The ErbB2 receptor and breast carcinoma cell migration: Memo is a novel mediator of cell motility

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Romina Marone

aus Bellinzona (TI)

Leiter der Arbeit: Prof. Dr. Nancy E. Hynes

Friedrich Miescher Institut for Biomedical Research, Basel

Basel, 2003

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Prof. Dr. Nancy E. Hynes, PD Dr. Patrick Matthias, Prof. Markus Affolter und Dr. Ali Badache.

Basel, den 18. November 2003

Prof. Dr. Marcel Tanner
Dekan

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I. SUMMARY

The ErbB family of receptor tyrosine kinases play important role in normal physiological processes occurring during development; moreover, their deregulated expression has been implicated in human cancer. Cancer patients, whose tumors have alterations in ErbB1 or ErbB2, tend to have a more aggressive disease associated with parameters predicting a poor clinical outcome, including tumor metastases. For tumors to metastasize, the cells have to possess specific characteristics, including the ability to migrate and to invade the surrounding basal membrane. The role of the Neu/ErbB2 receptor in cancer cell migration is the major topic of this thesis.

The Neu/ErbB2 receptor is often overexpressed in different human tumors, including breast and ovarian tumors. Clinical and *in vitro* studies revealed that Neu/ErbB2 plays important functions in tumor cell motility. Upon ErbB receptors activation via ligand-induced dimerization, receptors autophosphorylate specific tyrosines in the carboxy domain leading to activation of downstream signaling cascades, including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K) pathways. These pathways, which are known to be important for cell migration, are involved in actin cytoskeleton remodeling, leading to formation of lamellipodia and actin stress fibers.

In this work we used T47D breast carcinoma cells expressing Neu/ErbB2 add-back mutants harboring none or only one of the five major autophosphorylation sites, to study the contribution of individual Neu/ErbB2 tyrosine autophosphorylation sites in cell migration. We showed that activation of MAPK and PI3K in T47D cell failed to induce efficient cell motility in the absence of the Neu/ErbB2 tyrosines 1201 or 1227 phosphorylation. Moreover, we present evidence that efficient, long-term cell migration depends upon ongoing transcription and translation. Signaling downstream of tyrosine 1201 and 1227 is required for *de novo* synthesis of RNA and protein involved in cell migration. Further investigation of the function of these two tyrosines led to the identification of a novel protein that specifically interacts with the phosphorylated tyrosine 1227. We called this new protein Memo for mediator of ErbB2-driven cell

motility. Memo does not bind directly to the phosphorylated tyrosine 1227 of the Neu/ErbB2 receptor, but very likely via the adaptor molecule Shc. Memo is required for ErbB2-driven breast carcinoma cell migration, because its downregulation leads to decreased motility of cells expressing the receptor with the tyrosine 1227. Interestingly, we found that Memo is not only required for migration downstream of the ErbB receptors, but it may be a general mediator of growth factor-induced breast carcinoma cell migration.

Cell migration is a multistep process and we further defined at which step Memo is required. We found that upon Neu/ErbB2 activation, wild type cells, but interestingly also Memo-deficient cells form actin stress fibers and extend lamellipodia. However, Memo-deficient cells are not able to extend microtubules toward the cell cortex. There is increasing evidence that not only the actin cytoskeleton but also the microtubule cytoskeleton plays a crucial role for cell migration. For instance, microtubules are required for the polarization of the cells and also for the transport of proteins required for motility to the cell leading edge. Further studies have to be done to understand the exact role of Memo in microtubule outgrowth and its contribution to cell motility.

In summary, the work presented in the thesis shows the identification of a novel protein, Memo, which is required for breast carcinoma cell migration. We propose that Memo controls cell motility by transmitting extracellular chemotactic signals to the microtuble cytoskeleton.

II. ACKNOWLEDGMENTS

First, I would like to thank Prof. Nancy Hynes, for giving me the opportunity to perform my PhD thesis in her lab. I also thank Dr. Patrick Matthias and Prof. Markus Affolter to be member of my thesis committee, and Prof. Frederick Meins to be chairing my exam.

Many thanks to everybody in Nancy's lab, past and present, for the nice working atmosphere. I am especially thankful to Ali for the wonderful supervision and the helpful discussions during my whole PhD, to David and Ilja for the nice time that we spent together inside and outside the lab, to Francis for the help with cloning and maxi-preps and to Susanne for the enthusiasm that she had in helping me in the last part of my PhD.

Finally I thank my parents and my sisters for their support over all these years and Matthias for standing by my side in every situation. I am also grateful to Yasmina for the discussions and nice lunches that we had together and to Simona for the advices, the hours at the gym and the nice friendship.

III. INTRODUCTION

1. THE ERBB RECEPTOR FAMILY OF RECEPTOR TYROSINE KINASES

Within a multicellular organism, cells are continuously exposed to a flow of different signals coming from the environment and from the neighboring cells. These signals have to cross the membrane and to be converted into intracellular signals in order to be correctly interpreted and to exert their pleiotropic effects. During evolution different devices have been developed in order to achieve this challenge. One of these is the presence of receptors on the surface of the cells, which are responsible for the capture of the signals. One class of receptors is the family of receptor tyrosine kinases (RTKs) that can be divided in different subfamilies, based on the sequence homologies and conserved structural features. The subfamily I is formed by the ErbB or epidermal growth factor (EGF) receptors and include four members: EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. All members have in common a cysteine-rich extracellular ligand binding domain, a single hydrophobic transmembrane region and a cytoplasmic tail containing tyrosine kinase activity (Ullrich and Schlessinger, 1990). The signal-transducing tyrosine kinase activity of these receptors is inactive when they are in isolation. A number of different ligands activate the receptor by binding to the extracellular domain and inducing the formation of receptor homo- and heterodimers. Tyrosine residues on the receptors are cross- or autophosphorylated and serve as docking sites for signaling complexes which will then activate different signal transduction cascades (Olayioye *et al.*, 2000; Yarden and Sliwkowski, 2001).

1.1. ErbB receptors in evolution

The EGFR signaling module has been highly conserved in evolution. In the nematode *Caenorhabditis elegans*, only one receptor, LET-2, and one ligand, LIN-3, are present (Aroian *et al.*, 1990; Hill and Sternberg, 1992). This pathway plays a central during

development role in the determination of the fate of several types of cells. The first function identified was in vulval induction, which occurs when the LIN-3 ligand secreted by an anchor cell binds to LET-23 receptor on adjacent multipotent vulval precursor cells. These cells will assume a vulval fate, while the surrounding cells that did not receive the LET-23 activation will become part of the epidermis (Chang and Sternberg, 1999).

In the fruitfly *Drosophila Melanogaster*, while only one receptor, DER is present, the number of ligands is increased to five (Livneh *et al.*, 1985). DER plays a multitude of roles during development, leading to a multitude of cell fate choices: cell division, cell survival or cell migration (Schweitzer and Shilo, 1997; Shilo, 2003). To ensure a tight activation of these processes, four activating ligands, Spitz (Rutledge *et al.*, 1992), Keren (Reich and Shilo, 2002), Gurken (Neuman-Silberberg and Schupbach, 1993) and Vein (Schnepp *et al.*, 1996), in conjunction with a negative-feedback loop generated by the inhibitory secreted ligand Argos are present in the fly (Golembo *et al.*, 1996).

The four mammalian ErbB family members can be activated by multiple ligands, providing a higher specificity and expanded repertoire of potential responses, via the formation of various homo- or heterodimers. The ErbB receptors are expressed in a variety of tissues of epithelial, mesenchymal and neural origin and they play important roles during development in cell proliferation, differentiation and migration. In addition, deregulated expression of the receptors, especially of ErbB1 and ErbB2, is implicated in the formation of human cancers and is associated with an aggressive disease phenotype (Slamon *et al.*, 1987; Hynes and Stern, 1994).

1.2. ErbB receptor ligands

In mammals, ErbB receptors are activated by a large family of ligands called EGF-related peptides (Riese and Stern, 1998). The structural motif shared by all ligands is the EGF-like domain composed of six characteristically spaced cysteines, which will form three disulfide-linked bridges. This domain function as a receptor binding site and is alone sufficient for high affinity binding (Peles *et al.*, 1993; Jones *et al.*, 1999b). Most EGF-related peptides are synthesized as transmembrane precursors that have to be

proteolitically cleaved in order to release the soluble form (Massague and Pandiella, 1993). In mammals, it was shown that the ADAM (a disintegrin-like and metalloproteinase-containing protein) family of zinc proteases, endowed with metalloproteinase and disintegrin receptor-binding activity, are involved in the shedding of the membrane-anchored precursor form (Gee and Knowlden, 2003; Seals and Courtneidge, 2003). Moreover, other studies provide evidence that the matrix metalloproteinases MMP-3 and MMP-7 are able to cleave the precursor form of HB-EGF (Suzuki *et al.*, 1997; Yu *et al.*, 2002). Interestingly, in *Drosophila melanogaster*, three of the five DER ligands, Spitz, Keren and Gurken are also produced as transmembrane precursor molecules. Processing of these molecules is not carried out by metalloproteinases like the ADAMs, but by Rhomboids, which are seven-transmembrane serine proteases (Urban *et al.*, 2002).

The mammalian ErbB ligands can be divided into three groups according to the binding specificity (Figure 1).

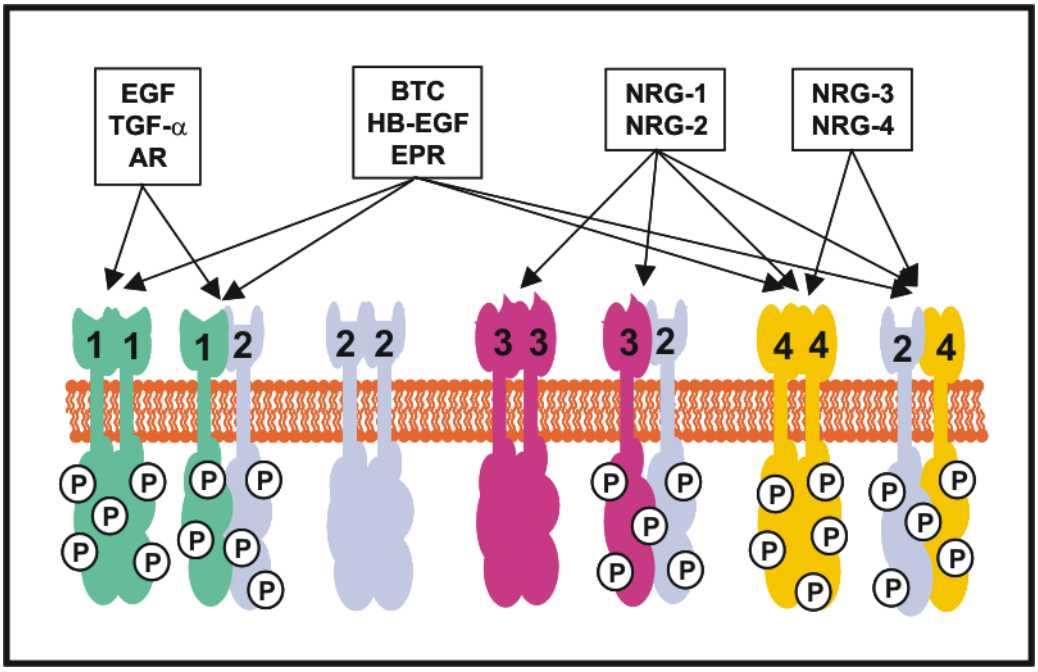

Figure 1: Binding specificity of the EGF-related peptide growth factors.

The first group comprises EGF, TGF-α and amphiregulin (AR), which specifically bind to ErbB1 (Harris *et al.*, 2003); the second group includes betacellulin (BTC), heparin-

binding EGF (HB-EGF) and epiregulin (EPR), which bind both ErbB1 and ErbB4 (Harris *et al.*, 2003). The third group comprises the neuregulins (NRGs). This group can be further subdivided in two subgroups based upon the binding of the NRG to ErbB3 and ErbB4 (NRG-1 and NRG-2) or only to ErbB4 (NRG-3 and NRG-4). The neuregulins are encoded by four different genes that can be alternative spliced leading to multiple NRG isoforms (Falls, 2003). NRG-1 is also known as neu differentiation factor (NDF), heregulin (HRG), acetylcholine receptor-inducing activity (ARIA) or glial growth factor (GGF), reflecting the biological system where the ligand was first described (Olayioye *et al.*, 2000). Interestingly, despite the large number of ligands identified for ErbB1, ErbB3 and ErbB4, no direct ligand for ErbB2 has been described yet (Klapper *et al.*, 1999). However, results from different studies support the idea that ErbB2 functions mainly in complex with the other family members, acting as a co-receptor. Interestingly, ErbB2-containing heterodimers are formed preferentially (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997) and are the most potent complexes concerning activation of signaling pathways (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995).

1.3. ErbB receptor dimerization

1.3.1. The extracellular domain

Dimer formation between multiple ErbB family members is a process driven by ligand binding to the extracellular domain of the receptors. New data from the crystal structure of ErbB1 (Garrett *et al.*, 2002; Ogiso *et al.*, 2002), ErbB2 (Cho *et al.*, 2003; Garrett *et al.*, 2003) and ErbB3 (Cho and Leahy, 2002) have provided a better understanding of the dimerization mechanism (Burgess *et al.*, 2003).

The ErbB receptor extracellular domain can be subdivided in four distinct subdomains, named I, II, III and IV. The subdomains I and III of ErbB1 have been identified as important in ligand binding, whereas the subdomain II of each receptor in the dimer forms a betahairpin arm and holds the body of the other, leading to a direct receptor–receptor interaction. Interestingly, in the ErbB1 dimer formed by two 1:1 receptor:ligand complexes, the two ligands are distant from each other and bind only a single ErbB receptor, thus they are monomeric (Lemmon *et al.*, 1997; Schlessinger, 2000). The

structure of ErbB2 reveals an activated conformation similar to that of the ErbB1 when complexed with a ligand, where the subdomains I and III are interacting, mimicking the bridging of the two domains by bound ligand in activated ErbB1 (Figure 2). Between the subdomains II and IV there is no interaction, since in ErbB2 three of the seven conserved residues important for stabilization of unactivated ErbB1 receptor are different, presumably reducing the strength of this interaction. These studies on the structure of ErbB2 explain the inability of the ErbB2 receptor to bind known ligands and why ErbB2 can interact with other ErbB receptors in the absence of direct ligand binding. Interestingly, overexpression especially of ErbB2, appears to force the equilibrium toward spontaneous homodimer formation leading to activation in the absence of ligands (Samanta *et al.*, 1994). This situation is often present in a variety of human cancers (Klapper *et al.*, 2000).

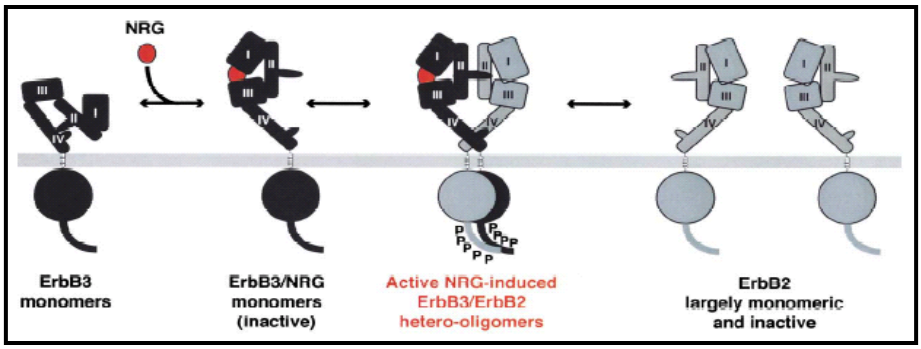

Figure 2: Model for NRG-induced heterodimerization of ErbB2 and ErbB3 (Burgess *et al.*, 2003).

ErbB3 structure is similar to the one of the inactive ErbB1. ErbB3 has impaired kinase activity due to substitutions in the kinase domain (Guy *et al.*, 1994) and therefore, in order to signal ErbB3 has to form dimers with the other ErbB receptors.

1.3.2. The intracellular domain

Although the extracellular domain appears to be responsible for the majority of interactions leading to ErbBs dimerization, evidence suggests that the intracellular domain plays more than a passive role in dimerization. It was proposed that dimerization

of ErbB receptors may be influenced by electrostatic charge distribution near the membrane proximal regions and that proper orientation of the receptors is critical for dimerization (Murali *et al.*, 1996). Moreover, results from one study proposed that the kinase domain is critical for dimerization (Chantry, 1995).

1.4. Intracellular signaling

The specificity and potency of intracellular signals are determined by the identity of the ligand and the dimer composition, but mainly by the multiple types of phospho-binding proteins that associate with the tail of each ErbB receptor in the dimer.

Ligand binding drives receptor dimerisation, leading to activation of the intrinsic kinase domain and subsequent autophosphorylation of specific tyrosine residues (Lemmon and Schlessinger, 1994; Jiang and Hunter, 1999). The identity of the ligand, as well as the heterodimer partners, determines which are the sites phosphorylated, and therefore, which adaptor proteins bind to the receptors (Olayioye *et al.*, 1998). The association of the adaptor molecules with the receptor's phosphorylated tyrosines occurs through their Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Interestingly, the amino acid sequence adjacent to the phosphorylated tyrosine is also important for the binding of the docking proteins (SH2 domains recognize residues carboxy-terminal to the phosphorylated tyrosine, whereas PTB domains the amino-terminal ones) (Pawson and Scott, 1997; Yaffe, 2002). The Shc/Grb2-activated mitogen-activated protein kinase (MAPK) pathway is a downstream target of all ErbB receptors (Olayioye *et al.*, 2000). Interestingly, not only the mammalian ErbB receptors, but also the *Drosophila* homologue DER and the *C. elegans* homologue Let-23 couple via Shc or Grb2 to the MAPK pathway (Diaz-Benjumea and Hafen, 1994; Moghal and Sternberg, 2003). In addition, the phosphatidylinosithol-3-kinase (PI3K) pathway is activated by all ErbB receptors, however the potency and kinetics of PI3K activation differs among the ErbB dimers probably because PI3K couple directly with ErbB3 and ErbB4, but indirectly with ErbB1 and ErbB2 (Prigent and Gullick, 1994; Soltoff and Cantley, 1996; Elenius *et al.*, 1999). Despite sharing some pathways, each receptor is coupled with a distinct set of signaling proteins (Table 1) (Olayioye *et al.*, 2000).

ERBB1	ERBB2	ERBB3	ERBB4
Grb2 (Batzer *et al.*, 1994)	**Grb2** (Ricci *et al.*, 1995; Dankort *et al.*, 1997)	**Grb7** (Fiddes *et al.*, 1998)	**p85** (Elenius *et al.*, 1999)
Nck (McCarty, 1998)	**Nck** (Dankort *et al.*, 2001b)	**Shc** (Prigent and Gullick, 1994)	**Shc** (Cohen *et al.*, 1996)
Crk (Hashimoto *et al.*, 1998)	**Crk** (Dankort *et al.*, 2001b)	**p85** (Prigent and Gullick, 1994)	
Shc (Batzer *et al.*, 1994)	**Shc** (Ricci *et al.*, 1995; Dankort *et al.*, 1997)		
Dok-R (Jones and Dumont, 1999)	**Dok-R** (Dankort *et al.*, 2001a)		
PLCγ (Chattopadhyay *et al.*, 1999)	**p34** (Dankort *et al.*, 2001a)		
PTB-1B (Milarski *et al.*, 1993)	**p150** (Dankort *et al.*, 2001a)		
SHP-1 (Keilhack *et al.*, 1998)	**Chk** (Zrihan-Licht *et al.*, 1998)		
Src (Stover *et al.*, 1995)			
Abl (Zhu *et al.*, 1994)			
Cbl (Levkowitz *et al.*, 1996)			

Table 1: Signaling proteins that associate directly with the ErbB receptors.

The principal process that turns off signaling downstream of the ErbB receptors is ligand-mediated receptor endocytosis. The kinetics of this process depends on the dimers composition. In contrast to the other ErbB receptors, activated ErbB1 is rapidly internalized and targeted to lysosomes (Baulida *et al.*, 1996). However, dimerization of ErbB1 with ErbB2 decreases the rate of endocytosis (Lenferink *et al.*, 1998; Wiley, 2003). Recent studies have shown a strong correlation between Cbl mediated ubiquitination of ErbB1 and accelerated degradation (Levkowitz *et al.*, 1999; Yokouchi *et al.*, 1999). This mechanism of negative regulation of ErbB1 is also present in the nematode *C. elegans*, where sli-1, the homologue of Cbl is involved in LET-23 degradation (Jongeward *et al.*, 1995).

1.5. ErbB receptors crosstalk with other receptors

The ErbB signaling network integrates not only the input from the multiple EGF-related peptides, but also from heterologous signals, such as hormones, neurotransmitters, lymphokines and stress inducers (Carpenter, 1999). Many of these transregulatory interactions are mediated by protein kinases that directly phosphorylate the ErbB receptors affecting their kinase activity or endocytic transport. The most extensively studied mechanism involves activation of ErbB by G-protein-coupled receptors (GPCRs) (Carpenter, 2000). Different groups showed that GPCR-dependent stimulation of the EGF receptor involve stimulation of membrane-bound metalloproteinase, which induce the extracellular release of the ErbB1 ligand heparin-bound-EGF (HB-EGF) (Fujiyama *et al.*, 2001; Pierce *et al.*, 2001; Asakura *et al.*, 2002). A similar activation could also occur for other growth factors, such as the precursor of transforming growth factor-α (pro TGF-α). This model of receptor tyrosine kinases transactivation is called triple-membrane-passing-signaling (TMPS) since it involves three signaling steps traversing the membrane (Figure 3) (Prenzel *et al.*, 1999; Wallasch *et al.*, 2002). Transactivation by GPCRs has been shown for other receptor tyrosine kinases, such as ErbB2 and platelet-derived growth factor receptor (PDGF). Moreover, additional pathways of ErbB1 transactivation that do not involve activation of metalloproteinases have been identified. It was proposed

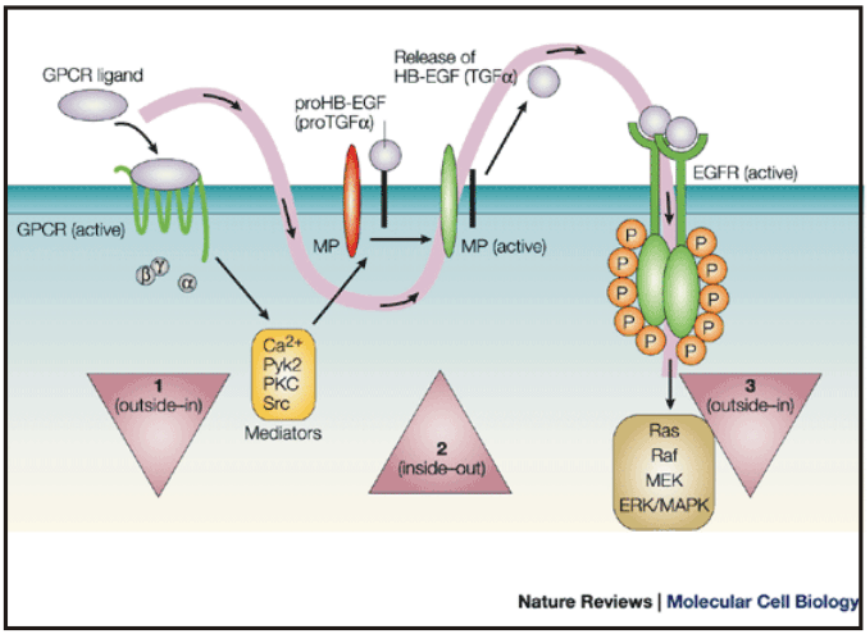

Figure 3: The triple-membrane-passing-signaling model (Wetzker and Bohmer, 2003).

that the tyrosine kinases Src and Pyk mediate ErbB1 transactivation downstream of GPCR activation. Both kinases can interact with ErbB1 and Src is able to directly phosphorylate and activate ErbB1 (Luttrell *et al.*, 1996; Biscardi *et al.*, 1999; Keely *et al.*, 2000). Alternatively, GPCR activation might lead to the production of hydrogen peroxide, which inactivates phosphatases that negatively control receptor tyrosine kinase activity.

1.6. ErbB receptors in mouse development

The ErbB network is a key developmental signaling pathway throughout evolution. The function of specific ligands and individual ErbB receptors in mammalian development was studied using knockout and transgenic mice.

ErbB1 and its ligands: Inactivation of ErbB1 is embryonic or perinatal lethal depending on the genetic background (Threadgill *et al.*, 1995). The mice show defects in the development of many organs, including lung, skin, brain and gastrointestinal tract (Miettinen *et al.*, 1995; Threadgill *et al.*, 1995; Sibilia *et al.*, 1998). Transgenic and *in*

vitro studies reveal a role for ErbB1 in promoting proliferation and differentiation of the epithelial component of those organs. Mice lacking transforming growth factor-α (TGF-α) have abnormal skin, hair and eye development, but in contrast to ErbB1 deficient mice, they show no brain abnormalities (Luetteke *et al.*, 1993; Mann *et al.*, 1993). The limited phenotype of TGF-α knock out mice suggest that each ErbB ligand has a distinct functional role and tissue specificity during development.

ErbB2, ErbB3, ErbB4 and the neuregulins: Mice defective in ErbB2, ErbB4 and NRG-1 die at embryonic day 10.5 due to defect in the cardiac traberculae formation, showing the importance of ErbB2/ErbB4 heterodimers in heart development (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). Mice lacking ErbB3 survive until embryonic day 13.5 and they suffer from valves heart malformation (Erickson *et al.*, 1997). Interestingly, mice lacking ErbB2, ErbB3 and NRG-1 have underdeveloped sympathetic ganglion chain, due probably to defective migration of neuronal progenitors from the neuronal crest (Britsch *et al.*, 1998). Moreover, a genetic rescue of ErbB2 knock out mice heart development by myocardial expression of ErbB2 cDNA, reveals the important role of ErbB2/ErbB3 heterodimers in peripheral nervous system development (Woldeyesus *et al.*, 1999).

1.5.1 ErbBs in development of the immature mammary gland

The mammary gland is an unusual organ because it undergoes postnatal development. In fact, female are born with a small ductal tree. Under the influence of systemic hormones, extensive ductal elongation and branching occurs at puberty and in adult animals. Further development in pregnancy includes continued ductal growth, formation of glandular structures and production of milk at parturition. Weaning induces involution, a program of cell death and remodeling to restore the gland to a prepregnancy-like state.

Each ErbB receptor has a unique pattern of expression in this organ. In the mouse, ErbB1 and ErbB2 are abundant prior to puberty and during subsequent developmental stages, whereas ErbB3 and ErbB4 display low levels prior to pregnancy (Schroeder and Lee, 1998; Sebastian *et al.*, 1998). All four ErbBs are expressed during pregnancy and lactation, but ErbB1 and ErbB2 are preferentially expressed in lactating ducts and alveoli, whereas ErbB3 and ErbB4 are more pronounced in alveoli (Schroeder and Lee, 1998).

ErbB1 and ErbB2 are phosphorylated and therefore active during puberty, late pregnancy and lactation (Sebastian *et al.*, 1998). During pregnancy all four receptors are phosphorylated (Schroeder and Lee, 1998; Sebastian *et al.*, 1998). These results suggest that ErbB1 and ErbB2 act at puberty, late pregnancy and lactation, whereas ErbB3 and ErbB4 are most active in pregnancy and lactation.

Most ErbB1-/- mice die after birth, however the prolonged survival of a fraction of them made it possible to determine that they are impaired in postnatal ductal formation (Wiesen *et al.*, 1999). These mice have a reduced proliferation of the mammary epithelium and stroma and they lose the periductal fibroblasts.

Comparison of single or combined knockout for EGF, TGF-α and AR showed that AR is important for normal ductal development (Luetteke *et al.*, 1999). The severity of the phenotype and the lack of a similar phenotype associated with loss of EGF or TGF-α identified AR as the foremost mammary regulator of ErbB1 at puberty (Luetteke *et al.*, 1999; Li *et al.*, 2002).

1.5.2. ErbBs and ErbB ligands in adult mammary gland development

ErbBs are active during adult mammary development. Transgenic mice expressing a truncated dominant negative (DN) ErbB1 receptor under the control of the mammary gland specific mouse mammary tumor virus (MMTV) display reduced ductal side branching (Xie *et al.*, 1997). Transgenic animals expressing a dominant negative MMTV-truncated ErbB2 have significant defects in mammary developmental late in gestation and early postpartum, with failure of alveolar expansion and production of milk (Jones and Stern, 1999). Lactation problems and early postpartum immature phenotype are also seen in mice expressing MMTV-DN-ErbB4 (Jones *et al.*, 1999a; Tidcombe *et al.*, 2003). ErbB3 is expressed and active during pregnancy, but an ErbB3 loss-of-function phenotype in the mammary gland has not been described yet.

Mammary organ culture experiments have suggested that NRG induces alveolar morphogenesis and lactational differentiation (Yang *et al.*, 1995). Targeted disruption of NRG1α, but not NRG1β transiently reduces alveolar maturation and proliferation of the mammary epithelium late in pregnancy (Li *et al.*, 2002).

The limited expression of ligands and activation of ErbB receptors during involution (the final developmental step) and the lack of effects on involution in ligands knockout, suggests that ErbBs do not contribute significantly to this developmental step.

1.7. The ErbB2/Neu receptor

ErbB2 is the second member of the ErbB family of receptor tyrosine kinases and is often overexpressed or amplified in different tumors. The human *c-erbB2* gene was isolated from human genomic DNA library screened with a viral *v-erbB* hybridization probes under low stringency (Coussens *et al.*, 1985; King *et al.*, 1985; Semba *et al.*, 1985). The human *c-erbB2* is localized to bands q12-q22 of chromosome 17. Sequence analysis of the cDNA confirmed that the *c-erbB2* gene was the human homologue of the rat *neu* and had significant homology with the *erbB* gene (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986). The extracellular portion of ErbB2 is 44% homologous to ErbB1 (Bargmann *et al.*, 1986b) (Figure 4). The kinase domain is highly conserved within the ErbB family, whereas the carboxy-terminus residues show the highest sequence variation.

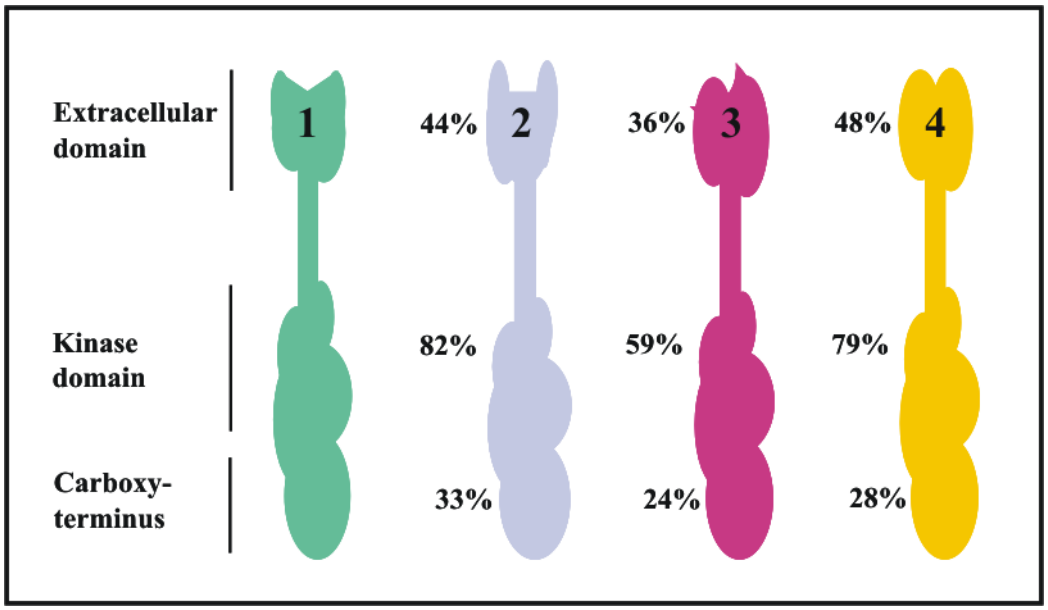

Figure 4: Domain homology between ErbB1 and the other ErbB family members.

1.7.1 ErbB2 and Neu in cancer

The rat *neu* oncogene was originally identified in cell lines derived from rat neuroectodermal tumors (Shih *et al.*, 1981). Further studies showed that the *neu* oncogene is associated with a specific antigen designated p185 (Padhy *et al.*, 1982; Schechter *et al.*, 1984), a phosphoprotein that is associated to the plasma membrane (Padhy *et al.*, 1982; Drebin *et al.*, 1984). The sequence suggested that the normal version of p185 is related to *erbB*. A comparison of cDNA clones isolated from both normal and transforming alleles indicates that the difference between the oncogenic and the proto-oncogenic form of *neu* is a single (T→A) point mutation resulting in an amino acid substitution (Val→Glu) at position 664 within the transmembrane domain of the receptor (Bargmann *et al.*, 1986a). Although the oncogenic point mutation identified in the rat *neu* is not found in human tumors, a polymorphism at codon 655 of *c-erbB2*, which results in Val→Ile has been identified (Papewalis *et al.*, 1991) and an association between the polymorphism and an increased risk of breast cancer was shown (Xie *et al.*, 2000). The human protein is overexpressed in a number of adenocarcinomas as a result of *c-erbB2* gene amplification or protein overexpression. ErbB2 overexpression leads to the spontaneous formation of ErbB2 homodimers, which activate different downstream signaling pathways. Observation of *c-erbB2* amplification was first described in human gastric tumors (Yamamoto *et al.*, 1986), but also appears to be associated with non-small cell lung (Weiner *et al.*, 1990), colon (Cohen *et al.*, 1989), ovarian (Slamon *et al.*, 1989) and pancreatic adenocarcinomas (Williams *et al.*, 1991). Overexpression of ErbB2 has been found in about 30% of invasive breast cancers (Slamon *et al.*, 1987; Slamon *et al.*, 1989). ErbB2 overexpression correlates with tumor size, spread of the tumor to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors, implying that ErbB2 confers a strong proliferative advantage to tumor cells (Ross and Fletcher, 1998). Moreover, ErbB2 overexpression is associated with resistance to anti-estrogen therapy and poor patient prognosis (Borg *et al.*, 1994).

1.7.2. Role of ErbB2 in tumor and metastases formation

Tumorigenesis is a multistep process that drives the progressive transformation of normal cells into highly malignant derivatives. During this progression the cells have to gain new

properties necessary for the malignant phenotype (Hanahan and Weinberg, 2000; Sledge and Miller, 2003). First, cancer cells have to acquire a proliferative potential that allow them to grow continuously and independently of growth signals. Moreover, the cells have to become insensitive to antiproliferative signals and should evade apoptosis. In addition, new blood vessels have to be formed in order to supply the tumor with oxygen and nutrients. The final step, metastases formation, is dependent on the capability of the cells to migrate and invade the surrounding tissue. ErbB receptors, as well as ErbB ligands, play distinct roles in each of these processes (Evan and Vousden, 2001; Green and Evan, 2002; Holbro *et al.*, 2003).

Different studies have been done in order to better understand the role of the ErbB receptors in cell proliferation, angiogenesis and cell motility (Holbro *et al.*, 2003). Since the central topic of this thesis is the role of ErbB2 in cell migration, only this aspect will be discussed in more details although if the knowledge is very limited.

Studies with transgenic mice have revealed that mice bearing either an activated form of Neu (NeuT or c-Neu with mutations in the extracellular region proximal to the transmembrane domain) or the wild type proto-oncogene under the control of the mouse mammary tumor virus (MMTV) promotor, frequently develop mammary tumors and lung metastases (Muller *et al.*, 1988; Bouchard *et al.*, 1989; Guy *et al.*, 1992; Siegel *et al.*, 1994; Siegel *et al.*, 1999). Metastases formation is very rapid in mice expressing the activated neu receptor, whereas the ones expressing the wild type proto-oncogene form metastasis only after a long latency. More studies using NeuT add-back mutants (mutants which have only one single tyrosine autophosphorylation site) were performed in order to better understand the role of the adaptor molecules, binding to specific NeuT sites, in cancer development and metastases formation (Dankort *et al.*, 2001b). Interestingly, two mice strains expressing only the second or the forth of the five add-back mutants efficiently form mammary tumors, but only one of them develops lung metastases, suggesting that metastases formation is more complex than tumor formation.

In vitro studies reveal that many types of tumor cells migrate or scatter in response to autocrine receptor activation (El-Obeid *et al.*, 1997) or ErbB ligands treatment (Adelsman *et al.*, 1999; Chausovsky *et al.*, 2000; Spencer *et al.*, 2000). Moreover, not only ErbB2 activation via a ligand but also ErbB2 overexpression is correlated with

increased cell motility and invasion by alterations in cell and cytoskeletal morphologies (De Corte *et al.*, 1994; Adam *et al.*, 1998; Grothey *et al.*, 2000).

In order to form metastases, carcinoma cells have to leave the primary tumor, process dependent on the ability of the cells to migrate. Afterward, they invade the surrounding basal membrane, process dependent on proteolysis, in order to reach and invade the blood vessels. Once in the blood, tumor cells circulate and they become trapped in the capillary of distant organs. At this point the cells will leave the blood stream and migrate into the organ. The cells start to proliferate in the target organ, forming the secondary tumor (Fidler, 2003). *In vitro*, HRG treatment of breast cancer cells was shown to induce the expression of the matrix metalloproteinase (MMP)-9 (Xu *et al.*, 1997) and of the membrane associated urokinase-type plasminogen activator (uPA) and its receptor (Mazumdar *et al.*, 2001), leading to an invasive phenotype. Clinical studies reveal that expression of MMP-2 and MMP-9 is associated with grade and stage of breast cancer (Monteagudo *et al.*, 1990; Zucker *et al.*, 1993; Kossakowska *et al.*, 1996). Moreover, uPA expression and the ratio of uPA to the plasminogen activator inhibitor-1 (PAI-1) are associated with impaired survival and local relapse (Prechtl *et al.*, 2000; Harbeck *et al.*, 2002). Inhibitors of MMP and uPA may have therapeutical potential, but clinical development has so far been limited due to toxicity (Bramhall *et al.*, 2001; Hidalgo and Eckhardt, 2001; Shepherd *et al.*, 2002).

1.8. ErbB as target for cancer therapy

The central role of ErbB2, but also of ErbB1 in the development of solid tumors and the detailed understanding of the underlying biochemistry has made the ErbB network a target for pharmacological intervention. Many different approaches have been taken.

Immunological strategies: a humanized monoclonal antibody to ErbB2 (Herceptin) (Hudziak *et al.*, 1989) has been approved for clinical use, both alone and in combination with chemotherapeutic agents (Baselga *et al.*, 1998; Pegram *et al.*, 1999). Herceptin induces ErbB2 downregulation and a proliferative block of the cells via induction of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the Rb-related protein p130 (Sliwkowski *et al.*, 1999; Lane *et al.*, 2000; Yakes *et al.*, 2002). Furthermore, it has been shown that in

vivo, Herceptin elicits an antibody-mediated cytotoxicity through engagement of Fc receptors and that this process contributes to its anti-tumor activity (Clynes *et al.*, 2000). The anti-tumor properties of Herceptin used alone and its increased efficacy when used in combination with cytotoxic agents have been confirmed using in vivo xenograft models (Baselga *et al.*, 1998; Pegram *et al.*, 1999).

In vitro approaches: One way it is to block transcription or translation by triple-forming oligonucleotides, designer transcription factor, antisense oligonucleotides or specific ribozymes (Ebbinghaus *et al.*, 1993; Noonberg *et al.*, 1994; Juhl *et al.*, 1997; Beerli *et al.*, 2000; Chiang *et al.*, 2000; Roh *et al.*, 2000). Another way is to interfere with the trafficking of the receptors to the cell surface using intracellular single-chain Fv fragments of antibodies (scFvs) (Beerli *et al.*, 1994; Neve *et al.*, 2000). An alternative approach is to affect receptor stability with e.g. geldanamycin (Basso *et al.*, 2002). However for clinical use, the most promising and advanced strategies include reversible and irreversible low molecular weight inhibitors that compete with ATP in the receptor kinase domain. Inhibitors capable of discriminating between ErbB receptors and other kinases have been developed (Fry *et al.*, 1998). The irreversible inhibitors bind to a conserved cysteine in the ATP-pocket, increasing selectivity of the inhibitor (Fry, 2003). At least five of these compounds are now being tested in human clinical studies (Baselga, 2002; Khalil *et al.*, 2003).

2. CELL MIGRATION

Cell migration plays a central role in the development and maintenance of multicellular organisms. In embryogenesis, cellular migration is very important in morphogenic processes ranging from gastrulation to development of the nervous system (Locascio and Nieto 2001). However, migration is also essential in the adult organism for normal physiological processes as well as pathological ones (Locascio and Nieto, 2001; Franz *et al.*, 2002). For example, during inflammation leukocytes have to migrate to the area of interest, where they mediate phagocytic and immune functions. Migration of fibroblasts and vascular endothelial cells is required during wound healing. In metastasis, tumor cells migrate from the initial tumor mass into the blood stream, which they will leave and finally migrate into the new site.

2.1. The motility cycle of a cell

Migration of cells over a substratum requires the coordination of several cellular processes which operate in a cycle. This cycle can be divided into five different steps (Figure 5):

1. Extension of the leading edge
2. Adhesion to the matrix
3. Contraction of the cell body
4. Release from the contact sites
5. Recycling of the membrane receptors from the rear to the front of the cell

Each of these steps is dependent upon one or more biochemical processes, which include protein and enzymatic components, extracellular-matrix receptors on the cell and physical forces.

1. Extension of the leading edge: the critical element of the extension process is directed actin assembly (Cramer *et al.*, 1994). The process of actin assembly must generate a protrusive force sufficient to extend the plasma membrane against compressive forces imposed by the environment, and by tension within the plasma membrane. The extensions formed are of two different types: flat, broad, sheet-like structures, called

lamellipodia, or thin, cylindrical, needle-like projections called filopodia. Cytoplasmic organelles are excluded from both of these structures, which contain actin and actin-associated proteins (Schmidt *et al.*, 1993).

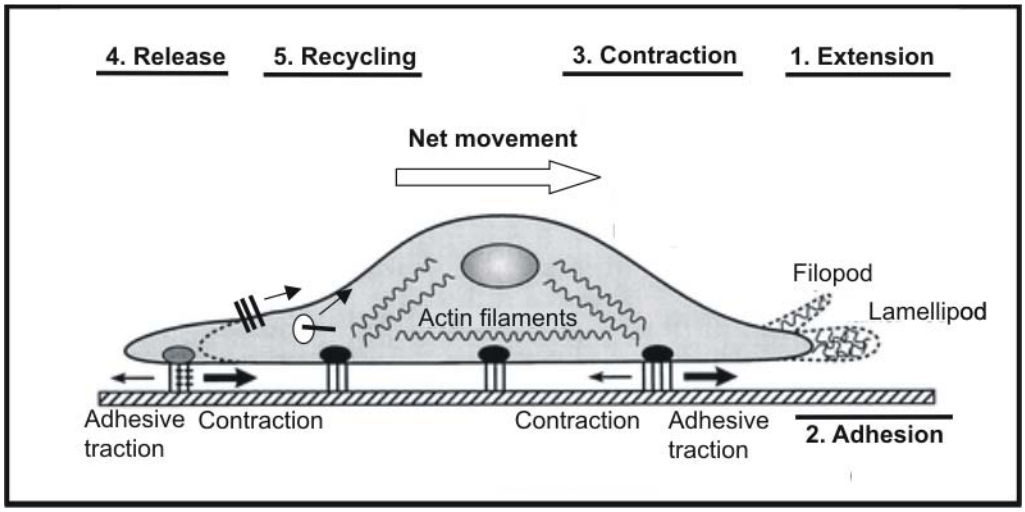

Figure 5: The five steps model of cell migration (Lauffenburger and Horwitz, 1996).

2. Adhesion to the matrix: extension of the leading edge to new extracellular matrix (ECM) molecules will enable receptors to bind and to initiate the adhesion process. The adhesion receptors-ECM complexes stabilize newly extended cellular domains and permit the cell to exert forces on the substrate. At the molecular level, integrins are the best characterized receptors for ECM molecules playing a role in cell migration (Holly *et al.*, 2000). Integrins are a family of heterodimeric transmembrane adhesion receptors that link ECM on the outside of the cell with the cell's cytoskeleton (Hynes, 1992).

Covalent modification of proteins by tyrosine phosphorylation is strongly implicated in the formation of adhesive structures. Upon adhesion to a substratum, a group of cytoskeletal-associated proteins are phosphorylated on tyrosines: focal adhesion kinase (FAK), paxillin and tensin are among the prominent and best characterized of these phosphoproteins that form the adhesive complexes (Lo *et al.*, 1994; Schaller and Parsons, 1994; Turner, 1994).

Moreover, also members of the Rho GTPases are important in the formation of new adhesions and stabilization of existing ones (Hall, 1998). Rac and Cdc42 appear to be important in the formation of new protrusions and small focal complexes, required for

adhesion at the cell periphery. Rho induces the maturation of the small focal complexes into the larger and highly organized focal adhesions.

Microtubules are regulators of focal adhesion and focal complex dynamics (Kaverina *et al.*, 1999; Waterman-Storer and Salmon, 1999). Depolymerization of microtubules leads to a decrease in the turnover of focal complexes, which results in reduced cell spreading and formation of large peripheral focal adhesions. Adhesions dissociate upon direct contact with microtubules and the cell either retracts the edge or forms new protrusions. Thus, microtubules appear to regulate the turnover of focal adhesions by targeting them directly and delivering signals to promote their turnover, initiating either protrusion or retraction (Palazzo and Gundersen, 2002).

3. Contraction of the cell body: at least two distinct types of force have to be generated independently by a motile cell. The first is the protrusive force required to extend membrane processes, lamellipodia and filopodia. Generation of this force is dependent on actin polymerization and not on myosin motor activity. The second force is a contractile force, needed to move the cell body forward. This force is dependent on active myosin-based motors (Cramer, 1999; Katoh *et al.*, 2001). Rac and Cdc42 appear to be important in regulating the contractile forces at the leading edge by modulating phosphorylation of the myosin light chain (MLC) (Bagrodia and Cerione, 1999) (Figure 5).

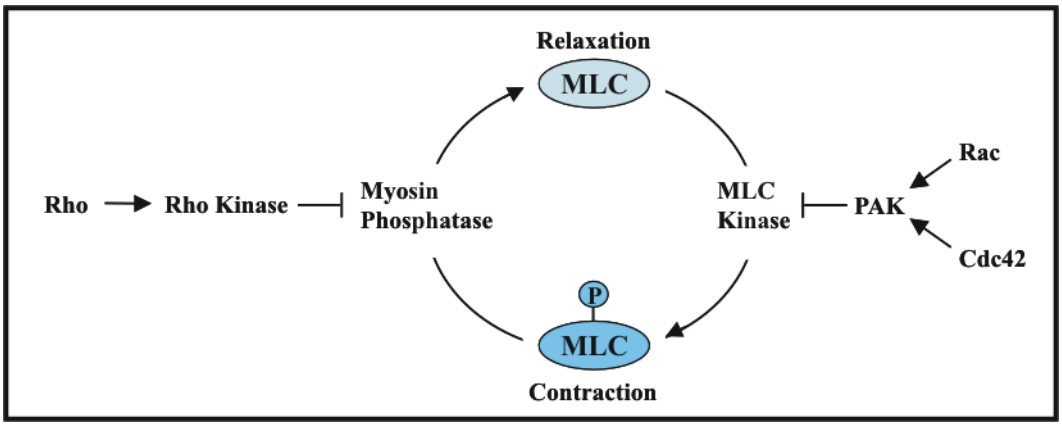

Figure 5: Regulation of the contractile forces dependent on MLC.

Phosphorylation of MLC by myosin light chain kinase (MLCK) promotes both their dimerization and their interaction with actin to drive contraction. Rho regulates the organization of actin into bundles, called stress fibers. Rho promotes tension through its action on MLC phosphorylation (Katoh *et al.*, 2001). However, in this case, Rho activates Rho kinase, which in turn inhibits the myosin phosphates, maintaining MLCs in a contractile state. The resulting contractile forces organize the actin in fibers and cluster the integrins, leading to tightly bundled actin and focal adhesions. It appears that Rho kinase requires Dia proteins, members of formin homology family, for proper formation of stress fibers (Nakano *et al.*, 1999; Watanabe *et al.*, 1999). Dia may contribute to stress fibers formation through interaction with profilin, a G-actin binding protein which promote actin polymerization and organization of actin filaments into stress fibers (Watanabe *et al.*, 1997; Alberts, 2001).

4. Release from the contact sites: upon cell body contraction, an asymmetry in the adhesion process has to be generated for forward migration. At the cell rear, adhesions need to be released, whereas at the front the formation of adhesion has to be controlled. Focal contact disassembly occurs through several mechanisms. Actin binding and severing proteins such as gelsolin and cofilin, cap actin filaments and cause actin filament breakage, thereby promoting filament turnover (Wear *et al.*, 2000). Phosphatases play important roles in rear release, for example by limiting the assembly of cytoskeletal proteins (Zeng *et al.*, 2003). Migratory defects have been reported in cells lacking Src family kinases (Klinghoffer *et al.*, 1999), FAK (Ilic *et al.*, 1995; Sieg *et al.*, 1999) and calpain (Huttenlocher *et al.*, 1997), all focal adhesion components. The defects appear to be caused by an inhibition of focal adhesion turnover, because focal adhesion formation is not impaired. Moreover, focal contacts are further weakened through the proteolytic cleavage of adhesion receptors by sheddases (Moss and Lambert, 2002) and the accumulation of collagen fragments that are generated while the cell moves forward (Carragher *et al.*, 1999).

5. Recycling of membrane receptors: in order to maintain a continuous retrograde flow of integrins on the cell surface, migrating cells must reload receptor at the leading edge.

Two different models have been suggested to explain the recycling of these proteins. Integrins detach from the substrate and become internalized via the endocytic vesicles and transported toward the leading edge (Bretscher, 1996), or there is a forward transport of the protein in the plasma membrane (Kucik *et al.*, 1989; Sheetz *et al.*, 1990; Regen and Horwitz, 1992).

2.2. The Rho GTPases: Rho, Rac and Cdc42

Rho GTPases regulate many important processes in eukaryotic cells. They are principally known for their role in regulating the actin cytoskeleton organization, but they also participate in the regulation of cell polarity, microtubule dynamics, vesicular transport pathways and gene transcription (Etienne-Manneville and Hall, 2002).

Rho GTPases cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. Guanine nucleotide exchange factors (GEFs) enhance the exchange of bound GDP for GTP, whereas GTPase-activating proteins (GAPs) increase the intrinsic rate of hydrolysis of bound GTP. In addition, the Rho GTPases are regulated further by guanine nucleotide dissociation inhibitors (GDIs), which can both inhibit exchange of GTP and hydrolysis of bound GTP preventing the interaction of the Rho GTPases with the plasma membrane. In the GTP-bound form they interact with downstream target proteins to induce cellular responses (Schmitz *et al.*, 2000).

The Rho GTPases Rho, Rac and Cdc42 regulate actin cytoskeleton polymerization, depolymerization and the activity of actin-associated myosins. These regulatory proteins are part of a hierarchical signaling cascade that initiate the formation of filopodia, lamellipodia, focal adhesion and stress fibers (Hall, 1998). Formation of filopodia and induction of polarization are regulated by Cdc42 (Kozma *et al.*, 1995; Nobes and Hall, 1995), while formation of lamellipodia and small adhesions is regulated by Rac, whose activation stimulates also membrane ruffling (Ridley *et al.*, 1992). Finally formation of actin stress fibers and of focal adhesion, highly organized adhesive complexes containing termini of stress fibers, is regulated by Rho (Ridley and Hall, 1992).

Moreover, recent evidence indicates that Rho GTPases might also affect the organization of microtubules (Waterman-Storer *et al.*, 1999). It was shown that Rac activation

promotes growth of microtubules (Wittmann *et al.*, 2003). Interestingly, previous work provide evidence that microtubule polymerization induces Rac activation (Waterman-Storer *et al.*, 1999). These results suggest that Rac and microtubules might constitute a positive feedback loop in which microtubules promote Rac activation, and Rac induces further microtubule growth reinforcing the polarization of migrating cells. Moreover, not only Rac, but also Cdc42 activation can mediate polarization of the microtubule network in migrating cells (Nobes and Hall, 1999). Microtubule depolymerization induces multiple cell morphological changes that include actin stress fiber formation and focal adhesion assembly, effects dependent on Rho activity (Liu *et al.*, 1998; Krendel *et al.*, 2002). Further investigations reveal that microtubule depolymerization in fact, induces activation of Rho.

2.3. The actin cytoskeleton in lamellipodia formation

Actin and actin-related proteins (Arps) are major determinants of cell morphology in eukaryotic, but also prokaryotic cells. Assembly of actin filaments drives the locomotion of many cell types including nerve growth cones, fibroblasts and leukocytes. Expansion of a dense network of actin filaments underlying the plasma membrane provides sufficient force to push forward the leading edge (Svitkina *et al.*, 1997). Actin polymerization also moves some cytoplasmic particles including endosomes (Merrifield *et al.*, 1999), pathogenic bacteria and viruses (Dramsi and Cossart, 1998), as well as drive engulfment during phagocytosis (Aderem and Underhill, 1999; Chimini and Chavrier, 2000).

The actin filaments are double helical polymers of globular subunits all arranged head-to tail to give the filament molecular polarity. One end is called the barbed end and the other the pointed end. The barbed end is favored for growth and actin filaments in cells are strongly oriented with respect to the cell surface, barbed end outward (Small *et al.*, 1978). Actin-based cellular motility can be explained by the treadmilling-type reaction (Figure 6). The actin-monomer-binding protein profilin and, in eukaryotic cells, sequestering proteins such as thymosin-β4 maintain a pool of unpolarized ATP-actin subunits in the cells (Goldschmidt-Clermont *et al.*, 1992; Vinson *et al.*, 1998). Extracellular stimuli such

as chemotactic factors bind to the plasma membrane receptors activating intracellular signaling molecules including the Rho GTPases (Van Aelst and D'Souza-Schorey, 1997; Schmitz *et al.*, 2000). Cdc42 binds and activates WASP/Scar family proteins, which are nucleation-promoting factors, by freeing them from autoinhibition (Bishop and Hall, 2000; Higgs and Pollard, 2001; Ridley, 2001). Active WASP/Scar proteins bring together an actin monomer and an Arp2/3 complex (Machesky *et al.*, 1999; Yarar *et al.*, 1999; Higgs and Pollard, 2001), an assembly of seven subunits including two actin-related proteins (Arp2 and Arp3) (Machesky *et al.*, 1994). The Arp2/3 complex sits on the "mother" filament and initiates the growth of a new "daughter" filament, polymerizing out from it at an angle of 70º (Mullins *et al.*, 1998; Amann and Pollard, 2001). The new branch grows rapidly at its barbed end by addition of actin-profilin complexes stored in the cytoplasm. As it grows, it pushes the plasma membrane and the cell forward.

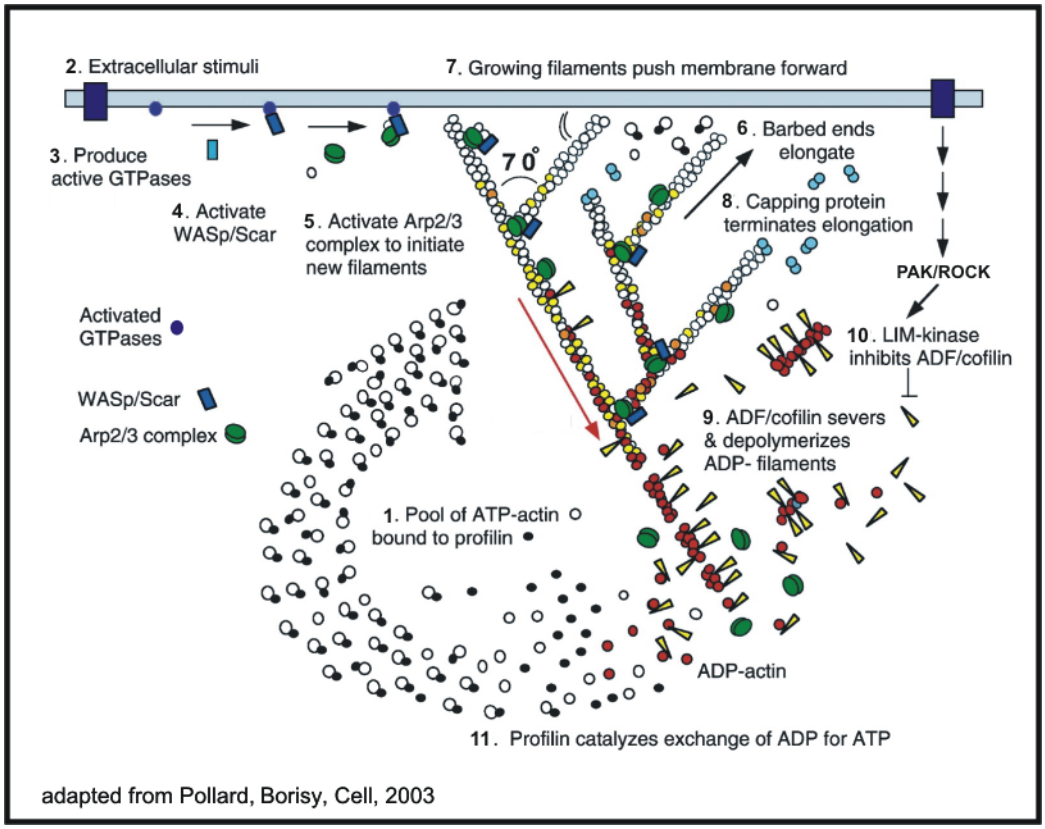

Figure 6: Treadmilling model for protrusion formation at the leading edge (Pollard and Borisy, 2003).

The system is set up to terminate the growth of the filaments automatically, before they grow so long that they do not push effectively. Then, the network dissociate so that the components can be recycled for the next step of polymerization. First of all, the new filament is capped by capping proteins, terminating, therefore, the elongation step (Sun *et al.*, 1999; Cooper and Schafer, 2000). Next, small proteins called actin-depolymerization factors (ADF)/cofilin bind to ADP-Pi-actin filaments and accelerate the dissociation of the gamma phosphate (Bamburg *et al.*, 1999). Dissociation of phosphate promotes dissociation of branches from Arp2/3 complexes and binding of ADF/cofilin to ADP-actin subunits. ADF/cofilin bound to filaments promotes severing of the filaments and dissociation of ADP-actin bound to ADF-cofilin (Blanchoin *et al.*, 2000). Moreover, Rho family GTPases activate p21-activated protein kinase (PAK) or Rho-associated kinase (ROCK), which stimulate LIM kinase to phosphorylate ADF/cofilin (Edwards *et al.*, 1999; Bishop and Hall, 2000; Schmitz *et al.*, 2000; Ridley, 2001). Phosphorylation inactivates ADF/cofilin prolonging the lifetime of newly formed actin filaments. Interestingly, activation of small GTPases stimulates not only the formation but also stabilization of new filaments. Finally, profilin, a nucleotide-exchange factor for actin, binds tightly to the actin-monomers, refilling the actin-monomer pool.

2.4. The microtubule cytoskeleton

Microtubules are dynamic structures that provide mechanical support for cell shape and act as tracks along which molecular motors move structures such as organelles, chromosomes or mitotic spindles around the cell. Moreover, microtubules remodeling is important in migrating cells, in order to establish and maintain cell polarity. Microtubules are hollow tubes composed of protofilaments of α- and β- tubulin dimers organized in a head-to-tail fashion. Tubulin polymerizes more quickly from the plus end, which is terminated by a β-subunit. The other, slow growing end, is terminated with an α-subunit and is called the minus end (Mitchison, 1993). In many cell types, the minus end of microtubules is embedded in a microtubule-organizing center (MTOC), whereas the plus end explores the cytoplasmic space. Microtubules have a dynamic behavior: individual microtubules alternate between periods of growth and shrinkage, a property called

dynamic instability (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997). The transition from growth to shrinkage is called catastrophe, and the one from shrinkage to growth is called rescue. The energy to drive microtubule polymerization comes from GTP hydrolysis. Tubulin is a GTPase whose activity is stimulated by polymerization (Erickson and O'Brien, 1992). Evidence coming from the atomic structure of tubulin shows that the β-subunit pocket can bind GTP, but lacks residues crucial for hydrolysis. These residues are given by the α-subunit when it binds to the end of the microtubule, triggering GTP hydrolysis (Nogales *et al.*, 1999). Microtubules at the growing end consist of sheets of protofilaments (Chretien *et al.*, 1995; Arnal *et al.*, 2000), whereas microtubules at the shrinking end are curled (Mandelkow *et al.*, 1991; Arnal *et al.*, 2000). Therefore, it seems that there is a structural transition associated with the switch between microtubule growing and shrinking. These structural changes are dependent on the GTP hydrolysis. In fact it was shown that GTP-tubulin form straight protofilaments that fit nicely into the wall of the microtubules, whereas in the GDP state the protofilaments are bent and they splay out from the microtubule lattice.

2.4.1. Microtubule end-binding proteins

GTP hydrolysis is known to occur very fast during microtubule polymerization and thermodynamic studies revealed that GDP-tubulin makes the microtubules very unstable. Some proteins were shown to modulate microtubule dynamics. These molecules are called microtubule-associated proteins or MAPs (Andersen, 2000). Two distinct classes of end-binding proteins have been described: the MCAKs (for mitotic centromere-associated kinesins, also called Kin I kinesins) which bind microtubule ends and destabilize them; and the plus-end-binding or tracking proteins (+TIPs) (Schuyler and Pellman, 2001), which also bind to the growing end of the microtubules, but stabilize microtubule growth.

MCAKs use energy from ATP in order to bind to the end of microtubules and they attach preferentially to the bend form of the tubulin dimer. MCAKs probably destabilize growing microtubules by inducing the formation of the curl, which then weakens the association of the terminal tubulin dimer, triggering its dissociation.

The prototype for +TIPs is CLIP-170 (Perez *et al.*, 1999). CLIP-170 binds to the microtubule plus end during their elongation and probably dissociates as the microtubule closes into a tube. Since the discovery of CLIP-170, more plus-end-binding proteins have been identified (Sawin, 2000; Schuyler and Pellman, 2001). For example the CLASP (CLIP associated proteins) proteins target microtubule end by binding to CLIP-170 and CLIP-115 (Akhmanova *et al.*, 2001). EB1 is also binding to tips of growing microtubules, where it stabilizes the polymer during mitosis preventing catastrophes (Tirnauer and Bierer, 2000). The adenomatous polyposis coli protein (APC) also accumulates at growing microtubule plus ends, specifically in protruding areas of the cells (Nathke *et al.*, 1996; Mimori-Kiyosue *et al.*, 2000). +TIPs have at least three different functions. First, they play a role in the regulation of microtubule dynamic behavior, modifying the probability of microtubule elongation, shrinkage and pausing (Brunner and Nurse, 2000; Komarova *et al.*, 2002a; Komarova *et al.*, 2002b). Second, +TIPs are involved in anchoring microtubules to cellular structures such as cortical actin which allow protein delivery at the cell periphery (Behrens and Nurse, 2002; Fukata *et al.*, 2002). Third, it was shown that +TIPs regulate dynein motor protein activity, which plays a role in the organization of the cytoskeletal architecture (Valetti *et al.*, 1999; Vaughan *et al.*, 1999).

2.4.2 Molecular motors

Molecular motors trigger most forms of movement in the cells. These motors transport a variety of cargos, power cell locomotion, drive cell division and when combined in large ensemble allow organisms to move. There are three classes of cytoplasmic motors: myosins, dyneins and kinesins. These proteins have a globular domain at one end, followed by a rod. The globular domain serves as a so-called “motor domain” that slides against the tracks using energy from ATP hydrolysis. The motor domains of kinesins and dyneins have ATP-binding and microtubule binding sites. They repeat cycles of attachment, sliding on the microtubules in an ATP-dependent manner, and move along the microtubules. Outside the motor domains, proteins from the same family can be quite different. These variable domains are the binding sites for the molecules to be transported (cargos); the diversity enables the motor proteins to participate in a wide variety of

intracellular transport. There is evidence that these cytoplasmic motors are involved not only in transport of organelles and vesicles (Lafont *et al.*, 1994; Kreitzer *et al.*, 2000), but also of messenger RNA and macromolecular complexes (Schliwa and Woehlke, 2003). Molecular motors are very important in the segregation of the chromosomes and in the motility of the mitotic spindle and in the cell scission (Scholey *et al.*, 2003). Moreover, there is increasing evidence that a growing number of diseases are linked to molecular motors (Hirokawa and Takemura, 2003; Schliwa and Woehlke, 2003).

Microtubule-dependent motor proteins are used for long-distance transport, for example from near the nucleus to the plasma membrane, whereas actin-dependent motor proteins are used for short distances. Both microtubules and actin filaments have polarity, and each motor protein moves unidirectionally. Most of the members of the kinesin superfamily move to the plus end of microtubules, whereas members of the dynein superfamily move to the minus end.

2.5. Microtubules, actin cytoskeleton and Rho GTPases interplay

Migrating cells are polarized with the lamellipodia facing the direction of migration. Protruding activity at the leading edge and retrograde flow of integrins coupled to adhesion of the lamellipodia near the leading edge are thought to be the driving force for cell motility. As described above, these activities are dependent on directed actin filament assembly and on the microtubule cytoskeleton. Although some small, specialized cell types such as keratocytes and leukocytes do not required microtubules for motility (Zigmond *et al.*, 1981; Euteneuer and Schliwa, 1984), microtubules are necessary for the persistent, polarized movement of larger cells such as fibroblasts or epithelial cells (Vasiliev, 1991). Depolymerization of microtubules stops cells migration and induces loss of cell polarity, so that ruffling activity normally restricted to the leading edge becomes reduced and redistributed to the entire cell (Vasiliev, 1991). Recent studies have shown that actin has a major influence on the organization of microtubules. There is evidence that microtubules are transported in the lamella of migrating cells and that this transport depends on actin (Waterman-Storer and Salmon, 1997). Moreover, other studies showed that, in the lamella, microtubules are coupled to actin retrograde flow,

whereas in the cell body microtubules are coupled to the anterograde motion of actin (Gupton *et al.*, 2002; Salmon *et al.*, 2002). Microtubule association to actin movement results in a gradient of microtubule assembly states in the cell, with plus-end growth at the leading edge and minus-end shortening in the cell body, behind the lamella. Several hypotheses have been proposed to explain how cells use actin-microtubules interactions to generate movement. One hypothesis is that cell motility depends on the structural linkage of microtubules to actin retrograde flow, which creates and maintains a regulatory Rho GTPases signaling gradient that triggers migration (Wittmann and Waterman-Storer, 2001). Microtubule growth at the leading edge could induce Rac activity in the cell front to drive lamellipodia protrusion and focal complexes formation. Rac activation, in turn promotes further microtubule growth reinforcing the polarization of a migratory cell in the absence of extracellular signals (Wittmann *et al.*, 2003). One candidate that can mediate Rac activation driven by microtubule growth in migrating cells is APC. APC localized at the microtubule plus end (Nathke *et al.*, 1996) and binds Asef, which is a Rac-specific GEF that stimulate lamellipodia formation and cell migration (Kawasaki *et al.*, 2003). Another candidate is IQGAP1, which binds Cdc42, Rac and actin, but also associates with growing microtubule plus end via CLIP-170 (Fukata *et al.*, 2002). Another hypothesis is that actin-microtubule interactions adjust toward the leading edge, which could then direct the delivery of signaling molecules or membrane components required for lamellipodia formation (Gundersen, 2002). It was shown that MTOC reorientation during cell migration is mediated by Cdc42 and the microtubule motor dynein (Etienne-Manneville and Hall, 2001; Palazzo *et al.*, 2001). Microtubules are crosslinked to specific sites defined by Cdc42 in the actin cortex and dynein may pull the MTOC in front of the nucleus.

Moreover, microtubules-actin interactions may mediate specific spatiotemporal regulation of focal contacts with the substrate to guide cell movement. In fact, it has been shown that during dynamic instability microtubules specifically target focal contacts and that the targeting frequency is inversely proportional to the focal contact lifetime (Kaverina *et al.*, 1999; Krylyshkina *et al.*, 2003). Further evidence illustrates that the microtubule motor kinesin may deliver putative factors that promote focal adhesion turnover (Krylyshkina *et al.*, 2002). Furthermore, microtubule shortening could activate

Rho behind the lamellipodia to drive actomyosin contraction and to promote stabilization of a subpopulation of microtubules, protecting them from breakage and thus maintaining a polarized microtubule cytoskeleton (Ren *et al.*, 1999). There are evidences that the Rho exchange factor GEF-H1 is regulated by an interaction with microtubules (Ren *et al.*, 1998; Krendel *et al.*, 2002). GEF-H1 is inactivated by microtubules binding and microtubules depolymerization can activate Rho by increasing the amount of free, active GEF-H1. Microtubules could serve to sequester GEF-H1 in the vicinity of adhesion sites and thereby reduce Rho activity, promoting adhesion turnover (Krendel *et al.*, 2002). Microtubules are probably guided to focal adhesions by structural links to adhesion-associated actin filaments, but the identity of these crosslinking proteins is still unknown (Salmon *et al.*, 2002; Krylyshkina *et al.*, 2003).

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IV. RESULTS

1. MEMO IS A NOVEL MEDIATOR OF ERBB2-DRIVEN CELL MOTILITY

Romina Marone, Daniel Hess, David Dankort, William J. Muller, Nancy E. Hynes and Ali Badache

Revised manuscript resubmitted to Nature Cell Biology

Data not shown and unpublished observations in the paper are numbered and shown in the appendix.

Memo is a novel mediator of ErbB2-driven cell motility

Romina Marone[1], Daniel Hess[1], David Dankort[2], William J. Muller[3], Nancy E. Hynes[1] and Ali Badache[1]

[1]Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland
[2]University of California San Francisco Comprehensive Cancer Center, San Francisco California, USA
[3] Molecular oncology group, Mc Gill University Health Center, Montreal, Quebec, Canada

Correspondence should be addressed to N.E.H.; email: hynes@fmi.ch

Abstract

The Neu/ErbB2 tyrosine kinase receptor has an important function in tumor cell motility, an essential characteristic of metastatic cells. In this study, we show that activation of a set of signaling molecules, including MAP kinase, phosphatidylinositol 3-kinase and Src, is required for Neu/ErbB2-dependent lamellipodia formation and for motility of breast carcinoma cells. Stimulation of these molecules, however, fails to induce efficient cell migration in the absence of Neu/ErbB2 Tyr1201 or Tyr1227 phosphorylation. We describe a novel mediator of ErbB2-driven cell motility (Memo) that interacts with a phospho-Tyr1227-containing peptide via the Shc adaptor protein. Upon Neu/ErbB2 activation, Memo-defective cells form actin fibers and grow lamellipodia, but fail to extend microtubules toward the cell cortex. Our data suggest that Memo controls cell migration by relaying extracellular chemotactic signals to the microtubule cytoskeleton.

The Neu/ErbB2 receptor tyrosine kinase is often overexpressed in human tumors of diverse origins including breast and ovaries[1,2]. Clinical studies have revealed that cancer patients whose tumors have alterations in ErbB2 expression tend to have more aggressive, metastatic disease, which is associated with parameters predicting a poor outcome[3]. In accordance with the clinical data, transgenic mice expressing activated Neu under the control of the mouse mammary tumor virus long terminal repeat develop metastatic mammary tumors[4-6]. Data from *in vitro* studies provide evidence that Neu/ErbB2 plays an important role in cancer cell motility and extracellular matrix invasion[7-10]. The molecular basis underlying ErbB2-dependent cell motility and metastases formation, however, still remains poorly understood.

Activation of ErbB2 via dimerization with other ligand-bound ErbB members results in phosphorylation of tyrosine residues in the cytoplasmic tail[11,12]. These phosphotyrosines serve as high affinity binding sites for molecules containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains such as the Shc and Grb2 adaptor molecules[13,14] or the p85 subunit of phosphatidylinositol 3-kinase (PI3K)[15]. These docking proteins transduce proliferative, transforming or migratory signals to the cell nucleus via activation of, for example, the Ras/mitogen-activated protein kinase (MAPK) and the PI3K pathways[16-19], both of which regulate different processes associated with cell migration, including formation of lamellipodia and actin stress fibers[20,21]. There is also evidence that p38 kinase and c-Src induce actin reorganization via phosphorylation of focal adhesion proteins[22,23]. The precise contribution of each pathway to ErbB receptor-regulated cell migration, however, remains to be determined.

The purpose of our study was to investigate the role of individual ErbB2 autophosphorylation sites in migration of human breast carcinoma cells. Our results show that Neu/ErbB2 lacking the five major autophosphorylation sites is impaired in stimulating migration and that two of the sites, Tyr1201 and Tyr1227, are fully able to restore the migratory phenotype of breast carcinoma cells. Moreover, we demonstrate that Memo, a newly identified protein which interacts with phosphorylated Tyr1227, mediates ErbB2-driven cell migration by controlling microtubule outgrowth.

Results

Role of specific ErbB2 tyrosine residues in heregulin-induced migration.

It has previously been shown that the T47D breast carcinoma cell line, which expresses moderate levels of the four ErbB receptors, is dependent upon ErbB2 activity for migration in response to EGF-related ligands[8]. We have now investigated the role of individual ErbB2 autophosphorylation sites in migration. For that purpose, ErbB2 was first functionally inactivated in T47D cells by expressing a single chain antibody (scFv-5R) that traps human ErbB2 in the endoplasmic reticulum[24], thus inhibiting its transfer to the plasma membrane, as confirmed by the absence of ErbB2 surface staining (data not shown, Appendix Figure 1), and preventing ligand-induced ErbB2 activation[25]. Migration of parental and scFv-5R-expressing cells (T47D-5R) in response to heregulin β1 (HRG) was measured in Boyden-like chambers. HRG binding to ErbB3 and ErbB4 leads to the formation of active ErbB2-containing heterodimers. HRG strongly stimulated migration of T47D cells, while T47D-5R cells were unable to migrate beyond basal levels (Fig. 1a), confirming the essential role of ErbB2 in EGF-related peptide induced migration[8].

T47D-5R cells were transfected with expression vectors encoding either wild type Neu, the rat homologue of ErbB2, or mutant Neu with a Phe residue substituted in each of the five autophosphorylation sites (termed NYPD for Neu tyrosine phosphorylation deficient) or Neu add-back mutants harboring only one of the five autophosphorylation sites, called YA, YB, YC, YD and YE, corresponding to Tyr1028, Tyr1144, Tyr1201, Tyr1227 and Tyr1253, respectively (nomenclature according to Dankort et al.[13]) (Fig. 1b). Cells expressing similar levels of wild type or mutant Neu were selected (data not shown, Appendix Figure 2) and their migration in response to HRG was evaluated. Neu efficiently replaced ErbB2 in T47D-5R cells, as demonstrated by their restored migratory response to HRG (Fig. 1c). In contrast, NYPD-, YA-, YB- and YE- expressing cells showed strongly reduced migration in response to HRG. It should be noted that each Neu mutant, despite lacking autophosphorylation sites, can interact with and transphosphorylate the other HRG- bound ErbB receptors, which likely explains the ability of these cells to migrate in response to HRG beyond the basal levels observed in the T47D-5R cells (Fig. 1a).

Intriguingly, migration of YC- and YD-expressing cells was equivalent to that of Neu-expressing cells (Fig. 1c), indicating that these two tyrosine residues couple to signaling pathways required for efficient cell migration. To verify their proposed role, tyrosine phosphorylated or non-phosphorylated peptides, corresponding to the region of Neu including the YC or YD residues, were used to compete for binding of signaling molecules to Neu. Peptide-mediated delivery[26] of a phospho-YC peptide into YC-expressing cells prevented HRG-induced migration (Fig. 2a), while the non-phosphorylated peptide did not interfere significantly with migration. Similarly, only the phospho-YD peptide efficiently inhibited migration of YD-expressing cells (Fig. 2b). Moreover, the phospho-YD peptide did not inhibit migration of YC-expressing cells (Fig. 2c) and conversely phospho-YC peptide did not interfere with migration of YD-expressing cells (Fig. 2d). These results not only confirm the requirement for phosphorylation of YC or YD tyrosine residues for cell migration, but also show that these two tyrosines recruit distinct signaling complexes.

HRG induces morphogenetic changes in T47D and NYPD cells

Cell motility can be viewed as a series of morphogenetic events based on remodeling of the actin cytoskeleton. Thus, we analyzed HRG-induced changes in cell morphology and cytoskeleton organization in migratory and non-migratory cells. HRG-treated T47D cells rapidly spread and formed membrane ruffles. Initially, cells extended lamellipodia in all directions, before showing a more polarized organization, paralleling the formation of actin stress fibers (Fig. 3a, upper panel). Surprisingly, NYPD cells, while greatly impaired in migration (Fig. 1c), displayed a normal morphogenetic response, extending and organizing lamellipodia after HRG treatment (Fig. 3a, lower panel). Lamellipodia formation is dependent on the activation of Rac, a member of the Rho GTPase family[27]. The kinetics of HRG-induced Rac activation was similar in T47D and NYPD cells: in both cell lines activity was transient, peaking 5 min after HRG addition (Fig.3b). These results are in accordance with the morphological results and provide further evidence that T47D and NYPD cells undergo comparable cytoskeletal rearrangements shortly after HRG treatment.

Cytoskeleton remodeling is widely used as a read-out for cell motility. The fact that HRG-triggered actin reorganization in T47D and NYPD cells was essentially identical, was in apparent contradiction with HRG's differential effect on migration of these cell lines. To minimize variations due to assay conditions, we analyzed lamellipodia formation in the same dual-chamber setting used to measure cell migration. T47D cells show a rapid increase in lamellipodia, apparent within an hour of HRG treatment, followed by a plateau and a second slower increase after 6 hrs (Fig. 3c & d). Interestingly, lamellipodia formation during the early time points (up to 4 hrs) was similar in Neu cells and in NYPD cells, but was strikingly altered at later times (Fig. 3d). These results confirm that early morphological and molecular changes, occurring in response to HRG are similar in migratory and non-migratory cells. Furthermore, they suggest that migration of NYPD cells is affected at a stage independent of lamellipodia formation.

Signaling pathways involved in ErbB2-dependent migration.

Our data show that the phosphorylated YC and YD residues of Neu/ErbB2 are crucial mediators of efficient, HRG-induced cell migration. Pathways implicated directly, or indirectly, in ErbB2-induced cytoskeleton remodeling and/or cell motility have previously been identified. These include the Ras/MAPK, PI3K, p38MAPK and Src kinase-dependent pathways[20-23]. Using selective kinase inhibitors on Neu cells, we determined that blocking each of these pathways led to a strong inhibition of HRG-induced migration (Fig. 4a). While each of these pathways is required for migration, it is, however, not sufficient. Indeed, stimulation of the MAPK, PI3K, p38MAPK (Fig. 4b) and Src (data not shown, Appendix Figure 3) pathways did not correlate with migration, since HRG activated each pathway as efficiently in, *e.g.*, NYPD or YA cells, as in Neu cells (Fig. 4b). As mentioned previously, Neu mutants can transphosphorylate the other ErbB receptors (ErbB3 or ErbB4), which likely explain activation of the examined pathways.

Interestingly, the kinase inhibitors also had a negative effect upon the low level of HRG-induced migration observed for NYPD cells, with blockade of the MAPK and PI3K

pathways having the strongest effect (Fig. 4c). Moreover, both inhibitors also prevented Neu and NYPD cells from forming lamellipodia in response to HRG (Fig. 4d). The severe loss of polarity of lamellipodia induced by inhibitors of these two pathways is likely to contribute to the loss of chemotaxis. Thus, activation of the MAPK and PI3K pathways is essential for early stages of migration, involving remodeling of the actin cytoskeleton. In contrast, we propose that phospho-YC or -YD provide links to novel signaling pathway(s) controlling stages of cell migration not directly connected to lamellipodia formation.

Identification of signaling molecules binding to the YC and YD residues

To search for novel proteins that might link phospho-YC and -YD to signaling pathways mediating ErbB2-dependent migration, tyrosine-phosphorylated peptides, corresponding to the regions of Neu including the YC or YD residues, were coupled to agarose beads and employed as affinity reagents. The corresponding non-phosphorylated peptides served as controls. We performed a large-scale systematic identification of proteins from T47D cell extracts that bound specifically to the phospho-, but not to the non-phosphorylated peptides, by high-pressure liquid chromatography-tandem mass spectrometry (LC-MSMS).

A number of proteins were identified, some of which have been reported to bind ErbB2/Neu, others being novel interactors. Previous studies have shown that the adaptor molecules Shc and Crk II associated with the phospho-YD and -YC residues, respectively[4,13]. We also identified these two proteins in the LC-MSMS screen, finding in addition that Shc bound both phosphorylated peptides. Furthermore, not only CrkII, but also the CrkI splice variant and the Crk-like protein bound the phospho-YC peptide. Phospholipase Cγ (PLCγ), which has not previously been reported to interact with either of these tyrosine residues, was found to associate with the phospho-YC peptide. Finally, an uncharacterized protein, CGI-27 or c21orf19-like protein, that we have named Memo (see below), associated specifically with the phospho-YD peptide. The binding specificity of each protein was confirmed in independent experiments using the phospho- and non-phosphorylated peptides as affinity reagents, followed by Western analysis (Fig. 5a). We

next investigated the *in vivo* interaction of Memo with ErbB2. Due to its high levels of expression, ErbB2 is constitutively activated in SKBr3 cells (Fig. 5b, P-ErbB2). In co-immunoprecipitation experiments, Memo associated with ErbB2 only when the latter was activated and not in cells treated with the ErbB2 kinase inhibitor PKI166[28] (Fig. 5b). We investigated the localization of ectopically-expressed Memo in SKBr3 cells. Memo was present at the plasma membrane of control SKBr3 cells (Fig.5c, upper panel). PKI166-mediated inhibition of ErbB2 activity led to decreased membrane staining, while upon addition of HRG, Memo was concentrated in specific ruffle-like areas of the plasma membrane (Fig.5c, upper panel). While immunolocalization using the Memo anti-serum was less sensitive and did not reveal membrane-bound Memo in control SKBr3 cells, it still showed recruitment of Memo to the plasma membrane upon addition of HRG (Fig.5c, lower panel).

The sequence of Memo does not contain SH2 or PTB phosphotyrosine-binding domains, known to interact with phosphotyrosines. The fact that Shc also interacted with the phospho-YD site raised the possibility that Shc, which has both a PTB and an SH2 domain, mediates the binding of Memo to phospho-YD. In immunoprecipitation experiments, ectopically expressed Memo was found to interact with Shc in SKBr3 cells (Fig. 5d). Moreover, both endogenous Memo and ErbB2 co-immunoprecipitated with Shc in T47D cells (Fig. 5e). Immunodepletion of endogenous Shc from reticulocyte lysates did not significantly affect the levels of *in vitro* transcribed Memo, present in large excess, yet binding of Memo to the phospho-YD peptide was strongly decreased (Fig. 5f). These results strongly suggest that the Shc adaptor protein is a mediator of Memo binding to the phospho-YD residue.

Role of phosphoYC/phosphoYD-binding proteins in cell migration.

The identified proteins were next tested for their role in ErbB2-dependent cell migration. The function of Shc and Crk in HRG-induced migration was tested using small interfering (si) RNAs to block their expression. Specific siRNA transfection of YC or YD cells led to a strong decrease in the level of Shc (and Crk, data not shown, Appendix Figure 4) relative to mock-transfected cells (Fig. 6a, inserts). In contrast to control cells,

migration of YC and YD cells with reduced expression of Shc (Fig. 6a) or Crk or decreased PLC activity (not shown, Appendix Figure 4) was strongly inhibited. Furthermore, siRNA-mediated knock-down of Shc and Crk levels or inhibition of PLC activity prevented Neu and NYPD cells from forming lamellipodia in response to HRG (Fig. 6b). Taken together, the results show that Shc, Crk and active PLCγ are required for HRG-induced cell migration. However, in contrast to the proteins we are seeking, they are involved in lamellipodia formation.

Memo, a mediator of ErbB2-driven cell motility

The function of Memo, the novel protein identified as a specific phospho-YD binder, is unknown. Furthermore, its sequence does not provide any information on a potential role in migration. Thus, we tested Memo's function using specific siRNA to knock-down its expression. Quantitative PCR revealed that Memo mRNA expression was ~ 80% lower in Memo siRNA transfected cells relative to cells treated with a control siRNA (Fig. 6c, insert). We have verified that the decrease in Memo RNA was paralleled by a decrease in Memo protein expression (data not shown, Appendix Figure 5). Importantly, loss of Memo strongly decreased migration of YD cells in response to HRG (Fig. 6c). Based upon these and the following results, it was named Memo for mediator of ErbB2-driven cell motility.

Inhibition of Memo's expression had a strong effect on HRG-induced migration of YD, but not YC cells, demonstrating that Memo acts specifically downstream of the YD tyrosine residue (Fig. 6e). In addition, Memo siRNA did not affect NYPD-expressing cells (Fig. 6e), whose migration is dependent on e.g. the MAPK and the PI3K pathways (Fig. 4). Importantly, HRG-induced formation of lamellipodia was not affected by a reduction in Memo levels (Fig. 6d), showing that, in contrast to the other identified signaling molecules, Memo is not involved in this step of cell migration. Based upon these results, we propose that Memo is a signaling molecule that links phosphorylated YD to stages of cell migration, independent of early cytoskeletal actin reorganization.

Our data demonstrate that Memo is required for migration of YD cells. HRG-induced migration of Neu-expressing cells (Fig. 6e) and parental ErbB2-expressing T47D

cells (Fig. 7a) was also dependent (by at least 50%) on Memo's expression. Thus, in the context of the wild type receptor, YC-dependent signaling is not able to fully offset the loss of functional Memo.

The SKBr3 and MDA-MB-231 cell lines are frequently used as experimental breast tumor models. Constitutive activation of ErbB2 in SKBr3 cells promotes constitutive signaling of the MAPK and PI3K pathways[29,30]. Despite this, SKBr3 cells display only low migration in the absence of ligands. In contrast, MDA-MB-231 cells show high basal motility in the absence of ligands and display metastatic growth in animal models. SKBr3 and MDA-MB-231 cells with reduced Memo levels showed decreased HRG-induced migration (Fig. 7a). Moreover, reduction in Memo's expression also lowered basal migration of MDA-MB-231 cells (Fig. 7a), which likely reflects the presence of autocrine activated ErbB2 in these cells (data not shown, Appendix Figure 6).

Finally, we examined the role of Memo downstream of other tyrosine kinase receptors. Fibroblast growth factor (FGF) 2 and, to a lower degree, insulin and epidermal growth factor (EGF) stimulated T47D cell migration (Fig. 7b). SiRNA-mediated knock-down of Memo did not affect insulin-dependent migration, but strongly reduced FGF2- and EGF-induced cell migration (Fig. 7b), indicative of a more widespread role for Memo in receptor tyrosine kinase-induced cell motility.

Memo is required for ErbB2-dependent microtubule outgrowth

Recent studies demonstrate the central role of the microtubule cytoskeleton for cell polarity and cell migration[31]. HRG induces the extension of microtubules from the centrosome to the cell periphery in T47D and SKBr3 cells (data not shown, Appendix Figure 7). However, when Memo's expression was inhibited, the network of microtubules induced by HRG was strongly reduced (Fig. 8a). The number of T47D and SKBr3 cells showing showing microtubule outgrowth is reduced from around 80% in control cells to 20% in cells transfected with Memo siRNA (Fig. 8b). Furthermore, actin stress fibers appeared to be increased when microtubule outgrowth was prevented. Interestingly, the central microtubule network, which is present regardless of ErbB2 activation, was not affected by the decrease in Memo's expression. This data show that

Memo is required for the ErbB2-dependent elongation of microtubules toward the cell cortex.

Discussion

The results presented here demonstrate that Neu/ErbB2-induced cell migration depends on the cooperative action of many signaling molecules working in concert to regulate discrete steps of the process. Ras/MAPK, PI3K, p38MAPK and Src-mediated signaling were all found to be indispensable for HRG-induced lamellipodia outgrowth and motility. In the absence of signaling pathway lying directly downstream of Tyr1201 and Tyr1227, however, their activation leads to only modest cell migration.

In this respect, we have identified a new mediator of Neu/ErbB2 signaling, Memo, which interacts specifically with phospho-Tyr1227 and is required for breast carcinoma cell migration. Memo corresponds to the CGI-27/c21orf19-like hypothetical protein, which was identified by comparative gene identification using the *C. elegans* proteome as a template[32], but was not attributed a function yet. It is conserved throughout evolution: proteins homologous to Memo exist in yeast, nematodes, drosophila and mammals. Its sequence shows no domain of identified function and does not provide information on how it might associate with phospho-Tyr1227. In fact, our results indicate that Memo binds to the phospho-Tyr1227 peptide via the Shc adaptor protein. Intriguingly, Memo does not bind to the phospho-Tyr1201 peptide, even though Shc does. This might be explained by the fact that Shc can associate with phosphorylated tyrosines using either the SH2 or the PTB domain[19,33,34] and thus, could adopt different conformations allowing the recruitment of different signaling molecules. Whether binding of Memo to Shc depends on the domain by which Shc interacts with the phosphotyrosine remains to be determined.

Unlike other investigated signaling molecules, Memo is not involved in stages of cell migration, such as lamellipodia formation, which are linked to remodeling of the actin cytoskeleton. We found that Memo is required for the extension of a microtubule network to the cell periphery. Microtubules grow out from the centrosome, their plus

ends exploring the cytoplasm through alternate phases of growth and shortening, a phenomenon termed dynamic instability. Microtubule dynamics can be modulated by two types of molecules; microtubule-associated proteins such as MCAK (mitotic centromere-associated kinesins), which bind to microtubule ends and destabilize them; and plus-end binding proteins, which favor microtubule growth through binding to the growing end, allowing microtubules to reach their target destination[35]. HRG triggers the growth of microtubules from the centrosome to the cell cortex. This does not occur in the absence of Memo. Thus, Memo could be a linker between extracellular chemotactic cues and the microtubule cytoskeleton, allowing the stabilization of outgrowing microtubules and the maintenance of cell polarity. Whether Memo prevents microtubule destabilization or promotes microtubule extension is still under investigation. Wittman et al.[36] recently described a population of central microtubules with little or no net growth. We also observed a population of stable central microtubules in both resting and ligand-activated cells. Interestingly, Memo is not required for the organization or maintenance of the central microtubules, indicating a specific role for Memo in stabilizing the most dynamic microtubules, extending toward the protruding membrane of migrating cells. Microtubules are also a key element for cell division. The fact that Memo is not required for microtubule organization in general, but specifically for microtubule outgrowth within lamellipodia, can explain why knocking down Memo's expression does not interfere with breast carcinoma cell proliferation (RM and AB, unpublished observations, Appendix Figure 8).

Both T47D and SKBr3 cells are capable of extending polarized lamellipodia in the absence of microtubule outgrowth. Similarly, nocodazole-treated cells do not grow microtubules, but are still capable of forming lamellipodia (RM and AB, unpublished observations, Appendix Figure 9) indicating that microtubule outgrowth is not required for early actin cytoskeleton remodeling. However, we have observed that in cells lacking Memo, while microtubule outgrowth is inhibited, the amount of actin stress fibers appears to be increased. It was previously shown that depolymerization of microtubules triggers the formation of stress fibers[37]. The Rho GTPase-exchange factor GEF-H1 was recently identified as a stress-fiber-inducing factor[38]; GEF-H1 stimulates Rho activity and stress fiber formation, only when it is not bound to microtubules. While the factors

leading to increased stress fibers when Memo is knocked down are not known, our observations highlight the dynamic interactions that take place between the actin and the microtubule cytoskeleton in migrating cells. Ongoing studies are aimed at understanding the role of Memo in the targeting of microtubules to cell cortical regions and how this contributes to cell polarization and directed cell motility.

Methods

Plasmid constructs, cell culture, cell transfection

T47D, SKBr3 and MDA-MB-231 breast carcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO Invitrogen AG, Basel, Switzerland). T47D-5R cells were obtained by infection of T47D cells with a pBabe-based retrovirus expressing the scFv-5R cDNA, as previously described[24]. The infected cells were selected in 1 mg/ml G418 (GIBCO) and clones were generated and tested for the absence of surface ErbB2 by FACS. Cells were then transfected with plasmids encoding Neu or Neu phosphorylation mutants[13] with a wild-type transmembrane sequence (V664) using FuGene (Roche Diagnostics Corporation, Indianapolis, IN, USA) and selected in 1 μg/ml puromycin (Sigma, St. Louis, MI, USA). In order to obtain cells expressing similar amounts of Neu receptor, cells were sorted after surface staining with a Neu specific antibody (Oncogene, Darmstadt, Germany). Memo cDNA, obtained by RT-PCR using RNA of T47D cells as a template, was first cloned into pGEM-T Easy (Promega Corporation, Madison, WI, USA), then subcloned into pcDNA3-derivatives containing the Myc epitope to generate the N-terminally tagged fusion proteins Myc-Memo. Constructs were verified by sequence analysis and transfected into SKBr3 cells using FuGene.

Migration assay

Cell migration was tested using 8 μm-pore polycarbonate membrane Transwell chambers (Corning Costar Products, Acton, MA, USA) as described previously[39]. In brief, the bottom side of the membrane was coated with 25 μg/ml rat tail collagen I (Roche). Serum starved cells were plated in the top Transwell chamber. Medium with or without 1 nM HRG-β1 (R&D systems, Inc., Minneapolis, MN, USA) was added to the bottom chamber and cells were allowed to migrate for 24 hours. Non-migrated cells were scraped off the top of the membrane. Migrated cells were fixed in 4% formaldehyde and stained in 0.1% crystal violet. Cells were counted under a microscope in ten high power fields. Migration was expressed as cell number per mm^2. In some instances, cells were pre-incubated for 60 minutes with the UO126 MEK inhibitor (50 μM; Promega), the LY294002 PI3K inhibitor (50 μM; Calbiochem-Novabiochem Corporation, San Diego, CA, USA), the SB203580 p38 MAPK inhibitor (20 μM; Calbiochem), the CGP77675 Src inhibitor (2.5 μM; kindly provided by M. Šuša and J. Green, Novartis, Basel, Switzerland) or the U-

73122 PLC inhibitor (2 μM; Calbiochem). Cells were then allowed to migrate for 8 hours before counting.

Immunoprecipitation and western blot

Cells were lysed in NP-40 lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 25 mM β-glycerol phosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml sodium vanadate and 100 μM phenylmethylsulfonyl fluoride) for 5 minutes on ice. For immunoprecipitation, equal amount of proteins were incubated for 1 hour with a Shc antibody from BD Transduction laboratories (Heidelberg, Germany) or an ErbB2-specific 21N polyclonal antiserum[40]. Immunocomplexes were collected with protein A-Sepharose (Sigma) and washed three times with lysis buffer and proteins were released by boiling in sample buffer. Proteins were blotted on polyvinylidene difluoride membranes (Millipore GmbH, Vienna, Austria) and probed with specific antibodies: P-p44/42, P-Akt/PKB and P-p38MAPK from New England Biolabs (Beverly, MA, USA), Myc from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Shc, 21N antiserum, phosphotyrosine-specific mAb[25] and affinity purified polyclonal antiserum directed against Memo (amino-acids 61 to 75). Proteins were visualized with peroxidase-coupled secondary antibodies using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Dübendorf, Germany).

Rac activity assay

Active Rac was detected using a glutathione-S-transferase (GST)-PAK- CRIB domain (CD) fusion protein (kindly provided by J. G. Collard, the Netherlands Cancer Institute, Amsterdam) as described previously[41]. Briefly, lysates from cells plated on collagen-coated dishes were incubated with bacterially produced GST-PAK-CD fusion protein bound to glutathione-coupled Sepharose beads. Proteins bound to the fusion protein were analyzed by Western blotting using an anti-Rac1 antibody (Upstate biotechnology, Lake Placid, NY, USA). Aliquots from the cell lysates were taken in order to analyze total amount of Rac1.

Immunofluorescence and actin staining

Cells were grown on glass coverslips (Falcon, Le Pont De Claix, France) coated with 25 μg/ml rat tail collagen I, serum starved overnight and stimulated with 1 nM HRG-β1 for different times. In some experiments the cells were pre-incubated for 60 minutes with the ErbB2 inhibitor, PKI166 (5 μM, kindly provided by P. Traxler, Novartis[28]). Cells were fixed in 4% formaldehyde in phosphate buffer saline (PBS) for 20 minutes, permeabilized in 0.2% Triton X-100 for 10 minutes, blocked with 1% bovine serum albumin in PBS for 20 minutes before addition of anti-Myc, anti-Memo or anti-α-tubulin antibody (kindly provided by W. Krek, ETH, Zürich) and the appropriate fluorophore-labeled secondary antibody (Molecular Probes, Leiden, The Netherlands). DNA was counterstained with 0.25 mg/ml Hoechst No. 33342 (Sigma). Actin was stained for 45 minutes with 2U/ml TRITC-labeled phalloidin (Sigma) or Alexa-Fluor 488 phalloidin (Molecular Probes). Images were recorded with an Axioskop Zeiss microscope coupled to a Sony 3CCD camera or an Olympus IX70 microscope linked to the DeltaVision workstation (Applied Precision, Issaquah, WA).

Purification of lamellipodia

Proteins localized in lamellipodia were specifically purified using the method described by Cho et al.[42]. Briefly, cells were plated on 3 μm porous polycarbonate membrane Transwell chamber (Costar) coated on the bottom side with rat tail collagen I. The lower chamber contained medium with or without 1 nM HRG-β1. Cells were allowed to extend lamellipodia through the pores for different times. Cell bodies remaining on the upper surface were removed and the lamellipodia extending to the lower surface were recovered in lysis buffer. Protein concentration was measured using Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

SiRNA and peptide transfection

Cells were transfected with siRNA using Oligofectamine (GIBCO) according to the manufacturer's instructions. The following 21-mer oligoribonucleotide pairs (obtained from Xeragon Inc., Huntsville, AL, USA) were used: for Shc (accession number HSU7377) nucleotide 236 to 256[43], for Memo (CGI-27; accession number AF132961) nucleotide 460 to 480 and for control LacZ (from D. Cappellen, FMI, Basel; accession number M55068) nucleotide 4277 to 4297. Cells were plated for migration assays 3 days after siRNA transfection and allowed to migrate for 24 hours. Cell lysates were also prepared 3 and 4 days after transfection and analyzed by Western blotting using a specific anti-Shc antibody. For Memo, RNA was extracted using RNeasy Mini (Qiagen, Cologne, Germany) and quantitative radioactive PCR[44] was performed using Memo specific primers (forward from nucleotide 90 to 111 and reverse from nucleotide 256 to 235).

Peptides were delivered into cells using the Chariot peptide carrier[26] (Active Motif, Rixensart, Belgium) according to the instruction manual. 16 aa-peptides spanning tyrosine residue 1201 of Neu (YC peptide) and tyrosine residue 1227 of Neu (YD peptide) were obtained from Neosystem (Strasbourg, France) in phosphorylated and non phosphorylated forms. Cells were plated for migration 30 minutes after peptide transfection and the assay was finished after 22 hours.

Pull-down assay and mass spectrometry

Phosphorylated and non-phosphorylated YC and YD peptides were coupled under anhydrous conditions to Affi-gel 10 agarose beads (Bio-Rad). Coupled beads were incubated with 0.5 mg (for Western blotting) or 12 mg (for mass spectrometry) T47D cell lysates. Proteins bound to the peptides were subjected to SDS-PAGE. For mass spectrometry the gels were stained with Coomassie Brilliant Blue R-250. Each lane of the gels was sliced and analyzed by LC-MSMS (LCQ Deca XP, Thermo Finnigan) and proteins identified by Turbo Sequest. Proteins identified by more than two peptides and binding specifically to the phosphorylated form of the peptides were selected for further analysis. Binding was confirmed by Western blotting using antibodies against CrkII and PLCγ (Santa Cruz) , Shc and Memo. In some experiments, Shc was immunodepleted from reticulocyte lysates expressing *in vitro* translated Myc-Memo using the anti-Shc antibody, before performing the pull-down assay.

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ACKNOWLEDGEMENTS
We thank F. Maurer for technical assistance, D. Cappellen for help with quantitative PCR and discussions, S. Kleiner for help with siRNA experiments, A. Brahmbhatt and R. Klemke for help with lamellipodia purification, W. Krek, G. Thomas and R. Chiquet for critical reading of the manuscript. This work was supported by the Novartis Research Foundation and grants from the Swiss Cancer League to A.B. and R.M.

Figure legends

Figure 1 **Tyr1201 (YC) and Tyr1227 (YD) are required for HRG-induced cell migration**. **a**, Parental T47D and T47D lacking functional ErbB2 (T47D-5R) were assayed for their ability to migrate in response to HRG. A typical experiment is shown; bars represent the average number of cells migrated per mm^2, error bars represent s.d. **b**, Schematic representation of Neu, NYPD and the add-back mutants used in this study; YA, YB, YC, YD and YE correspond to Tyr1028, Tyr1144, Tyr1201, Tyr1227 and

Tyr1253 of Neu, respectively. **c**, T47D-5R cells expressing the different Neu constructs were tested for their ability to migrate in response to HRG.

Figure 2 **Effect of YC- or YD-containing synthetic phosphopeptides on HRG-induced cell migration. a-d**, YC- and YD-expressing cells were transfected with YC, phosphorylated YC (pYC), YD or phosphorylated YD (pYD) peptides as indicated and migration of YC- and YD-expressing cells in response to HRG was analyzed.

Figure 3 **Neu and NYPD cells both form lamellipodia upon HRG stimulation**. **a**, T47D and NYPD cells were treated for different times with HRG, then F-actin was visualized with TRITC-labeled phalloidin. **b**, Rac activity in HRG-stimulated T47D and NYPD cells was measured via pull-down of GTP-bound Rac1 using beads coupled to a GST-PAK-CD fusion protein (upper panels). The total amount of Rac1 is shown in the lower panels. **c**, Lamellipodia formed in response to HRG were collected from T47D cells plated on a 3 μm porous membrane Transwell chamber and their concentration was measured and plotted with time. **d**, The concentration of lamellipodia from HRG-treated Neu and NYPD cells was determined as in **c**. The average of two independent experiments is shown.

Figure 4 **Activity of the MAPK, PI3K and p38MAPK pathways is required, but is not sufficient for Neu/ErbB2 dependent cell migration. a**, HRG-induced migration of Neu cells was tested in the presence of MEK, PI3K, p38 or Src inhibitors. **b**, Activation of the MAPK, PI3K and p38MAPK pathways in response to HRG was assayed in the NYPD- and add back mutant-expressing cells. Cell extracts were analyzed by Western blotting and membranes were probed with P-MAPK, P-PKB and P-p38 antibodies. **c**, HRG-dependent migration of NYPD cells was tested in the presence of MEK, PI3K, p38 or Src inhibitors. **d**, Effect of MEK and PI3K inhibitors on HRG-induced lamellipodia formation in Neu and NYPD cells

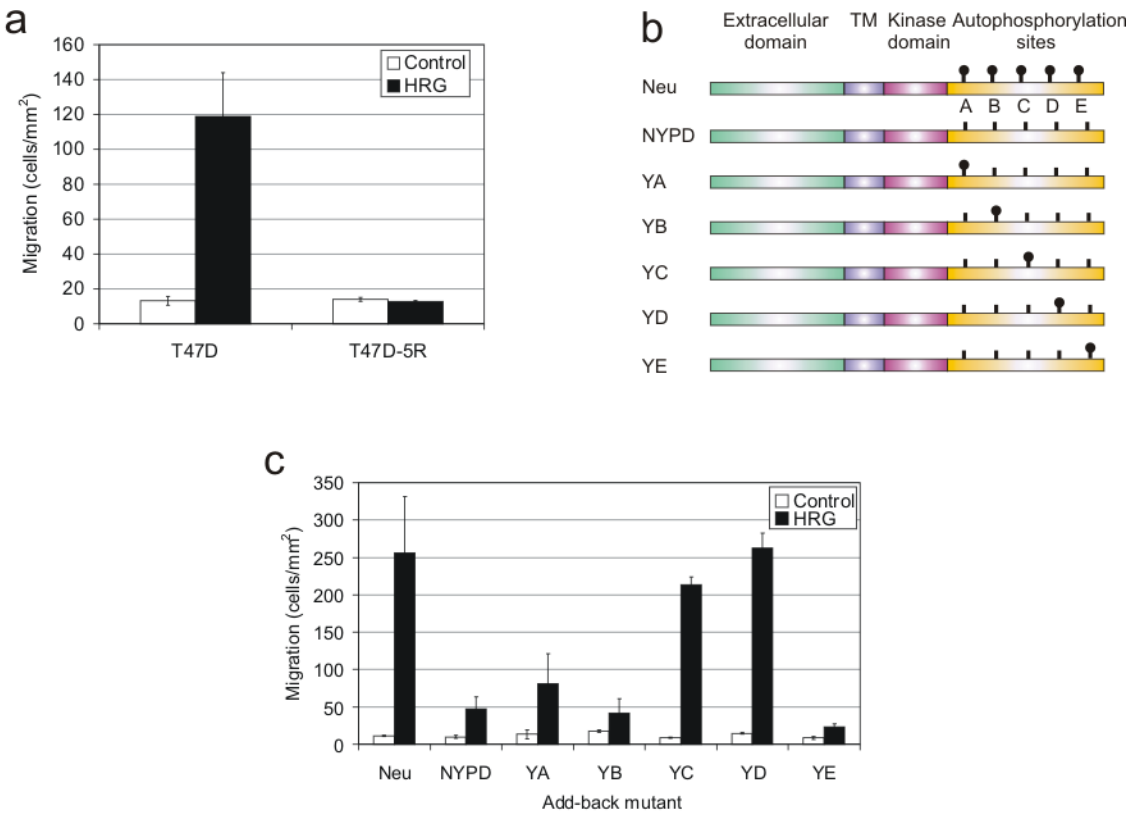
Figure 5 **Memo interacts with ErbB2 Tyr1227 via the Shc adaptor molecule . a**, Binding of Shc, CrkII, PLCγ and Memo to YC, pYC, YD and pYD peptides was

analyzed by peptide pull-down, followed by Western blotting with the respective antibodies. Whole cell extracts (W) were also loaded on the gel. **b**, Myc-Memo-expressing SKBr3 cells were pretreated or not with the ErbB2 inhibitor PKI166. Cell lysates were immunoprecipitated with ErbB2, analyzed by Western blotting and the membrane probed with antibodies against Myc, phosphotyrosine or ErbB2. **c**, Memo cellular localization in Myc-Memo-expressing and untransfected cells was visualized using respectively an antibodies against Myc (upper panel) or Memo (lower panel) in control, PKI166- or HRG-treated SKBr3 cells. Arrows indicate the cell membrane. **d**, Myc-Memo-expressing SKBr3 cells were pretreated or not with PKI166, before Shc immunoprecipitation and probing with anti-Myc and anti-Shc antibodies. **e,** T47D cells were stimulated or not with HRG and cell extracts analyzed by immunoprecipitation of Shc and probing for ErbB2, Memo and Shc. **f**, Shc was immunodepleted from reticulocyte lysates expressing *in vitro* translated Myc-Memo and Myc-Memo interaction with the pYD peptide was tested in control and Shc-depleted lysates.

Figure 6 **Memo is required for ErbB2-driven cell motility, but not for lamellipodia formation. a**, HRG-dependent migration of YC and YD cells was tested after Shc siRNA transfection. Protein extracts were collected 3 and 4 days (3d and 4d) after transfection. The effect of Shc siRNA on Shc expression was verified by Western blotting using a Shc specific antibody (insert). **b**, HRG-dependent lamellipodia outgrowth was visualized in Neu and NYPD cells in the presence of Shc or CrkII siRNA or a PLC inhibitor. **c**, HRG-dependent migration of YD cells treated with control (LacZ) or Memo siRNA was analyzed. RNA was collected 3d and 4d after transfection and Memo mRNA was measured by quantitative PCR (insert). **d**, HRG-induced lamellipodia formation in YD cells was observed in the presence of control (LacZ) or Memo siRNA. **e**, Effect of Memo siRNA on HRG-induced migration of Neu, NYPD, YC and YD cells was measured.

Figure 7 **Memo is a mediator of growth factor-induced breast carcinoma cell motility. a**, HRG-dependent migration of T47D, SKBr3 and MDA-MB-231 cells was tested after Memo siRNA transfection. **b**, Effect of Memo siRNA on migration of T47D cells in response to HRG, FGF2, insulin and EGF.

Figure 8 **Memo is required for ErbB2-dependent microtubule outgrowth. a**, The actin and the microtubule cytoskeleton were visualized in HRG-stimulated T47D cells treated with control or Memo siRNA using Alexa-Fluor 488-phalloidin and an anti-α-tubulin antibody, respectively. Arrows indicate the cell membrane. **b**, Microtubule outgrowth was evaluated in cells treated with HRG for 30 min, in the presence of control or Memo siRNA. For T47D cells n=321 and 347 for LacZ and Memo siRNA respectively and for SKBr3 cells n=348 and 256 for LacZ and Memo siRNA, respectively.



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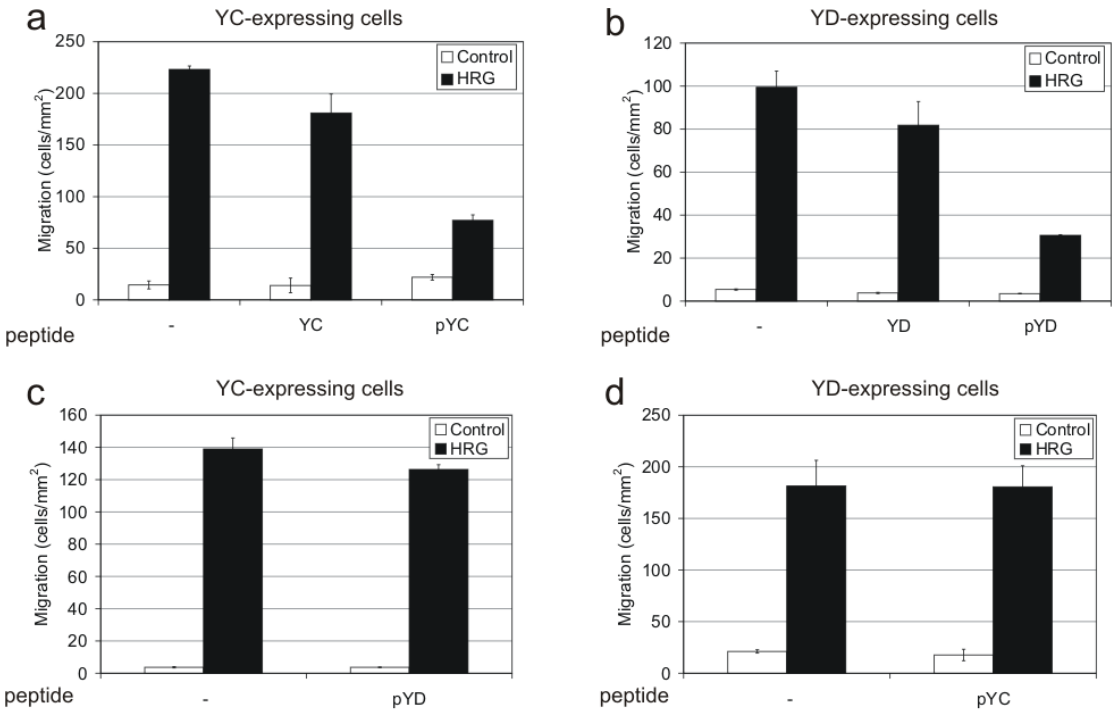

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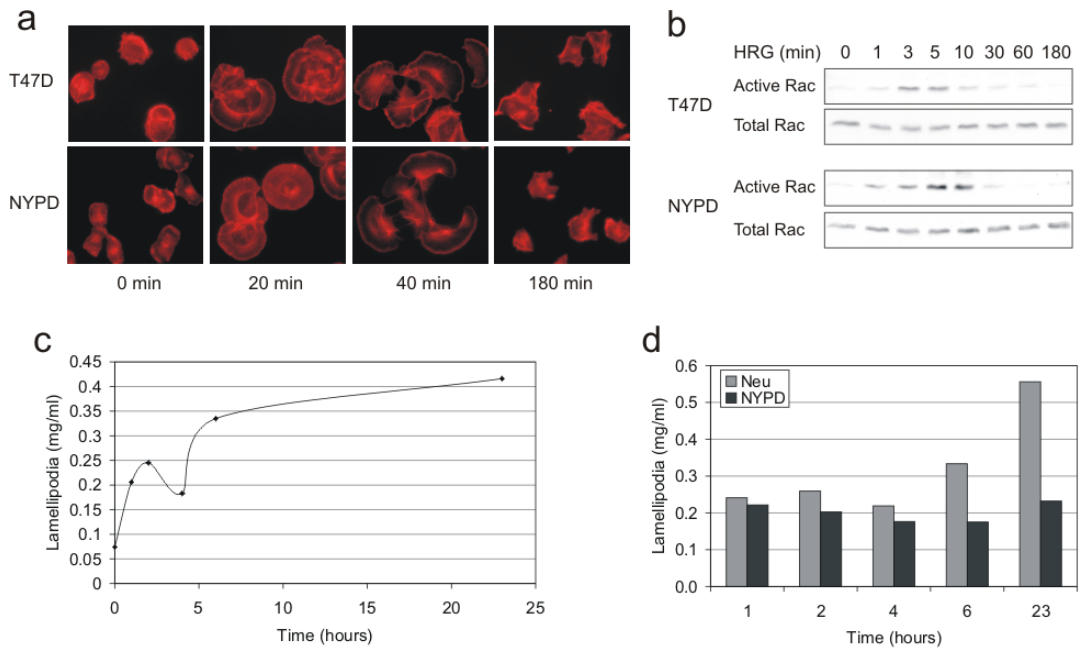

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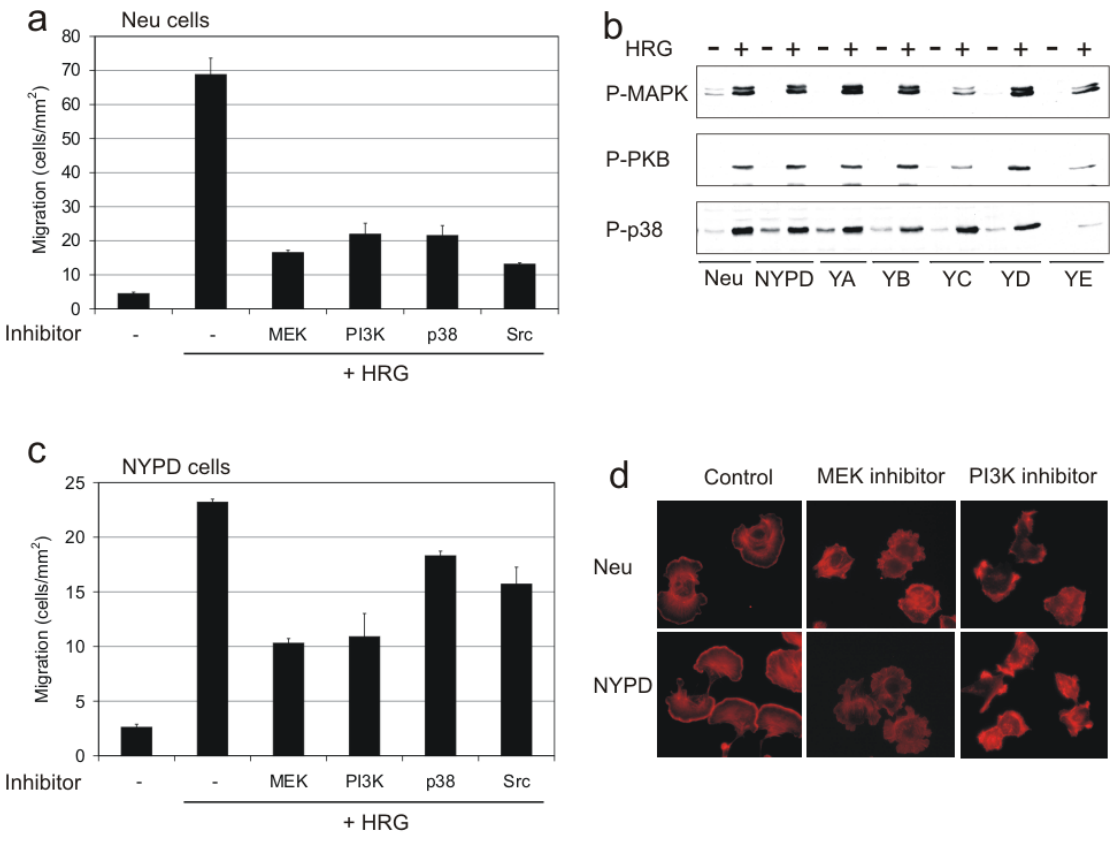

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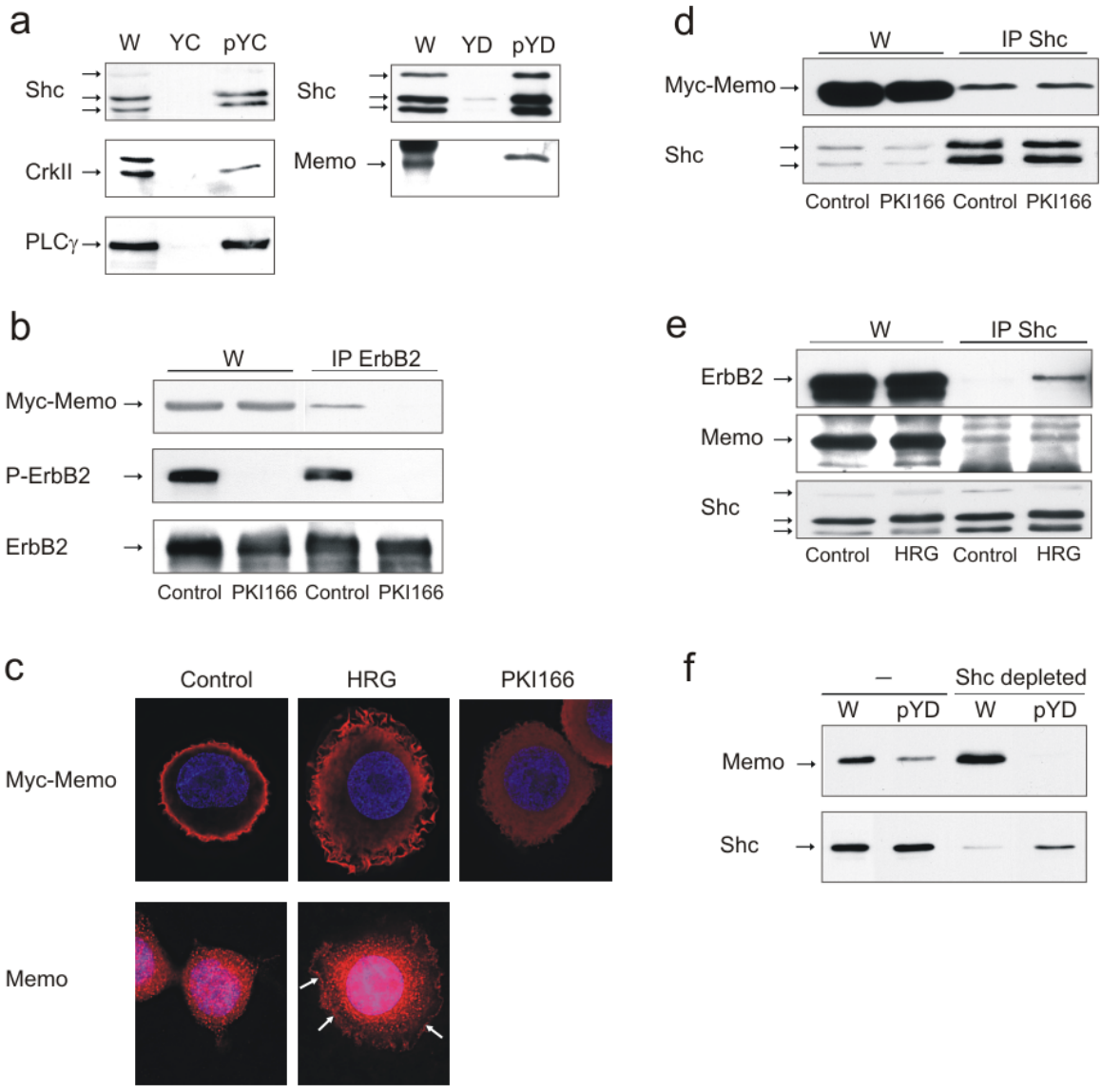

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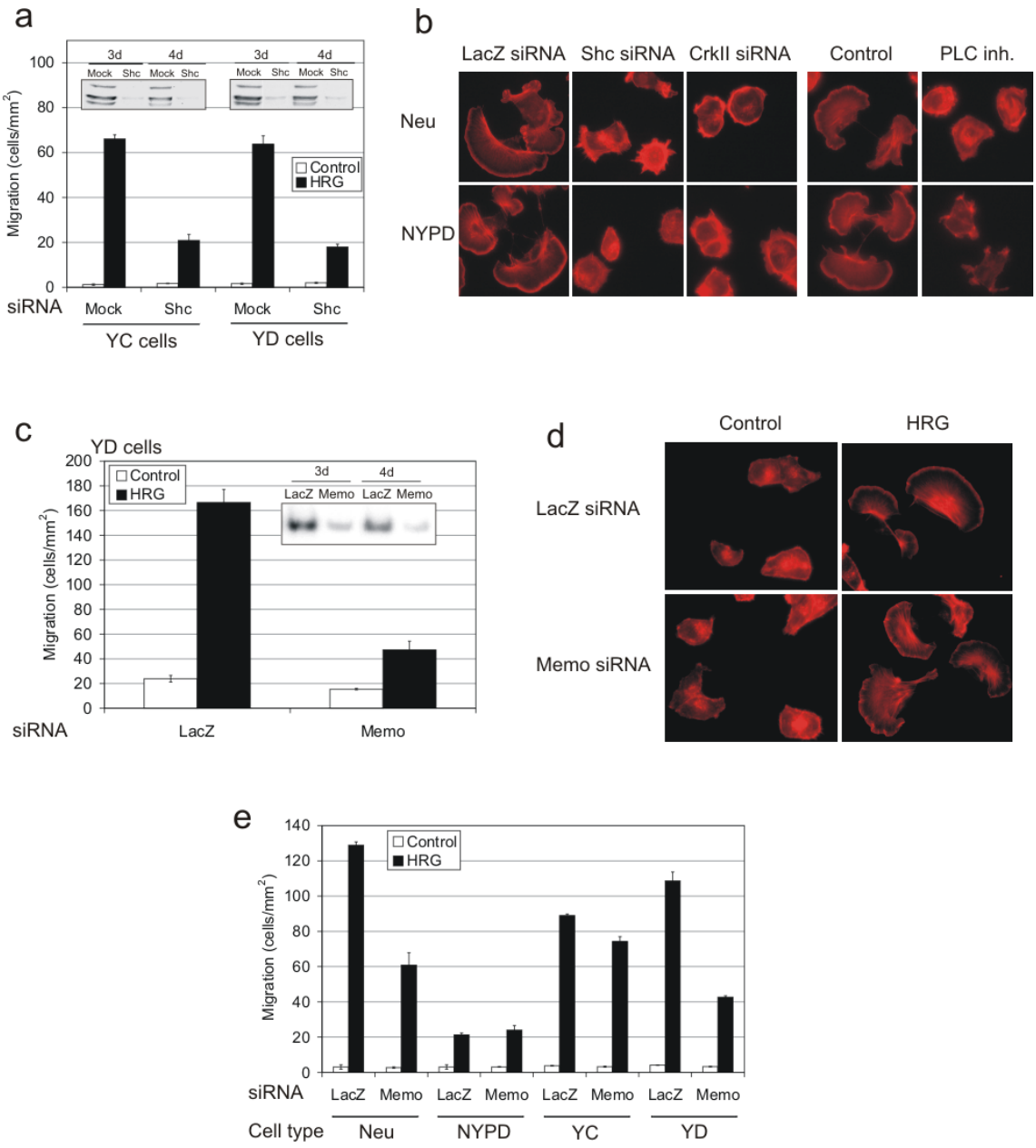

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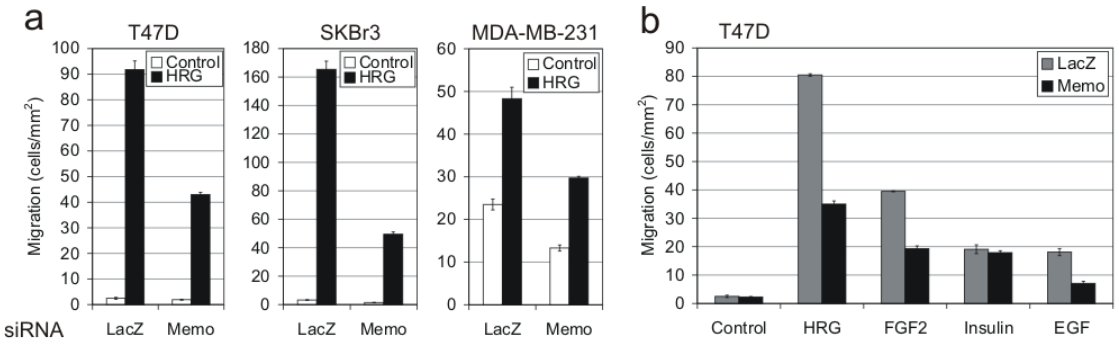

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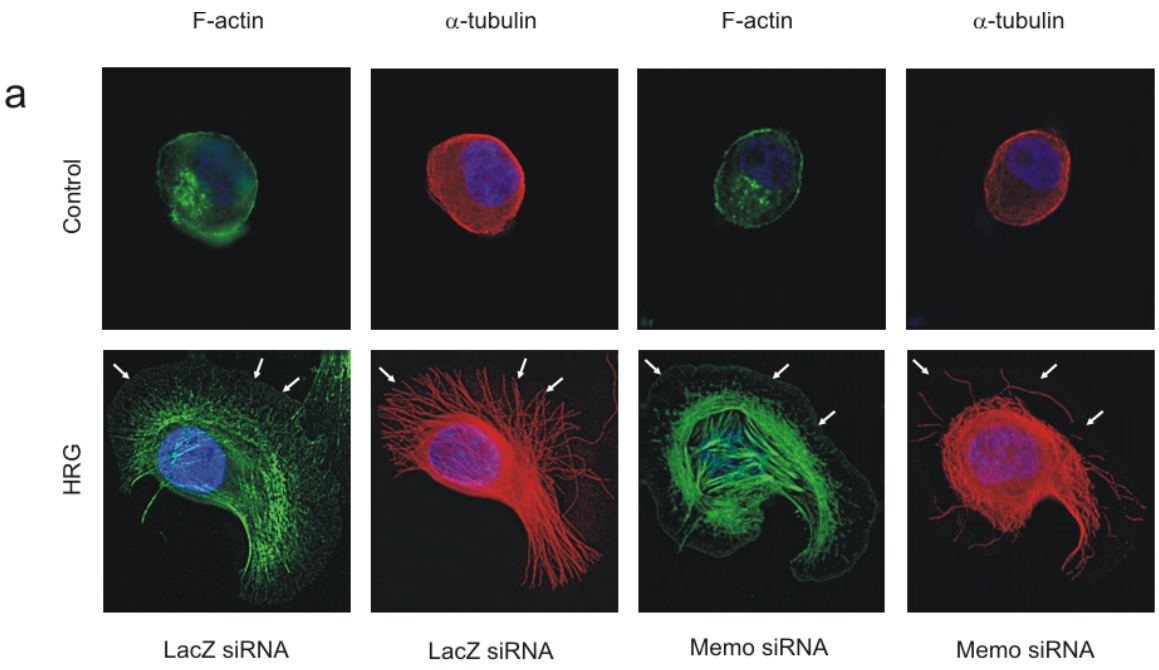


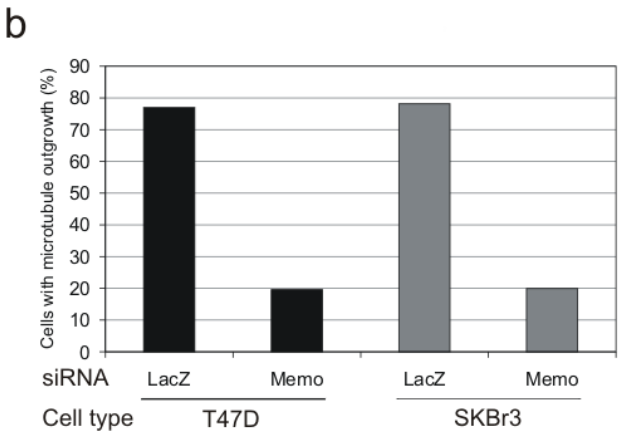


Figure8 (Hynes)

1.1. Appendix

Figure 1. Surface staining of T47D and T47D-5R cells

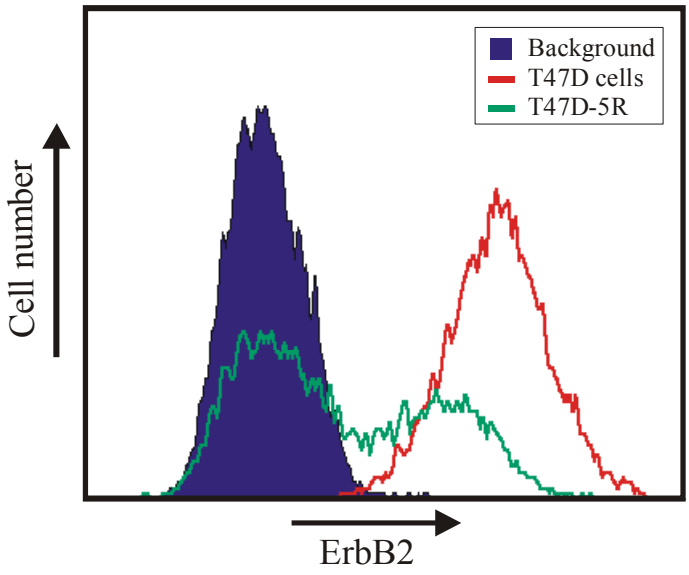


Figure 2. Surface staining of wild type Neu or Neu add-back mutant expressing cells

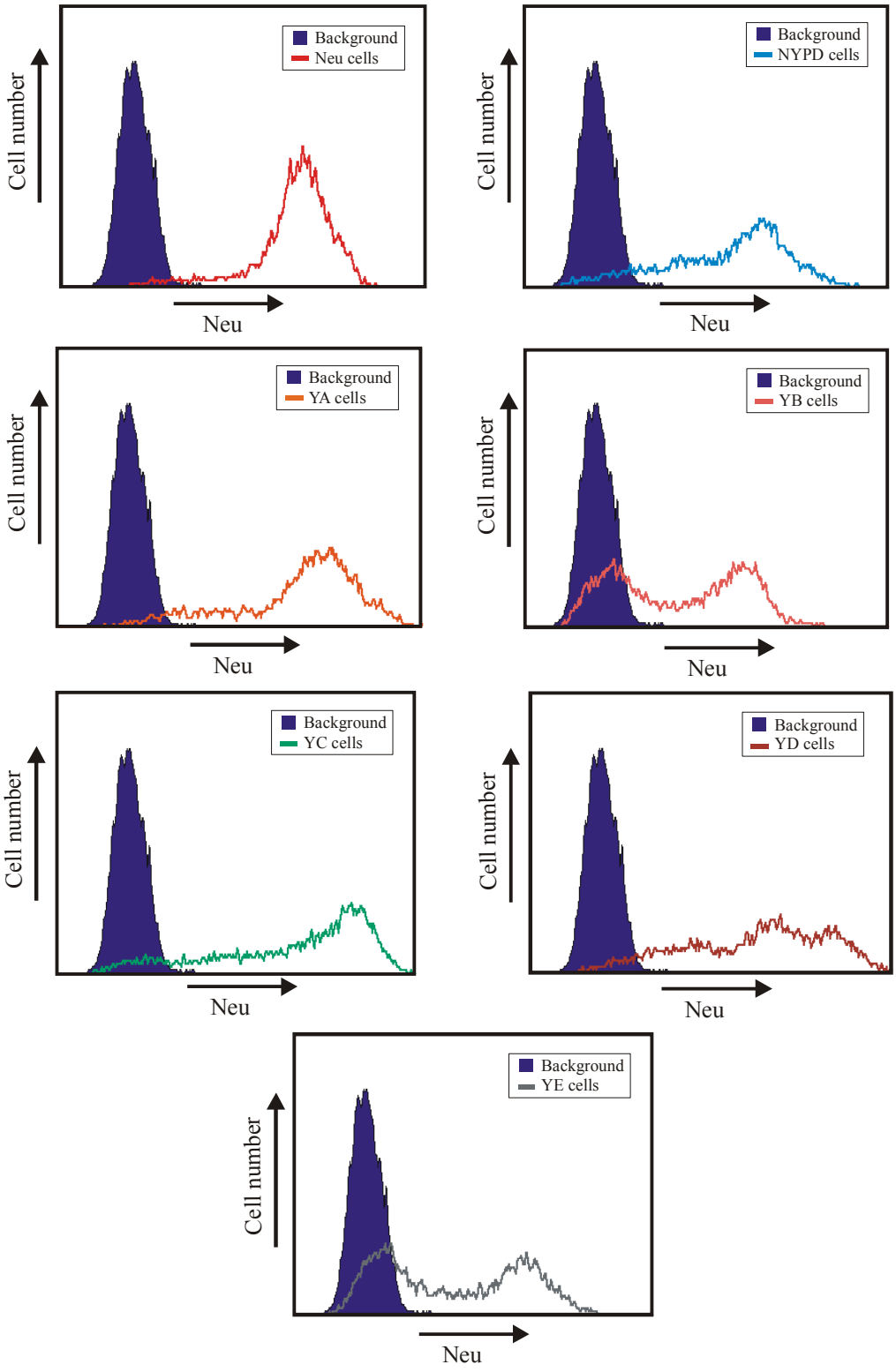


Figure 3. Activation of Src in T47D and NYPD cells

- Immunopreciptiation of the Src substrate cortactin followed by western blot for phosphotyrosine

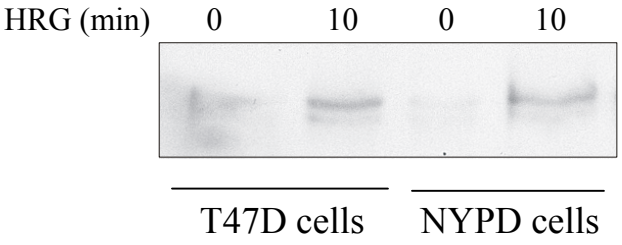
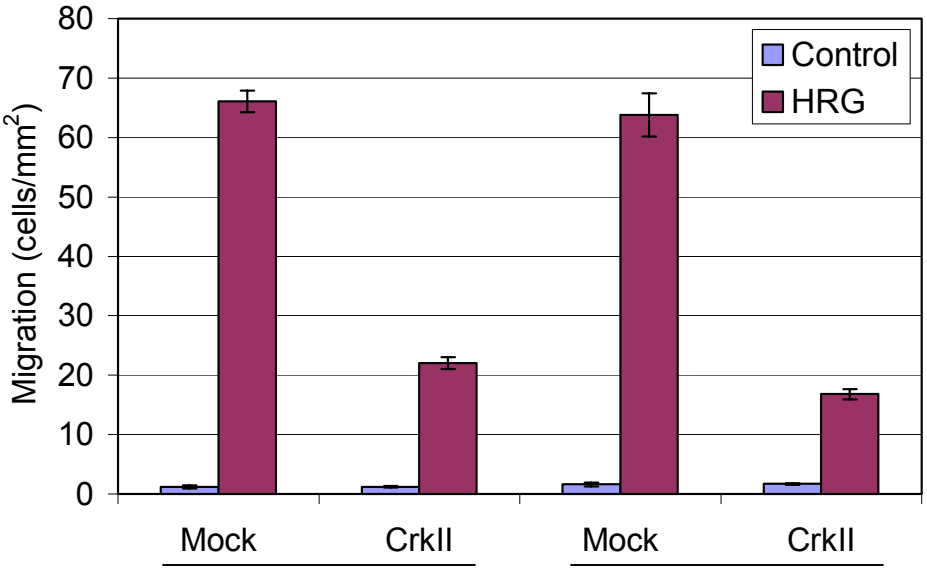


Figure 4. Downregulation of CrkII and inactivation of PLC in YC cells: effects on cell migration

- CrkII western blot of YC and YD cells transfected with mock or CrkII siRNA.

YC cells YD cells

- Migration assay of YC and YD cells transfected with mock or CrkII siRNA.



YC cells YD cells

• Migration assay of YC and YD cells treated with the U-73122 PLC inhibitor.

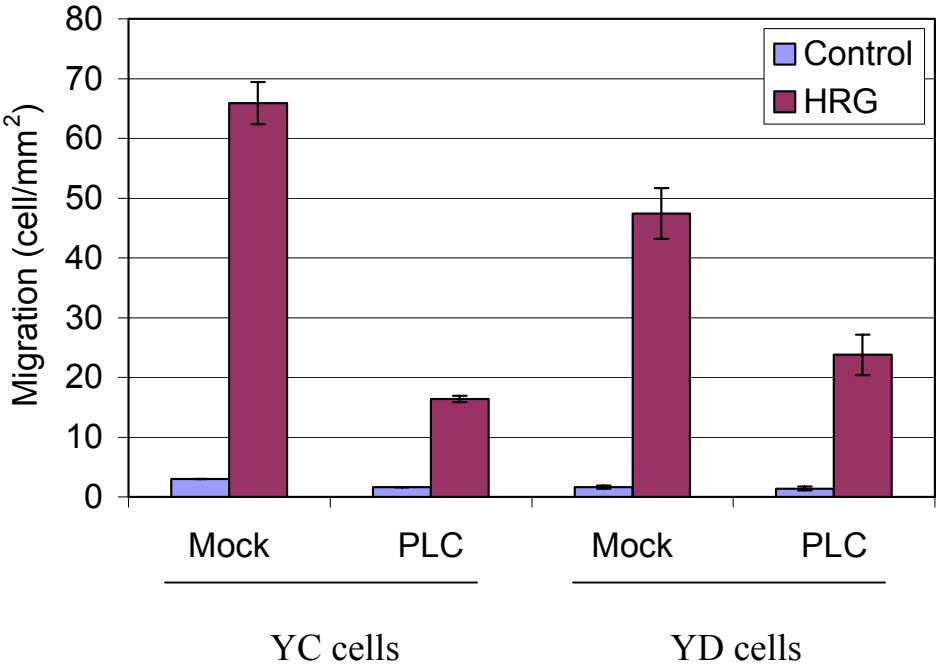


Figure 5. Memo protein levels

Western blot of T47D cells transfected with LacZ or Memo siRNA with an antibody against Memo

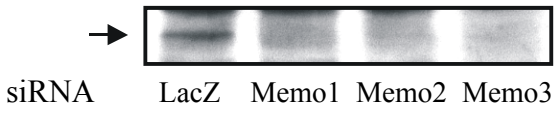
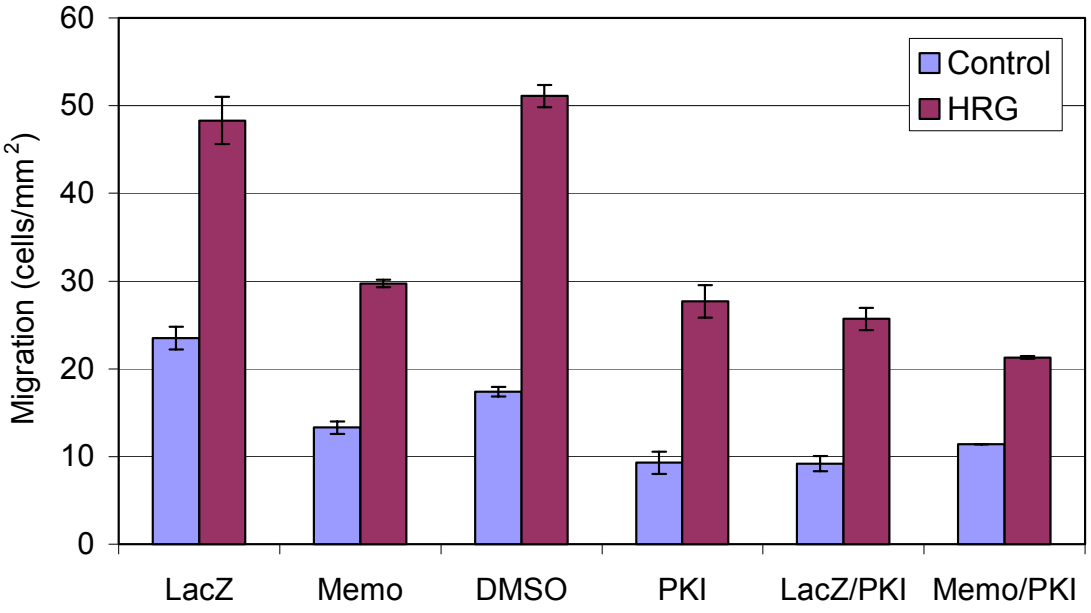


Figure 6. Migration of MDA-MB-231 cells was tested with Memo siRNA and /or the ErbB2 kinase inhibitor PKI166



Treatment of the MDA-MB-231 cells with PKI166 lowers the HRG-induced migration, but also the basal cell migration, which is dependent on the autocrine activated ErbB2 in these cells. Interestingly, the combination of PKI166 with the downregulation of Memo does not have an addictive effect, suggesting that Memo acts downstream of ErbB2.

Figure 7. Microtubule staining of T47D cells, which were stimulated with HRG for different times, using an anti-tubulin antibody

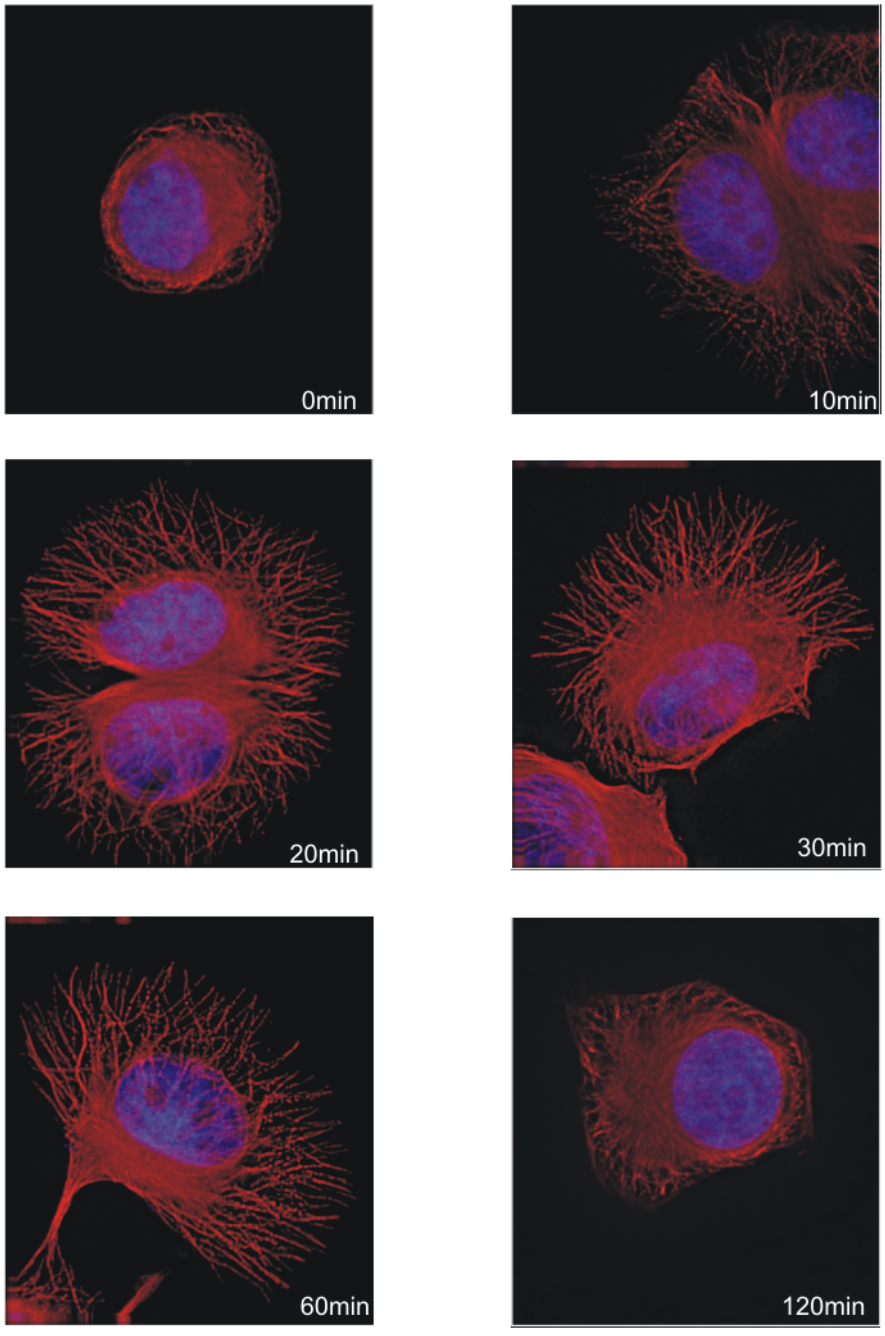


Figure 8. Proliferation of T47D, SKBr3 and MDA-MB-231 cells transfected or not with Memo siRNA in the presence or absence of HRG

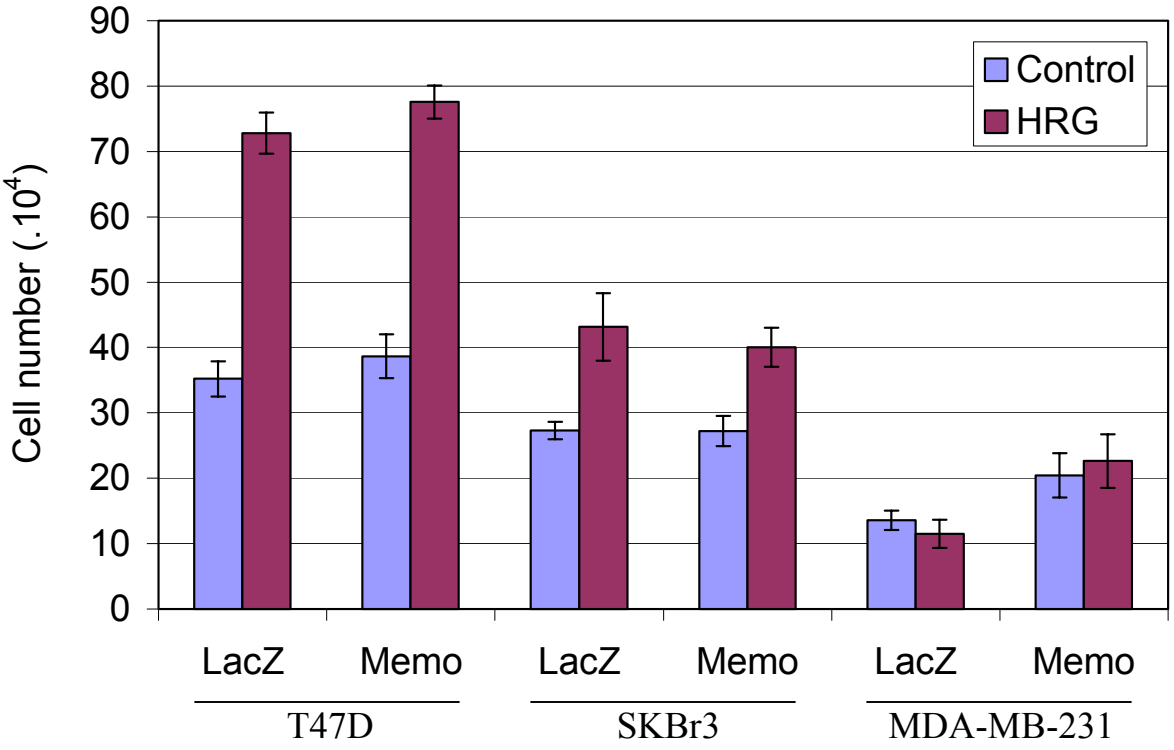
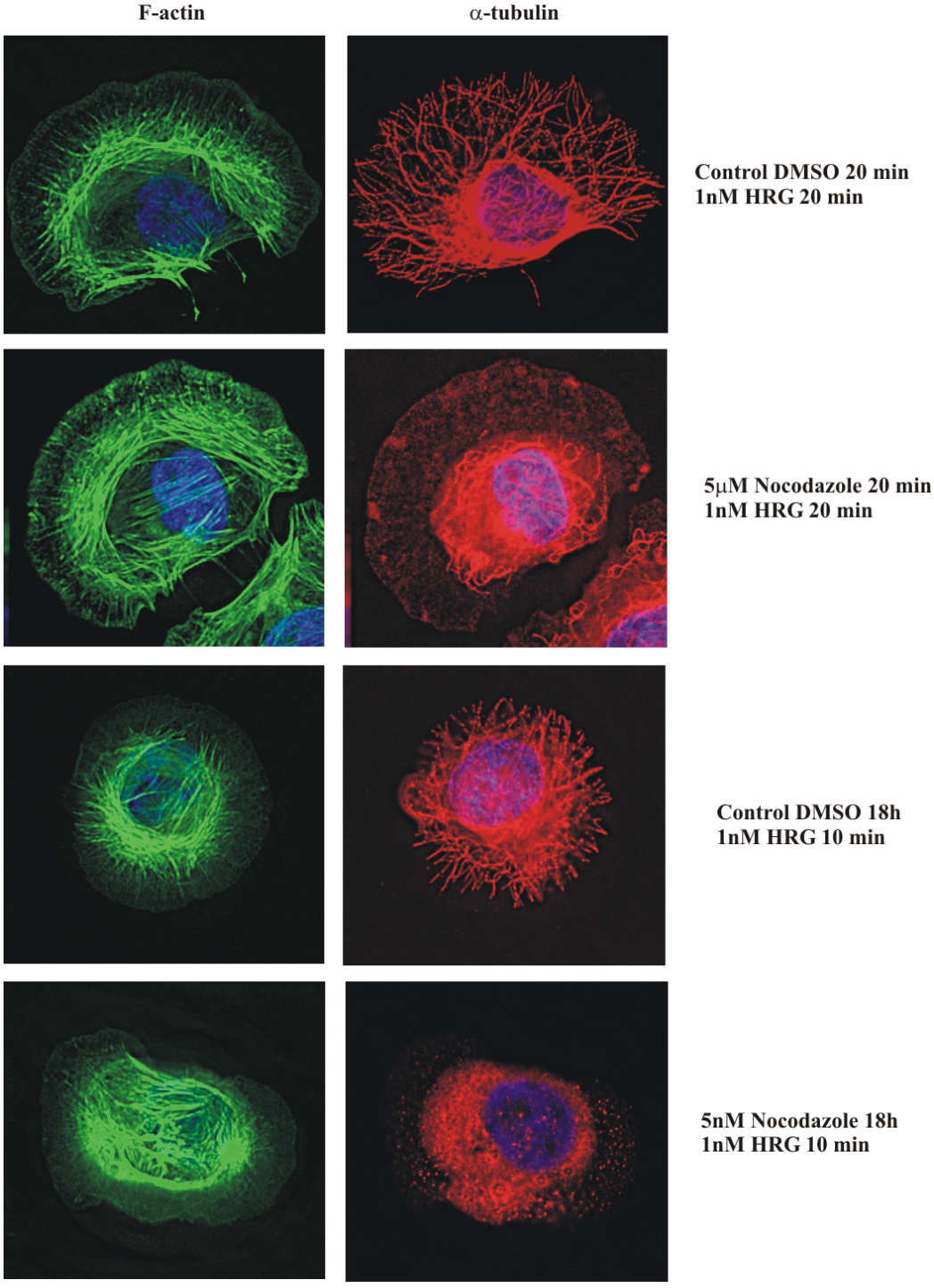


Figure 9. Effect of nocodazole on lamellipodia formation of T47D cells



Nocodazole mediated disruption of the microtubule network has no effect on the ability of the cells to form lamellipodia. Interestingly, long-term nocodazole treatment also leads to the disruption of the central microtubules, but has no consequence on the actin cytoskeleton remodeling.

Additional materials and methods

Surface staining

Cells were trypsinized and washed in PBS prior to staining. Approximately 10^6 cells were then resuspended in 500 µl of PBS containing 5 µg/ml of the ErbB2 mAb FSP77 (Appendix Figure 1) or 1 µg/ml of the Neu mAb (Appendix Figure 2). After incubation on ice for 1 hour, cells were washed three times in PBS. Bound antibodies were stained for 1 hour with fluorescent-labeled anti-mouse secondary antibody (from Amersham Pharmacia Biotech). Finally, the cells were washed three times in PBS, resuspended in 500 µl of PBS, and analyzed for their fluorescence with a Becton Dickinson FACScan.

SiRNA transfection

Cells were transfected with siRNA using Oligofectamine (GIBCO) according to the manufacturer's instructions. For CrkII (accession number NM_016823) the 21-mer oligoribonucleotide pair from nucleotide 454 to 474 was used. For Memo (accession number AF132961) the following 21-mer were used: Memo1 from nucleotide 460 to 480, Memo2 from nucleotide 232 to 252 and Memo3 from nucleotide 701 to 721. Cells were plated for migration assays 3 days after siRNA transfection and allowed to migrate for 24 hours. Cell lysates were also prepared 3 and 4 days after transfection and analyzed by Western blotting using a specific anti-CrkII antibody (Santa Cruz).

Proliferation assay

T47D, SKBr3 and MBA-MD-231 cells were transfected with LacZ or Memo siRNA as described before. Cells were trypsinized 3 days after siRNA transfection and counted. Same numbers of cells ($2*10^5$ for T47D and SKBr3 cells and 10^5 for MDA-MB-231) were plated in triplicate in the presence or not of 1nM HRG-β1. Cells were allowed to proliferate for 3 days and then were trypsinized and counted in a hemocytometer. (Appendix Figure 8)

Immunofluorescence of T47D cells after nocodazole treatment

Cells were grown on glass coverslips coated with 25 μg/ml rat tail collagen I, serum starved overnight and pre-incubated for 18 hours or 20 minutes with nocodazole at concentrations of 5nM or 5μM, respectively (Sigma). The cells were then stimulated with 1 nM HRG-β1 for different times. Cells were fixed in 4% formaldehyde in PBS for 20 minutes, permeabilized in 0.2% Triton X-100 for 10 minutes, blocked with 1% bovine serum albumin in PBS for 20 minutes before addition of the anti-α-tubulin antibody and the Alexa Fluor 594 goat anti-rat secondary antibody. DNA was counterstained with 0.25 mg/ml Hoechst No. 33342 (Sigma). Actin was stained for 45 minutes with 2U/ml Alexa-Fluor 488 phalloidin (Molecular Probes). Images were recorded with an Olympus IX70 microscope linked to the DeltaVision workstation (Applied Precision, Issaquah, WA). (Appendix Figure 9)

1.2. Information about Memo

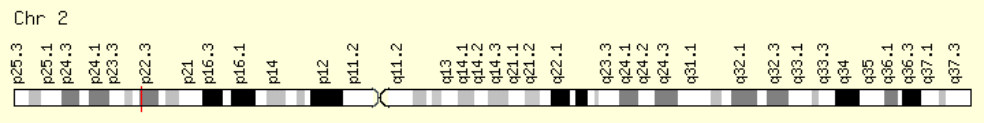
Memo= mediator of ErbB2-driven cell motility
(CGI-27 or c21orf19 like proteins)

1.2.1. Characterization of Memo

- Memo was discovered by comparative genome identification:
  The C. elegans proteome was used as scaffold to assist in novel human gene identification from human EST nucleotide databases [32].

- Memo encodes a predicted protein of 297 residues (molecular weight 33.7kD).

- The human gene maps to chromosome 2 [2p23.2]. There are also two pseudogenes, one on chromosome 6 and the other one on chromosome 21.



- The Memo gene contains 9 exons.

- Regarding the protein structure, Memo has no domain of identified function.

1.2.2. Memo in the evolution

Memo is very well conserved during evolution, from yeast to mammals.

Sequence identity:

Human	**100%**
Mouse	**99%**
Zebrafish	**88%**
Drosophila	**65%**
C. elegans	**53%**
S. Pombe	**42%**
S. cerevisiae	**40%**

1.2.3. Memo sequence alignment (alignment done with T-coffee):

Symbol code: * Identical
: Highly similar
. Poorly similar

Color code: BAD AVG GOOD

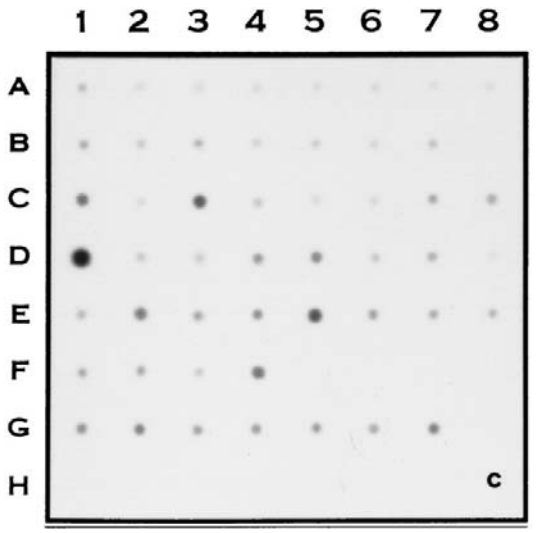
```
Human          MSNRVV---CREASHAGSWYTASGPQLNAQLEGWLSQVQSTKRPARAIIAPHAGYTYCGS
Mouse          MSNRVV---CREASHAGSWYTASGPQLNAQLEGWLSQVQSTKRPARAIIAPHAGYTYCGS
Zebrafish      MSNRMV---CREASHAGSWYTASGSQLNAQLEGWLSQAQSIAGPARAIIAPHAGYTYCGA
Drosophila     ----MS---ARRATHAGSWYTDSGAELSRQLDRWLGAADLSHGPARAIIAPHAGYTYCGA
C. elegans     MSLNGFGEHTRSASHAGSWYNANQRDLDRQLTKWLDNA-GPRITARALISPHAGYSYCGE

Cons                  * *:******. .  :*. **  **. .    .***:*:*****:***

Human          CAAHAYKQVDPSITRRIFILGPSHHVPLSRCALSSVDIYRTPLYDLRIDQKIYGELWKTG
Mouse          CAAHAYKQVDPSVTRRIFILGPSHHVPLSRCALSSVDIYRTPLYDLRIDQKIYGELWKTG
Zebrafish      CAAHAYKQVDPSITRRVFILGPSHHVPLSRCALSPAEVYRTPLYDLRIDQKVYADLWKTG
Drospohila     CAAFAYRQVSPVVVKRIFILGPSHHVRLRGCALSVAKKYRTPLYDLKIDAQINSELEKTG
C. elegans     TAAYAFKQVVSSAVERVFILGPSHVVALNGCAITTCSKYRTPLGDLIVDHKINEELRATR

Cons           **.*::** .  ..*:******* * *  **::  . ***** ** :* ::  :*  *

Human          MFERMSLQTDEDEHSIEMHLPYTAKAMESHKDEFTIIPVLVGALSESKEQEFGKLFSKYL
Mouse          MFERMSLQTDEDEHSIEMHLPYTAKAMESHKDEFTIIPVLVGALSESKEQEFGKLFSKYL
Zebrafish      MFERMSLQTDEDEHSIEMHLPYTAKAMENHKDEFSIVPVLVGALSGSKEQEYGKLLSKYL
Drosophila     KFSWMDMKTDEDEHSIEMHLPYIAKVMEDYKDQFTIVPILVGSLNPEQEAQYGSLLSSYL
C. elegans     HFDLMDRRDEESEHSIEMQLPFIAKVMGSKR--YTIVPVLVGSLPGSRQQTYGNIFAHYM

Cons           *. *. : :*.******:**: **.* . :  ::*:*:***:*  .::  :*.::: *:

Human          ADPSNLFVVSSDFCHWGQRFRYSYYDE-SQGEIYRSIEHLDKMGMSIIEQLDPVSFSNYL
Mouse          ADPSNLFVVSSDFCHWGQRFRYSYYDE-SQGEIYRSIEHLDKMGMSIIEQLDPVSFSNYL
Zebrafish      ADPSNLFIISPDFCHWGQRFRYTYYDE-SQGEIYRSIEHLDKMGMGIIEQLDPISFSNYL
Drosophila     MDPTNLFVISSDFCHWGHRFSYTYYDS-SCGAIHKSIEKLDKQGMDIIESLNPHSFTEYL
C. elegans     EDPRNLFVISSDFCHWGERFSFSPYDRHSSIPIYEQITNMDKQGMSAIETLNPAAFNDYL

Cons           ** ***::*.******.** :: **  *   *:..* ::** **. ** *:* :*.:**

Human          KKYHNTICGRHPIGVLLNAITELQ-KNGMN-MSFSFLNYAQSSQCRNWQDSSVSYAAGAL
Mouse          KKYHNTICGRHPIGVLLNAITELQ-KNGMN-MSFSFLNYAQSSQCRSWQDSSVSYAAGAL
Zebrafish      KKYHNTICGRHPIGVLLNAVAELK-KNGID-MNFSFLNYAQSSQCRNWSDSSVSYAAGAL
Drosophila     RKYNNTICGRHPIGVMLGAVKALQ-DQGYDKMSFKFLKYAQSSQCQDIEDSSVSYASGSL
C. elegans     KKTQNTICGRNPILIMLQAAEHFRISNNHT-HEFRFLHYTQSNKVRSSVDSSVSYASGVL

Cons           :* :******:** ::* *   :: .:.  .* **:*:**.: :.  *******:* *

Human          TVH--
Mouse          TVH--
Zebrafish      IVH--
Drosophila     VFEM-
C. elegans     FVHPN

Cons           ..
```

Human: Q9Y316
Mouse: Q91VH6
Zebrafish: AAH44360
Drosophila: Q9VG04
C. elegans: Q22915

1.2.4. Memo RNA expression in different breast cell lines

1 = HB2 human mammary epithelial cells
2 = MCF10A human mammary epithelial cells
3 = MCF7 human breast carcinoma cells
4 = SKBr3 human breast carcinoma cells
5 = BT474 human breast carcinoma cells
6 = MDA-MB-231 human breast carcinoma cells
7 = MDA-MB-361 human breast carcinoma cells
8 = MDA-MB-453 human breast carcinoma cells
9 = ZR 7.5.1 human breast carcinoma cells
10 = T47D human breast carcinoma cells
11 = SKBr3 RT-

1.2.5. RNA master blot analysis of Memo in human tissues (from Lai *et al.*[32])



The tissue distribution on the blot from left to right (1-8) in order was:

A: whole brain; amygdala; caudate nucleus; cerebellum; cerebral cortex; frontal lobe; hippocampus; medulla oblongata.

B: occipital pole; putamen; substantia nigra; temporal lobe; thalamus; subthalamic; nucleus; spinal cord.

C: heart; aorta; skeletal muscle; colon; bladder; uterus; prostate; stomach.

D: testis; ovary; pancreas; pituitary gland; adrenal gland; thyroid gland; salivary gland; mammary gland.

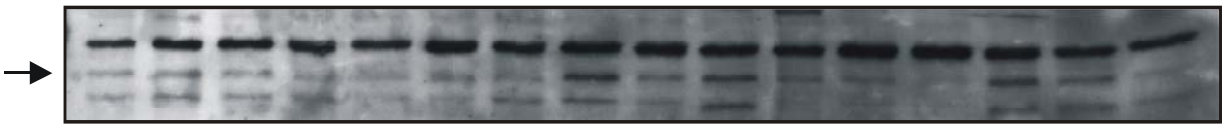
E: kidney; liver; small intestine; spleen; thymus; peripheral leukocyte; lymph node; bone marrow

F: appendix, lung, trachea, placenta.

G: fetal brain; fetal heart; fetal kidney; fetal liver; fetal spleen; fetal thymus; fetal lung.

H: yeast total RNA; yeast tRNA; *E. coli* rRNA; *E. coli* DNA; Poly r(A); human C0t DNA; human DNA; human DNA

1.2.5. Memo protein levels in different cells lines



1 = T47D human breast carcinoma cells
2 = MDA-MB-453 human breast carcinoma cells
3 = LS174 TRI human colorectal cancer cells
4 = HeLa human cervical cancer cells
5 = MCF7 human breast carcinoma cells
6 = MDA-MB-231 human breast carcinoma cells
7 = BT-474 human breast carcinoma cells
8 = DLDI TR7 human colorectal cancer cells
9 = HB2 human mammary epithelial cells
10 = Colon26 mouse colon cancer cells
11 = MKN7 human gastric cancer cells
12 = DU4476 human breast carcinoma cells
13 = SKBr3 human breast carcinoma cells
14 = NOG8 mouse mammary cells
15 = HC11 mouse epithelial cells
16 = Mouse embryonic fibroblasts (Mef)

2. ERBB2-DRIVEN LONG-TERM MIGRATION REQUIRES *DE NOVO* RNA AND PROTEIN SYNTHESIS

Romina Marone, Nancy E. Hynes and Ali Badache

Unpublished results

Introduction

Extracellular ligands bind to specific receptors on the cell inducing their activation. The receptors, upon dimerization become phosphorylated and stimulate intracellular pathways leading to cellular responses, such as cell cycle progression, changes in metabolism, cytoskeletal architecture, protein trafficking, adhesion and migration, which may involve changes in gene expression. Induction of cell motility by multiple growth factors, including EGF-related peptides has been linked to different pathways, including the mitogen-activated protein kinase (MAPK) pathway or the phosphatidylinositol-3-kinase (PI3K) pathway (Pawson, 1995; Schlessinger, 2000; Yarden and Sliwkowski, 2001). Activation of these signaling cascades is known to initiate specific transcriptional programs in the nucleus, which involve proto-oncogenes such as *fos*, *jun*, and *myc*, members of the family of zinc-finger-containing transcription factors that includes Sp1 and Egr1, signal transducers and activators of transcription (STATs) as well as Ets family members. Moreover, the Rho GTPases are known to regulate the expression of the transcription factor SRF (serum response factor) via their ability to induce actin polymerization (Hill *et al.*, 1995). The coordinated action of two Rho effector targets, mDia and Rho kinase, is required to regulate SRF activity by affecting actin dynamics (Geneste *et al.*, 2002). There is evidence that the levels of SRF activation correlate with the ratio of F-actin (polymerized actin) to G-actin (unpolymerized actin) (Sotiropoulos *et al.*, 1999). A recent study has identified the transcription factor MAL as an actin binding protein, that functions as an SRF coactivator and whose translocation from the cytoplasma to the nucleus depends on its dissociation from actin monomers (Miralles *et al.*, 2003).

Because most of the migration studies look at short-term events, the contribution and importance of transcription or translation for cell migration is not very well understood.

The intention of this study was to investigate the requirement of *de novo* RNA and protein synthesis for the migration of Neu or Neu add-back mutant expressing cells. Our results indicate that following HRG stimulation, post-translational events trigger moderate levels of migration. Efficient long-term migration requires *de novo* RNA and

protein synthesis, which is in turn dependent on signaling events activated downstream of the tyrosines YC and YD.

Results

We have analyzed HRG-induced cell migration of Neu and NYPD cells, expressing respectively, the wild type Neu receptor and the tyrosine-deficient Neu receptor, over a time course of 24 hours. Neu cells started to migrate within 2 hours of HRG treatment and the number of migrated cells increased up to 24 hours (Figure 1a). After 2 to 3 hours of HRG treatment the number of migrated NYPD cells was the same as the number of Neu cells. Starting at 4 hours, the migration of NYPD cells increased at a reduced rate in comparison to Neu cells, during the 24 hours time course. We have then investigated the possibility that efficient, long-term migration requires *de novo* RNA and protein synthesis, using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of RNA polymerase II or cycloheximide (CHX), respectively. Migration of Neu cells upon HRG stimulation was strongly decreased in the presence of CHX (Figure 1b) or in the presence of DRB (Figure 1b). Likewise, migration of the YC (cells expressing the receptor with the tyrosine 1201) and YD (cells expressing the Neu receptor with the tyrosine 1227) cells was sensitive to both inhibitors (Figure 1c and data not shown). In contrast, migration of NYPD cells as well as YE cells was not affected by CHX or DRB treatment (Figure 1c and data not shown). Importantly, these two inhibitors reduced the migration rate of the Neu, YC and YD cells to that observed for the NYPD and YE cells (Figure 1c). To rule out that decreased migration was due to toxicity of the inhibitors, we separately analyzed the proliferation of the cells in the presence of the inhibitors, by counting the cells before and after the migration assay (data not shown). No differences in cell proliferation were detected between the control and the treated cells, suggesting that the doses used and the duration of the treatment are not toxic to the cells. Interestingly, the breast carcinoma cells SKBr3 also showed reduced migration upon treatment with CHX or DRB (Figure 1d). These results suggest that following HRG

stimulation, post-translational events trigger low levels of migration, whereas efficient long-term migration is dependent on *de novo* RNA and protein synthesis. We propose that activation of signaling pathways downstream of the YC and YD tyrosines is required, for induction of transcription and translation, these processes are deficient in the NYPD and YE cells.

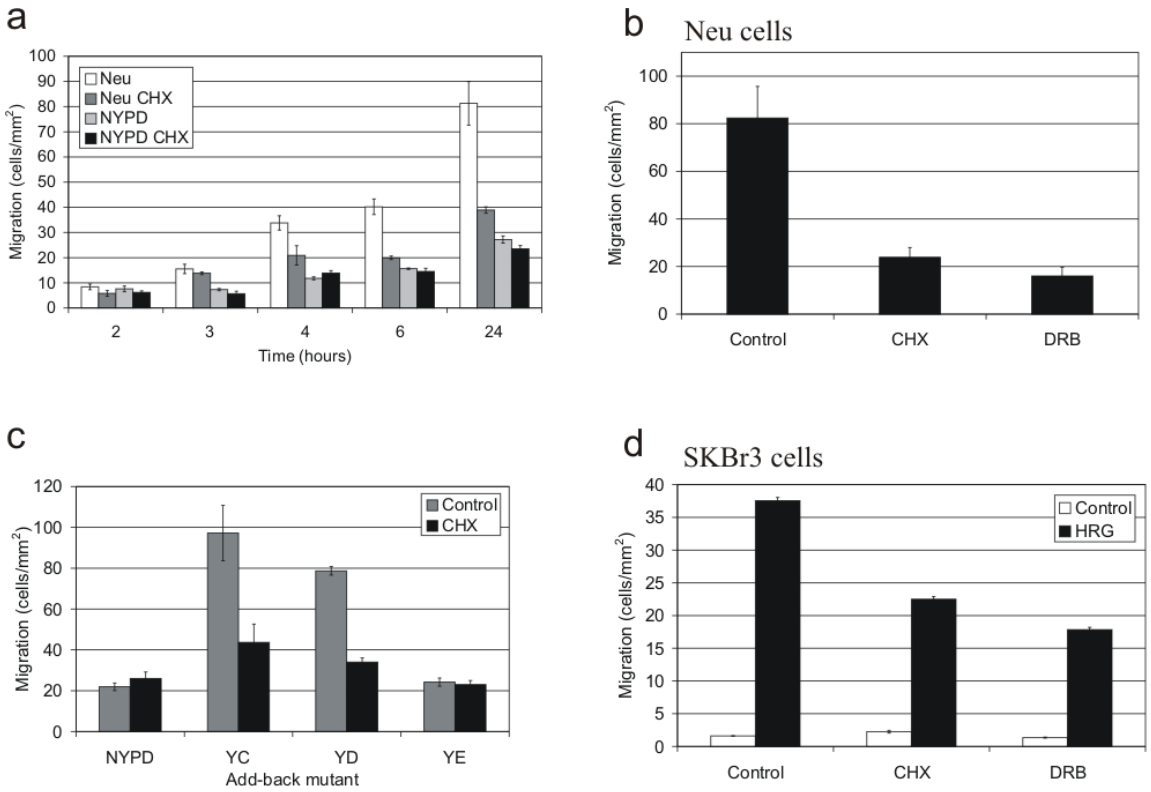


Figure 1: a, Migration of Neu and NYPD cells in response to HRG was assayed in the presence or absence of cycloheximide (CHX). **b,** Migration in response of HRG of Neu cells was measured after 24 hours in the presence or absence of CHX or DRB. **c,** HRG-dependent migration of NYPD, YC, YD and YE was tested over 24 hours in the presence or absence of CHX. **d,** SKBr3 cells migration in response to HRG was assayed over 8 hours in the presence or absence of CHX or DRB.

Memo, the new protein that we identified and described in chapter 1 of the results, acts downstream of the YD tyrosine and is not involved in early steps of cell migration such as lamellipodia formation. In order to investigate the possibility that Memo and induction of *de novo* protein synthesis are acting on the same pathway, we treated cells with cycloheximide and/or Memo siRNA. Cycloheximide treatment of YD cells or downregulation of Memo using siRNA in YD cells decreased migration to the same extent (Figure 2). Moreover, the combination of the two treatments does not have an additive effect on YD cell migration (Figure 2), suggesting that Memo requires *de novo* protein synthesis in order to trigger efficient, long-term cell migration.

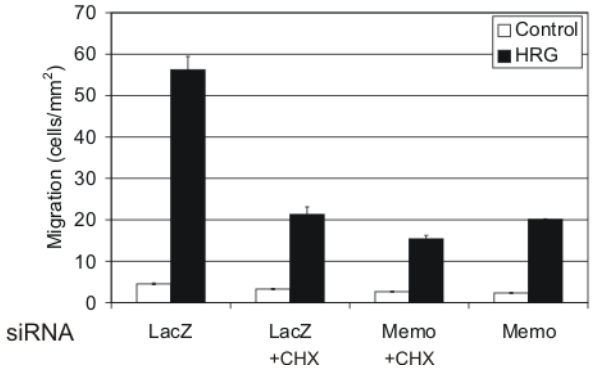


Figure 2: Effect of Memo siRNA on migration of YD cells treated with cycloheximide (CHX).

Further studies have to be done in order to identify the transcriptional program triggered by the signaling pathways, which are induced specifically downstream of the YC or YD tyrosines.

Discussion

Our data demonstrate that *de novo* RNA and protein synthesis are required for migration of Neu, YC and YD cells as well as SKBr3 cells. Interestingly, the low migration levels

of NYPD and YE cells are not affected by the treatment with the two inhibitors. Migration of these cells depends on the activation of the MAPK and PI3K pathways (see figure 4c of results chapter I). In fact, treatment of the cells with a MAPK or a PI3K inhibitor further reduces their migration levels and also prevents lamellipodia formation, suggesting that activation of the MAPK and PI3K pathways is essential for early stages of migration. Probably, NYPD and YE cells are impaired in migration because they are not able to activate signaling pathways leading to transcription and translation of key genes required for efficient cell motility.

In the literature there is increasing evidence that stimulation of cells with different growth factors leads to transcriptional activation of different genes required for cellular motility. In fact, it was shown that the increased migration seen in HRG-stimulated MCF-7 breast cancer cells was associated with transcriptional upregulation of the focal adhesion protein paxillin (Vadlamudi *et al.*, 1999). Perhaps, increased levels of paxillin promote cell motility via regulation of the dynamic disassembly and subsequent reassembly of the focal adhesions. Another group provided evidence for a link between EGF signaling, the transcription factor AP-1 (activator protein-1) and cell motility (Malliri *et al.*, 1998). They propose a model in which a subset of AP-1 target genes provides the molecular bridges between growth factor stimulation and activation of Rac and Rho, which in turn regulate morphological and motile responses. In addition, it was shown that constitutive expression of the transcription factor Fra-1 and Fra-2 increases DNA binding and functional activity of AP-1 (Kustikova *et al.*, 1998). Fra-1 expression leads to an increase in random cell migration and in invasion. Moreover, other data reveal that the transcription of NFAT (nuclear transcription factor of activated T-cells) is induced by integrin clustering in the presence of chemoattractants, resulting in enhanced cell motility (Jauliac *et al.*, 2002).

We have shown that Memo binds to the phosphorylated YD and mediates migration by controlling the microtubule cytoskeleton outgrowth. Moreover, we also found that migration downstream of Neu, YC and YD cells is dependent on *de novo* protein synthesis. Interestingly, the NYPD cells are able to extend the microtubule network to the cell periphery upon HRG treatment, but the low level of migration of these cells is not dependent on *de novo* protein synthesis. Thus, we propose the hypothesis that both

microtubule outgrowth and synthesis of new proteins are required for cell motility. This proposition is supported by the fact that microtubules can act as tracks along which molecular motors transport their cargo molecules (Schliwa and Woehlke, 2003). Moreover, microtubules together with microtubule-associated proteins are required for ER-to-Golgi (Presley *et al.*, 1997), as well as for Golgi-to-plasma membrane (Kreitzer *et al.*, 2000) transport of secretory and plasma membrane proteins. Therefore, we can speculate that microtubules transport the newly synthesized proteins to the cell leading edge, triggering, as a result, efficient cell motility.

Further studies have to be performed in order to identify which are the molecules required for efficient cell migration and to better understand the role of Memo and microtubules in the migratory process.

Additional materials and methods

Cell migration was tested using 8 μm-pore polycarbonate membrane Transwell chambers (Corning Costar Products, Acton, MA, USA) as described in chapter 1. In these experiments, cells were pre-incubated for 60 minutes with cycloheximide (10 μg/ml; from Sigma, St. Louis, MI, USA) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (20 μg/ml; from Fluka Chemie GmbH, Buchs, Switzerland). After the plating of the cells with medium with or without HRG, the cells were allowed to migrate in the presence of the inhibitors for different times before counting.

For cell proliferation same number of cells were plated and treated with or without the inhibitors. The following day, cells were trypsinized and counted using a hemocytometer.

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V. DISCUSSION

Upon ligand binding, ErbB receptors form homo- and heterodimers triggering autophosphorylation on specific tyrosine residues in their cytoplasmic tail. These phosphorylated tyrosines provide docking sites for intracellular signaling molecules containing SH2 and PTB domains and consequently, signaling pathways are activated. The ability of the activated receptors to induce proliferation, migration, differentiation or transformation is dependent on the receptors' intrinsic pattern of phosphorylatable carboxyterminal tyrosines. In order to identify the signaling pathways involved in Neu/ErbB2-dependent cell migration, we generated stable T47D breast carcinoma cell lines expressing Neu mutants. This was accomplished by functionally inhibiting the endogenous ErbB2 receptor using the human ErbB2-specific single chain antibody scFv-5R (Beerli *et al.*, 1994). Then, ErbB2 was substituted with wild type Neu, the rat homologue of ErbB2, or with Neu mutants harboring none (NYPD) or only one of the five major autophosphorylation sites (YA to YE) (Dankort *et al.*, 1997). Using these cell lines, we were able to show that cell migration depends on the collaboration of different signaling pathways. In fact, the Ras/MAPK, PI3K, p38MAPK and Src-mediated signaling pathways are all required for lamellipodia formation, an early step in the migration process. Interestingly, we provide evidence that in the context of the Neu/ErbB2 receptor, the tyrosines 1201 or 1227 are necessary for breast carcinoma cell migration. In the absence of the signaling pathways activated downstream of these two tyrosines, cells display only modest motility.

We performed affinity purification experiments in order to identify by mass spectrometry the signaling molecules interacting with the ErbB2's tyrosines 1201 and 1227. Using this method we discovered a novel mediator of Neu/ErbB2-dependent cell migration: Memo. Memo is not involved in cell migration stages linked to remodeling of the actin cytoskeleton, such as lamellipodia formation, but is required for outgrowth of the microtubule cytoskeleton. Memo interacts specifically with the phospho-tyrosine 1227 via the adaptor molecule Shc. Interestingly, Shc binds to the tyrosine 1201 as well, but Memo is not. The Shc adaptor protein contains both an SH2 and a PTB domain and could

therefore interact with the receptor's phosphorylated tyrosines in different ways (Ricci *et al.*, 1995; Dankort *et al.*, 2001; Ravichandran, 2001). Interestingly, the amino acid sequence around the tyrosine 1201 is a perfect recognition code (hy-X-N-P-X-pY, where hy is an hydrophobic amino acid) for binding of PTB containing proteins (Pawson and Scott, 1997; Sudol, 1998), whereas the sequence around Tyr1227 is no recognition code for neither PTB nor SH2 containing proteins (Songyang *et al.*, 1993). It is possible that Shc adopts different conformations upon receptor binding, allowing recruitment of Memo only when it is bound to Tyr1227. We are currently investigating the domains by which Shc interacts with tyrosine 1201 and 1227 respectively and also the regions of Shc and Memo, which are involved in the formation of the Shc/Memo complex.

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). Memo is very well conserved throughout evolution; in fact Memo homologues exist in yeast, nematodes, drosophila and mammals. The sequence of Memo does not provide any information about the protein function, since it does not contain any characterized domain. We found that Memo is required for the extension of microtubules to the periphery. We took advantage of the fact that Memo is conserved in yeast, to get a hint of its function. A close look at the proteome of *S. cerevisiae* provided evidence that the *S. cerevisiae* Memo homologue (Accession Number YJR008w) interacts with the Arp1 protein (actin-related protein 1), also called centractin. Arp1 is the most abundant component of the dynactin (dynein activator) complex (Schroer, 1994; Karki *et al.*, 2000). Another member of the complex is the cytoplasmic dynein, a motor protein which transport the cargoes along the microtubules (Schliwa and Woehlke, 2003). The functional interaction between cytoplasmic dynein and dynactin is critical for distinct cellular processes such as vesicle transport, mitotic spindle assembly and orientation (Echeverri *et al.*, 1996; Roghi and Allan, 1999). Moreover, cytoplasmic dynein has been implicated in the interactions between microtubules and the cell cortex in different physiological contexts, e.g. microtubule cytoskeletal reorientation during wound healing (Etienne-Manneville and Hall, 2001; Palazzo *et al.*, 2001). Interestingly, cytoplasmic dynein and dynactin subunits have been found associated with the growing microtubule

plus end and colocalize with CLIP-170 (Vaughan *et al.*, 1999; Xiang *et al.*, 2000). CLIP-170 is a linker between membranes and microtubules and it stabilizes the microtubules during their growing phase (Howard and Hyman, 2003). These studies could help to explain the phenotype of the Memo knockdown cells. In fact, we found that these cells are impaired in migration, probably because of disrupted microtubule outgrowth and we speculate this lead to inefficient transport of the newly synthesized proteins to the cell leading edge. The dynactin complex, but also the kinesin are required for the ER-to-Golgi and the Golgi-to-plasma membrane transport of the secretory and the plasma membrane proteins (Presley *et al.*, 1997; Kreitzer *et al.*, 2000). However, one requirement for these molecular motors to work is an intact microtubule cytoskeleton, a structure that is disrupted in Memo knockdown cells. Moreover, Memo could play a role in the stabilization of the growing microtubules and a possible interaction with plus-end binding proteins such as CLIP-170 or with dynactin needs to be investigated. Interestingly, Memo is not involved in the control of the stable central microtubule network since downregulation of Memo expression in the cells does not disrupt this microtubule population. Microtubules, together with multiple mitotic motors are important during cell division, for processes such as mitotic spindle assembly and orientation or chromosome segregation (Scholey *et al.*, 2003). Proliferation of breast carcinoma cells is not affected by Memo downregulation, probably because the central microtubule network is present in the Memo knock down cells. Therefore, it seems that cell division is not dependent on Memo, even though the dynactin complex plays crucial roles in this process.

Cell migration plays important role in the development and maintenance of multicellular organisms. Cell motility during development is fundamental to the establishment of the embryonic architecture, which depends on processes such as gastrulation, neural crest formation and also morphogenetic movements that shape the embryo (Locascio and Nieto, 2001; Franz *et al.*, 2002). Our studies provide evidence that Memo is required for cell migration and therefore it could play a role during development. Moreover, results from different experiments show that Memo RNA and protein is expressed in human and mouse tissues as well as cell lines. However, nothing is known about the expression

pattern of Memo during development. Therefore, some experiments could be done in order to identify the expression of Memo during the different developmental stages.

The ErbB receptors are important during development and targeted inactivation of components of the ErbB signaling network highlighted the importance of the receptor-ligand interactions especially in mid-gestation inductive processes. Apparently, the ErbB network is primarily involved in mesenchyme-epithelial crosstalk and in neuronal effects on target cells, including muscle, astroglia, oligodendrocytes and Schwann cells (Britsch *et al.*, 1998; Buonanno and Fischbach, 2001). NRG-1 is synthesized by mesenchymal or neuronal cells, which influence the differentiation, proliferation and migration of adjacent epithelial or non-neuronal cells, respectively. Evidence for the essential role of ErbB receptors in mid-gestation was provided by the embryonic lethality of ErbB2-, ErbB4- and NRG-1-deficient mice at around day 10 post-fertilization due to aberrant cardiac development (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). In addition to cardiac disorders, ErbB4 deficient mice displayed severe defects in the development of the cranial sensory ganglia due to aberrant migration of some neural crest cells and aberrant axon pathfinding into the adjacent mesenchyme. Moreover, it was shown that migration of neural crest cells to the mesenchyme lateral of the dorsal aorta, in which they differentiate into sympathetic neurons, depends on NRG-1, ErbB2 and ErbB3 (Britsch *et al.*, 1998). All these data provide some evidence of the importance of the ErbB receptors and migration in development. It would be interesting to study the role of Memo during development, in order to understand in which developmental steps Memo is required.

The ErbB receptors play central roles during mammary gland development, process dependent on cell proliferation, migration, differentiation and apoptosis. Data from transgenic mice reveal that ErbB1 KO mice have a normal mammary gland ductal tree at birth (Wiesen *et al.*, 1999). A fraction of the ErbB1 KO mice survive after birth and this made it possible to determine that these mice, in the puberty have a reduced proliferation of mammary epithelium and stroma and a loss of periductal fibroblasts (Wiesen *et al.*, 1999). Moreover, the mammary tree of MHC (myosin heavy chain)-ErbB2-rescued ErbB2 KO animals is normal prior to birth (Stern, 2003). Mammary ductal development of the cardiac-rescued MHC-ErbB4 ErbB4 KO is also normal at birth, but during

pregnancy the mammary lobuloalveoli fail to differentiate correctly (Tidcombe *et al.*, 2003). We showed using breast carcinoma cells that Memo is required for cell motility, but not for cell proliferation. Therefore some experiments could be done to investigate the role of Memo during mammary gland development, especially during the invasion of the mammary epithelium into the mammary fat pad, where migration is required.

Tumorigenesis is a multistep process and occurs as the result of uncontrolled cell division, contributing to initial tumor formation, which is followed by metastatic spread. The process of metastasis involves an intricate interplay between cell proliferation, adhesion, proteolysis, migration and angiogenesis. As cancer cells become metastatic, they activate signaling cascades that regulate gene expression, cytoskeletal reorganization, cell adhesion and survival (Fidler and Kripke, 1977; Poste and Fidler, 1980; Kang *et al.*, 2003). These changes allow them to become more invasive and migratory and to better survive in different microenvironments. Breast carcinoma cells metastasize often to bone and to visceral organs (Thomas *et al.*, 1979; Price and Zhang, 1990; Boyce *et al.*, 1999; Yoneda *et al.*, 2000; Liotta, 2001; Mundy, 2002). The human breast carcinoma cell line MDA-MB-231 is particularly appealing to explore Memo's role in metastasis formation. The orthotopic metastasis model closely resembles the situation in breast carcinoma patients. In this model the human mammary tumor cells MDA-MB-231 are injected into the inguinal mammary fat pad of SCID (severe combined immunodeficiency) mice (Singh *et al.*, 1997; Muller *et al.*, 2001). The mammary cancer cells form tumors 7-10 days after injection and subsequently they develop bone and visceral metastasis 3-4 weeks after inoculation. Using this model it would be possible to study the role of Memo in the early steps of breast tumor formation and even more interesting, due to crucial role of Memo in migration, during the whole metastatic process. We showed that Memo is required for MDA-MB-231 cell migration, but not for proliferation *in vitro*. Using this model, it would be possible to verify the role of Memo for cell proliferation in vivo. In fact, tumor growth is dependent on cell division and in the animals it would be possible to measure the size of the mammary tumor, which reflect cell proliferation. In another model, the experimental bone metastasis model, the cells are directly introduced into the arterial circulation through the left ventricle of the heart in young female nude mice (Yoneda *et al.*, 1994; Sung *et al.*, 1997; Kang *et al.*, 2003). The

carcinoma cells develop radiologically distinctive osteolytic bone metastasis 3-4 weeks after cell inoculation. This model is suitable to specifically study the events involved in the formation of bone metastasis. However, it has the disadvantage that it lacks the critical early steps occurring between tumor formation at the primary site and entry into the blood stream. Moreover, visceral organ metastases, which most patients already have developed at the time of detection of bone metastasis, are rarely formed. In this model, the cells in order to metastasize have to reach the bone via the blood circulation and ultimately to arrest in the capillary bed in bone. Afterward, they have to extravasate and to destroy the bone in order to form metastases. Extravasation is a process dependent on specific adhesion of the tumor cells to the blood vessel endothelial cells and on the subsequently migration of the cancer cells between the endothelial cells. Results from our experiments reveal that Memo is required for MDA-MB-231 cell migration. Therefore, this model appears to be appropriate for investigate the role of the Memo for migration during the bone metastasis process.

In summary, some more work needs to be done to better comprehend the contribution of Memo during the cell migratory process. Moreover, the use of animal models could help to improve the knowledge regarding the function of Memo not only during tumor and metastases formation, but also during the animal developmental phases.

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VI. ABBREVIATIONS

+TIP	Plus-end-binding protein
ADAM	A Disintegrin-like and metalloproteinase-containing protein
ADF	Actin-depolymerization factor
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
AR	Amphiregulin
ARIA	Acetylcholine receptor-inducing activity
Arp	Actin-related protein
BTC	Betacellulin
CHX	Cycloheximide
CLASP	CLIP associated protein
DN	Dominant negative
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
ECM	Extracellular matrix
EGF(R)	Epidermal growth factor (receptor)
EPR	Epiregulin
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GGF	Glial growth factor
GPCR	G-protein coupled receptor
HB-EGF	Heparin-binding EGF
HRG	Heregulin
MAPK	Mitogen-activated protein kinase
MCAK	Mitotic centromere-associated kinesin
MHC	Myosin heavy chain

MLC(K)	Myosin light chain (kinase)
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MTOC	Microtubule-organizing center
NDF	Neu differentiation factor
NRG	Neuregulin
PAI-1	Plasminogen activator inhibitor-1
PAK	p21-activated protein kinase
PDGF(R)	Platelet-derived growth factor (receptor)
PI3-K	Phosphatidylinosithol-3-kinase
PLCγ	Phospholipase Cγ
PTB	Phosphotyrosine-binding
ROCK	Rho-associated kinase
RTK	Receptor tyrosine kinase
SH2/3	Src-homology 2/3
STAT	signal transducers and activators of transcription
TGF-α	Transforming growth factor-α
Tyr	Tyrosine
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

Curriculum Vitae

Name	Romina Marone
Date of birth	December 26, 1976
Place of birth	Bern
Nationality	Swiss
Marital status	Single

Working address
Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
4058 Basel
Switzerland
Phone: +41 61 697 80 89
Fax: +41 61 697 39 76
email: romina@fmi.ch

Private address
An der hohlen Gasse 12
4058 Basel
Switzerland
Phone: +41 61 681 51 69

Educational record

Sept. 1991- June 1995:	High school in Bellinzona (Tessin). Final exam: federal matura, type C
Oct. 1995 - Apr. 2000:	Student in Biology at the ETH of Zurich.
Oct. 1999 - Apr. 2000:	Practical part of the diploma thesis in Prof. Dr M. Fussenegger's lab, ETH of Zurich.
Jun. 2000 – Mar. 2004:	Ph.D. in Prof. Dr. N. E. Hynes' lab, Friedrich Miescher Institute, Basel.

External fundings

Sept. 2001- Mar. 2004	Krebsliga Schweiz

Languages Italian (mother language), German, English, French

Publication list

- Kaufmann H, **Marone R**, Olayioye M.A., Bailey J.E., Fussenegger M. Characterization of an N-terminally truncated cyclin A isoform in mammalian cells. *J Biol Chem*. **276**, 29987-93 (2001).

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  Manuscript resubmitted to Nature Cell Biology.

Meetings

- 10 March 2001: oral presentation at the USGEB Young Investigator Meeting, Lausanne, Switzerland

- 27-28 September 2001: Swiss Cell Cycle workshop, Vue-des-Alpes, Switzerland

- 7-9 March 2002: Poster presentation at the USGEB Annual Meeting, Lugano, Switzerland

- 29 June- 3 July 2002: Poster presentation at ELSO Meeting, Nice, France

- 2-5 May 2003: Poster presentation at the EMBO Workshop Mechanism of Cell Migration, Heidelberg, Germany

- 7 May 2003: oral presentation at the SPO (Schwer Punkt Onkologie) Symposium, Augst, Switzerland

- 13-17 December 2003: Poster presentation at the ASCB meeting in San Francisco, USA

Awards

- 10 March 2001: prize for the best USGEB abstract at the USGEB Young Investigator Meeting, Lausanne, Switzerland

- 15 November 2002: prize for the best poster at the Swiss Society for Oncology Annual Meeting, Lausanne, Switzerland

- 26 March 2004: prize ASRIB-Roche 2003, Lugano, Switzerland