinoma models used in our study.

The connection between PLCγ1, which appears to be important for directional migration, and Memo, remains to be explored in more detail. However, an increasing body of evidence suggests that both proteins "cooperate" to induce efficient cell migration. In the Boyden chamber assays performed with breast tumor cells, individual or simultaneous down regulation of Memo and PLCγ1 strongly impaired chemotaxis of cells, while chemokinesis, which reflects general movement, was only affected in double KD cells. These results are in accordance with those obtained using the Dunn chamber model, where individual knock down of Memo or PLCγ1 moderately inhibited general movement of cells and strongly affected cell directionality, whereas double knock-down, similarly to cofilin KD, resulted in a total blockade of cell movement. These results indicate an additive effect of Memo and PLCγ1 down regulation, and suggest that these two proteins act in concert to promote cell migration, either in the same or in parallel pathways.

In our study, we also used T47D breast carcinoma cell lines in which endogenous ErbB2 had been functionally inhibited and substituted with Neu mutants harboring none (NYPD) or only one of the two major autophosphorylation sites (YC or YD) that were found to be important for cell migration (Marone et al., 2004). In accordance with the previous report showing that parental T47D, YC and YD cells, but not NYPD cells, were able to migrate upon HRG stimulation, we observed that T47D, YC and YD cells migrated towards the HRG source in the Dunn chamber model, whereas NYPD cells were totally blocked in their movement (data not shown).

Furthermore, phosphorylation of PLCγ1 was induced by HRG in T47D, YC and YD cells, but not in NYPD cells, suggesting that cooperation between effectors downstream of the YC and

YD autophosphorylation sites is required for PLCγ1 activation (data not shown). These results also suggest that PLCγ1 phosphorylation is required for cell migration. In accordance with this hypothesis, Chen and colleagues have previously investigated the role of PLC-γ1 in EGF-induced cell movement or migration into an acellular area created by wounding a monolayer of 3T3 cells. Based on the analysis of transfected EGF receptor deletion mutants, pharmacological inhibition of PLC activity with the drug U73122, antisense reduction of PLC-γ1 levels, and expression of a dominant negative fragment of PLC-γ1, this group also concluded that PLC-γ1 function is required for cell migration (Chen et al., 1994). Subsequently, gelsolin was identified as a downstream target of EGF-induced PLC-γ1 activity (Chen et al., 1996).

Gelsolin is a Ca^{2+}- and PIP_2-regulated actin filament severing and capping protein that has a role in actin remodeling (Sun et al., 1999). Interestingly, gelsolin null fibroblasts exhibit defective chemotaxis and wound healing (Azuma et al., 1998). Furthermore, similarly to our breast tumor cell models with a KD of Memo or PLCγ1, gelsolin null fibroblasts have pronounced actin stress fibers (Witke et al., 1995), a phenotype that is consistent with an inability to sever and remodel actin filaments. Accordingly, reduction of ADF/cofilin protein levels in B16F1 cells also induced an increase in the number and thickness of stress fibers (Hotulainen et al., 2005). ADF/cofilins catalyze depolymerization of actin filaments and thus increase the rate of actin turnover by providing new actin monomers for polymerization (Carlier et al., 1997). In a study performed by Hotulainen and colleagues, direct evidence was provided that actin filament treadmilling rates in stress fibers were severely diminished in cofilin knockdown cells (Hotulainen et al., 2005).

Based on their sub-cellular localization and interactions with focal adhesions, stress fibers of cultured mammalian cells can be divided into three classes (Naumanen et al., 2008). Ventral stress fibers are contractile actin bundles, which are typically associated with focal adhesions at both their ends (Small et al., 1998). Ventral stress fibers are responsible for the tail retraction and other cell shape changes due to increased contractility (Chen, 1981) and they also work against membrane tension at cell borders (Thery et al., 2006). Transverse arcs are curved actin bundles which are typically not directly attached to focal adhesions, but they are connected to the substrate via dorsal stress fibers. In migrating cells, they flow from the leading edge towards the cell center (Small et al., 1998) (Hotulainen and Lappalainen, 2006) and increased contractility enhances their motility. The contractile force is then transmitted to the substrate via dorsal stress fibers, which are actin filament bundles that attach to focal adhesion at one end and rise towards the dorsal section of the cells at the other end, but do not contract. Our results suggest that Memo or PLCγ1 down regulation in breast tumor cells,

affected the organization of ventral and transversal stress fibers, reflecting enhanced contractility.
Also of note is the fact that simultaneous down regulation of Memo and PLCγ1 resulted in a more striking effect on actin stress fiber organization, strengthening the hypothesis of a "collaboration" between these two proteins.

Considering these effects on actin organization, and given that Memo was found to be important for PLCγ1 phosphorylation, we hypothesize a possible function for Memo whereby it mediates activation of downstream PLCγ1 effectors such as cofilin and perhaps even gelsolin. However, the mechanism whereby Memo influences PLCγ1 activation remains to be explored. It is worth mentioning that this "collaborative" connection between Memo and PLC is conserved through evolution, since a synthetic lethal interaction was identified for yeast *MEMO* and *PLC1* gene products.

Rho-family GTPases, including Cdc42, Rac1 and RhoA, were shown to play a central role in establishing cell polarization (Fukata et al., 2003). In fact, activation of Rac maintains the lamellipodium. Simultaneously, a Rho-dependent pathway determines the trailing edge (Xu et al., 2003). This antagonistic response with Rho that is suppressed at the front of the cell whereas Rac is suppressed at the back, allows cells to maintain polarity in one direction. During the course of our studies, we found that RhoA activation was delayed in Memo knock out mouse fibroblasts, suggesting a possible disorganization in the establishment of cell polarity during cell migration. Furthermore, Rho GTPases also capture and stabilize microtubules (MT) through their effectors (e.g. IQGAP1, mDia and Par6) near the cell cortex, leading to polarized cell morphology and directional cell migration (Fukata et al., 2003) (Watanabe et al., 1999) (Palazzo et al., 2001) (Cook et al., 1998) (Ishizaki et al., 2001). MT minus ends are anchored in the MTOC, whereas MT plus ends are directed to the cell periphery and continually alternate elongation and shrinkage phases (Burbank and Mitchison, 2006). Thus, when the cells are polarized, the plus ends of MTs are targeted, captured and stabilized near the leading edge, where MTs play critical roles in directed transport of specific proteins and vesicles for cell polarization. As an example, Yamana and colleagues showed that depletion of mDia1 by RNA interference impaired directed migration of rat C6 glioma cells by inhibiting both cell polarization and adhesion turnover (Yamana et al., 2006). In fact, the Rho-mDia1 pathway was shown to regulate polarization by aligning MT and actin filaments and delivering Apc/Cdc42 to the front of migrating cells where they work together for cell polarization. Also, Rho-mDia was found to promote MT-mediated Src localization to focal adhesions where it participates in the formation and adhesion turnover of focal contacts.

Interestingly, Memo knock down in breast tumor cell lines resulted in a dramatic alteration of MT organization such that MT failed to extend to the periphery of the cell (Marone et al., 2004). Considering this particular model, we hypothesize a possible role for Memo on Rho activation and consequently on MT-mediated molecular transport, contributing to directed cell migration and Src localization. Also of note is the fact that HRG or EGF-induced Src and Erk phosphorylation was impaired in cells that lost Memo. We hypothesize that Memo loss might affect FAK/Src-induced recruitment or activation of molecules such as Erk2 and calpain-2 which regulate focal adhesion disassembly (McLean et al., 2005). This model could explain why Memo deficient cells exhibit a defect in focal adhesion disassembly. Also, Zhang and colleagues suggested a role for FAK in recruiting PLCγ1 to the plasma membrane at sites of cell-matrix adhesion through the FAK autophosphorylation site Tyr-397 which mediates a direct interaction with PLCγ1 (Zhang et al., 1999). This interaction was shown to promote PLCγ1 enzymatic activity, possibly by releasing the repression caused by intramolecular interactions of the PLCγ1 Src homology domains and/or by positioning it for phosphorylation by associated Src-family kinases. Considering that PLCγ1 phosphorylation is impaired in Memo-deficient cells, and despite showing no effect on FAK Y397 phosphorylation, it is possible that Memo affects Src-mediated PLCγ1 phosphorylation at focal contact sites.

Although MTs do not appear be essential for actin/dependent ruffling and lamellipodia formation, MTs were shown to be required for tail retraction during cell movement (Ballestrem et al., 2000) (Wehrle-Haller and Imhof, 2003). Interestingly, when scored for their ability to migrate in a shallow gradient of HRG that was created in a Dunn chamber, Memo knock down breast tumour cells exhibited a tail retraction defect, suggesting an involvement of Memo at different stages of the migratory process. This tail retraction defect that we observed in knock down cells and also in Memo KO MEFs was similar to the one observed in B16 cells treated with the microtubule-disrupting reagent nocodazole in combination with PMA (phorbol 12-myristate 13-acetate), a known inducer of the reorganization of the actin cytoskeleton (Ballestrem et al., 2000). Also similarly to the nocodazole-treated B16 cells, and probably because of the driving force created by polymerizing actin at the leading edge and the stable contact at the rear, Memo knock down cells were sometimes torn apart, resulting in fragments separated form the main cell body, leaving a trace of actin-containing membrane behind. However, the tail retraction defect observed in Memo deficient cells is independent of impaired PLCγ1 phosphorylation since inhibition of PLCγ1 by siRNA transfection of breast tumour cells did not affect the retraction step during cellular movement (data not shown). We favour the hypothesis that through its

effect on FAK/Src signaling, Memo loss could affect focal adhesion disassembly, resulting in a tail retraction defect.

In the course of our studies, we also found that Memo is involved in cell adhesion, another important step of the metastatic process. The ability of a cell to adhere to its neighbor and to the extracellular environment is an essential process that defines in part a normal multicellular organism. During the process of tumor metastasis, adhesion molecules provide a selective advantage for migration of the tumor cell to a distant site (Cooper and Pienta, 2000). Adhesion complexes are platforms where many molecules meet to organize the cytoskeleton and trigger signaling (Lo, 2006). Integrins are a widely expressed family of cell adhesion receptors that are composed of αβ heterodimeric units, and are expressed on a wide variety of cells (Arnaout et al., 2007; Mousa, 2008). They play a critical role in the connection between focal adhesions and proteins of the extracellular matrix (Hynes, 2002). Furthermore, upon engagement of integrin cell adhesion receptors, FAK is activated and initiates several signaling events. In fact, the FAK-Src complex acts to recruit and/or phosphorylate a number of signaling proteins and is involved in regulation of adhesion. Memo possibly affects cellular adhesion by regulating FAK/Src complex signaling. Moreover, in an interesting study, Tvorogov and colleagues showed that PLCγ1 deficient cells have an adhesion, spreading and migration defect (Tvorogov et al., 2005). In addition, they demonstrated that integrin engagement by fibronectin induces tyrosine phosphorylation of PLCγ1 at Tyr783 and that this signaling event requires Src activity. Furthermore, mutagenesis of Tyr783 abrogated the capacity of PLCγ1 to facilitate adhesion. Considering the effect of Memo on PLCγ1 and Src phosphorylation, a possible role for Memo on the regulation of integrin function remains to be explored.

Cell adhesion can also be regulated by members of the tetraspanin family (Lazo, 2007) (Levy and Shoham, 2005). Human tetraspanin proteins are a group of highly hydrophobic membrane proteins that can form complexes in cholesterol-rich microdomains on the cell surface. These complexes are composed of a core of several tetraspanin proteins that organize other membrane proteins such as integrins, and some growth factor receptors. Tetraspanin proteins and their complexes were shown to affect cellular adhesion and motility, interactions with stroma or affect signaling by growth factors. These proteins have been mostly studied in cells of lymphoid lineage, but they are present in all cell types. Interestingly, among the candidates obtained in our YTH screen, the tetraspanin CD151 was identified as a potential interacting partner of Memo (Table 1 in Appendix). CD151-α6 integrin complexes were recently shown to play a functional role in basal-like mammary tumor progression (Yang et al., 2008). Also,

both α3β1- and α6β4-dependent cell adhesion to laminin-5 were impaired in CD151-silenced cells (Winterwood et al., 2006). Furthermore, those cells displayed markedly impaired motility, accompanied by unusually persistent lateral and trailing edge adhesive contacts. In another study, Yamada and colleagues demonstrated that CD151 KD cells have aberrant membrane protrusions and exhibit reductions in the tyrosine phosphorylation of FAK, Src, p130Cas and paxillin (Yamada et al., 2008). Considering these results and some similar effects obtained in Memo defective cells, we suggest a possible connection between Memo and CD151 that would be important for cell adhesion and migration processes.

Finally, another possible role for Memo during the metastatic process has to be explored in more detail; this is regulation of MMPs. In fact, MMPs contribute to cancer development by degrading ECM molecules, thereby facilitating cancer cell migration/invasion across tissue boundaries (Steeg, 2006). Interestingly, expression of MMP9 (RNA and protein) was impaired in Memo deficient cells, suggesting that proteolysis of the ECM during invasion might be decreased in those cells. Considering that ErbB2 overexpression was shown to activate transcription and enhance secretion of MMP9 proteases, leading to increased membrane degradation and invasiveness of ErbB2-overexpressing breast cancer cells (Tan et al., 1997), Memo appears to be a potential downstream ErbB2 mediator of cellular invasiveness.

To conclude, our study suggests the involvement of Memo at different stages of the metastatic process and reveals a potential role for Memo on the regulation of critical components of multiple signaling pathways that regulate proliferation, survival, angiogenesis and metastasis. As an example, aberrant activation of Src family members is common in solid tumor malignancies and may contribute to the development and/or progression of these tumors. As a result, Src has emerged as a potential therapeutic target in the prevention of tumor spreading and inhibitors have been developed and tested for the treatment of different types of solid tumors (Kopetz et al., 2007) (Angelucci et al., 2006) (Hiscox and Nicholson, 2008). Inhibitors of FAK have also entered clinical studies for the treatment of solid tumors (Brunton and Frame, 2008) (McLean et al., 2005). PLCγ1 was also shown to function as a key molecular switch in the initiation of signaling cascades that regulate tumor migration (Wells and Grandis, 2003). Considering a potential role for Memo in the regulation of these three important actors of tumor progression, Memo might also become a biochemically relevant target in cancer research.

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----------ABBREVIATIONS----------

A

Abp1	actin-filament-binding protein
ADAM	A Disintegrin-like and metalloproteinase-containing protein
ADF	Actin-depolymerization Factor
AIP1	actin-interacting protein 1
AngII	Angiotensin II
Apc	Adenomatous polyposis coli
AR	Amphiregulin
Arp	Actin-related protein
ARP2/3	Actin-related protein-2/3

B

bFGF	basic fibroblast growth factor
BSA	Bovine serum albumine
BTC	Betacellulin

C

Ca++	Calcium
CAP	cyclase-associated protein
CBB	Coomassie Brilliant Blue
Cbl	E3 ubiquitin ligase
Cdc42	cell division cycle 42
CH	calponin homology
CH1	central proline rich domain1
CIN	chronophin
CloNAT	Nourseothricin
CoCl2	Cobalt chloride

D

DAG	1, 2-diacylglycerol
DN	dominant-negative
Dok	docking protein 1

E

ECM	Extracellular matrix
EGF	Epidermal Growth Factor
Ena	Enabled
Eph	ephrin
EPR	Epiregulin
ER	oestrogen receptor
ERK	Extracellular signal-Regulated Kinase

F

FA	Focal adhesions
F-actin	Filamentous actin
FAK	Focal Adhesion Kinase
FCS	Fetal Calf Serum
FGF	fibroblast growth factor
FZD	seven-pass membrane receptor Frizzled

G

Gab1	GRB2-associated binding protein 1
G-actin	Globular actin
Gal	Galactose
GAP	GTPase-activating protein
GFP	Green fluorescent protein
GPCR	G-protein-coupled-receptors
Grb2	Growth factor receptor-bound protein 2
GST	glutathione S-transferase

H

HB-EGF	Heparin-Binding EGF
HER2	human EGFR-related 2
His	Histidine
HRG	Heregulin
HU	Hydroxyurea

I

IgG	Immunoglobulin G
IP	immunoprecipitation
IP3	inositol 1, 4, 5-trisphosphate

J

Jak2	Janus tyrosine kinase 2
JNK	Janus kinase 1

K

KD	knock-down
KO	Knock-out

L

LAP	Lysophosphatidic acid
LIMK	LIM domain kinase

M

MAPK	Mitogen-activated protein kinase
mDia1	mammalian homologue of the *Drosophila* gene *Diaphanous 1*
MEFs	mouse embryonic fibroblasts
Memo	Mediator of ErbB2-driven cell Motility
MLC(K)	Myosin Light Chain (Kinase)
MMP	Matrix Metalloproteinase
MMS	Methyl methanesulfonate
MRCK	myotonic dystrophy kinase-related Cdc42-binding kinase
MT	Microtubules
MTOC	Microtubule-organizing center

N

Neu	rat ErbB2 homologue
NGF	nerve growth factor
NRG	Neuregulin
NSCLC	non-small cell lung cancer

O

ORFs	Open reading frames

P

PAK	p21-activated protein kinase
PDGF	Platelet-derived growth factor
PEI	polyethylenimine
Pi	inorganic phosphate
PI3K	Phosphatidylinosithol-3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLCg1	Phospholipase Cg
PP1	Protein phosphatase 1
PTB	PhosphoTyrosine-Binding domain
PtdIns(3,4,5)*P*3	phosphatidyl-inositol 3-4-5 triphosphate
PTEN	phosphatase and tensin homologue deleted on chromosome ten

R

ROCK	Rho.associated kinase
ROS	Reactive oxygen species
RTK	Receptor Tyrosine Kinase

S

S. Cerevisae	*Saccharomyces cerevisiae*
SCAR	suppresor of cAR
SD	Minimal synthetic defined bases
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2/SH3	Src Homology 2/ Src Homology 3
SHP-1	Shatterproof 1
SLS	Synthetic Lethal Screen
Sos	Son of sevenless
SSH	Protein phosphatase Slingshot
STAT	Signal Transducers and Activators of Transcription

T

TAPtag	Tandem Affinity Purification tag
TESK	Testicular kinase
TGF-a	Transforming Growth Factor a
TK	Tyrosine Kinase
TKI	tyrosine kinase inhibitors
Trp	Tryptohpane
Tyr	Tyrosine

V

VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
v-erbB	erythroblastoma viral gene product
VPC	vulval precursor cells

W

W	Whole cell lysates
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP family Verprolin-homologous protein
WH2	WASP-homology-2
WIP	verprolin/WASP-interacting protein
WT	wild type

Y

YFP	Yellow fluorescent protein
YPD	Yeast Peptone Dextrose
YTH	Yeast-2-Hybrid

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CURRICULUM VITAE

Name: MEIRA Maria
Date of birth: June 25th, 1978
Nationality: French
Marital status: Single

Private address: 6A, rue de l'Horticulture
68330 Huningue, FRANCE
Tel: +33.6.81.21.37.76
Email: maria.meira@fmi.ch

EDUCATION

2003/2008 : **Ph.D in Prof. Dr.N. E. Hynes´ lab**,
Friedrich Miescher Institut, Basel- Switzerland

2001/2002 : **Diplôme d'Etudes Approfondies (Diploma) in Pharmacology and Pharmacochemistry,**
Université Louis Pasteur de Strasbourg- France

2000/2001 : **Maîtrise de Biochimie générale,**
Université Louis Pasteur de Strasbourg- France

1999/2000 : **Licence de Biologie,**
Université Louis Pasteur de Strasbourg- France

1998/1999 : **Diplôme d'Etudes Universitaires Générales en Biologie(DEUG),**
Université Louis Pasteur de Strasbourg- France

1996/1998 : **PCEM 1** (First year of Medical Studies),
Université Louis Pasteur de Strasbourg- France

1996 : **Baccalauréat Scientifique,** (High School Diploma) Mulhouse-France

LANGUAGES

- **French** : Native language, spoken and written
- **Portuguese**: Mother tongue, spoken and written
- **English** : Spoken and written
- **Spanish**: Spoken and written
- **German**: Basics

CURRICULUM VITAE

ORAL COMMUNICATIONS and POSTERS

September 2003: **Meira M.**, Fumagalli S. & Thomas G., "Effect of the PI3K inhibitor Wortmannin on the S6K1 signalling and on the translation of 5'TOP mRNAs": **poster** presented at the "FMI Annual Meeting 2004", Pontresina – Switzerland.

September 2004: **Meira M.**, Masson R. & Hynes N.E., "Genetic and cellular approaches for the detection of ErbB2 receptor interacting proteins": **poster** presented at the "FMI Annual Meeting 2004", Crans-Montana – Switzerland.

September 2005: **Meira M.**, Masson R. & Hynes N.E., "Using yeast to study Memo, a novel ErbB2 effector protein": **poster** presented at the "FMI Annual Meeting 2005", Pontresina – Switzerland.

February 2006: **Oral & poster** presentation at the USGEB Meeting- Geneva- Switzerland "Using mammalian and yeast systems to study Memo, a novel ErbB2 effector protein"

September 2006: **Meira M.**, Masson R. & Hynes N.E., "Yeast and mammals, two systems to study Memo, a novel ErbB2 effector protein": **poster** presented at the "FMI Annual Meeting 2006", Murten – Switzerland.

December 2006: **Meira M.**, Masson R. & Hynes N.E., "Using yeast and mammals to study Memo, a novel ErbB2 effector protein": **poster** presented at the "Targeting the Kinome" meeting, Basel- Switzerland.

December 2006: **Meira M.**, Masson R. & Hynes N.E., "Yeast and mammals, two different systems to study Memo, a novel ErbB2 effector protein": **poster** presented at the 46th annual meeting of the American Society for Cell Biology, San Diego CA, USA.

September 2007: **Meira M.**, Masson R. & Hynes N.E.,"Memo, at the front and the rear of a migrating cell": poster presented at the"FMI Annual Meeting 2007", Grindelwald-Switzerland.

PUBLICATIONS

Bennasroune A, Gardin A, Auzan C, Clauser E, Dirrig-Grosch S, **Meira M**, Appert-Collin A, Aunis D, Cremel G, Hubert P.: Inhibition by transmembrane peptides of chimeric insulin receptors", *Cell Mol Life Sci*. 2005 Sep;62(18):2124-31.

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