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NOVEL PLAYERS IN THE INTEGRIN SIGNALING ORCHESTRA: TCPTP AND MDGI

by

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Nature does nothing in vain.
-Aristotle

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ABSTRACT

Metastases are the major cause of cancer deaths. Tumor cell dissemination from the primary tumor utilizes dysregulated cellular adhesion and upregulated proteolytic degradation of the extracellular matrix for progeny formation in distant organs. Integrins are transmembrane adhesive receptors mediating cell–cell and cell–matrix interactions that are crucial for regulating cell migration, invasion, proliferation, and survival. Consequently, increased integrin activity is associated with augmented migration and invasion capacity in several cancer types. Heterodimeric integrins consist of an α- and β-subunit that are held together in a bent conformation when the receptor is inactive, but extension and separation of subdomains is observed during receptor activation. Either inside-out or outside-in activation of receptors is possible through the intracellular molecule binding to an integrin cytoplasmic domain or extracellular ligand association with an integrin ectodomain, respectively. Several regulatory binding partners have been characterized for integrin cytoplasmic β-domains, but the regulators interacting with the cytoplasmic α-domains have remained elusive.

In this study, we performed yeast two-hybrid screens to identify novel binding partners for the cytoplasmic integrin α-domains. Further examination of two plausible candidates revealed a significant coregulatory role of an integrin α-subunit for cellular signaling processes. T-cell protein tyrosine phosphatase (TCPTP) showed a specific interaction with the cytoplasmic tail of integrin α1. This association stimulated TCPTP phosphatase activity, leading to negative regulation of epidermal growth factor receptor (EGFR) signaling and diminished anchorage-independent growth. Another candidate, mammary-derived growth inhibitor (MDGI), exhibited binding to several different integrin cytoplasmic α-tails through a conserved GFFKR sequence. MDGI overexpression in breast cancer cells altered EGFR trafficking and caused a remarkable accumulation of EGFR in the cytoplasm. We further demonstrated *in vivo* that MDGI expression induced a novel form of anti-EGFR therapy resistance. Moreover, MDGI binding to α-tails retained integrin in an inactive conformation attenuating integrin-mediated adhesion, migration, and invasion. In agreement with these results, sustained MDGI expression in breast cancer patients correlated with an increased 10-year distant disease-free survival. Taken together, the integrin signaling network is far from a complete view and future work will doubtless broaden our understanding further.

Key words: integrin, cancer, EGFR, protein tyrosine phosphatase, TCPTP, MDGI

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TIIVISTELMÄ

Etäpesäkkeiden muodostuminen aiheuttaa valtaosan syöpään sairastuneiden menehtymisistä. Integriinit ovat solukalvon läpäiseviä tarttumisreseptoreja, joiden avulla solu muodostaa kontakteja muiden solujen ja ympäristönsä kanssa. Tarttumisaktiivisuudellaan integriinit säätelevät muun muassa solujen liikkumista, invaasiota ja jakautumista. Monissa syövissä integriinien aktiivisuus on kohonnut. Rakenteeltaan integriinit ovat heterodimeerejä koostuen α- ja β-alayksiköistä, joiden muodostama inaktiivinen reseptori on solukalvolla kumarassa, suljetussa muodossa, mutta reseptorin aktivoituessa alayksiköt ojentuvat ja muodostavat avoimen, sitoutumisherkän kolmiulotteisen rakenteen. Reseptorin aktivoituminen voi tapahtua solukalvon yli molempiin suuntiin joko solunsisäisen molekyylin sitoutuessa integriinin sytoplasmanpuoleiseen osaan tai solunulkoisen ligandin tarrautuessa integriiniin. β-alayksikön sytoplasmaosaan tiedetään sitoutuvan lukuisia säätelymolekyylejä, mutta α-alayksikköön sitouvat molekyylit ovat pääosin tuntemattomia.

Tämän väitöskirjatyön lähtökohtana oli tunnistaa säätelymolekyylejä, jotka sitoutuvat integriinin α-alayksikön sytoplasmaosaan. Vuorovaikutusparien muodostumista analysoitiin aluksi hiivassa, jonka perusteella kahden lupaavimman vuorovaikutuskandidaatin tarkastelua jatkettiin nisäkässoluissa. T-solun proteiinityrosiinifosfataasi (TCPTP) sitoutui spesifisesti α1-integriiniin, mikä aktivoi fosfataasiaktiivisuuden johtaen epidermaalisen kasvutekijän reseptorin (EGFR) signaalinvälityksen vaimenemiseen sekä heikentyneeseen kykyyn kasvaa ilman kiinnitysalustaa. Kiinnitysalustasta riippumatonta kasvua pidetään yleisenä pahanlaatuisen kasvun merkkinä. Toinen kandidaateista oli rintaperäinen kasvunestäjä (MDGI), joka kykeni sitoutumaan usean integriinin α-sytoplasmaosan kanssa todennäköisesti α-alaosissa konservoituneen GFFKR-sekvenssin välityksellä. MDGI-proteiinin ilmentäminen rintasyöpäsolulinjassa aiheutti massiivisen EGF-reseptorin soluunoton, minkä todensimme aiheuttavan lääkeresistenssiä koe-eläinmallissa EGFR-pohjaiselle vastaaineterapialle. Lisäksi MDGI-proteiinin sitoutuminen integriiniin säilytti integriinin inaktiivisessa konformaatiossaan heikentäen näin integriinivälitteistä solun adheesiota, liikkumista ja migraatiota. Lopuksi rintasyöpäpotilaiden näytteiden immunohistokemiallinen värjäys paljasti, että potilailla, joilla MDGI ilmentyi syövästä huolimatta, oli parempi 10-vuotisennuste. Näiden tulosten perusteella integriinin α-alayksiköllä on merkittävä rooli solun signaloinnissa, mitä alamme vasta vähitellen ymmärtää.

Avainsanat: integriini, syöpä, epidermaalinen kasvutekijän reseptori (EGFR), proteiinityrosiinifosfataasi, TCPTP, MDGI

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ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADMIDAS	Adjacent to MIDAS
Akt	Protein kinase B
ATP	Adenosine triphosphate
BTC	Betacellulin
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CIS	Carcinoma in situ
DAG	Diacyl glycerol
DDFS	Distant disease-free survival
DNA	Deoxyribonucleic acid
Dok1	Docking protein 1
DSP	Dual-specificity phosphatase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Epiregulin
ERK	Extracellular signal-regulated kinase
FABP	Fatty acid-binding protein
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FERM	Protein 4.1, ezrin, radixin, moesin
FHL	Four and one-half LIM domain protein
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GFFKR	Glycine-phenylalanine-phenylalanine-lysine-arginine sequence
Grb2	Growth factor receptor-bound 2
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolyzing enzyme
HER	Human epidermal growth factor receptor
HB-EGF	Heparin-binding EGF-like growth factor
ICAM	Intercellular adhesion molecule
ICAP-1	Integrin cytoplasmic domain-associated protein-1
IGF-1R	Insulin-like growth factor-1 receptor
ILK	Integrin-linked kinase
IP_3	Inositol (1,4,5)-trisphosphate
JAK	Janus kinase
KRAS	Kirsten rat sarcoma 2 viral oncogene homolog
LAMP1	Lysosomal-associated membrane protein 1

LBP	Lipid-binding protein
LCFA	Long-chain fatty acid
LIMBS	Ligand-induced metal-binding site
MAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MDGI	Mammary-derived growth inhibitor
MIDAS	Metal-ion-dependent adhesion site
MMP	Matrix metalloproteinase
MPTP	Murine protein tyrosine phosphatase
mRNA	Messenger RNA
NLS	Nuclear localization sequence
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDZ	Postsynaptic density-95, discs large, zonula occludens-1
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PLA	Proximity ligation assay
PLC-γ	Phospholipase C-γ
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PSI	Plexin-semaphorin-integrin domain
PTB	Phosphotyrosine-binding domain
PTPB1	Protein tyrosine phosphatase B1
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
Rac	Ras-related C3 botulinum toxin substrate 1
RCP	Rab-coupling protein
RGD	Arginine-glycine-aspartic acid sequence
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SFK	Src-family kinase
SH2	Src-homology 2 domain
Shc	Src-homology domain 2 containing
SHP2	SH2-domain containing protein tyrosine phosphatase 2
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TC45, TC48	TCPTP isoforms 45 kDa and 48 kDa
TCPTP	T-cell protein tyrosine phosphatase
TGF-α	Transforming growth factor-α
TKI	Tyrosine kinase inhibitor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-III:

I Mattila E, Pellinen T, **Nevo J**, Vuoriluoto K, Arjonen A, Ivaska J: Negative regulation of EGFR signalling through integrin-alpha1beta1-mediated activation of protein tyrosine phosphatase TCPTP. *Nature Cell Biology*. 7(1): 78-85, 2005.

II **Nevo J,** Mattila E, Pellinen T, Yamamoto DL, Sara H, Iljin K, Kallioniemi O, Bono P, Heikkilä P, Joensuu H, Wärri A and Ivaska J: Mammary-derived growth inhibitor alters traffic of EGFR and induces a novel form of cetuximab resistance. *Clinical Cancer Research.* 15(21): 6570-81, 2009.

III **Nevo J**, Mai A, Tuomi S, Pellinen T, Pentikäinen OT, Heikkilä P, Lundin J, Joensuu H, Bono P and Ivaska J: Mammary-derived growth inhibitor (MDGI) interacts with integrin α-subunits and suppresses integrin activity and invasion. *Oncogene*, 2010 Aug 30 [Epub ahead of print].

INTRODUCTION

Cell adhesion is one of the most fascinating biological phenomena due to the fact that it is involved in several pivotal normal and pathological situations, such as the formation of complex organs, extravasation of immune cells into tissues during host defense, and migration of metastatic cells from the primary tumor to secondary organs. The major transmembrane receptors responsible for cell–cell and cell–matrix interactions are integrins, which integrate extracellular and intracellular cues into conformational changes of the integrin receptor itself. Inactive integrins stay in a bent conformation, but activated integrins are fully extended and ready to bind to the extracellular ligands. What makes integrins so exceptional among the transmembrane receptors is their capacity for bi-directional signaling across the plasma membrane. Thus, not only the binding of the receptor extracellular domain to abundantly expressed extracellular ligands, but also the binding of intracellular signaling molecules to the integrin cytoplasmic domain can trigger changes in integrin conformation and modulate integrin activity. This leads to alterations in outside-in and inside-out signaling, respectively. Increased integrin activity is associated with augmented migration and invasion capacity in several cancer types, thus emphasizing the attractive role of integrins for targeted anti-adhesive therapies. To date, the clinical use of anti-integrin therapy is limited to compounds inhibiting aggregation of platelets or suppressing immune cell response in autoimmune diseases. However, several encouraging results have emerged from clinical studies with anti-integrin molecules targeted at different malignancies.

Epidermal growth factor receptor (EGFR) belongs to a family of receptor tyrosine kinases (RTKs) that are capable of generating multiform downstream signaling pathways in the presence of ligand binding. EGFR signaling plays a pivotal role in numerous cellular responses, including differentiation, proliferation, migration, and survival. In some cases, EGFR downstream signaling pathways overlap with those originating from the integrins (Moro et al., 1998). Integrin clustering on the plasma membrane induces an accumulation of other cell surface molecules as well and EGFR has been shown to form a bigger complex with integrins (Moro et al., 2002). However, the detailed mechanism as to how EGFR and integrins regulate each other is not fully understood. Several structural and signaling molecules have been shown to bind directly to the cytoplasmic β-tails of integrin α/β heterodimers and to regulate integrin signaling. Interestingly, binding partners for the integrin cytoplasmic α-tails have remained elusive. The aim of this study was to identify novel interaction partners for the cytoplasmic α-tails of the collagen-binding integrins and to determine the significance of these novel partners in the integrin biology.

REVIEW OF THE LITERATURE

1. Cancer

Tumors arise from normal tissues via a multistep process including different gradations of abnormality. The first sign of this progress is an excessive number of normal cells, known as hyperplasia. A minimal deviation from normal is seen in the next stage, called metaplasia, in which cells are displaced by other types of normal cells that are not usually located in this site within a tissue. A typical example of metaplasia is a replacement of squamous epithelium in the esophagus by secretory epithelial cells normally found within the stomach. This condition is called Barrett's esophagus. A further degree of abnormality is reached with dysplastic cells which look abnormal due to the variations in their nuclear size, shape, and staining. Dysplastic cells have also lost their differentiation capacity and tissue architecture, and they are a clear indication of premalignancy. When dysplastic cells gain ground and form a carcinoma in situ (CIS), abnormality increases further. Although CIS lacks invasion into surrounding tissues, it may later progress into an invasive primary carcinoma. Primary tumor mass is rarely lethal, but can develop insidiously and may affect normal tissue function, for example, by the physical pressure exerted from the expanding tumor masses. However, primary tumors are estimated to be responsible for about 10% of deaths from cancer (Chau and Ashcroft, 2004). Of course, these numbers depend somewhat on the tissue in focus. Moreover, primary tumors can only grow to a limited mass size without the vasculature needed for supplying indispensable oxygen and nutrients. Neoangiogenesis is required when the primary tumor mass exceeds 2 mm (Gimbrone et al., 1972). Additionally, for the resolution of the limited diffusion of nutrients or oxygen, neoangiogenesis also functions as a prerequisite for dissemination of primary tumor cells into distant organs. Metastases are the cause of 90% of human cancer deaths (Hanahan and Weinberg, 2000). When the invasive primary carcinoma outgrows locally into the surrounding microenvironment and the epithelial-originated cells penetrate the basement membrane underneath, then local metastases start to progress. Interestingly, distant metastases seem to be organ-specific, thereby breast carcinoma can be later found mainly in the bones, lungs, brain, and liver, but prostate cancer favors just bones (Nguyen et al., 2009).

Ten years ago, Hanahan and Weinberg described six hallmarks of cancer that are shared by most types of human cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These acquired capabilities can be gained through various mechanistic strategies, but they are responsible for driving the progressive transformation of normal cells into highly malignant derivatives. For instance, genomic instability results in mutations, which can cause either the gain of function of potential oncogenes or the loss of function of tumor suppressor genes, and enables the formation of acquired capabilities (Hanahan and Weinberg, 2000). Several of these enumerated characteristics are

regulated by growth factor receptors or integrins, which have opened them up as appealing targets for cancer therapy.

2. Integrins

2.1. Structural overview

Since the naming of the integrin receptor family in 1986 (Hynes, 1987; Tamkun et al., 1986), these adhesive receptors have been under intensive research and continuous progress in understanding the structure of integrins has emerged after the first crystal structure of the extracellular portion of integrin αVβ3 was solved in 2001 (Xiong et al., 2001). Integrins are cell surface transmembrane receptors consisting of non-covalently associated α- and β-subunits that mediate cell–cell and cell–extracellular matrix (ECM) crosstalk. To date, 18 α-subunits and 8 β-subunits are known to form 24 different heterodimeric combinations in vertebrates, each having a different ligand binding preference. Based on their ligand specificities, the integrin family can be divided into four subgroups (Figure 1): collagen receptors (α1β1, α2β1, α10β1, and α11β1), laminin receptors (α3β1, α6β1, α7β1, and α6β4), RGD receptors (α5β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, and αIIbβ3), leukocyte-specific receptors (αLβ2, αMβ2, αXβ2, αDβ2, α4β7, and αEβ7), and a pair of related integrins (α4β1 and α9β1) (Hynes, 2002). RGD receptors recognize a tripeptide sequence (arginine-glycine-

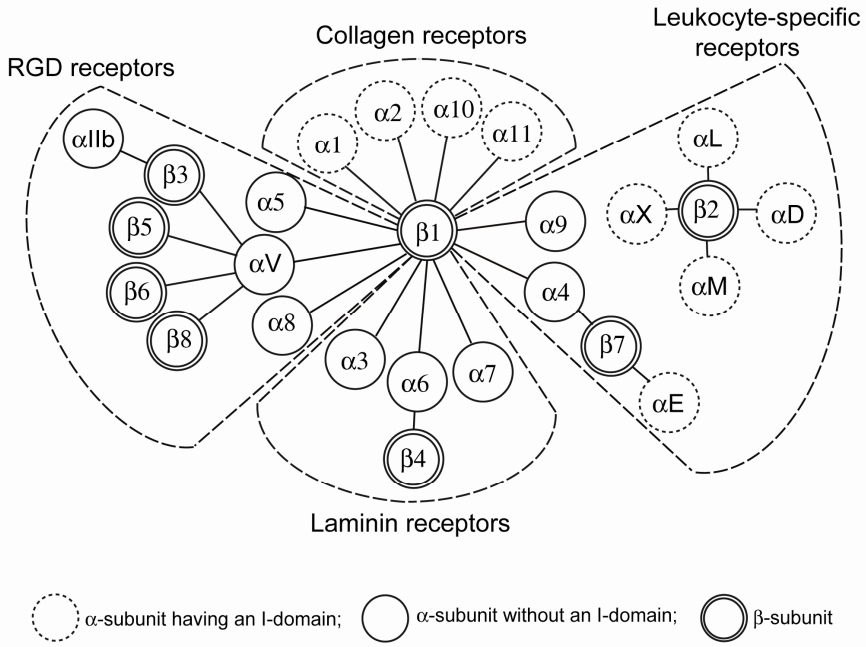

Figure 1: The integrin superfamily. Different integrin α- and β-subunits form 24 heterodimers which can be further classified into subfamilies based on their ligand specificity. Dashed circles represent integrin α-subunits containing an I-domain. See the text for further details. The figure is modified from Barczyk et al., 2010; Gahmberg et al., 2009.

aspartic acid) in molecules such as fibronectin, vitronectin, fibrinogen, the von Willebrand factor, entactin, tenascin, bone sialoprotein, and osteopontin (Ruoslahti, 1996). Leukocyte-specific receptors instead take part in adhesion of leukocytes to the endothelium and extravasation from blood vessels and recognition of phagocytic material by macrophages and neutrophils, thus being crucial for host defense (Hyun et al., 2009). Whereas ECM components function as major ligands for most of the integrins, leukocyte-specific integrins bind to intercellular adhesion molecules (ICAMs) to mediate interaction with endothelial cells or to complement factor iC3b for phagocytosis of apoptotic cells or pathogens (Barczyk et al., 2010). Some integrin family members (α4β1, αDβ2, and α4β7) are also capable of docking with vascular cell adhesion molecules (VCAMs) for cell–cell contact formation (Barczyk et al., 2010).

Structurally, integrins consist of a large extracellular domain and a short rod-like cytoplasmic domain joined together by a hydrophobic single-spanning transmembrane domain (Carrell et al., 1985; Weisel et al., 1992). The extracellular domain of the integrin α-subunit contains an αI-domain, a β-propeller, a thigh domain, a calf-1 domain, and a calf-2 domain forming a large, ligand-binding, globular headpiece and a thin tailpiece (Figure 2). Only nine (α1, α2, α10, α11, αL, αM, αX, αD, and αE) out of the 18 different integrin subunits contain an I-domain (αI-domain) also known as an "inserted" domain, but other domains are expressed ubiquitously (Barczyk et al., 2010). The αI-domain has the metal-ion-dependent adhesion site (MIDAS) which takes part in ligand binding and requires divalent cations for the bond formation (Lee et al., 1995; Liddington and Bankston, 1998). Magnesium and manganese ions are known to stimulate ligand binding, the latter increasing integrin activity to a maximum (Loftus et al., 1994). Calcium ions can act in a biphasic manner, promoting ligand binding to MIDAS at low concentrations (≈ 0.1 mM) and inhibiting ligand binding at higher concentrations (1-10 mM) (Chen et al., 2006; Legler et al., 2001).

The extracellular domain of the integrin β-subunit can be divided into five subdomains named a βI-domain, a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, a four cysteine-rich epidermal growth factor (EGF)-like repeat-containing domain, and a β-tail domain (Figure 2). Additionally, the βI-domain has a MIDAS site which takes part in ligand binding in those integrins lacking the α-subunit I-domain (Xiong et al., 2001; Xiong et al., 2002). The βI-domain also has two additional cation-binding sites named as a region adjacent to MIDAS (ADMIDAS) and a ligand-induced metal-binding site (LIMBS), which are able to modulate ligand binding either negatively or positively, respectively, both in the αI-domain and βI-domain (Valdramidou et al., 2008). Physiologically, ADMIDAS and LIMBS bind calcium ions (Xiao et al., 2004), and calcium ion replacement with a manganese ion at ADMIDAS induces ligand binding (Chen et al., 2003). The LIMBS mediates the synergistic effects of low calcium concentrations with suboptimal magnesium concentrations to support adhesion, whereas the ADMIDAS mediates the negative regulatory effects of higher calcium concentrations (Chen et al., 2003). The amino terminal domains of integrin α- and β-subunits (in most cases the β-propeller and βI-domain, respectively) assemble by non-covalent interactions to form a binding site for ligands.

Integrin cytoplasmic domains are diminutive in their size when compared to the large extracellular domains, but they have been under intensive research for their signaling properties. However, the β4-tail is a notable exception, with a long cytoplasmic domain of 1072 residues compared to other β-tails of around 50 residues (Hogervorst et al., 1990). Integrin α-subunit cytoplasmic tails are comprised of 13 to 56 residues and share the conserved GFF(K/R)R [glycine-phenylalanine-phenylalanine-lysine/arginine-arginine] sequence in their membrane-proximal region (GFFAH [glycine-phenylalanine-phenylalanine-alanine-histidine] sequence in α10, GFFRS [glycine-phenylalanine-phenylalanine-arginine-serine] in α11), but distal sequences are divergent (Lehnert et al., 1999a; Lehnert et al., 1999b; Sastry and Horwitz, 1993). Cytoplasmic tails of the integrin β-subunit contain the conserved HDR(R/K)E [histidine-aspartic acid-arginine-arginine/lysine-glutamic acid] sequence, which is proposed to take part in forming a salt bridge with a conserved sequence of an α-subunit (Vinogradova et al., 2002). Almost all cytoplasmic β-tails have a membrane-proximal NPxY [asparagine-proline-any amino acid-tyrosine] and a membrane distal NxxY [asparagine-any amino acid-any amino acid-tyrosine] motif, which are recognized by several phosphotyrosine-binding (PTB) domains containing proteins capable of forming different signaling complexes (Calderwood et al., 2002).

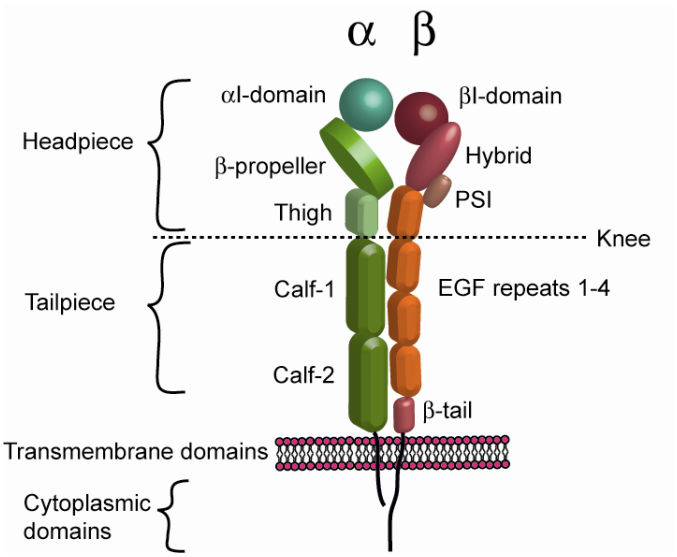

Figure 2: Schematic illustration of the integrin structure. Integrin α- and β-subunits consist of extracellular, transmembrane, and intracellular domains. The large extracellular domain can be further subdivided into several smaller domains. Note that not all integrin heterodimers contain an αI-domain. See the text for further details. The figure is modified from Gahmberg et al., 2009.

2.2. Activation of the receptors

Integrin receptor activation displays changes in the ligand-binding affinity of the receptor ectodomain and is crucial for the regulation of cell adhesion and migration. Activation occurs via conformational changes in the receptor structure and can be triggered by inside-out or outside-in mechanisms, where either intracellular molecule binding to the integrin cytoplasmic domain or extracellular ligand association with the integrin ectodomain, respectively, causes conformational transitions.

Inactive integrins stay in a bent, low affinity conformation on the cell surface where the globular headpiece points towards the plasma membrane generating an upturned V-shape topology and where different integrin subunits are closely associated (Xiong et al., 2001) (Figure 3). In this inactive, resting state, integrins were considered to be unable to bind their ligands. Intriguingly, in some circumstances the bent conformation is able to engage ligands such as fibronectin fragments (Adair et al., 2005). Extensive work done with the leukocyte and platelet integrins points out the importance of the inactive integrin receptor conformation, as circulation of leukocytes and platelets in blood is possible only with minimal interactions with other cells and the surrounding vessel wall. When integrins are activated, they show extended, high affinity conformation and are ready to bind their ligands (Takagi et al., 2002) (Figure 3). In the example of resting leukocytes and platelets, this means extravasation into peripheral tissues and a formation of a hemostatic or pathological thrombus, respectively (Kasirer-Friede et al., 2007; Rose et al., 2007).

A detailed analysis of the integrin ectodomain structures under the electron microscope has revealed two main extended conformations, one with a closed headpiece and the other with an open headpiece (Takagi et al., 2002; Takagi et al., 2003) (Figure 3). A closed headpiece is thought to represent an intermediate affinity state, which can be achieved by straightening up the bent integrin conformation. This state is also called primed conformation, because it is already able to bind extracellular ligands. For the high affinity conformation, the hinge between the βI-domain and the hybrid domain should swing out, resulting in a separation of the integrin α- and β-subunits (Xiao et al., 2004). This separation takes place both in the transmembrane domains (Zhu et al., 2007) and cytoplasmic tails (Kim et al., 2003) of the integrin α- and β-subunits and it is thought to be the final step in integrin activation. The *in vitro* demonstrations of specific mechanical interactions between integrin α and β cytoplasmic tails have remained controversial, but speculations of their existence *in vivo* are universal (Hughes et al., 1996). There are several potential hydrophobic and electrostatic forces between integrin α and β cytoplasmic tails. Furthermore, Vinogradova and co-workers have shown a formation of the salt bridge between the conserved membrane-proximal sequences of integrin α and β cytoplasmic tails (Vinogradova et al., 2002; Vinogradova et al., 2004), but other studies have failed to detect the formation of such a bridge (Li et al., 2001; Ulmer et al., 2001). Intriguingly, mutational studies of conserved membrane-proximal sequences in both integrin α- and β-tails resulted in a constitutive activation of integrins (Hughes et al., 1996; O'Toole et al., 1994) and introduction of an artificial

clasp between the cytoplasmic tails of α- and β-subunits constrained the integrin, making it inactive (Lu et al., 2001). Similar results have been shown with transmembrane sequences, because the introduction of an artificial disulfide bridge between the integrin α and β transmembrane sequences not only prevented the separation of integrin subunits, but also abolished the activating effect of mutations in the conserved membrane-proximal sequences (Luo et al., 2004). This implies that interactions between the transmembrane and cytoplasmic domains of integrin α- and β-subunits regulate integrin activation. Lau and co-workers corroborated this recently with their nuclear magnetic resonance (NMR) studies of αIIbβ3 integrin transmembrane domains by revealing two different clasps between the integrin α/β heterodimer (Lau et al., 2009). These clasps consist of either packing the interactions of three glycine residues in together or of unique electrostatic and hydrophobic bridges located at the outer and inner leaflet of the lipid bilayer, respectively. Thus, perturbations at either the cytoplasmic face or separation of the integrin ectodomains can disrupt the clasps and lead to dissociation of transmembrane domains. The sequences that take part in forming the inner membrane clasp are highly conserved between integrins, suggesting that the integrin activation mechanism is likely to be shared. Conversely, sequences forming the outer membrane clasp are less conserved, and this might explain differences between different integrin subclasses in the activation process.

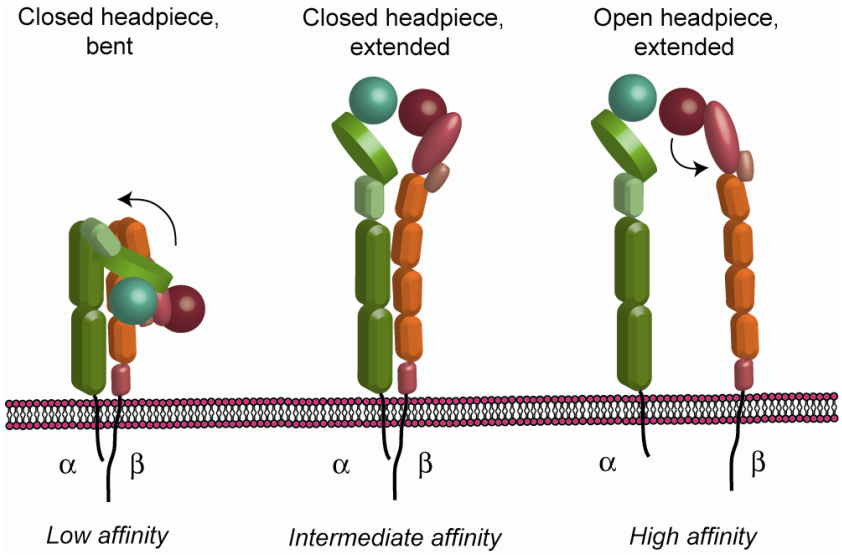

Figure 3: Schematic illustration of the rearrangements during integrin activation. In the inactive conformation, integrin α- and β-subunits stay close to each other and the globular headpiece points towards the plasma membrane. Straightening of the heterodimer generates an extended conformation where the hybrid domain of the β-subunit further swings out resulting in the high affinity conformation. Separation of the integrin α- and β-subunits is the final step in the integrin activation process. The figure is modified from Gahmberg et al., 2009.

In live cells, the first visualization of the dynamic nature of integrin intersubunit association was revealed with fluorescence resonance energy transfer (FRET) studies, where a decrease in fluorescence resonance energy transfer between two different fluorophore-fused integrin C-terminal domains was observed during integrin activation (Kim et al., 2003). In the resting state, the intact αL- and β2-subunits were close to each other, resulting in an efficient FRET. Recent progress concerning the crystal structure of the integrin αIIbβ3 transmembrane domain embedded in the phospholipid bicelles (Lau et al., 2008; Lau et al., 2009) and a computational Rosetta modeling combined with disulfide scanning of intact integrins on the cell surface under physiological conditions (Zhu et al., 2009) have greatly advanced our understanding of integrin transmembrane activation. While integrin α and β transmembrane subunits dissociate from each other, the α-subunit seems to maintain a similar conformational structure, but the β-subunit helix is tilted due to embedding an additional 5-6 residues into the lipid bilayer. Based on the computational modeling combined with disulfide scanning, the formation of the disputed salt bridge seems not to be absolutely necessary for the association of integrin cytoplasmic domains, but instead it is suggested to be important for the priming of initial α/β heterodimer formation (Wang and Luo, 2010; Zhu et al., 2009). The salt bridge is thought to form between the arginine (R) residue in the conserved GFFKR sequence of the α-subunit and the aspartic acid residue (D) in the conserved HDR(R/K)E sequence of the β-subunit (Vinogradova et al., 2002). Zhu and co-workers showed in the computational Rosetta modeling that these two residues are in close proximity to each other, but only about 30% of the representative structures were at a distance, which would enable the formation of such a hydrogen bond (Zhu et al., 2009). They also demonstrated the lysine-716 residue (LLxxxHDRRE; where x is any amino acid) of the β3-integrin to be critical for the association of the α/β heterodimer and that mutations of this residue activated integrin for ligand binding. The positively charged L716 residue is also able to change the tilt angle of the transmembrane domain in the membrane when α- and β-subunits are separated (Zhu et al., 2009).

2.3. Signal transduction across the plasma membrane

Integrins are unusual among the transmembrane receptors due to their ability to signal bidirectionally, either mediating stimulus from the extracellular matrix into the cytoplasm (outside-in signaling) or transmitting the effect of intracellular molecule binding into the structural changes in the integrin ectodomain (inside-out signaling) (Hynes, 2002). Extended integrins are able to bind their extracellular ligands, but lateral separation of α- and β-subunits is required for integrin signaling function.

2.3.1. Outside-in signaling

Integrin adhesiveness to the surroundings is regulated by two major processes: affinity and avidity. Affinity regulation is a conformational change of individual integrin ectodomains, as discussed earlier with the three different conformations: inactive

(bent), primed (extended, closed headpiece), and active (extended, open headpiece). In an intact cell, integrins are in a dynamic equilibrium between these different conformational states. Avidity regulation is the clustering of several receptors together on the cell membrane where hundreds of weak interactions can then sum. The collagen-binding integrin recognition sequence GFOGER [glycine-phenylalanine-pyrrolysine-glycine-glutamic acid-arginine] in native collagens (Knight et al., 2000) is a simple example of multivalent ligand binding and clustering of integrins together. However, clustering alone is not enough, because antibody-induced clustering was not sufficient to induce outside-in signaling when dissociation of integrin α- and β-subunits was blocked with a disulfide bond between their transmembrane domains (Zhu et al., 2007).

The integrin α-subunit plays a central role in determining ligand specificity (Barczyk et al., 2010). Extracellular ligand binding at the intersection of integrin α- and β-subunits triggers conformational changes leading to outside-in signaling. αI- and βI-domains take part in ligand binding, but in the absence of the αI-domain, the β-propeller is involved in linking (Gahmberg et al., 2009). As mentioned earlier, integrins can be classified according to their extracellular ligand preferences (Barczyk et al., 2010). Despite the fact that some integrins show a high ligand-binding specificity, others bind several different types of ligands. Prototypic ligands for collagen-binding integrins are type I, IV, and IX collagens, whereas RGD receptors bind ligands containing the RGD sequence, such as fibronectin, vitronectin, and fibrinogen (Barczyk et al., 2010). The laminin receptor subfamily prefers different laminin isoforms as the ligand, but leukocyte-specific receptors bind to ICAMs and VCAMs to mediate cell–cell contacts (Barczyk et al., 2010). A fully detailed mechanism as to how integrin is activated upon extracellular ligand binding remains elusive.

Integrin outside-in signaling changes can be divided into three temporal stages (Legate et al., 2009): immediate events containing the phosphorylation of specific protein substrates and accumulation of the lipid second messengers phosphatidylinositol (4,5)-bisphosphate (PIP_2) or phosphatidylinositol (3,4,5)-trisphosphate (PIP_3); short-term effects taking place within 60 minutes and consisting of mainly actin cytoskeleton reorganization leading to changes in the cell shape; and long-term effects having consequences for cell survival, proliferation, and differentiation. Due to the fact that integrin cytoplasmic tails themselves lack enzymatic activity, they must bind accessory molecules to mediate downstream signaling from outside-in. Zaidel-Bar and co-workers have identified the integrin adhesome *in silico* consisting of 156 different molecules including kinases, phosphatases, GTPases (guanosine triphosphate hydrolyzing enzymes), GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), as well as adaptor, cytoskeletal, and actin-binding proteins based on the extensive literature searches and protein–protein interaction databases (Zaidel-Bar et al., 2007). The adaptor proteins, which interact directly with integrin cytoplasmic tails, function in these adhesomes as a major linker, serving as docking sites for all other molecules. The exact spatio-temporal order of adhesome organization is not known, but extracellular ligand binding to the integrin heterodimer and

subsequent lateral dissociation of integrin α- and β-subunits, together with concomitant actin cytoskeleton reorganization, is thought to lead to outside-in signaling.

2.3.2. Inside-out signaling: β-tails

In addition to extracellular ligands, intracellular molecules can bind to cytoplasmic domains of integrins and activate them via the inside-out mechanism. Adaptor proteins binding to short cytoplasmic β-tails of integrins can be categorized into three groups: structural adaptors, scaffolding adaptors providing binding sites for additional proteins, and catalytic adaptors (Legate and Fässler, 2009).

A. Structural adaptors

Talin, filamin, and α-actinin are structural adaptors able to form a single bridge between the integrin cytoplasmic β-tail and actin cytoskeleton. PIP_2 activates cytosolic talin by disrupting the autoinhibitory interaction between the head and rod domains of talin, thus unmasking the talin phosphotyrosine-binding (PTB) motif for docking to the integrin β-subunit membrane-proximal NPxY sequence (Goksoy et al., 2008). Talin binds to non-phosphorylated β1, β2, β3, and β7 integrin tails, but tyrosine phosphorylation of the membrane-proximal NPxY motif decreases affinity, when the PTB domain containing scaffolding adaptor docking protein 1 (Dok1) is able to outcompete talin (Anthis et al., 2009). Talin has a key role in integrin activation via inside-out signaling and thus Dok1 serves as a negative regulator of integrin affinity (Wegener et al., 2007). Integrin cytoplasmic domain-associated protein-1 (ICAP-1) is another molecule able to compete with talin via binding to the membrane-distal NxxY motif of β1-integrin and thus impair integrin activation (Millon-Frémillon et al., 2008). *Icap-1*-deficient mouse embryonic fibroblasts displayed defects in cellular spreading and migration which correlated with the focal adhesion redistribution defects and increased integrin affinity (Millon-Frémillon et al., 2008).

Due to the limited length of the integrin cytoplasmic β-tails many adaptor-binding sites overlap. Filamin binds to the non-phosphorylated serine/threonine-rich sequence between the membrane-proximal NPxY and membrane distal NxxY motifs, partially overlapping with the talin binding sequence (Kiema et al., 2006). The integrin β7-tail contains three threonine residues in this intervening region (Thr783, Thr784, and Thr785), and phosphorylation-mimicking mutations at any of these sites disrupted the filamin interaction allowing talin to bind (Kiema et al., 2006). Filamin binding is known to inhibit integrin affinity for its ligands and integrin-mediated cell migration (Calderwood et al., 2001). A novel cytoskeletal adaptor molecule, migfilin, is able to regulate filamin binding to the integrin cytoplasmic tail by masking the binding residues in filamin and so promote integrin activation via talin binding (Ithychanda et al., 2009). Molecular scaffolding protein 14-3-3 also competes for binding to the intervening serine/threonine-rich sequence and phosphorylation of the Thr758 residue in the integrin β2-tail led to elimination of filamin interaction and induced 14-3-3 binding (Takala et al., 2008). Interestingly, 14-3-3 was also able to outcompete talin binding when the Thr758 residue was phosphorylated.

α-actinin binds directly to the membrane-proximal sequence of integrin β1, β2, and β3 cytoplasmic tails (Otey et al., 1993; Pavalko and LaRoche, 1993), which overlaps with the talin binding sequence. α-actinin is a structural protein capable of crosslinking actin filaments into bundles and networks, but also serving as a docking site for other cytoskeletal proteins, such as vinculin. Vinculin instead is able to bind both to talin and actin (Gilmore and Burridge, 1996), thus strengthening intermolecular associations at cell–cell and cell–ECM contacts. α-actinin is shown to be crucial for assembly and maturation of nascent adhesions via formation of an actin–α-actinin template (Choi et al., 2008).

Non-muscle myosins are actin-based motor proteins taking care of integrin redistribution within the cell and are capable of direct interaction with the membrane-proximal NPxY motif of integrin β1, β3, and β5 cytoplasmic tails (Zhang et al., 2004). Zhang and co-workers have demonstrated impaired integrin-mediated adhesion and filopodia elongation in myosin-deficient cells. Consistent with that, myosin10 overexpression stimulated the formation and elongation of filopodia in an integrin-dependent manner and relocalized β1- and β3-integrins with myosin10 to the tips of filopodia (Zhang et al., 2004).

B. Scaffolding adaptors

Scaffolding adaptors Dok1 and 14-3-3 have already been mentioned for their ability to outcompete some structural adaptors from preferred binding sites. Dok1 binds to the membrane-proximal NPxY motif of integrins β2, β3, β5, and β7 favoring the phosphorylated tyrosine residue (Legate and Fässler, 2009). Also 14-3-3 favors phosphorylated residues while binding to the intervening serine/threonine-rich sequence between the membrane-proximal NPxY and membrane distal NxxY motifs of β1, β2, and β3 cytoplasmic tails (Legate and Fässler, 2009).

Kindlins, which are proteins named after the gene mutated in Kindler syndrome characterized by rare skin blistering disorders and poikilodermas (Siegel et al., 2003), are able to bind directly to the membrane distal NxxY motif of β1, β2, and β3 cytoplasmic tails (Moser et al., 2009). Kindlins also function as scaffolding adaptors, linking integrins indirectly to the actin cytoskeleton via integrin-linked kinase (ILK)–parvin and migfilin–filamin protein complexes (Montanez et al., 2008; Tu et al., 2003). Despite their distinct binding motifs in the integrin β cytoplasmic tails, talin and kindlin seem to cooperatively regulate integrin affinity for its ligands, or in other words, inside-out signaling. Kindlin-2$^{-/-}$ mammalian cells showed impaired integrin activation in the presence of normal talin expression levels (Montanez et al., 2008). Conversely, kindlin-2 overexpression was not sufficient to change integrin activity, but coexpression of talin and kindlin-2 resulted in a synergistic increase in αIIbβ3 affinity (Montanez et al., 2008). Detailed analysis of whether talin and kindlin binding to the cytoplasmic β-tail is sequential or simultaneous remains to be determined.

C. Catalytic adaptors

In addition to the above-mentioned structural and scaffolding adaptors, there are catalytic adaptors binding to the integrin cytoplasmic β-tails (Legate and Fässler, 2009). Src is a prototype of Src-family kinases (SFKs) belonging to the group of cytoplasmic non-receptor tyrosine kinases and able to bind constitutively to the integrin β cytoplasmic tails (Arias-Salgado et al., 2003). Focal adhesion kinase (FAK) is also capable of binding to integrin β cytoplasmic tails either directly or via association with Src (Chen et al., 2000; Shen and Guan, 2001). The major downstream signaling pathway from the Src-FAK complex is the Ras-MAPK (mitogen-activated protein kinase) pathway, which regulates multiple cellular functions including cell survival, proliferation, and differentiation.

Integrin-linked kinase (ILK) has been shown to be necessary, but not sufficient, for integrin αIIbβ3 inside-out activation (Honda et al., 2009). ILK knockdown suppressed integrin activation, but ILK overexpression in talin knocked-down parental cells was not enough to rescue an inactivated integrin phenotype. ILK has been shown to bind directly to the cytoplasmic domains of integrin β1 and β3 (Hannigan et al., 1996), and ILK is also able to interact directly with kindlin-2 (Montanez et al., 2008). Recently, the controversy of ILK kinase activity was clarified by establishing knock-in mouse strains with point mutations in ILK (Lange et al., 2009). Lange and co-workers demonstrated that ILK is a non-receptor serine/threonine pseudokinase whose kinase activity was dispensable for mammalian development, but the scaffold protein function of ILK was vital for kidney development (Lange et al., 2009). Based on these facts, ILK should now be classified as a scaffolding adaptor.

2.3.3. Inside-out signaling: α-tails

The adaptor molecules binding to the integrin cytoplasmic α-tails are not so well characterized, but paxillin is reported to bind directly to the α4 cytoplasmic tail enhancing FAK phosphorylation and cell migration, but reducing cell spreading (Liu et al., 1999). Cell spreading is also documented to decrease when paxillin binds directly to the integrin α9 cytoplasmic tail (Liu et al., 2001). The intracellular calcium-binding protein, calreticulin, binds directly to the conserved KxGFFKR sequence of several integrin α-subunits (Leung-Hagesteijn et al., 1994). Calreticulin is speculated to stabilize the integrin active conformation due to the facts that it interacts with the active α2β1 integrin conformation and calreticulin-deficient cells showed impaired cell spreading and adhesion (Coppolino et al., 1995; Coppolino et al., 1997). Moreover, a small GTPase, Rab21, which regulates both endocytosis and exocytosis in cells, is able to associate with the conserved membrane-proximal sequence of integrin α-tails and thus control the endosomal traffic and adhesion of integrins (Pellinen et al., 2006).

Protein phosphatase 2A (PP2A) is a ubiquitously expressed serine/threonine phosphatase which has been shown to interact directly with the membrane-proximal KVGFFKR sequence of αIIb integrin and thus negatively regulate αIIbβ3-mediated adhesion to fibrinogen when overexpressed in human embryonal kidney 293

(HEK293) cells (Gushiken et al., 2008). Intriguingly, PP2A is recruited to associate with integrin β1 in skeletal myoblasts (Kim et al., 2004). The reason for this might be the broad substrate specificities of several phosphatase enzymes.

CIB1, a calcium- and integrin-binding protein 1, interacts directly with the membrane-proximal αIIb-tail sequence inhibiting integrin αIIbβ3 activation (Yuan et al., 2006). FHL2 and FHL3 (four and one-half LIM domain proteins 2 and 3) are expressed in striated muscle and the double zinc-finger containing LIM domain is named after the initials of Lin11, Isl-1, and Mec-3 in which LIM was first discovered (Samson et al., 2004). In muscle, FHL2 and FHL3 mediate a wide range of protein–protein interactions via their LIM domain, regulating the function of cytoskeletal proteins and transcription factors (Samson et al., 2004). Samson and co-workers used a yeast two-hybrid interaction assay to show binding between FHL2 and the intracellular domains of integrins α3, α7, or β1. Additionally, FHL3 was able to pair with the integrin α3, α7, and β1 cytoplasmic domains.

Taken together, there is an increasing interest towards identifying novel binding partners for the integrin cytoplasmic α-tails, since a plethora of adaptor molecules have been shown to bind directly to the cytoplasmic β-tails. However, some discrepancies in the above-mentioned examples may be explained by the cell-specific functions of different integrin subclasses, technical challenges due to the integrin heterodimeric structure *in vivo*, and a vast number of molecules located in the integrin adhesome. Many interactions are also low in their affinity or short-lived, and thus difficult to study.

2.4. Integrins in cell migration and invasion

Cell adhesion to the extracellular matrix via integrin ectodomains causes mechano-transduction across the plasma membrane where flexible integrin internal tails interact with the actin cytoskeleton via several adaptor molecules. Much debate surrounds the naming of these intracellular adhesion-based structures, but they are called focal complexes (transient structure) and focal adhesions (mature structure) in adherent fibroblasts, immunological kinapses (asymmetric and mobile structures) and synapses (symmetric and stabile structures) in activated T-lymphocytes, podosomes in adherent macrophages and osteoclasts, and invadopodia in cancer cells (Gimona et al., 2008; Shattil et al., 2010).

Treadmilling F-actin dynamics are crucial for cell movement in which continuous protrusion and retraction dynamics are seen at the cell edge. Lamellipodia are integrin-rich structures at the leading edge of mobile cells capable of *de novo* polymerization of F-actin. Thus, integrins do not just organize preformed actin filaments, but also recruit the actin nucleating protein Arp2/3 (actin-related protein 2/3) complex and formins to synthesize new actin filaments (Butler et al., 2006). Actin dynamics are finely controlled by several molecules, but the central signaling complex downstream from

integrins is Src/FAK, which regulates the Rho GTPase family via controlling the activity of GEFs and GAPs. Integrin-mediated adhesion causes autophosphorylation of the Tyr-397 residue in FAK, generating a binding site for the SH2 (Src-homology 2) domain of Src (Mitra and Schlaepfer, 2006). Src then phosphorylates several tyrosine residues in FAK, creating additional protein–protein binding sites (Mitra and Schlaepfer, 2006). Rac (Ras-related C3 botulinum toxin substrate 1), Cdc42 (cell division cycle 42), and RhoA (Ras homolog gene family, member A) are the most important members of the Rho GTPase family at adhesion sites for the regulation of cytoskeletal actin organization and they shuttle between an active GTP-bound form and an inactive GDP-bound form. During integrin-mediated cell spreading, Cdc42 and Rac are activated to regulate the assembly of filopodia and lamellipodia, respectively (DeMali et al., 2003). RhoA is suppressed during spreading, but activated later when contractility is needed for the generation of tension (DeMali et al., 2003). The spatio-temporal coordination of protrusion, contraction as well as assembly and disassembly of the cell–ECM adhesions are required during the cell movement. Thus, a subtle balance between the activity of Rac and RhoA is required at the leading front and retracting rear, respectively. Indeed, Rac can restrain RhoA activity and vice versa. High Rac activity is needed at the leading edge to promote the formation of lamellipodia and to suppress RhoA-induced contractility, but high RhoA activity is required at the retracting rear to suppress the formation of multiple Rac-induced lamellipodia that would interfere with the directionality of movement, and sustain the high turnover rate of the matrix adhesions (Huveneers and Danen, 2009). Not surprisingly, fibroblasts lacking either FAK or Src have impaired cell motility (Ilic et al., 1995; Klinghoffer et al., 1999).

Cell invasion requires adhesion to the matrix, proteolysis of the surrounding ECM components to make the track, and finally, migration. Cells can adopt either an amoeboid or mesenchymal shape and utilize non-proteolytic or proteolytic migration strategies, respectively (Frampton and Plosker, 2009). Amoeboid cells are low both in β1- and β3-integrin expression levels as well as in adhesiveness to collagen and a number of focal contacts, which all together enable them to migrate faster than mesenchymal cells (Friedl and Wolf, 2003). However, the therapeutic paradigm in inhibiting the different migration strategies is that cells are able to switch between amoeboidal and mesenchymal migration due to their plasticity if only one strategy is blocked (Wolf et al., 2003).

Pericellular proteolysis of ECM components is mainly mediated by matrix metalloproteinases (MMPs). Some MMPs are able to bind directly to integrins, such as pro-MMP-1 with an I-domain of integrin α2β1 in migrating keratinocytes (Dumin et al., 2001) and MMP-2 with integrin αVβ3 in experiments done *in vitro* (Brooks et al., 1996). Instead, MT1-MMP (membrane-type 1-MMP), which co-localized with integrins β1 and αVβ3 on collagen-coated beads added to endothelial cells, has been shown to be able to interact directly with type I collagen (Gálvez et al., 2002; Tam et al., 2002). Integrin receptor occupancy can also directly regulate MMP levels. In melanoma cells, triple-helical peptide ligands of type IV collagen for integrin α2β1

were able to induce the expression levels of MMP-1, -2, -3, -13, and -14 (Baronas-Lowell et al., 2004). In mammary epithelial cells and breast cancer cells, the synthetic fibronectin-derived peptide was sufficient to induce MMP-1-dependent invasion via α5β1 (Jia et al., 2004).

Pericellular ECM proteolysis is followed by intracellular binding of active motor protein non-muscle myosin II (NMII) to actin filaments and thus formation of the contractile actomyosin complex (Vicente-Manzanares et al., 2009). Not only contraction, but also disassembly of the focal contacts at the trailing edge is needed for gliding the cell body slowly forward. Calcium-dependent cytoplasmic protease calpain 2 plays a critical role in adhesion disassembly due to its ability to cleave talin (Franco et al., 2004) or cytoplasmic β1, β2, β3, and β7 integrin tails (Pfaff et al., 1999). Finally, integrin receptors are either internalized via endocytic vesicles for recycling towards the leading edge (Bretscher, 1996) or deposited onto substratum behind the retracting rear on the migratory path of the cell (Friedl et al., 1997). Although the direct evidence of integrin receptor removal from the retracting rear to the leading edge via long-range transport is lacking, several studies indicate that endosomes are crucial in controlling the spatial restriction of integrins (Pellinen and Ivaska, 2006).

Taken together, cell motility is a dynamic process between the cell, its substratum, and intracellular cytoskeleton-associated contractile apparatus where membrane-traversing integrins integrate ECM and the actin cytoskeleton. Migration and cell polarity require controlled adhesion turnover where fresh adhesions are needed at the leading edge and adhesions at the rear need to be released to allow cell body retraction. Adhesion remodeling also means that integrins are constantly trafficked by the endosomal pathway. In addition to mediating adhesion, integrins are able to control actin dynamics and matrix remodeling.

2.5. Integrins in health and disease

Migratory cells are crucial for early embryonal development where the cell needs to break loose from its original neighbors and colonize new sites in the embryo. Similarly, cancer cells disseminate from the primary tumors to colonize distant organs.

2.5.1. Collagen-binding integrins in development

Knockout mice carrying the germline deletions of the collagen-binding integrin α-subunits (α1, α2, α10, or α11) express very mild phenotypes and fail to result in any overt defects in normal development. However, embryonic fibroblasts derived from the α1-null mice are defective in spreading and migration to type IV collagen and laminin (Gardner et al., 1996). The α2-null mice instead express diminished mammary gland branching morphology (Chen et al., 2002), delayed thrombus formation following carotic injury (He et al., 2003), and defective innate immunity mediated by mast cells (Edelson et al., 2004). The α10-deficient mice show mild cartilage defects and growth

retardation of the long bones (Bengtsson et al., 2005) and α11-null mice have defects in incisor eruption (Popova et al., 2007). Viable phenotypes described herein most probably illustrate the compensatory role of other members in the integrin subfamilies. Conversely, β1-deficient mice die after implantation (Fässler and Meyer, 1995; Stephens et al., 1995). It is noteworthy that β1 takes part in the formation of several α/β1 heterodimers.

2.5.2. Integrins in cancer progression

Compelling evidence shows that integrins have a pivotal significance for cell migration, but discrepancies as to how integrin expression levels correlate with invasion exist. Several integrins are found to associate with different cancer types (Table 1). αVβ3- and αVβ5-integrins are crucial for angiogenesis (Friedlander et al., 1995), but the increased expression of αVβ3 is associated with augmented metastasis capacity and decreased survival in several tumor types, such as melanoma (Nip et al., 1992), breast carcinoma (Liapis et al., 1996), prostate carcinoma (Zheng et al., 1999), pancreatic carcinoma (Hosotani et al., 2002), cervical cancer (Gruber et al., 2005), and glioma (Bello et al., 2001). On the contrary, αVβ3-integrin expression can also impede invasion in melanoma (Boukerche et al., 1994) and ovarian cancer (Kaur et al., 2009). αVβ3 is, additionally, also important for macrophage function during host defense. Macrophage infiltration into the primary tumor was significantly reduced in integrin β3-null mice, thereby enhancing tumor growth (Taverna et al., 2004).

Disparities in studies with integrin αVβ3 emphasize that integrins do not function as isolated units on the cell surface, but the repertoire of all integrin heterodimers and other cell surface receptors present on a given cell dictate the outcome. The highly invasive melanoma cells showed increased levels of integrin α2β1 (Klein et al., 1991), but re-expression of integrin α2β1 in a poorly differentiated breast carcinoma abrogated the malignant phenotype and reduced the *in vivo* tumorigenicity of the cells (Zutter et al., 1995). Impaired α2β1-integrin levels also correlated with a poorly differentiated phenotype in colorectal adenocarcinoma (Pignatelli et al., 1990).

Integrin α5β1 binds to fibronectin that is present at high concentrations in walls of the tumor blood vessels and in the tumor interstitial stroma. High α5β1 expression in melanoma is associated with augmented lymph node metastasis (Laidler et al., 2000). Additionally, upregulated α5β1 expression is seen in lung cancers where higher expression correlates with poorer outcome (Adachi et al., 2000). Expression of integrin αVβ6 is downregulated in healthy adult epithelia, but upregulated in several different cancers, such as colon, ovarian, and cervical carcinomas, where it is linked with more aggressive disease outcomes (Ahmed et al., 2002; Bates et al., 2005; Hazelbag et al., 2007). Moreover, high integrin α6β4 expression levels correlated with reduced survival in breast carcinoma (Friedrichs et al., 1995), but deletion of the β4-subunit impaired tumor invasion and progression (Guo et al., 2006).

Elevated integrin β1 expression levels have been documented in the gene expression profile of invasive carcinoma cells in primary rat mammary tumors (Wang et al., 2004). A cohort study of 249 breast cancer patients revealed that the highest β1-integrin intensity score was associated with a significantly reduced 10-year overall survival and a decreased disease-free survival (Yao et al., 2007). Furthermore, targeted disruption of β1-integrin in the mammary epithelium of a transgenic mouse model of human breast cancer revealed a dramatic reduction in the number of hyperplastic mammary lesions (White et al., 2004). Together, these results demonstrate that β1-integrin has a significant role in tumorigenesis, at least in some subsets of cancers.

Integrin	Role in cancer
α2β1	Upregulated expression in invasive melanoma (Klein et al., 1991). Reduced expression in breast carcinoma (Zutter et al., 1995) and colorectal adenocarcinoma (Pignatelli et al., 1990).
α5β1	Upregulated expression correlates with invasive melanoma (Laidler et al., 2000) and poor survival in lung cancer (Adachi et al., 2000).
α6β4	Upregulated expression correlates with poor survival in breast carcinoma (Friedrichs et al., 1995).
αVβ3	Upregulated expression correlates with metastases in melanoma (Nip et al., 1992), breast carcinoma (Liapis et al., 1996), prostate carcinoma (Zheng et al., 1999), pancreatic carcinoma (Hosotani et al., 2002), cervical cancer (Gruber et al., 2005), and glioma (Bello et al., 2001). Upregulated expression correlates with reduced metastasis capacity in melanoma (Boukerche et al., 1994) and ovarian cancer (Kaur et al., 2009).
αVβ6	Upregulated expression correlates with aggressive disease in colon carcinoma (Bates et al., 2005), ovarian cancer (Ahmed et al., 2002), and cervical carcinoma (Hazelbag et al., 2007).

Table 1: Integrin heterodimers are associated with tumorigenesis. See the text for further details.

2.6. Integrins in targeted drug therapy

The importance of integrins in cell adhesion and especially in cell migration has made them an appealing target for cancer therapy. Because excessive integrin inhibition can be deleterious, as seen with the β1-null phenotype (Fässler and Meyer, 1995; Stephens et al., 1995), which can form several α/β1 heterodimers, selective integrin-activity inhibitors are needed.

There are already a few selective adhesion molecule inhibitors in clinical use, such as Abciximab (ReoPro®), a humanized monoclonal function-blocking antibody against the integrin β3-subunit, which blocks platelet fibrinogen receptor αIIbβ3 and thus

inhibits the formation of platelet aggregates. It is used mainly during percutaneous coronary interventions (Cohen, 2009). Another antiplatelet therapeutic is tirofiban (Aggrastat®), which is a non-peptide, synthetic inhibitor against integrin αIIbβ3 and is used for patients with unstable angina or myocardial infarction (Juwana et al., 2010). Natalizumab (Tysabri®) instead is a humanized monoclonal antibody against integrin α4 and is used for therapy in active remitting-relapsing multiple sclerosis patients to block leukocyte integrins' α4β1 and α4β7 function and reduce inflammation (Sangalli et al., 2010). Efalizumab (Raptiva®) is a humanized monoclonal antibody against αL-integrin in lymphocytes suppressing the immune system of psoriasis patients (Frampton and Plosker, 2009). Both natalizumab and efalizumab are associated with an increased risk of a severe demyelinating disease called progressive multifocal leukoencephalopathy (PML), and thus Raptiva was withdrawn from the market in April 2009 (Major, 2010).

One milestone of anti-integrin therapy was bypassed recently, when cilengitide entered into phase III clinical trials for glioblastoma patients. Cilengitide, the first in a new class of integrin inhibitors, is a cyclic RGD peptide selective for integrins αVβ3 and αVβ5. Glioblastomas are aggressive brain tumors rich in blood vessels and usually linked with poor prognosis. Preferential susceptibility of glioblastomas to the cilengitide therapy was elucidated in nude mice, where brain tumor cells were injected simultaneously into the forebrain (orthotopic) and the subcutis (heterotopic) (MacDonald et al., 2001). Cilengitide-treated mice showed no evidence of tumors or only scant residual tumor cells in the orthotopic tumor model, but tumor cells in the heterotopic model lacked growth inhibition. Thus, it seems that the ECM composition of the tumor microenvironment is able to dictate the outcome of the therapy. Cilengitide is currently also being tested for prostate and lung cancer, and melanoma in phase II trials (Albert et al., 2006; Bradley et al., 2010).

Also other potential candidates are in the pipeline: Volociximab is a chimeric monoclonal antibody that inhibits the functional activity of integrin α5β1, thereby decreasing tumor growth and tumor blood vessel density (Bhaskar et al., 2008). Volociximab is currently under phase II clinical trials for non-small cell lung cancer (NSCLC), melanoma, pancreatic cancer, and ovarian cancer (www.clinicaltrials.gov). Moreover, ATN-161 is a non-RGD-based small peptide that blocks the binding of integrin α5β1. ATN-161 is a five-amino acid peptide PHSCN [proline-histidine-serine-cysteine-asparagine] derived from the synergy region of human fibronectin where a cysteine residue replaces an arginine in the original sequence. When it was tested in phase I clinical trials for patients with solid tumors, one-third of the patients manifested for prolonged stable disease (Cianfrocca et al., 2006). Earlier work done with the mouse models proved that ATN-161 can inhibit breast cancer growth and formation of soft tissue and skeletal metastases (Khalili et al., 2006), and reduce the number of colon cancer liver metastases in combination with chemotherapy (Stoeltzing et al., 2003). ATN-161 is currently under phase II studies for malignant glioma in combination with chemotherapy (www.clinicaltrials.gov).

A totally distinct avenue for therapeutic intervention is based on the use of integrins for targeted drug delivery. Hood and co-workers used a nanoparticle coupled with an organic integrin αVβ3-targeting ligand to deliver a mutant *RAF1* gene to angiogenic blood vessels in tumor-bearing mice (Hood et al., 2002). This targeted gene delivery to the subcutaneous mouse model of human melanoma cells resulted in apoptosis of the tumor-associated endothelium and a rapid, sustained regression of tumors. The advantages of this model are high local concentrations and low systemic exposure. Drug delivery can be targeted to tumor cells, tumor vessels, or to receptors shared by both, but the low penetration capacity of drugs limits the use of targeted therapy. Drugs generally penetrate only from three to five cell diameters from the blood vessels, thus exposing more distantly located tumor cells only to low drug concentrations and preparing the way for resistance development (Hambley and Hait, 2009). The laboratory of E. Ruoslahti has recently discovered a unique tumor-homing peptide named internalizing RGD (iRGD), where the RGD motif delivers the peptide to the αV-integrins on the tumor endothelium and a proteolytic cleavage then exposes a C-terminal binding motif CRGDK [cysteine-arginine-glycine-aspartic acid-lysine] for neuropilin-1, which mediates compound penetration into extravascular tumor parenchyma (Sugahara et al., 2009).

Altogether, integrins remain a promising target for some special types of tumors, but several questions remain to be answered. Structural similarities make them a challenge for designing integrin heterodimer-specific antagonists and a subtle regulation between active and inactive conformation states makes the picture even more complicated. Importantly, many of the integrin heterodimers are active in both normal cells and cancer cells, thus tailoring the therapy will be of critical importance.

3. Epidermal growth factor receptor

3.1. Structure and ligands

Early studies with the radioisotope-labeled epidermal growth factor (EGF), which was originally isolated from the submaxillary glands of adult male mice, exhibited specific binding to human fibroblasts grown in culture and plausible competition-binding assays showed that the reduction in the amount of bound, labeled EGF to the cells was directly proportional to the amounts of unlabeled EGF added (Carpenter et al., 1975). Further competitive-binding assays with a variety of other polypeptide hormones, loss of cell-bound radioactivity as a function of time, and the recognition of monoiodotyrosine from the cell culture medium after incubation with ^{125}I-EGF led to the prevailing idea by S. Cohen that there has to be a specific receptor for EGF on the cell surface (Carpenter et al., 1975), but the sequence of this receptor was not published until 1984 (Ullrich et al., 1984). EGFR was the first growth factor receptor identified and was a prototype for the receptor tyrosine kinase (RTK) family. Abundantly used synonyms for EGFR are HER1 (human EGFR) or ErbB1, based on the sequence homology with the avian erythroblastosis virus oncogenic factor, v-erbB.

Stanley Cohen received the 1986 Nobel Prize in Physiology and Medicine together with Rita Levi-Montalcini for their discovery of nerve growth factor (NGF) (Cohen and Levi-Montalcini, 1957) and for his own subsequent work on the isolation of EGF from mouse saliva and human urine (Cohen, 1962; Cohen and Carpenter, 1975).

The three other members in the EGFR family are HER2 (Bargmann and Weinberg, 1988; Stern et al., 1986), also known as ErbB2 or neu after the sequence homology with the neuroblastoma-causing *neu* proto-oncogene in rats, HER3 (ErbB3) (Kraus et al., 1989; Plowman et al., 1990), and HER4 (ErbB4) (Plowman et al., 1993). These receptors share a common structural overview, having a large ligand-binding and dimerization-arm-containing ectodomain, a short single-span transmembrane domain, and a large intracellular domain including the kinase activity domain and a C-terminal tail for docking of downstream signaling molecules. In detail, the extracellular domain is comprised of four subdomains I-IV (Lax et al., 1988). Subdomains I and III, which can be alternatively named as L1 and L2, are leucine-rich ligand-binding domains, instead of cysteine-rich subdomains II and IV (also known as CR1 and CR2) that are involved in the receptor dimerization and activation of the tyrosine kinase subdomain (Ward et al., 1995; Ward and Garrett, 2001). Adjacent to the transmembrane domain is the intracellularly located juxtamembrane domain that is capable of regulating kinase activity and the intracellular dimerization of the EGFR family (Aifa et al., 2005). The juxtamembrane domain is also shown to harbor the nuclear localization sequence (NLS), thereby mediating the nuclear localization of the EGFR family (Hsu and Hung, 2007), and taking part in the receptor downregulation via recycling and endosomal sorting (Kil et al., 1999). The juxtamembrane domain is followed by the bi-lobular intracellular kinase domain consisting of N-terminal and C-terminal lobes separated by the ATP-binding cleft (Stamos et al., 2002). However, the HER3 receptor is a catalytically inactive kinase with several altered sequences in the N-terminal lobe (Guy et al., 1994). Recently, HER3 was shown to have residual kinase activity under some conditions, a fact that may explain the role of HER3 in the resistance to EGFR- or HER2-targeted therapies via heterodimer formation (Shi et al., 2010). In addition to harboring several tyrosine residues which act as docking sites for downstream signaling molecules, the intracellular C-tail has a direct regulatory effect on the kinase domain, which may be mediated by extensive contacts with N-terminal and C-terminal lobes of the kinase domain (Bose and Zhang, 2009). HER4 is unique among other members due to alternative mRNA splicing that generates two different extracellular juxtamembrane (JM) isoforms, JM-a or JM-b, and two cytoplasmic (CYT) isoforms, CYT-1 or CYT-2, which can be combined into four distinct HER4 isoforms (Elenius et al., 1997; Elenius et al., 1999).

The EGFR ligands are EGF, the transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR), and epigen (EPG) (Harris et al., 2003). HB-EGF, BTC, and EPR can also bind to HER4, but major ligands for HER3 and HER4 receptors belong to another group of growth factors called neuregulins (NRGs). The NRG gene family consists of four members, *NRG1*, *NRG2*, *NRG3*, and *NRG4*, which can be translated to several

isoforms (Falls, 2003). However, NRG3 and NRG4 fail to activate HER3 signaling (Hobbs et al., 2002). All the EGFR ligands are produced as transmembrane precursors on the cell surface where they are released by several proteases (Sahin et al., 2004). The principal sheddases for ligand precursors are a disintegrin and metalloproteinase 10 (ADAM10) and ADAM17 (Sahin et al., 2004). Mature growth factor peptides contain the consensus sequence, which is also known as the EGF motif, consisting of six spatially conserved cysteine residues (Harris et al., 2003). While most EGFR ligands are soluble and may act in an autocrine, paracrine, or endocrine fashion, the membrane-anchored peptide can also mediate juxtacrine signaling (Singh and Harris, 2005).

3.2. Ligand-induced oligomerization and signaling

In the absence of bound ligand, there is an intramolecular interaction between the EGFR extracellular domains II and IV and the receptor adopts a monomeric, inactive, tethered structure (Ferguson et al., 2003) (Figure 4). When the ligand binds, having contact both with domains I and III, the monomer extends, unmasking the dimerization arms of domains II and IV for interactions with another ligand-bound extended monomer (Ogiso et al., 2002). This makes EGFR unique among the RTKs, because ligands are not present at the dimerization interface and the dimerization is mediated exclusively by receptor contacts. HER2 is the only orphan member of the EGFR family adopting the extended, constitutive active structure with exposed dimerization arms even without ligand binding (Garrett et al., 2003). In fact, HER2 has no known soluble ligands and thus it forms only heterodimers with ligand-bound EGFR, HER3, or HER4 (Lemmon, 2009). Recently, a significant number of dimerization studies have been extensively reviewed by M.A. Lemmon: robust formation of homodimers seems to be restricted only to EGFR and HER4. However, EGFR/HER2 heterodimers form with a similar affinity to EGFR homodimers, but heterodimerization between EGFR and HER3 or HER4 is significantly weaker (Lemmon, 2009). Based on the mutation studies, when breaking the autoinhibitory tether between domains II and IV, the unmasked dimerization arms are not sufficient to induce dimer formation without ligand binding (Dawson et al., 2005). Thus, ligand-induced structural rearrangements outside the region of the dimerization arms are also critical for pairing of the extended monomers.

Ligand-induced conformational changes in the extracellular domain followed by subsequent dimerization of the monomeric extended receptors bring autoinhibited intracellular kinase domains into close proximity. Kinase domains form an asymmetric dimer, where the N-terminal lobe of the monomer A is activated by the C-terminal lobe of the monomer B, and this leads to subsequent *trans*-autophosphorylation of monomer B itself (Zhang et al., 2006). *Trans*-autophosphorylation of tyrosine residues within the C-terminal tail of the monomer generates docking sites for several downstream signaling molecules containing phosphotyrosine-binding (PTB) or Src-homology 2 (SH2) domains.

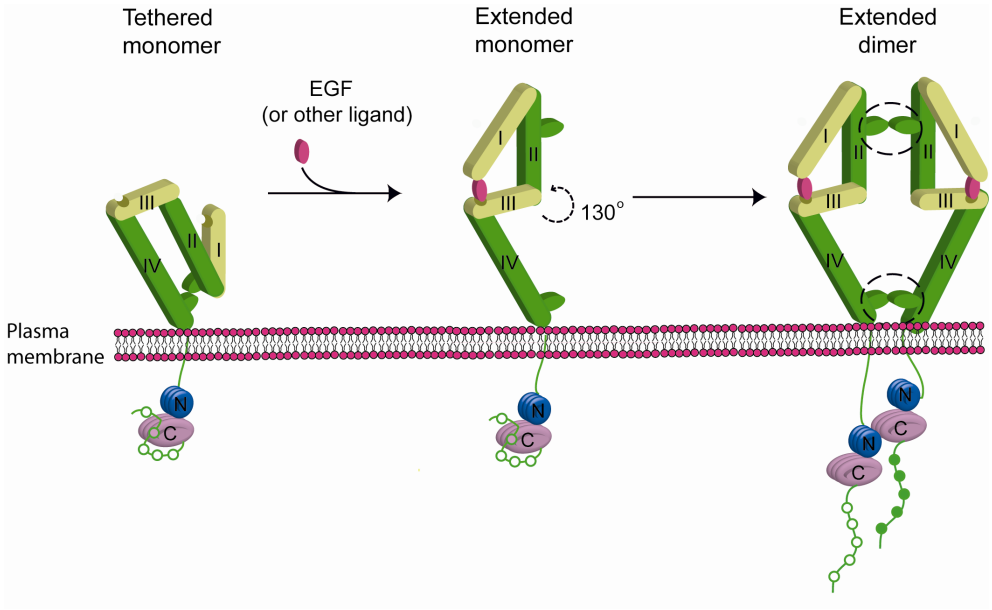

Figure 4: EGFR dimerization and activation. The inactive, tethered EGFR monomer extends when the ligand binds either to domains I or III, causing breakage of the link between the dimerization arm and rotation of the angle between domains II and III to bring ligand-binding domains I and III into close proximity allowing a simultaneous ligand binding. The extended conformation exposes the dimerization arm for interaction with another extended monomer. The dimerization arm contacts are ringed with a dashed line. Dimerization allows autophosphorylation of the intracellular kinase domains, leading to subsequent phosphorylation of tyrosine residues in the C-terminal tail. Phosphorylated tyrosine residues are indicated with closed circles. The figure is modified from Lemmon, 2009.

A plethora of downstream substrates are recruited to the phosphorylated C-terminal tail of EGFR. Schulze and co-workers have identified the phosphotyrosine interactome for the four cytosolic tails in the EGFR family by using quantitative proteomics combined with pull-down experiments (Schulze et al., 2005). Adaptor proteins, named growth factor receptor-bound 2 (Grb2) and Src-homology domain 2 containing (Shc) proteins, are capable of interacting with all EGFR family members, and subsequently recruit Ras, leading to the activation of MAPK cascades. HER4 shows a striking similarity with the EGFR for adaptor binding and they both recruit STAT5 (signal transducer and activator of transcription 5) that can locate to the nucleus and function as a transcription factor, and Crk (CT10 regulator of a tyrosine kinase; where CT10 refers to chicken tumor virus 10 (Birge et al., 2009)), which can further serve as a docking site for several other signaling complexes. HER3 was revealed to be the major docking partner for phosphatidylinositol 3-kinase (PI3K), having six binding sites for it, instead of just one binding site in the CYT-1 isoform of HER4 and a total lack of sites in EGFR or HER2 (Schulze et al., 2005). PI3K is able to further activate Akt, also known

as protein kinase B (PKB). Intriguingly, the cytoplasmic terminus of EGFR is the only family member having a recognition site for the ubiquitin ligase Cbl (Schulze et al., 2005). Dimer formation with different partners modifies which downstream signaling pathways are activated.

Many studies have also shown that phospholipase C-γ (PLC-γ) is rapidly activated during EGF stimulation (Blagoev et al., 2004; Chattopadhyay et al., 1999), but the direct interaction between EGFR and PLC-γ remains controversial (Chattopadhyay et al., 1999; Schulze et al., 2005). PLC-γ catalyzes the cleavage of phosphatidylinositol (4,5)-bisphosphate (PIP_2) into the second messengers diacyl glycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3). DAG is able to activate protein kinase C (PKC), which enhances the downstream signaling of multiple complexes, and IP_3 mediates calcium release mainly from the endoplasmic reticulum, causing an increase in the cytosolic calcium concentration.

Interestingly, all the EGFR family members can be found in the nucleus as full-length forms (Wang and Hung, 2009). In addition, HER4 is the only EGFR family member where ligand-binding induced proteolytic processing generates an intracellular domain (ICD) that can directly translocate to the nucleus (Zhou and Carpenter, 2000). Several studies argue that the EGF–EGFR complex can translocate to the nucleus and bind to the cyclin D1 promoter, thus functioning as a transcription factor (Lin et al., 2001). EGFR can also associate with other transcription factors, such as STAT3 and STAT5, to promote their binding to the DNA (Hung et al., 2008; Lo et al., 2005). EGF-dependent co-operation of the EGFR and transcription factor E2F1 is also documented (Hanada et al., 2006). The full-length EGFR in the nucleus retains its kinase activity and is shown to phosphorylate the proliferating cell nuclear antigen (PCNA), which is essential for DNA replication and damage repair (Wang et al., 2006). Increased PCNA tyrosine phosphorylation correlated with poor breast cancer patient outcome. Overall, nuclear localization of EGFR is associated with a significant increase in local recurrence and a decrease in disease-free survival in several cancer types (Wang and Hung, 2009). The proposed mechanisms for EGFR family nuclear localization are endosome-mediated translocation, where the NLS sequence of the juxtamembrane region in the EGFR family is recognized (Hsu and Hung, 2007), or retro-translocation of endosomes to the endoplasmic reticulum, where EGFR is then released to the cytosol by the Sec61 translocon (Liao and Carpenter, 2007). Nuclear receptor localization seems to be a remarkable player in genotoxic stresses induced by radiotherapy or chemotherapy, causing resistance to targeted therapies. The clinical ramification of this phenomenon has been proved recently, when the HER1 and HER2 dual tyrosine kinase inhibitor, lapatinib, was shown to inhibit the nuclear translocation of these receptors, thus sensitizing cancer cells to fluoropyrimidine-based chemotherapy (Kim et al., 2009).

3.3. Terminating the signaling

Studies in cultured fibroblasts with isotopically labeled methionine followed by anti-EGFR immunoprecipitation revealed a half-life of about 10 hours for EGFR degradation (Stoscheck and Carpenter, 1984b). When EGF was added, enhanced degradation was seen within 5 minutes and the half-life of the prelabeled receptor was decreased to 1.2 hours. This was the first direct evidence of ligand-induced EGFR downregulation to control the duration of signaling. However, in EGFR overexpressing cells the half-life of receptor degradation seems to be at least twofold; $T_{1/2} = 20$ hours (Stoscheck and Carpenter, 1984a). In an elegant study, where EGF-stimulated cells were cultured in the presence of isotopically labeled arginine and subjected to immunoprecipitation with anti-tyrosine antibodies followed by mass spectrometry analysis, Blagoev and co-workers later demonstrated a potent EGFR phosphorylation occurring within a minute after adding the ligand (Blagoev et al., 2004). Phosphorylation of the ubiquitin ligase Cbl was temporally similar to EGFR (Blagoev et al., 2004).

Clathrin-coated pit-mediated endocytosis is the major pathway for EGFR internalization and internalization is inhibited when clathrin heavy chains or dynamin is depleted using small interfering RNAs (siRNAs) (Huang et al., 2004). On the contrary, high ligand concentration or receptor overexpression may also lead to clathrin-independent endocytosis, most probably due to saturation of the internalization process, because clathrin knockdown in the presence of high EGF impaired EGFR endocytosis only by 50% (Sigismund et al., 2005). These results argue for the existence of two alternative pathways for endocytosis, the exclusive clathrin-dependent at the low EGF concentrations, and at the high EGF, both clathrin-dependent and clathrin-independent pathways. Of note, non-clathrin mediated endocytosis is significantly slower and it is unclear whether high ligand concentrations are present *in vivo*. Grb2 binding to EGFR followed by subsequent recruitment of ubiquitin ligase Cbl to the receptor are necessary for the clathrin-mediated endocytosis (Jiang et al., 2003). Interestingly, in spite of the fact that Cbl functions as ubiquitin ligase, ubiquitination of the EGFR is not required for the clathrin-mediated endocytosis (Huang et al., 2007). The non-clathrin pathway depends on the cholesterol-rich lipid rafts/caveolae and instead requires ubiquitination, where ubiquitin-interacting motif (UIM) containing proteins, such as epsin, can bind (Sigismund et al., 2005). When internalized, clathrin-coated vesicles rapidly shed their coat and fuse with early endosomes. Due to a mildly acidic pH in early endosomes, EGF–EGFR complexes stay both dimerized and phosphorylated (Sorkin and Carpenter, 1991), and intact receptor complexes are either rapidly recycled back to the cell surface (Sorkin et al., 1991) or remain in these endosomes during their maturation into multivesicular bodies (MVBs), also known as late endosomes. At least some of the EGF–EGFR dimers also remain unmodified in late endosomes, where they can recycle to the cell surface via a slow recycling pathway (Sorkin et al., 1991). Late endosomes fuse with primary lysosomes, finally causing the degradation of EGF–EGFR complexes. Consequently, under normal conditions, EGFR is predominantly localized at the plasma membrane resulting in only

a small endosomal pool. Of note, the TGF-α–EGFR complex already dissociates in the early endosomes due to lower receptor–ligand affinity, which leads to receptor dephosphorylation and fast recycling back to the cell surface (French et al., 1995). Thus, TGF-α stimulus does not cause prominent degradation and downregulation of EGFR.

HER2, HER3, and HER4 are internalized and targeted to lysosomes less efficiently than EGFR, thus EGFR heterodimerization with these other family members may impair EGFR endocytic trafficking as well (Baulida et al., 1996). Upon internalization, EGFR/HER2 dimers are mostly recycled back to the plasma membrane evading lysosomal targeting, thus enhancing EGFR signaling (Lenferink et al., 1998). HER2/HER3 dimers are very potent signaling complexes due to their defective endocytosis and enhanced recycling (Citri et al., 2003). Prolonged HER2/HER3 heterodimer activation is further increased via HER2's ability to enhance dimerization partner affinity for its ligands (Citri et al., 2003). HER4 is internalized very slowly, and instead of rapid endocytosis, HER4 undergoes a two-step proteolytic process in response to ligand binding. First, the tumor necrosis factor-alpha converting enzyme (TACE), also known as ADAM17, proteolytically sheds the ectodomain resulting in a membrane-anchored cytoplasmic domain (Rio et al., 2000), which is subsequently processed by a second membrane-localized protease, γ-secretase (Ni et al., 2001). This process liberates the intracellular HER4 fragment for translocation to the cytoplasm and nucleus for signaling purposes.

In addition to endocytosis, dephosphorylation of tyrosine residues by different protein tyrosine phosphatases (PTPs) will antagonize RTK signaling (Tiganis, 2002). Dephosphorylation can take place when the catalytic kinase domain of EGFR is cytoplasmically exposed, such as at the plasma membrane or in endosomes. Finally, EGFR sequestration to intraluminal vesicles from the perimeter membrane of a multivesicular body removes the kinase domain from the cytoplasm and terminates signaling (Eden et al., 2009).

3.4. EGFR crosstalk with integrins

Overlapping MAPK and PI3K/Akt downstream signaling pathways from the integrin receptor and EGFR suggest that these two signaling cascades co-operate. Sustained synergistic extracellular signal-regulated kinase (ERK) activity has been documented when both growth factor stimulation and integrin-mediated adhesion are present, but adding growth factors on fibroblasts in suspension resulted only in transient ERK activation (Roovers et al., 1999). In addition to this, cell adhesion to immobilized matrix proteins alone was sufficient to induce phosphorylation of EGFR and subsequent activation of ERK signaling in the absence of growth factor receptor ligands (Moro et al., 1998). Moro and co-workers further documented that adhesion-induced EGFR phosphorylation represented only a partial activation and phosphorylation could be amplified 5-fold by adding EGF (Moro et al., 1998).

Interestingly, adhesion-induced phosphorylation on EGFR residues involved different residues than the classical EGF-induced phosphorylation (Boeri Erba et al., 2005; Moro et al., 2002). An increasing number of studies argue that several growth factors are able to bind to integrins directly, such as a latent form of TGF-β to integrin αVβ6 (Munger et al., 1999), vascular endothelial growth factor (VEGF) to integrin α9β1 (Vlahakis et al., 2007), and NRG-1 to integrins αVβ3 and α6β4 (Ieguchi et al., 2010). However, no such direct binding between integrins and EGF has yet been documented.

EGFR has been shown to form a complex with β1- or β3-integrins when cells are plated on dishes precoated with matrix proteins or integrin antibodies (Moro et al., 2002). The formation of this complex was inhibited in the presence of Src inhibitor or p130Cas knockdown (Moro et al., 2002). Also, HER2 has been demonstrated to co-operate with α6β4-integrin in mammary carcinogenesis by promoting the formation of a multimeric complex including HER2, Src, and α6β4, which results in a subsequent activation of transcription factors c-Jun and STAT3 (Guo et al., 2006). Targeted deletion of the integrin β4 cytoplasmic domain delayed tumor formation significantly in the HER2-dependent mouse mammary model, thereby enhancing the potency of HER2-targeted therapy (Guo et al., 2006). The formation of the integrin–EGFR family complex can activate growth factor receptors either in a collaborative or direct way (Yamada and Even-Ram, 2002). In both cases, integrin clustering markedly recruits other membrane proteins as well, such as EGFR. In the collaborative mechanism, integrin and EGFR both bind to their individual ligands, enhancing signaling compared to signaling at one receptor type at a time (Yamada and Even-Ram, 2002). The direct mechanism occurs without ligand binding to the growth factor receptor via recruitment of other kinases, such as Src, to adherent clustered integrins (Yamada and Even-Ram, 2002). ECM is thought to be more stable by nature when compared to soluble growth factors, and thus integrins are thought to function as proprioceptors, mediating mechanical signals from the surroundings to inform the cell when growth factor signals are favorable (Schwartz and Ginsberg, 2002). Indeed, proper, strong, and sustained growth factor receptor signaling is enabled only in adherent cells (Schwartz and Ginsberg, 2002).

EGFR has been shown to remain phosphorylated when internalized, thereby continuing signaling from the endosomes (Di Guglielmo et al., 1994). Recently, α5β1-integrin and EGFR have been shown to form a proinvasive complex with Rab-coupling protein (RCP) in the recycling endosomes (Caswell et al., 2008). The Rab family consists of small GTPase proteins that regulate intracellular vesicle targeting. Blocking the αVβ3 adhesion with the cyclic peptide cilengitide increased recycling of the RCP protein complex back to the plasma membrane, potentiating both EGFR phosphorylation and subsequent downstream signaling to Akt, thereby promoting cell migration in 3D (Caswell et al., 2008).

3.5. EGFR in health and disease

EGFR family members have a significant role in cell differentiation, proliferation, survival, and migration. Thus, aberrations in receptor expression or signaling can lead to severe consequences.

3.5.1. EGFR in development

A lack of one EGFR family member has already been shown to lead to the embryonic or early postnatal death due to severe defects in implantation, central nervous system, or cardiac function. The timing of lethality in EGFR-null mice depends on the genetic background of the mouse strain (Sibilia and Wagner, 1995). EGFR knockout mice have defects in various epithelia such as skin, hair follicle, eye, lung, liver, kidney, and intestine (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995) and they develop a progressive postnatal neurodegeneration (Sibilia et al., 1998). Newborn EGFR-null mice had the phenotype of retarded growth, open eyelids and rudimentary waved whiskers (Sibilia and Wagner, 1995; Threadgill et al., 1995). Mice expressing tyrosine kinase defective EGFR, known as the *waved-2* phenotype, also had wavy hair with irregular hair angles and open eyelids (Luetteke et al., 1994). Sibilia and co-workers have shown with knock-in approaches that human EGFR in mice is partially able to rescue lack of murine EGFR (Sibilia et al., 2003). These humanized EGFR mice were viable, but growth retarded, and expressed similar defects in skin and hair follicles compared to EGFR-null mice. However, the neurodegeneration was totally rescued in these humanized mice.

3.5.2. EGFR in cancer

Mechanistically, EGFR function can be dysregulated via receptor mutation, receptor deletion, receptor overexpression, autocrine ligand stimulation, alterations in the dimerization process, limited or enhanced endocytosis of activated receptors, limited receptor turnover, and deficient phosphatase activity (Sebastian et al., 2006). The most common EGFR deletion, truncated EGFR variant III (EGFRvIII), lacks the major part of the ligand-binding ectodomain, resulting in constitutive, ligand-independent activation of the EGFR receptor kinase domain (Sebastian et al., 2006).

EGFR is overexpressed and activated in several cancer subtypes such as colon, breast, lung, ovarian and cervical carcinomas, squamous cell carcinoma of head and neck, and gliomas due to mutations, deletions, or amplifications of the receptor (Bauknecht et al., 1989; Libermann et al., 1985; Sainsbury et al., 1987; Salomon et al., 1995; Tong et al., 1998; Veale et al., 1987). HER2 is overexpressed in 25-30% of breast cancers (Slamon et al., 1987), where it correlates with an aggressive tumor phenotype including tumor size, lymph node involvement, aneuploidy, a high percentage of dividing cells, and lack of an estrogen receptor (Moasser, 2007). High HER2 levels are also linked to subsets of gastric, esophageal, and endometrial cancers (Mimura et al., 2005; Morrison et al., 2006; Yano et al., 2006). Currently, the pivotal role of HER3 seems to be its function as a co-receptor for amplified HER2. In fact, HER3 has been documented to

be the rate-limiting step in HER2-driven tumor growth, because HER3 knockdown was sufficient to induce a rapid tumor regression in HER2 overexpressing breast cancer cells *in vivo* (Lee-Hoeflich et al., 2008). Increased HER3 expression levels in melanoma correlated with enhanced cell proliferation, migration, and invasion, thus causing tumor progression and reduced patient survival (Reschke et al., 2008). Recently, a conditional knockout mouse model of HER3 revealed upregulation of caspase-3 activity and prevention of tumorigenesis in the mouse intestinal epithelium (Lee et al., 2009). Interestingly, HER3 knockout led to concomitant loss of HER4 in the epithelium as well. Lee and co-workers further reproduced these results in human colon cancer cells resistant to the EGFR therapy due to KRAS mutation, where siRNA knockdown of either HER3 or HER4 caused apoptosis (Lee et al., 2009). Knockdown of HER3 in human colon cancer cells resulted in a marked attenuation of HER4 levels similar to results obtained from mice, thereby suggesting that HER3/HER4 dimerization has a clinical significance in colon cancer (Lee et al., 2009). HER3 is also unleashed in lung and ovarian carcinomas (Tanner et al., 2006; Yi et al., 1997). Of note, increased HER4 expression is linked with a more favorable outcome in breast cancer (Abd El-Rehim et al., 2004; Aqeilan et al., 2007; Koutras et al., 2008), but mutational activation of HER4 is seen in melanomas and NSCLCs (Ding et al., 2008; Prickett et al., 2009). At least in a subset of breast cancers seems the subcellular localization of HER4 expression to be critical, because membranous location is associated with favorable survival when compared to nuclear HER4 (Junttila et al., 2005). Increased HER4 expression is also seen in colorectal cancer, where it is linked with a higher risk of recurrence (Baiocchi et al., 2009). HER4-positive squamous cell carcinomas of the head and neck show enhanced survival when compared to the HER4-negative subset, but nuclear localization in the HER4-positive subgroup resulted in a significant reduction in outcome (Xu et al., 2008).

Taken together, overexpression of EGFR family members is linked to cancer progression and poor prognosis in several human malignancies. In addition, several distinct dimers of activated monomers can be formed due to homo- and heterodimerization processes, making EGFR family a challenge for targeted therapies.

3.6. EGFR family for targeted cancer therapy

The two classes of anti-EGFR agents have been developed and they are directed either against the extracellular domain of the receptor (monoclonal antibodies, MAbs) or the intracellular kinase domain (tyrosine kinase inhibitors, TKIs). MAbs prevent ligand binding to the ectodomain and subsequent ligand–receptor internalization is replaced by MAb–receptor endocytosis. TKIs, instead, are small molecules competing with ATP (adenosine triphosphate) for binding, thereby preventing kinase activity and intracellular signaling.

3.6.1. Monoclonal antibodies against EGFR or HER2

The chimeric monoclonal antibody cetuximab (Erbitux®), targeting the EGFR ectodomain and blocking ligand binding, has shown efficacy in advanced colon cancer, squamous cell carcinoma of the head and neck, and NSCLC patients (Baselga and Arteaga, 2005). Cetuximab binding to domain III overlaps with the EGF binding site and also sterically prevents the ectodomain adopting the extended conformation, thereby inhibiting dimerization (Li et al., 2005). A fully human EGFR antibody, panitumumab (Vectibix®), is used for the treatment of metastatic colorectal cancer with disease progression (Wu et al., 2008). Panitumumab also blocks ligand binding and further receptor activation (Yang et al., 2001). Of note, panitumumab is an antibody of the IgG2 subtype, thereby lacking the enhanced cellular cytotoxicity mechanism of several other antibodies mediated by host immune cells (Wu et al., 2008). Nimotuzumab is a humanized affinity-optimized EGFR antibody preventing ligand binding to the receptor as well. Nimotuzumab has a lower affinity constant when compared to cetuximab or panitumimab and its documented mechanism of action seems to require bivalent binding, where both antibody arms simultaneously bind to separate antigens (Ramakrishnan et al., 2009). This limits nimotuzumab binding only to cells expressing moderate to high levels of EGFR, thereby having less severe adverse effects in the clinic as well (Ramakrishnan et al., 2009). Nimotuzumab is currently under phase III clinical trials for pancreatic and cervical cancer. It has already been granted approval for use in squamous cell carcinoma of the head and neck or glioma in several countries, but it is not approved in the United States or Canada (www.ymbiosciences.com). A fully human EGFR antibody, zalutumumab (HuMax®-EGFr), was approved for fast-track status by the U.S. Food and Drug Administration (FDA) in 2006 for monotherapy in squamous cell carcinoma of the head and neck refractory to the conventional therapies. Zalutumumab binding locks EGFR in an inactive conformation, thus blocking ligand binding and further receptor dimerization (Rivera et al., 2009). Currently, several clinical trials of zalutumumab in combination with conventional chemotherapy and radiotherapy are ongoing.

A humanized HER2 monoclonal antibody, trastuzumab (Herceptin®), is in clinical use for HER2-positive breast cancer patients in combination with traditional chemotherapy and is associated both with improved disease-free and overall survival (Chang, 2010). Trastuzumab does not block dimerization or activation of HER2 (Agus et al., 2002). Several conflicting results have been obtained from the intensive studies done with trastuzumab, but the prevailing current ideas to describe the mechanism of action are the disruption of ligand-independent constitutive HER2/HER3 complexes (Junttila et al., 2009), the inhibition of HER2 ectodomain shedding (Molina et al., 2001), and the immunological targeting of HER2 (Carter et al., 1992). Currently, the efficacy of neoadjuvant trastuzumab therapy in combination with chemotherapy has been estimated in several ongoing clinical trials and the results of such trials are encouraging. Combined neoadjuvant trastuzumab therapy increased pathologic complete response rates up to 65% compared to 26% in the chemotherapy-alone group (Chang, 2010). Pathologic complete response is based on histopathological analysis of

residual tumor from breast or lymph node samples and correlates strongly with the outcome. Another humanized HER2 antibody, pertuzumab (Omnitarg®), binds to the HER2 dimerization arm, blocking the dimerization and subsequent activation of the receptor (Agus et al., 2002). The combination therapy of pertuzumab with traditional chemotherapy and trastuzumab for patients having HER2-positive metastatic breast cancer is currently under phase III clinical trials (www.clinicaltrials.gov). Ertumaxomab is a trifunctional bispecific antibody targeting both HER2 on tumor cells and CD3 on T-cells and stimulating the immune response by recruiting cytotoxic T-cells via the Fc region (Kiewe et al., 2006). Phase I clinical trials in breast cancer patients showed antitumor efficacy, but also a strong immunological response (Kiewe et al., 2006). However, ertumaxomab is no longer under clinical development. A different approach is to combine antibody to drug, as it has been done with trastuzumab-DM1 (T-DM1). Targeted delivery of chemotherapy into HER2-positive cells has indicated substantial clinical activity (Krop et al., 2010) and T-DM1 has progressed to phase III clinical trials for metastatic HER2-positive breast cancer.

Monoclonal therapies against EGFR and HER2 have become a part of standard treatment of many cancer types, but only a subset of patients seems to benefit from therapy. However, costs of therapy are significant. This has led to intensive attempts to identify predictive markers of therapy response and to evolve optimized combination therapies for patients to improve outcomes.

3.6.2. Tyrosine kinase inhibitors against EGFR

The first-generation EGFR TKIs are synthetic anilinoquinazolines that reversibly inhibit ATP binding to the tyrosine kinase domain of the EGFR. The second-generation irreversible TKIs are also ATP-mimetics, but they bind covalently to the cysteine residue in the ATP-binding pocket of the kinase domain. Among the second-generation agents are also dual, irreversible TKIs targeted to both EGFR and HER2.

Increased response to reversible EGFR TKIs, gefitinib (Iressa®) or erlotinib (Tarceva®), is associated with activating EGFR mutations in NSCLC patients. The predominant activating mutations are an in-frame deletion mutation in exon 19 (delE746_A750) and a single-point substitution mutation L858R in exon 21 (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). These mutations occur only in 10-15% of Caucasians, but in 30-40% of Asian NSCLC patients (Engelman and Jänne, 2008). However, even if there is a strong initial response to gefitinib or erlotinib, it usually lasts only for 6-12 months, leading to a progressive disease (Engelman and Jänne, 2008). The above-mentioned phenomenon, where some cancers show an apparent dependency on one or a few genes for the maintenance of the malignant phenotype, is called oncogene addiction (Weinstein and Joe, 2008). Described mechanisms for the acquired EGFR TKI resistance are a secondary point mutation T790M in the EGFR kinase domain (Kobayashi et al., 2005), amplification of the *MET* oncogene (Engelman et al., 2007), or overexpression of the MET receptor ligand HGF (hepatocyte growth factor) (Yano et al., 2008). The tyrosine kinase receptor MET causes acquired

resistance by activating PI3K signaling via HER3 in an EGFR-independent manner (Engelman et al., 2007). Interestingly, PI3K activation driven by MET can also be HER3-independent (Yano et al., 2008). HER3 signaling is also important for gefitinib resistance in HER2 overexpressing breast cancer cells *in vitro* (Sergina et al., 2007). HER3 is the principal EGFR family member involved in recruiting PI3K/Akt and thus evading EGFR TKI therapy. Recently, compensatory signaling mechanisms via insulin-like growth factor-1 receptor (IGF-1R) to PI3K has been documented (Buck et al., 2008). In this study, Buch and co-workers demonstrated that individual inhibition of either EGFR or IGF-1R resulted in activation of the residual receptor. They further proved that even the inhibition of MAPK downstream of EGFR was sufficient to enhance IGF-1R-directed PI3K/Akt signaling (Buck et al., 2008). It has now been shown by several studies that a lack of inhibition in downstream PI3K/Akt pathway signaling results in EGFR TKI resistance (She et al., 2003). Interestingly, Zhou and colleagues have demonstrated, by using NSCLC cells, that gefitinib insensitivity can also result from a neuregulin-1 autocrine loop and a selective dual ADAM inhibitor, INCB3619, which blocks the function of both ADAM10 and ADAM17 as sheddases, enhancing gefitinib inhibition (Zhou et al., 2006). Without INCB3619 inhibition, HER3 heterodimers maintained their signaling and NSCLC cells evaded the proper response to EGFR TKI in the presence of HER2.

Lapatinib (Tykerb®) is a dual-specific kinase inhibitor against EGFR and HER2. It is in clinical use for advanced HER2-positive breast cancer in combination with capecitabide, if earlier treatment with an anthracycline, a taxane, or trastuzumab has failed (Geyer et al., 2006). Recently, lapanitib combination with trastuzumab was reported to be plausible compared to lapatinib alone in HER2-positive metastatic breast cancer (Blackwell et al., 2010). Preclinical studies have previously documented the synergy where lapatinib alone caused inhibition of HER2 phosphorylation, a decrease in receptor ubiquitination, and the subsequent accumulation of inactive receptors at the cell membrane, but trastuzumab alone instead enhanced phosphorylation, ubiquitination, and degradation of HER2 (Scaltriti et al., 2009). When combined, lapatinib induced HER2 accumulation at the cell surface and thus augmented antibody-dependent cellular cytotoxicity (Scaltriti et al., 2009).

BIBW-2992 is a dual EGFR/HER2 TKI, binding irreversibly to the kinase domains (Li et al., 2008), and has progressed already to phase III clinical trials for NSCLC and HER2-positive metastatic breast cancer patients (www.clinicaltrials.gov). HKI-272 (neratinib) is an irreversible pan-TKI, which is being used in several ongoing clinical trials. Recently, neratinib showed substantial clinical efficacy in phase II trials in patients with advanced HER2-positive breast cancer (Burstein et al., 2010) and has now progressed to phase III trials. On the contrary, phase II studies with neratinib in advanced NSCLC patients have been disappointing, potentially because of dose limitation followed by diarrhea (Sequist et al., 2010).

Despite encouraging activity in several studies *in vitro* and *in vivo* to inhibit EGFR and HER2 activity, EGFR TKIs have shown only a very limited clinical anti-tumor

activity. Clinical ramifications have mainly been modest delays in tumor progression and have been seen only in a small proportion of patients. Early studies notoriously overestimated the clinical potential of anti-EGFR therapy by pushing the idea of rapid and sustained inhibition. It is becoming increasingly apparent that combination therapy strategies designed to undermine the persistence in the EGFR family signaling via heterodimerization are needed. Improved strategies for integrating anti-EGFR family agents with conventional therapies and/or targeted therapies with other approaches such as downstream signaling molecule inhibitors are required.

4. T-cell protein tyrosine phosphatase (TCPTP)

4.1. General characteristics of protein tyrosine phosphatases

Phosphorylation is one of the most frequent phenomena for posttranslational protein modifications in eukaryotic cells. In this reversible process, a phosphate group ($-PO_4^{2-}$) originating from ATP is added to a specific serine, threonine, or tyrosine residue of the protein. This modifies the function of the protein in question either positively or negatively. Signaling cascades of sequential protein phosphorylation events are fundamental for signal transduction in cells. Phosphorylation of a single residue can have numerous consequences, such as kinase domain activation, modification of domain conformation for subsequent protein–protein interactions, or labeling the protein for degradation in the ubiquitin pathway. Tyrosine kinases are responsible for the phosphate group transfer to tyrosine residues and serine/threonine kinases can phosphorylate both serine and threonine residues. About one-third of human proteins are estimated to be exposed to phosphorylation (Cohen, 2001). The *in vivo* phosphoproteome analysis in EGF-stimulated HeLa cells revealed a total of 2244 phosphoproteins with 6600 different phosphorylation sites, where the relative abundances for phosphorylated tyrosine, threonine, and serine residues were 2%, 12%, and 86%, respectively (Olsen et al., 2006). However, nature shows a tendency to maintain balance. Thus, it is the phosphatases that function as antagonists for kinases by removing the added phosphate groups. The human genome contains 90 genes encoding protein tyrosine kinases (PTKs) and 107 genes for protein tyrosine phosphatases (PTPs), thus revealing the astonishing similarity in their number (Alonso et al., 2004). Of the 107 PTP genes, 81 are catalytically active and the remainder are known to be inactive or to dephosphorylate mRNA and inositol phospholipids (Alonso et al., 2004). Of the 90 PTK genes, 85 are considered to be catalytically active (Alonso et al., 2004).

The PTPs can be categorized as classical protein tyrosine phosphatases and dual-specificity phosphatases (DSPs) that both have the active site signature motif HCX_5R [histidine-cysteine-X_5-arginine; where X indicates any amino acid] (Tonks, 2006). The cysteine residue of this motif functions as a nucleophile and is important in the catalysis to form the phosphate-binding loop, which is further stabilized by the arginine

residue (Tiganis and Bennett, 2007; Tonks, 2006). Another important motif for PTP catalysis is the conserved flexible WPD [tryptophan-proline-aspartic acid] loop, where the invariant aspartic acid residue acts as a general acid/base catalyst during the dephosphorylation (Tiganis and Bennett, 2007). Classical PTPs are phosphotyrosine-specific phosphatases, but DSPs can dephosphorylate serine and threonine residues and phosphatidyl inositol phospholipids, in addition to tyrosine residues (Andersen et al., 2004).

Thus far, 38 members have been identified as belonging to the classical PTPs in the human genome (Andersen et al., 2004) and these can be further subdivided into 21 transmembrane receptor-like and 17 non-transmembrane PTPs (Tonks, 2006). Of these, receptor-like PTPs have variable extracellular domains, a single transmembrane domain, and two intracellular PTP domains. The membrane-proximal PTP domain is, in most cases, catalytically active and followed by the more distally located pseudo PTP domain (Andersen et al., 2001). However, the pseudo PTP domain plays a crucial role in the dimerization of the receptor-like PTPs and in controlling protein–protein interactions or binding specificity (Blanchetot et al., 2002). Extracellular domains of the receptor-like PTPs often contain immunoglobulin-like domains and fibronectin type III-like domains, which mediate cell–cell and cell–matrix contacts (Tonks, 2006). Non-transmembrane PTPs are localized intracellularly and they can primarily be found in the cytoplasm, endoplasmic reticulum, nucleus, or in close proximity to the plasma membrane (den Hertog et al., 2008). In addition to the catalytic PTP domain responsible for phosphatase activity, non-transmembrane PTPs' structure possesses variable domains taking care of protein–protein interactions, thereby regulating catalytic processes, targeting to substrates, and controlling subcellular localization (Alonso et al., 2004). Examples of these non-catalytic domains include: FERM (the protein 4.1, ezrin, radixin, moesin), PDZ [PSD-95 (postsynaptic density-95), Dlg (discs large), ZO-1 (zonula occludens-1)], SH2, and proline-rich sequences (Alonso et al., 2004). The group of dual-specificity phosphatases is very heterogenous and encoded by 65 distinct genes, making them structurally diverse (Tonks, 2006).

4.2. Cellular function of TCPTP

Ubiquitously expressed T-cell protein tyrosine phosphatase (TCPTP), which is also known as PTPN2 (PTP non-receptor type 2), belongs to a group of non-transmembrane PTPs. The structure of TCPTP is very simple, having just an N-terminal phosphatase catalytic domain followed by the C-terminal non-catalytic sequence that lacks the above-mentioned FERM, PDZ, SH2, and proline-rich domains (Tonks, 2006). The TCPTP transcript is alternatively spliced, generating two protein isoforms named TC45 and TC48 after their molecular masses of 45 kDa and 48 kDa, respectively (Cool et al., 1989; Mosinger et al., 1992). Of these isoforms, TC48 is localized predominantly to the endoplasmic reticulum, but TC45, which lacks the hydrophobic C-terminus, is targeted to the nucleus by a nuclear localization sequence (Lorenzen et al., 1995). TC45 is the major transcript in humans (Kamatkar et al., 1996). Interestingly, it has

been demonstrated that an EGF stimulus is able to mediate TC45 isoform exit from the nucleus and accumulation in the cytoplasm, where EGFR and Shc were identified to be substrates for TC45 (Tiganis et al., 1998). Tiganis and co-authors documented the change in the TC45 localization within only 5 minutes after the addition of growth factor and the maximum action was reached within 15 to 30 minutes (Tiganis et al., 1998). However, the effect was transient, leading to nuclear return within the next 6 hours (Tiganis et al., 1998). A similar change in the nucleocytoplasmic distribution of TC45 is also reported in the presence of specific cellular stresses, such as hyperosmotic stress (Lam et al., 2001).

Cleavage of the C-terminal hydrophobic sequence increased phosphatase activity of the TC48 isoform by 30-fold *in vitro* (Zander et al., 1993). Similar results were also obtained by Hao and colleagues by using limited tryptic proteolysis of the TC45 isoform to generate a 33 kDa fragment containing the catalytic domain (Hao et al., 1997). This 33 kDa isoform displayed 20- to 100-fold augmentation in phosphatase activity compared to full-length TCPTP (Hao et al., 1997). They further demonstrated that an increase in the phosphatase activity of the 33 kDa isoform could be reversed by addition of the non-catalytic C-terminal fragment of TC45 (Hao et al., 1997). In agreement with this, a monoclonal antibody against the C-terminal epitope of TCPTP was able to fully activate TC45 (Hao et al., 1997). These studies argue for the existence of intramolecular interaction between the catalytic and non-catalytic domains of TCPTP.

Catalytic domains of TCPTP and non-transmembrane PTP1B (protein tyrosine phosphatase 1B) display a 72% sequence identity (Tiganis and Bennett, 2007), and they share a preference for the tandem tyrosine phosphorylated substrates, such as the insulin receptor and Janus kinase (JAK) family members (Salmeen et al., 2000). In addition to the insulin receptor, several other tyrosine kinase receptors can function as substrates for TCPTP, such as platelet-derived growth factor β (PDGFRβ) (Persson et al., 2004), colony stimulating factor 1 receptor (CSF1R) (Simoncic et al., 2006), MET (Sangwan et al., 2008), and the above-mentioned EGFR (Tiganis et al., 1998). TC45 is mainly located in the nucleus without any specific stimulus, where it can dephosphorylate several members of the STAT transcription factor family, such as STAT1 (ten Hoeve et al., 2002), STAT3 (Yamamoto et al., 2002), STAT5 (Aoki and Matsuda, 2002), and STAT6 (Lu et al., 2007a). However, TCPTP nuclear exit is crucial for the negative regulation of JAK1 and JAK3 (Simoncic et al., 2002). In addition, SFK is dephosphorylated exclusively by the cytoplasmic TCPTP (van Vliet et al., 2005). The importance of TCPTP nuclear exit for dephosphorylation was demonstrated by Van Vliet and co-workers with a GFP-TCPTP construct whose expression is restricted to the nucleus due to its large molecular size (>60 kDa), which does not allow for its passive diffusion through the nuclear pore complex (van Vliet et al., 2005).

Although TCPTP is expressed ubiquitously, expression can vary at different cell cycle stages. Studies done in synchronized NIH3T3 cells transfected with a murine homolog

of human TCPTP, known as MPTP, revealed variation in MPTP RNA levels during the cell cycle (Tillmann et al., 1994). MPTP levels increased approximately 13-fold from basic levels found at G_0 when the cells progressed to late G_1, but a rapid decrease in levels was seen during the S phase followed by a new increase in G_2 (Tillmann et al., 1994). MPTP and TCPTP share a 93% amino acid identity (Tillmann et al., 1994). TCPTP-deficient mouse embryonic fibroblasts (MEFs) showed enhanced progression into the S phase due to elevated SFK-mediated PI3K signaling (Shields et al., 2008).

4.3. The role of TCPTP in health and disease

In spite of ubiquitous expression, TCPTP is present in higher amounts in cells of hematopoietic origin. Consistent with this, TCPTP-deficient mice displayed hematopoietic abnormalities and died from severe anemia at 3-5 weeks after birth (You-Ten et al., 1997). $TCPTP^{-/-}$ offspring started to exhibit growth retardation at 10-14 days after birth and at 2 weeks of age these mice acquired a hunched posture, piloerection, runting, and diarrhea (You-Ten et al., 1997). A closer analysis of TCPTP-deficient mice revealed severe splenomegaly, enlarged lymph nodes, and augmented total cellularity both in spleen and lymph nodes, but a significant reduction in the total cellularity of bone marrow (You-Ten et al., 1997). TCPTP-deficient mice showed impaired T- and B-cell functions and erythropoiesis. Interestingly, bone marrow transplantation from wild type mice to $TCPTP^{-/-}$ mice did not rescue these animals, suggesting a defective microenvironment in the bone marrow (You-Ten et al., 1997). However, bone marrow transplantation from $TCPTP^{-/-}$ mice was sufficient to rescue irradiated wild type recipients (You-Ten et al., 1997).

Based on our current knowledge, TCPTP expression is not linked to any diseases. However, the balance between phosphorylation and dephosphorylation is very fundamental and thus vulnerable to perturbations. Some PTPs have been linked to diseases like cancer, autoimmunity disorders, and diabetes. Type 2 diabetes is a multifactorial disease in which PTP1B expression and activity have been documented to be elevated (Dadke et al., 2000). Dadke and co-workers used skeletal muscles isolated from insulin-resistant type 2 diabetic rats to observe a significant reduction in insulin-stimulated insulin receptor autophosphorylation when PTPB1 levels were increased (Dadke et al., 2000). PTPB1-deficient mice showed enhanced insulin sensitivity and resistance to diet-induced obesity, whereas $PTPB1^{+/+}$ mice gained weight rapidly and were insulin resistant (Elchebly et al., 1999). Moreover, three independent screening studies of the human genome have associated mutations in the PTP1B locus with type 2 diabetes (Di Paola et al., 2002; Echwald et al., 2002; Mok et al., 2002). In addition, missense mutations of *SHP2* (SH2-domain containing protein tyrosine phosphatase 2), which belongs to the non-transmembrane PTPs, have been linked to an autosomal dominant Noonan syndrome characterized by multiple developmental abnormalities including dysmorphic facial features, short stature, cardiac defects, skeletal malformations, and mental retardation (Tartaglia et al., 2001). These mutations are the gain-of-function type, causing the release of an autoinhibitory

interface between the catalytic domain and N-terminus, thereby inducing excessive SHP2 activity (Tartaglia et al., 2001). However, PTPs' association with cancer is complicated and may vary depending on the cellular context and type of tumor. Receptor-like PTPα, which is capable of activating Src persistently, is overexpressed in late-stage colorectal tumors (Tabiti et al., 1995) and breast carcinomas (Ardini et al., 2000), but surprisingly, PTPα expression correlated with a less aggressive tumor phenotype in breast carcinomas (Ardini et al., 2000).

5. Mammary-derived growth inhibitor (MDGI)

Mammary-derived growth inhibitor (MDGI), also known as fatty acid-binding protein 3 (FABP3), belongs to the family of cytoplasmic FABP proteins which exhibit high affinity binding of long-chain fatty acids and other hydrophobic ligands.

5.1. General characteristics of fatty acid-binding proteins

The first fatty acid-binding protein (FABP) was extracted in 1972 from rat jejunal supernatant (Ockner et al., 1972). Since then, nine separate genes have been identified in mammals, which encode small (≈ 15 kDa) cytoplasmic proteins, FABP1-FABP9, sharing an amino acid sequence identity of 20-70% (Hertzel and Bernlohr, 2000). In spite of their moderate sequence similarity, all FABPs share a common tertiary structure, forming a β-barrel where the ligand-binding pocket is located inside the barrel (Hertzel and Bernlohr, 2000). This ligand-binding cavity is water-filled and thought to be lined on one side with the helix-turn-helix motif that can function as a gatekeeper for ligand entry (Sacchettini et al., 1992). As their name implies, FABPs avidly bind a single saturated or unsaturated long-chain fatty acid (LCFA) in a non-covalent, reversible manner, but with differences in ligand selectivity and binding affinity (Hertzel and Bernlohr, 2000). An LCFA contains a chain of 14 or more carbon atoms in its structure, where the carboxylic acid end is oriented inside the cavity of FABPs. The only exception in the FABP family is FABP1, because it simultaneously binds two fatty acids or hydrophobic molecules instead of a single fatty acid preferred by the other FABP members (Cistola et al., 1989).

In addition to their numerical nomenclature, FABPs have been named after the tissues in which they have originally been discovered or where they are predominantly expressed (Table 2). This can be confusing, since various FABPs are expressed in several tissues. FABP1 is also known as L-FABP due to its predominant expression in the liver, but it can also be found in the intestine, lung, and kidney (Hertzel and Bernlohr, 2000). FABP2 is mainly located in the intestine (I-FABP), FABP3 in the heart and muscle tissue (H-FABP), FABP4 in the adipose tissue (A-FABP), FABP5 in the epidermis (E-FABP), FABP6 in the ileum (IL-FABP), FABP7 in the brain (B-FABP), FABP8 in the myelin of Schwann cells (M-FABP), and FABP9 in testis (T-FABP) (Hertzel and Bernlohr, 2000). In the oversimplified model, FABPs are only involved in the intracellular transport and storage of fatty acids in an aqueous milieu.

However, several FABPs have tissue-specific functions: assimilation of dietary lipids and reuptake of bile acids in the small intestine; biosynthesis of lipoproteins, cholesterol, and bile acids in the liver; regulation of lipid storage in the form of triacylglycerols in the adipose tissue; efficient utilization of fatty acids in the energy metabolism of cardiac and skeletal muscles; and maintenance of sensitive phospholipid membranes in the nervous system are all sophisticated examples of FABP function in different cell types (Storch and Thumser, 2010).

Gene name	Common name	Other names	Expression
FABP1	Liver	L-FABP hFABP	Liver, intestine, kidney, lung, stomach
FABP2	Intestinal	I-FABP	Intestine, stomach, liver
FABP3	Heart/ muscle	H-FABP MDGI cFABP	Heart, mammary gland, skeletal muscle, brain, testes, ovary, lung, stomach, placenta, kidney
FABP4	Adipocyte	A-FABP aP2	Adipose tissue, macrophages
FABP5	Epidermal	E-FABP K-FABP PA-FABP	Skin, lens, adipose tissue, lung, endothelial cells, mammary cells, brain, stomach, tongue, placenta, heart, skeletal muscle, intestine, testis, retina, kidney
FABP6	Ileal	IL-FABP I-BABP ILBP	Ileum, ovary, adrenals, stomach
FABP7	Brain	B-FABP BLBP	Brain, olfactory bulb, glia cells, mammary gland
FABP8	Myelin	M-FABP PMP2 MLBP	Schwann cells, brain
FABP9	Testis	T-FABP TLBP	Testis

Table 2: The fatty acid-binding protein family. Naming and tissue distribution of different FABPs. Data adapted from Haunerland and Spener, 2004; Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002.

FABPs have been speculated to regulate transcriptional activity of nuclear hormone receptors by increasing the availability of ligands in the nucleus. Recently, L-FABP was shown to interact directly with peroxisome proliferator-activated receptor-alpha (PPARα), both in cultured primary hepatocytes and liver homogenates of mice (Hostetler et al., 2009). PPARs regulate transcription of several genes involved in fatty acid and glucose metabolism, as well as cell differentiation, and dysfunction of PPARs is associated, for instance, with diabetes, obesity, and hyperlipidemia (Hostetler et al., 2009). *In vitro* studies revealed a direct interaction of A-FABP and E-FABP with PPARγ and PPARβ, respectively (Tan et al., 2002). In studies done by Tan and co-workers, extensive relocalization of A-FABP and E-FABP was observed in kidney fibroblast cells during stimulation with selective PPAR ligands, which leads to enhanced transcriptional activity of PPAR isotypes in a ligand- and receptor-selective manner (Tan et al., 2002).

In addition to the above-mentioned protein–protein interactions with PPAR, A-FABP has also been demonstrated to interact with membranes. The surface of A-FABP possesses a positive net charge and analysis with Fourier transform infrared (FTIR) spectroscopy showed a preferred interaction with anionic phospholipids (Gericke et al., 1997). The degree of electrostatic interactions correlated directly with the rate of fatty acid transfer, but neutralization of A-FABP-positive surface charges by acetylation markedly diminished interaction (Gericke et al., 1997; Smith and Storch, 1999). In the collisional transfer mechanism of fatty acids, FABP and the membrane are in direct contact with each other. Site-directed mutagenesis of specific lysine residues in the gatekeeper helix-turn-helix motif significantly affected electrostatic interactions between H-FABP and the acceptor membrane (Herr et al., 1996). Collisional interaction of I-FABP with the phospholipid membrane has also been observed, where membranes can function either as an acceptor or a donor of a hydrophobic ligand (Thumser and Storch, 2000). However, L-FABP exchanges fatty acids via a diffusional transfer lacking electrostatic interaction with membranes (Thumser and Storch, 2000).

The expression pattern of many FABPs is related to cellular development and differentiation. B-FABP expression is maximal during early neuronal differentiation and migration of neuronal cells in the developing mouse nervous system, but B-FABP is also specifically found in the adult nervous system to a lesser extent (Kurtz et al., 1994). Perpetually regenerating intestinal epithelium represents a differentiation model, where a multipotent stem cell located at the basal portion of the crypt gives rise to progenies undergoing differentiation to absorptive cells, Paneth cells, Goblet cells, and enteroendocrine cells while migrating to the villus tip. I-FABP expression is spatially regulated along the duodenal–colonic and crypt–villus axes (Sweetser et al., 1988). Expression of I-FABP is highest in the distal jejunum with progressive decreases occurring toward either the proximal colon, where the levels are only 3% of those in the distal jenunum, or the proximal duodenum (Sweetser et al., 1988). Furthermore, I-FABP expression is tightly restricted only to differentiating cells exiting from the small intestinal and colonic crypts, so the crypt cell population remains negative for I-FABP, but enterocytes and Goblet cells maintain expression throughout their life span

(Sweetser et al., 1988). A-FABP is expressed at the late stage of adipocyte differentiation and induction of gene transcription was observed in the presence of fatty acid supplementation (Amri et al., 1991). Supplementation of long-chain fatty acids also overcame glucose deprivation-induced transcriptional inhibition of A-FABP (Amri et al., 1991).

Although the expression of FABP family members seems to be controlled accurately during development, deletion of particular FABP genes has not led to overt changes in phenotype. Instead, many knockout models have shown substantial compensatory upregulation of other FABPs or intracellular lipid-binding proteins (iLBPs). All generated FABP knockout mice develop normally and are fertile. *L-FABP*$^{-/-}$ mice express a drastic reduction in fatty acid-binding capacity in the liver, which causes diminished intracellular triacylglycerol stores (Martin et al., 2003). However, serum levels for triacylglycerols and fatty acids remained unaltered in these mice and only cholesterol accumulation in the liver was observed, most likely due to a highly increased expression of sterol carrier protein-2 (SCP-2) (Martin et al., 2003). *I-FABP*$^{-/-}$ mice showed elevated plasma insulin levels, but normal levels of glucose (Vassileva et al., 2000). Interestingly, *I-FABP*$^{-/-}$ mice also expressed gender-specific changes, because only male animals had enlarged livers (Vassileva et al., 2000). Male mice also weighed more and had elevated levels of plasma triacylglycerols, regardless of their dietary fat content (Vassileva et al., 2000). *A-FABP*$^{-/-}$ and control mice similarly developed diet-induced obesity, but null mice failed to develop insulin resistance and diabetes (Hotamisligil et al., 1996). In line with glucose and insulin tolerance, null mice did not show increased plasma levels for triacylglycerols or insulin, and fasting glucose and cholesterol remained low (Hotamisligil et al., 1996). Thus, ablation of A-FABP uncoupled obesity from insulin resistance, which has led to intensive examination in the obesity field. Interestingly, loss of A-FABP expression in adipocytes caused a marked increase in expression of E-FABP (Hotamisligil et al., 1996). However, this was reversed in *E-FABP*$^{-/-}$ mice, where the loss of E-FABP expression in adipocytes was compensated for by the A-FABP overexpression (Owada et al., 2002). E-FABP-null mice showed increased H-FABP expression in the liver during neonatal development and they demonstrated impaired water-permeability barrier recovery of the skin after damage by acetone treatment (Owada et al., 2002). Despite intensive work with knockout models, fully detailed tissue-specific functions of FABPs have remained confusing. In the future, the creation of double knockout or multiple knockout models may help improve our understanding.

5.2. Cellular function of MDGI

Mammary-derived growth inhibitor (MDGI) was originally purified from lactating bovine mammary glands and was named after its ability to reversibly inhibit proliferation of human mammary carcinoma cells *in vitro* (Böhmer et al., 1987). Later, MDGI was identified as H-FABP (FABP3) (Phelan et al., 1996; Specht et al., 1996). H-FABP and E-FABP are the most ubiquitously expressed FABPs. H-FABP is present

in large amounts in muscle tissues, both cardiac and skeletal, but it is also expressed in the mammary gland, ovary, placenta, kidney, lung, stomach, testis, and brain (Haunerland and Spener, 2004). H-FABP expression is upregulated during mouse cardiomyocyte differentiation (Tang et al., 2004). Additionally, H-FABP mRNA was found to be absent in virgin mammary tissue, but pregnancy significantly induced mRNA expression reaching a maximum at lactation (Kurtz et al., 1990). During midpregnancy, H-FABP expression was strongest in the alveolar cells of developing lobuloalveolar structures, but in terminally differentiated lactating mammary glands, H-FABP was expressed predominantly in ductal epithelial cells (Kurtz et al., 1990). Additionally, there was a clear geographical gradient in MDGI expression; thus, proximal glandular tissue of lactating mammary glands showed increased H-FABP levels compared to distal regions (Kurtz et al., 1990). Culturing of whole organ mammary glands from virgin mice in the presence of recombinant H-FABP resulted in a differentiated phenotype, indicating smaller ducts with numerous side branches compared to control glands (Yang et al., 1994). Furthermore, elevated β-casein levels in H-FABP-treated glands evidenced for functional differentiation (Yang et al., 1994). MCF7 cells transfected with H-FABP proliferated markedly less on plastic, exhibited significant reduction in colony formation in soft agar, and showed impaired *in vivo* tumorigenicity in nude mice relative to control cells (Huynh et al., 1995). In addition, T47D cells transfected with H-FABP showed inhibition of proliferation on plastic (Huynh et al., 1995). Interestingly, an 11-amino acid C-terminal peptide of H-FABP, which was designated as P108, was able to promote fully similar morphological and functional differentiation in the developing mammary gland as full-length recombinant H-FABP (Yang et al., 1994). Transfection of the P108 delivery construct into MCF7, MDA-MB-231, and MDA-MB-468 cells inhibited tumorigenesis in athymic nude mice, but failed to inhibit cell proliferation on plastic (Wang and Kurtz, 2000). These results argue for the ligand or fatty acid-binding independent function of H-FABP. However, H-FABP lacks a signal sequence to direct protein to the extracellular space. Thus, the biological relevance of studies done with the recombinant protein is unclear as is the manner in which secretion could occur in cells without a signal sequence. Hormonal induction of H-FABP expression has been documented in cultured mammary gland explants of virgin mice, but lack of induction was observed in cells cultured as monolayers (Yang et al., 1994).

Immunohistochemical staining of human breast tissue with H-FABP revealed an absence of staining in invasive ductal carcinomas, even though H-FABP was present in normal epithelial cells (Huynh et al., 1995). The human H-FABP gene has been mapped to chromosome 1p32-p35, which has been identified as a common site of loss of heterozygosity in sporadic breast tumors. However, further sequence analysis of the second allele in 30 sporadic breast tumors showed no mutations in the coding region of H-FABP (Phelan et al., 1996). In contrast, hypermethylation of the *H-FABP* gene was detected in human breast cancer cell lines *in vitro* and primary breast tumors *in vivo* (Huynh et al., 1996).

H-FABP knockout mice are fertile and have a normal phenotype (Binas et al., 1999). The only major difference in *H-FABP*$^{-/-}$ mice was in a severely reduced uptake of LCFA in cardiac muscle, which switched cardiac metabolism towards glucose oxidation (Binas et al., 1999). Physiological consequences of this switched metabolism included rapid fatigue during exercise and regional cardiac hypertrophy in older animals (Tang et al., 2004). When *H-FABP*$^{-/-}$ mice were stressed with the repetitive swimming test, null mice showed higher levels of exercise intolerance and even sudden death (Binas et al., 1999). Interestingly, deletion of the *H-FABP* gene led to no overt changes in phenotype of the mammary gland (Clark et al., 2000). H-FABP has been proposed as a promising marker for the early detection of acute myocardial infarction, but it is not used routinely in clinics (Murray and Alpert, 1994). However, H-FABP has been shown to function as an independent prognostic marker for survival after acute coronary syndrome (Bathia et al., 2009).

5.3. FABPs in disease

B-FABP overexpression is linked with Down syndrome, where it has been speculated to be associated with severe neurological disorders (Sánchez-Font et al., 2003). In contrast, diminished H-FABP levels have been measured in brains of patients with Down syndrome and Alzheimer's disease (Cheon et al., 2003). A single nucleotide polymorphism, Ala54Thr in A-FABP, has been reported to double the A-FABP affinity for its ligands, and this substituted allele correlated with high body-mass index (BMI), increased basal insulin levels, and raised triglyceride levels in obese Japanese, aboriginal Canadians, and Guadeloupe Indian populations (Hertzel and Bernlohr, 2000).

Several FABPs have been examined for their ability to function as predictive markers. Elevated urine I-FABP levels are associated with intestinal ischemia and systemic inflammatory response syndrome (Lieberman et al., 1998). Furthermore, increased L-FABP plasma levels were detected during acute and chronic hepatitis, but a simultaneous decrease of L-FABP levels was observed in hepatic cells (Kamisaka et al., 1981). The content of various FABPs has also been proved to decrease during tumor progression and cellular dedifferentiation, but this is not unambiquous for all FABPs. Immunohistochemical staining of L-FABP showed immunopositivity only in one-third of human colorectal mucinous adenocarcinomas, although normal colon epithelium expressed L-FABP ubiquitously (Carroll et al., 1990). Gradual loss of L-FABP expression was also associated with human hepatocellular malignancies (Suzuki et al., 1990). In the rat colon cancer model, a 10- and 50-fold decrease was observed in mRNA levels of I-FABP and L-FABP, respectively, compared with the control tissue (Davidson et al., 1993). Furthermore, loss of A-FABP expression correlated with progression of human bladder transitional cell carcinomas (Celis et al., 1996). A-FABP is expressed ubiquitously in normal urothelium and expression levels also stayed high in low-grade tumors, but decreased drastically in their high-grade counterparts (Celis et al., 1996). In contrast, human lipoblastomas, hibernomas, and liposarcomas showed a

strong immunopositivity for A-FABP in immunohistochemical stainings, but staining was absent for lipomas (Bennett et al., 1995). Moreover, B-FABP expression in glioblastomas has been associated with more invasive tumors and shorter survival (Kaloshi et al., 2007). Ubiquitously expressed E-FABP is upregulated in several different conditions, such as benign papillomas, squamous cell carcinomas (SCCs) of the skin, and psoriatic skin (Krieg et al., 1993; Madsen et al., 1992). Interestingly, E-FABP was found in the urine of stage I/II and stage III cutaneous melanoma, but only in females, and urinary levels of E-FABP were not detectable in stage IV melanoma patients (Brouard et al., 2002). In contrast, elevated E-FABP mRNA and protein levels were measured in malignant prostate and breast carcinoma cell lines (Jing et al., 2000). Increased E-FABP levels were also found in endometrial cancer patients, where immunopositivity correlated with poorer differentiation (Li et al., 2008). Cytoplasmic immunostaining of E-FABP was absent in 94% of benign prostatic hyperplasia, but present in 96% of prostate adenocarcinomas (Morgan et al., 2008). Thus, a positive correlation was observed between the intensity of cytoplasmic staining and the increased Gleason scores of the prostate carcinomas (Morgan et al., 2008). Transfection of the parental PC-3M prostate cell line with E-FABP siRNA caused decreased proliferation, a diminished number of colonies in the soft agar assay, and a significantly reduced tumor incidence in nude mice (Morgan et al., 2008). In conclusion, various FABPs seem to be associated with different cancer types and the above-mentioned results argue both for tissue-specific and overlapping functions for diverse FABPs.

FABP	Role in tumorigenesis
L-FABP	Downregulated expression in colon cancer (Carroll et al., 1990) and liver cancer (Suzuki et al., 1990).
I-FABP	Downregulated expression in colon cancer (Davidson et al., 1993).
A-FABP	Downregulated expression in progressed bladder cancer (Celis et al., 1996) and lipomas (Bennett et al., 1995). Strong expression in lipoblastomas, hibernomas, and liposarcomas (Bennett et al., 1995).
E-FABP	Overexpressed in benign papillomas (Krieg et al., 1993), squamous cell carcinomas of the skin (Krieg et al., 1993), prostate cancer (Jing et al., 2000; Morgan et al., 2008), breast cancer (Jing et al., 2000), and endometrial cancer (Li et al., 2008).
B-FABP	Overexpression linked with aggressive glioblastomas (Kaloshi et al., 2007).

Table 3: The role of different FABPs in tumorigenesis. See the text for further details.

AIMS OF THE STUDY

Integrins are α/β heterodimers capable of bi-directional signaling across the plasma membrane. An activated integrin receptor shows extended conformation and is primed to function in mediating cell–cell and cell–matrix contacts. Ligation of the active integrin triggers signaling which regulates cell proliferation, migration, and invasion. On the contrary, an inactive integrin receptor adopts a passive bent conformation on the plasma membrane. Several binding partners to the cytoplasmic domain of integrin β-subunits have been identified and their role in integrin-mediated signaling has been studied extensively, but interaction partners of integrin cytoplasmic α-tails have remained poorly investigated.

The specific aims of this doctoral thesis were:

1. To identify novel binding partners for the collagen-binding integrin α-subunit cytoplasmic domains.

2. To determine the function of these novel binding partners at cellular level.

3. To study the role of these novel binding partners in integrin biology and tumorigenesis.

MATERIALS AND METHODS

More detailed information on methods is available in the original publications (I-III).

Expression vectors

The following expression vectors were generated:

Expression vector	Application	Used in
pGBKT7-α1	yeast two-hybrid screen	I
	mating experiments	III
pGBKT7-α2	yeast two-hybrid screen, mating experiments	III
pGBKT7-α10, pGBKT7-α11	mating experiments	III
pGEX4T1-α1cyt, pGEX4T1-α2cyt, pGEX4T1-α10cyt, pGEX4T1-α11cyt, pGEX4T1-SHP2	GST expression vectors	I
pGEX6P1-TCPTP, pGEX6P1-TCPTP 37K, pGEX6P1-TCPTPΔ179-353, pGEX6P1-TCPTPΔ93-353, pGEX6P1-TCPTPΔ1-253	GST expression vectors	I
pRNA-U6.1/TCPTP	TCPTP shRNA construct	I
pEGFP-MDGI	plasmid transfection	II, III
pGEX6P1-MDGI	GST expression vector for producing recombinant MDGI protein	III

Expression vectors with the following inserts were kindly provided:

pcDNA3.1/zeo-α1 (A. Pozzi, Vanderbilt University, Nashville, TN, USA; used in I). X2C5PFneo (M. Hemler, Dana-Farber Cancer Institute, Boston, MA, USA; used in I). EGFR-FLAG (Y. Daaka, Duke University Medical Center, Durham, NC, USA; used in II).

Cell lines

Commercial cell lines	Species	Source	Used in
HeLa	human	cervical adenocarcinoma	I, II
PC-3	human	prostate adenocarcinoma	I
B104-1-1	mouse	embryonal fibroblasts	I
Saos-2	human	osteosarcoma	I
HT1080	human	fibrosarcoma	I, II
NCI-H358	human	bronchioalveolar carcinoma	II
hTERT-HME1	human	breast epithelial	II
MCF10A	human	breast epithelial	II
MCF7	human	breast adenocarcinoma	II, III
SKBr3	human	breast adenocarcinoma	II, III
MDA-MB-231	human	breast adenocarcinoma	II, III
MDA-MB-468	human	breast adenocarcinoma	II, III

Stable cell lines	Description	Used in
Saos-2-α2α1	Expresses chimeric α2-integrin with α1-integrin cytoplasmic tail.	I
Saos-2-α2α5	Expresses chimeric α2-integrin with α5-integrin cytoplasmic tail.	I
NCI-H358 GFP	Control cell lines expressing GFP.	II
MDA-MB-468 GFP		II, III
MDA-MB-231 GFP		III
NCI-H358 GFP-MDGI	Cell lines expressing full length MDGI with N-terminal GFP tag.	II
MDA-MB-468 GFP-MDGI		II, III
MDA-MB-231 GFP-MDGI		III

$\alpha1^{-/-}$ MEF and $\alpha1^{+/+}$ MEF cells (mouse embryonal fibroblasts, used in I) were a generous gift from H. A. Gardner, The Scripps Research Institute, La Jolla, CA, USA (Pozzi et al., 1998).

Primary antibodies

Antigen epitope	Species	Antibody	Used in
TCPTP	mouse	CF4, Calbiochem	I: IP, WB, IF
α1-integrin	mouse	MAB1973, Chemicon	I: IP, IF
			I & III: F
α2-integrin	mouse	MCA2025, Serotec	I: IP, IF
			I & III: F
α3-integrin	mouse	MAB1952, Chemicon	I & III: F
α4-integrin	mouse	MAB16983, Chemicon	III: F
α5-integrin	mouse	MAB1999, Chemicon	I: IP
			I & III: F
	mouse	MCA1187, Serotec	III: IF
α6-integrin	mouse	MAB1964, Chemicon	I: IP, F
	rat	MCA699, Serotec	III: F
αV-integrin	mouse	L230, Prof. J. Heino	III: F
β1-integrin	mouse	MB1.2, Chemicon	I: IP
	mouse	MAB2252, Chemicon	II & III: WB
	mouse	610468, Transduction Lab.	II & III: WB
	mouse	P5D2, Abcam	I, II & III: F
			III: IF
	mouse	9EG7, BD Biosciences	III: F
	mouse	HUTS-21, BD Biosciences	III: F
	mouse	12G10, Abcam	III: IF
	mouse	AIIB2, Hybridoma Bank	III: IP
β3-integrin	mouse	MCA728, Serotec	III: F
β4-integrin	mouse	MAB2060, Chemicon	III: F, IF
SHP2	rabbit	sc-7384, Santa Cruz Biotechnology	I: IP, WB
EGFR	rabbit	#2232, Cell Signaling Technology	I & II: WB
			II: IHC, IF
	mouse	LA1, Upstate	II: IF, F
pY1068-EGFR	rabbit	#2234, Cell Signaling Technology	I: WB
			III: WB, IF

(Continued on the following page)

Antigen epitope	Species	Antibody	Used in
pY845-EGFR	rabbit	#2231, Cell Signaling Technology	I: WB
pY992-EGFR	rabbit	#2235, Cell Signaling Technology	I & II: WB
pY1148-EGFR	rabbit	#4404, Cell Signaling Technology	II: WB
ErB2	rabbit	sc-284, Santa Cruz Biotechnology	II: WB
	mouse	sc-74241, Santa Cruz Biotechnology	II: F
pY1248-ErB2	rabbit	#2247, Cell Signaling Technology	II: WB
ErbB3	rabbit	sc-285, Santa Cruz Biotechnology	II: WB
pY1289-ErbB3	rabbit	#4791, Cell Signaling Technology	II: WB
ErbB4	rabbit	sc-283, Santa Cruz Biotechnology	II: WB
pY1284-ErbB4	rabbit	#4757, Cell Signaling Technology	II: WB
pT308-Akt	rabbit	#9275, Cell Signaling Technology	II:WB
pS473-Akt	rabbit	#9271, Cell Signaling Technology	II: WB
phospho-p44/42 MAPK	rabbit	#9101, Cell Signaling Technology	II: WB
MDGI	mouse	16915, Abcam	II: WB
	mouse	16916, Abcam	II: IHC
GFP	rabbit	A11122, Molecular Probes	II & III: WB
			III: IF
Rab5	rabbit	sc-309, Santa Cruz Biotechnology	II: IF
Rab7	rabbit	sc-10767, Santa Cruz Biotechnology	II: WB
Rab21	mouse	sc-81917, Santa Cruz Biotechnology	II: WB
FLAG	mouse	F-3165, Sigma-Aldrich	II: IF
EEA1	rabbit	07-292, Upstate	II: WB
caveolin-1	rabbit	sc-894, Santa Cruz Biotechnology	II: IF
clathrin	rabbit	sc-28276, Santa Cruz Biotechnology	II: IF
LAMP1	goat	sc-8098, Santa Cruz Biotechnology	II: IF
vinculin	mouse	V9131, Sigma-Aldrich	III: IF
kindlin	rabbit	ab68041, Abcam	III: IF
α-tubulin	mouse	12G10, Hybridoma Bank	I, II & III: WB

Abbreviations: F, *flow cytometry*; IHC, *immunohistochemistry*; IP, *immunoprecipitation*; WB, *Western blot*; IF, *immunofluorescence.*

Reagents and chemicals

Compound	Application	Supplier	Used in
Mouse E17 cDNA library	yeast two-hybrid screen	Clontech	I, II
DiFMUP	phosphatase substrate	Molecular Probes	I
FDP	phosphatase substrate for IF	Molecular Probes	I
PhosSTOP	phosphatase inhibitor	Roche	II, III
Complete	protease inhibitor	Roche	I, II, III
Phosphatase inhibitor cocktail	Ser/Thr PTP inhibitor	Sigma-Aldrich	I
WST-1	proliferation assay substrate	Roche	I, II
EGF	growth factor	Sigma-Aldrich	I, II
Alexa-555-EGF	EGF internalization	Molecular Probes	II
Alexa-546-transferrin	receptor internalization	Molecular Probes	II
Cycloheximide	protein synthesis inhibitor	Sigma-Aldrich	II
5-aza-2'-deoxycytidine	DNA demethylase	Sigma-Aldrich	II
Tyrphostin AG1478	EGFR TKI	Sigma-Aldrich	II
Erbitux® (cetuximab)	EGFR MAb	Merck	II
Alexa Fluor-647 phalloidin	detects F-actin in IF	Molecular Probes	III
DAPI	stains DNA in IF	Vector Laboratories, Inc.	I, II, III
DABCO	anti-fade reagent in IF	Sigma-Aldrich	I, II, III
Anti-biotin-HRP	detects biotin in WB	Cell Signaling Technology	I, II
Anti-human-HRP	detects human IgG in WB	Amersham Biosciences	II
Type I collagen	matrix for coating	Sigma-Aldrich	I, III
Fibronectin	matrix for coating	Calbiochem	I, III
Poly-L-lysine	matrix for coating	Sigma-Aldrich	I
Matrigel	3D matrix for cell culturing	BD Biosciences	II, III
Cell recovery solution	releases cells from matrigel	BD Biosciences	II
CellTracker green	fluorescent probe	Molecular Probes	I
Fugene 6	transfection reagent	Roche	I
Oligofectamine	transfection reagent	Invitrogen	I
Lipofectamine 2000	transfection reagent	Invitrogen	II, III

Abbreviations: PTP, *protein tyrosine phosphatase*; TKI, *tyrosine kinase inhibitor*; MAb, *monoclonal antibody*; IF, *immunofluorescence*; WB, *Western blot*.

Animals

Female athymic Nude-nu mice were used for the xenograft studies in I and II (Harlan Scandinavia, Allerod, Denmark).

Peptides

Name	Description	Used in
α1-TAT	FITC-YGRKKRRQRRRWKLGFFKRPLKKKMEK	I
α1-b-TAT	YGRKKRRQRRRRPLKKKMEKRPLKKKMEK	I
Scr-TAT	FITC-YGRKKRRQRRRLKGWRFKLKPKFKEMK	I
α1-peptide	RPLKKKMEKRPLKKKMEK	I
α2-peptide	KLGFFKRKYEKMTKNPDEIDETTELSS	I
α1-cyt	WKIGFFKRPLKKKMEKRPLKKKMEK	III
α2-cyt	WKLGFFKRKYEKM	III
β1-cyt	WKLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK	III

siRNAs

Target	Sense sequence or name	Supplier	Used in
TCPTP	GGCACAAAGGAGUUACAUCTT	Ambion	I
TCPTP	GGAGUUACAUCUUAACACATT	Ambion	I
SHP2	GGAGUUGAUGGCAGUUUUUTT	Ambion	I
SHP2	GGCCUAGUAAAAGUAACCCTT	Ambion	I
Scr ctrl	non-specific control duplex II	Dharmagon	I

Methodology

Method	Used in
Cell culture	I, II, III
3D culture	II, III
Transfection	I, II, III
Generation of stable cell lines	I, II
Yeast two-hybrid screen	I, III
Yeast mating	III
DNA cloning	I, II
Protein-protein interaction assay	I
Immunoprecipitation	I, II, III
Western blot analysis	I, II, III
Immunofluorescence	I, II, III
Immunohistochemistry	II, III
Flow cytometry	I, II, III
Phosphatase assay	I
Bead binding assay	I
Soft agar assay	I
Proliferation assay	I, II
Sensitivity to serum withdrawal	II
Adhesion assay	I, III
RNA extraction	II
Reverse transcriptase-PCR	II
Percoll gradients	II
Internalization and recycling assay	II
In vivo animal model	II
Quantitative interaction analysis (Biacore)	III
In situ proximity ligation (PLA)	III
Inverted 3D invasion assay	III
Live-cell microscopy	III
Molecular modeling	III
Statistical analysis	I, II, III

RESULTS

1. TCPTP and MDGI are novel interaction partners for the integrin cytoplasmic α-subunits (I, III)

In order to identify novel binding partners for the collagen-binding integrin cytoplasmic α-domains, we performed a yeast two-hybrid screen with integrin α1 and α2 cytoplasmic tails containing the conserved membrane-proximal GFFKR sequence (III: Fig 1b). The screening was done with a 17-day-old mouse embryo cDNA library (Clontech). Among the several binding candidates for the cytoplasmic α1-integrin, TCPTP was the focus of our attention based on the preliminary literature searches. For the integrin α2 candidates, MDGI held ample interest due to speculations about its ability in functioning as a tumor suppressor. Other members of the FABP family may also be of interest, as FABP4 emerged from a similar yeast two-hybrid screen carried out in our laboratory with the α10 cytoplasmic tail and FABP7 emerged from a screen with the α11-tail (J. Nevo, unpublished data).

Immunofluorescence studies carried out in mammalian cells expressing endogenous TCPTP and integrin α1 revealed matrix-dependent co-localization. When PC-3 and HeLa cells were plated to type I collagen, partial co-localization of TCPTP and α1-integrin at the peripheral areas of the cell membrane were observed (I: Fig 1a, 1b, Suppl 1a). On the contrary, on poly-L-lysine-plated PC-3 cells, diffuse staining of TCPTP was detected both in the cytoplasm and nucleus (I: Fig 1a), and on fibronectin, TCPTP was found mainly diffusively in the cytoplasm of HeLa cells (I: Fig 1b, Suppl 1a). The collagen-binding integrin subfamily consists of α1β1, α2β1, α10β1, and α11β1 heterodimers. Endogenous HeLa cell lysate incubation with different GST fusion proteins of the collagen-binding integrin cytoplasmic domain showed a specific interaction only between GST-α1cyt and TCPTP (I: Fig 1e). This direct association could be competed out when a synthetic α1-peptide lacking the conserved GFFKR sequence was added to the reaction mixture (I: Suppl 1d). These results suggest a direct association between TCPTP and the α1-specific cytoplasmic sequence lacking the conserved GFFKR shared with all integrin α-subunits. To study this association further, we tested immunoprecipitations in different conditions. It has been reported previously that TCPTP exits from the nucleus to the cytoplasm in response to mitogenic stimulus (Tiganis et al., 1998). Indeed, not only the plating of cells on type I collagen, but also serum stimulation induced TCPTP association with integrin α1 (I: Fig 1c, 1d). These results suggest a direct association between TCPTP and the α1-specific cytoplasmic sequence at the plasma membrane in response to cell adhesion to collagen or serum stimulus.

Mating experiments done in yeast cells revealed the ability of MDGI to interact with all collagen-binding integrin cytoplasmic α-domains (III: Fig 1a). Further studies with purified recombinant MDGI and cytoplasmic integrin peptides confirmed the binding

of MDGI to α1 or α2, but showed no interaction with the cytoplasmic β1-peptide (III: Fig 1c). Due to the fact that integrins are heterodimeric receptors, several different integrin α-subunits pair with β1, forming α/β1 heterodimers in intact cells. Immunoprecipitation of all these heterodimers with anti-β1-antibody showed co-precipitation with overexpressed MDGI (III: Fig 1d). To study whether MDGI also interacts with non-collagen-binding integrins, we used the *in situ* proximity ligation assay (PLA) technique that generates an antibody-based signal when two proteins are in close proximity (≈ 20-100nm) to each other in an intact cell (Söderberg et al., 2006). First, cell surface integrin levels of stable GFP and GFP-MDGI expressing MDA-MB-231 cells were analyzed with FACS to define the integrin subunit expression pattern in these cells (III: Suppl 1). In agreement with the previous data, a positive PLA signal was detected between MDGI and β1 (III: Fig 2a). Interestingly, a positive PLA signal was also observed between MDGI and α5 (III: Fig 2b) or β4 (III: Fig 2c). Of these, α5 dimerizes with β1 to form the major fibronectin-binding integrin and β4 pairs with α6 to bind laminin. Results from these experiments indicate that MDGI binds directly to several integrin α-domains, suggesting interaction via a conserved GFFKR sequence as being shared between all integrin α-subunits.

2. EGFR signaling is modulated via TCPTP activation or MDGI-induced trafficking (I, II)

A balanced action of protein tyrosine kinases and protein tyrosine phosphatases is critical for signaling pathways regulating vital cellular functions. TCPTP phosphatase activity in HeLa cell lysate was increased by 2.4-fold when cells were plated on type I collagen compared to poly-L-lysine (I: Suppl 2a). Similar augmentation in the phosphatase activity was also achieved by addition of the synthetic cytoplasmic α1-peptide to the TCPTP immunoprecipitated lysates, but addition of α2-peptide had no influence (I: Fig 2a). Based on previous *in vitro* studies, TCPTP is speculated to have an autoinhibitory intramolecular interaction between the catalytic N-terminal and non-catalytic C-terminal domains (Hao et al., 1997). In line with this, a truncated 37 kDa TCPTP isoform lacking the C-terminal regulatory segment was not activated by the addition of the synthetic α1-peptide (I: Fig 2b). Further studies with different GST-TCPTP deletion mutants showed that cytoplasmic α1-peptide interacts directly with the N-terminal part of TCPTP, thus inhibiting the formation of a proposed inhibitory autoregulatory interaction between the C- and N-terminal parts (I: Fig 2c, 2d). Thus, TCPTP association with the integrin α1 cytoplasmic domain modulates TCPTP conformation, leading to increased phosphatase activity.

TCPTP has several known substrates *in vivo* and one of them is EGFR (Tiganis et al., 1998). To study the TCPTP phosphatase activity *in vitro*, we treated cells on plastic, fibronectin, or type I collagen with EGF. In line with the previous data, phosphorylation of several different EGFR tyrosine residues was substantially inhibited only on collagen (I: Fig 3a, 3c). This effect was strongest during the early EGF stimulus at 5 minutes (I: Fig 3b). Specificity of the effect was confirmed by

TCPTP siRNA knockdown, which caused a clear increase in phosphorylation of EGFR-Y1068 (I: Fig 3d). To be sure of the α1-integrin specificity, we used $\alpha1^{-/-}$ and $\alpha1^{+/+}$ mouse embryonal fibroblasts, where α1-deficiency caused a clear augmentation in EGFR tyrosine phosphorylation (I: Fig 3e). Moreover, adhesion of parental HT1080 cells lacking the α1-integrin failed to inhibit EGFR phosphorylation on collagen (I: Suppl 3b), but this was restored with the transient expression of integrin α1 (I: Suppl 3c). Taken together, integrin α1 specifically activates TCPTP, causing attenuated EGFR phosphorylation.

In contrast to the ubiquitously expressed TCPTP, MDGI expression in cell culture conditions is lost due to epigenetic silencing (II: Fig 1a, 1b, 1c). However, immunohistochemical stainings showed that MDGI is expressed in histologically normal human mammary epithelium and at varying levels in human breast cancer samples (II: Fig 1d). The clinical relevance was confirmed with mRNA expression microarrays containing eight normal human breast epithelium and 553 breast cancer samples. MDGI mRNA levels were increased in 5% of cancer samples and similar in 40% of cancer samples compared to normal tissue samples (II: Fig 1e). However, the median mRNA levels of MDGI were significantly lower in breast cancer samples in comparison to normal tissue (II: Fig 1e). We were able to reverse MDGI mRNA expression in cultured cells with demethylase treatment to some extent (II: Fig 1c), but established a stable GFP-MDGI expressing MDA-MB-468 breast cancer cell line for further studies (II: Fig 1a, 1b).

The parental MDA-MB-468 cell line has *EGFR* amplification, thereby expressing EGFR at $1x10^6$ per cell (www.lgcstandards-atcc.org). Interestingly, immunofluorescence stainings showed a marked accumulation of EGFR in the perinuclear region of MDA-MB-468 cells stably expressing GFP-MDGI compared to control cells having EGFR mainly on the plasma membrane (II: Fig 3a). Moreover, EGF stimulation induced EGFR re-localization from the plasma membrane to the perinuclear compartment in control cells, but in GFP-MDGI cells, EGFR remained unaltered (II: Fig 3a). Detailed analysis revealed that GFP-MDGI colocalized in the cytoplasm with caveolin-1 and lysosomal-associated membrane protein 1 (LAMP1) (II: Fig 3b). In line with these results, EGFR cell surface expression in GFP-MDGI cells analyzed with FACS was reduced by 36±4% compared to control cells (II: Fig 2b) and GFP-MDGI cells bound less labeled EGF on their surface (II: Suppl 2). Importantly, total protein levels of EGFR remained similar between the cell lines (II: Fig 2a). Moreover, cell lysate fractionation studies on a continuous sucrose gradient confirmed GFP-MDGI accumulation in the endosomal fractions (II: Fig 3d, Suppl 5a). Changes in the cell surface expression levels of EGFR indicate alterations in the receptor trafficking. Indeed, biotinylation of cell surface proteins showed accumulation of endocytosed biotinylated EGFR within 30 minutes in GFP-MDGI expressing cells, but in control cells, EGFR was mainly recycled back (II: Fig 3c). EGFR recycling depends on receptor dimerization (homo- or heterodimerized) and phosphorylation status, but we were not able to detect any differences in the expression level or phosphorylation of other EGFR family members, such as ErbB2, ErbB3, or ErbB4 (II: Fig 2d). Moreover,

phosphorylation of EGFR itself was similar between GFP and GFP-MDGI expressing cells (II: Fig 3c). To study the specificity of the observed cytosolic EGFR accumulation, we used FACS measurements to analyze cell surface levels of other transmembrane receptors known to be endocytosed. Cell surface levels of β1-integrin (II: Fig 2b) and ErbB2 (II: Fig 2e) remained equivalent in GFP-MDGI expressing cells and control cells. In addition, endocytosis of labeled transferrin was not altered by GFP-MDGI expression (II: Suppl 3). Each transferrin protein contains two iron-binding sites and cellular iron uptake is mainly mediated via endocytosis of transmembranic transferrin receptors. These results demonstrate that a change in the receptor trafficking seems to be EGFR-specific. Earlier studies have shown that the EGF–EGFR complex remains dimerized and phosphorylated in late endosomes (Sorkin and Carpenter, 1991; Sorkin et al., 1991) and signals originating from endosomal EGFR were sufficient for cell proliferation and survival (Wang et al., 2002). In line with this, internalized EGFR remained phosphorylated in GFP-MDGI expressing cells (II: Suppl 4a). By treating cells with protein synthesis inhibitor, we were able to demonstrate that the EGFR turnover rate was similar between GFP and GFP-MDGI expressing cells (II: Suppl 5b). As a conclusion, in GFP-MDGI expressing cells, EGFR is mainly located in the cytoplasm, where it remains phosphorylated and evades proteolytic degradation.

3. Reversion of hallmarks of transformed cells: Changes in the anchorage-independent growth or cell morphology (I, II, III)

Only transformed cells are able to grow without solid substratum, thus anchorage-independent cellular growth is an indication of tumorigenicity. Earlier studies with the glioblastoma cells expressing mutant EGFR have demonstrated that TCPTP is able to inhibit anchorage-independent growth, but the mechanism has remained unknown (Klingler-Hoffmann et al., 2001). This led us to culture HeLa cells, which express endogenous TCPTP, in a soft agar assay for colony formation. TCPTP phosphatase activity was stimulated further with the delivery of α1-integrin cytoplasmic peptide into cells. To overcome problems with the plasma membrane permeability, α1-integrin cytoplasmic peptide was fused with a small TAT peptide (GRKKRRQRRR) that is a membrane-permeable peptide originally identified from the transactivator of transcription (TAT) of the human immunodeficiency virus (HIV). Delivery of α1-TAT into HeLa cells in soft agar significantly reduced the colony size compared to control cells (I: Fig 4d). α1-TAT peptide conjugation with fluorescein isothiocyanate (FITC) enabled the intracellular follow-up (I: Fig 4a). Attenuated EGFR phosphorylation was detected in the presence of α1-TAT (I: Suppl 4c). Next, the proliferation of cells grown in suspension was studied more carefully. Delivery of α1-TAT peptide efficiently inhibited both serum- and EGF-induced proliferation of HeLa cells (I: Fig 4a). Serum-induced proliferation was also decreased in tumorigenic mouse B104-1-1 cells in the presence of α1-TAT (I: Fig 4c). However, TCPTP downregulation in HeLa cells with specific short hairpin RNA (shRNA) abolished the decrease in proliferation (I: Fig 4b). Interestingly, proliferation of adherent HeLa cells was insensitive to α1-TAT peptide

treatment (I: Suppl 4d). Taken together, this suggests that α1-integrin cytoplasmic peptide activates TCPTP phosphatase activity *in vitro*, which leads to inhibition of anchorage-independent growth, but has no effect on adherent cells, most likely due to prevailing integrin-induced survival signals.

Transformed cells grow in disorganized colonies when cultured in a 3D matrix, such as when embedded in reconstituted matrigel, but normal human breast epithelial cells are able to form a structurally and functionally differentiated acinar phenotype in such growth conditions (Petersen et al., 1992). Since MDGI expression was lost in all cultured cell lines, we wanted to test whether cell propagation in 3D cultures would restore endogenous MDGI manifestation. This was not the case (data not shown), but MDGI overexpression in MDA-MB-468 cells caused a partial phenotype reversion from disorganized colonies observed in parental cells to organized acinar-like structures (III: Fig 5). The proportion of acinar-like structures was 42±8% higher in GFP-MDGI cells compared to control cells (III: Fig 5). When MDA-MB-468 cells were grown in matrigel for 3 days, stable GFP-MDGI expressing cells proliferated by 50±10% less compared to control cells (II: Fig 4a). Nevertheless, under standard cell culture conditions, GFP and GFP-MDGI expressing cells proliferated in a similar manner (II: Suppl 1b). Taken together, MDGI overexpression in 3D cultures is sufficient to induce partial reversion of the malignant phenotype and growth inhibition.

4. MDGI induces resistance to anti-EGFR antibody therapy (II)

MDGI-induced retardation of proliferation in matrigel led us to investigate the role of EGFR in this process. As mentioned earlier, under standard cell culture conditions, GFP-MDGI expressing cells have 36% less EGFR on their plasma membrane according to the FACS analysis (II: Fig 2b). We decided to repeat FACS with cells that were first grown for 3 days in matrigel and then released from the matrix with a recovery solution without enzymatic digests for subsequent analysis. Surprisingly, an even more impressive decrease was observed in the plasma membrane levels of EGFR in GFP-MDGI expressing cells. GFP-MDGI cells had 91±1% less EGFR on their cell surface compared to control cells, but the plasma membrane levels of integrin β1 remained similar between the cell lines (II: Fig 4b). Western blot analysis revealed an equal total EGFR protein level in both GFP and GFP-MDGI expressing cells released from the matrigel (II: Fig 4c), so receptor trafficking seems to be the reason for the diminished membrane localization of EGFR. To study further the specificity of this observed phenomenon, we measured the cell surface levels of HER2 from cells grown either in matrigel or standard cell culture conditions on plastic. FACS values were comparable between control and GFP-MDGI expressing cells both in matrigel and on plastic (II: Fig 4d). Interestingly, culturing in 3D induced HER2 localization to the plasma membrane, but in the light of Western blot analysis from the same samples, total HER2 protein levels were also increased in matrigel samples by 3.3±0.15-fold compared to plastic (II: Fig 4d).

Anti-EGFR therapy is based on the monoclonal antibodies targeted for the extracellular domain of the receptor or on small molecule inhibitors against the intracellular kinase domain of the receptor. The cytoplasmic localization of EGFR in GFP-MDGI cells would imply resistance to antibody-based therapy, but membrane-permeable small molecules could still enter their targets. Stable GFP or GFP-MDGI expressing MDA-MB-468 cells were grown in matrigel for 72 hours and treated with two different anti-EGFR antibodies. Cetuximab (Erbitux®) is an anti-EGFR antibody blocking the binding of EGF and is in clinical use for advanced colon cancer and squamous cell carcinoma of the head and neck in Finland. LA1, instead, is an EGFR-neutralizing monoclonal antibody used only in preclinical settings. Treatment with either of them significantly inhibited proliferation in control cells, but neither blocked proliferation in GFP-MDGI expressing cells further from that restrained by MDGI expression alone (II: Fig 5a). In line with our preliminary hypothesis, the small molecule tyrosine kinase inhibitor, AG1478, managed to inhibit proliferation in GFP-MDGI cells as well (II: Fig 5b).

Next, we generated stable GFP and GFP-MDGI expressing NCI-H358 cells to repeat findings in another cell line. NCI-H358 cells are originally from bronchioalveolar carcinoma that represents a subgroup of non-small cell lung cancer (NSCLC). NSCLC is currently under intensive phase III studies for cetuximab therapy. Total protein levels of β1-integrin and EGFR remained equivalent between stable GFP and GFP-MDGI expressing NCI-H358 cells (II: Fig 5c). When stable NCI-H358 cell lines were treated with cetuximab or AG1478 on plastic, cetuximab inhibited proliferation only in control cells, but AG1478 worked on both cell lines (II: Fig 5d).

In order to understand the molecular mechanisms underlying anti-EGFR antibody resistance in GFP-MDGI cells, downstream signaling was examined more carefully. In stable GFP expressing MDA-MB-468 cells, pretreatment with LA1 antibody inhibited EGF-induced phosphorylation of ERK and Akt, but in GFP-MDGI cells phosphorylation remained unaltered (II: Fig 5e). As expected, pretreatment with tyrosine kinase inhibitor, AG1478, inhibited EGF-induced phosphorylation of ERK and Akt both in GFP and GFP-MDGI cells (II: Fig 5f). It was slightly confusing that EGF-induced downstream signaling was comparable between GFP and GFP-MDGI cells, although GFP-MDGI cells have approximately one-third of the EGFR internalized in standard cell culture conditions. Nevertheless, EGF-induced phosphorylation of EGFR was diminished on collagen-plated GFP-MDGI cells compared to control cells (II: Fig 5f). To examine the kinetics of the anti-EGFR antibody in MDGI expressing cells, we performed the internalization assay with biotinylated cetuximab in stable GFP and GFP-MDGI expressing MDA-MB-468 cells. First, cells were allowed to internalize biotinylated cetuximab for 15 or 30 minutes at +37°C and then the biotin remaining on the cell surface was cleaved. Recycling samples were exposed to an additional incubation period of 15 minutes at +37°C followed by a second cleavage of cell surface biotin. In GFP-MDGI expressing cells cultured on plastic, biotinylated cetuximab was internalized more efficiently and recycling was reduced compared to control cells (II: Fig 5g). This implies that

attenuated cetuximab recycling in GFP-MDGI cells might be partly responsible for cetuximab ineffectiveness in standard cell culture conditions. Nevertheless, as mentioned earlier, in 3D culture conditions, approximately 90% of EGFR is internalized at any given moment when MDGI is overexpressed. Taken together, these results demonstrate that MDGI expressing cells are resistant to cetuximab therapy *in vitro*, but the fully detailed mechanism remains elusive.

To investigate the role of MDGI *in vivo*, we performed animal studies with nude mice injected orthotopically into the fourth pair of mammary fat pads. Stable GFP or GFP-MDGI expressing MDA-MB-468 breast cancer cells were used for injection. Surprisingly, GFP and GFP-MDGI expressing tumors grew at similar rates (II: Fig 6a). In the second study, we administered cetuximab twice a week starting 10 days after tumor cell inoculation. Cetuximab treatment efficiently blocked tumor growth of the control cells, but MDGI expressing tumors were resistant to therapy (II: Fig 6b). Postmortem examination of tumors confirmed the difference in tumor sizes, thus MDGI tumor masses were 45±7% higher compared to GFP tumors in the treated animals (II: Fig 6c). Subsequent analysis of lysated tumors revealed comparable levels of retained EGFR and phosphorylated ERK both in GFP and GFP-MDGI expressing tumors (II: Fig 6d). EGFR downregulation at protein level or sustained MAPK activation in the presence of EGFR have been documented to cause acquired resistance to cetuximab therapy (Ciardiello et al., 2004; Lu et al., 2007b). Thus, acquired resistance would be different to the observed inherent resistance of MDGI expressing cells since our *in vitro* findings suggest that EGFR localization is the main difference between control and MDGI cells. This was confirmed with immunofluorescence stainings of paraffin-embedded tumor samples. MDGI expressing tumors had less EGFR on their plasma membrane and more diffuse EGFR staining in their cytoplasm compared to control tumors (II: Fig 6e). In conclusion, MDGI-induced EGFR redistribution is a novel *in vivo* mechanism for cetuximab resistance.

5. MDGI modulates integrin activity and attenuates integrin-mediated cell adhesion, migration, and invasion (III)

Integrins can be activated via extracellular ligand binding to their ectodomains or intracellular molecule association with their cytoplasmic domains. Talin and kindlins are well-characterized cytoplasmic proteins capable of binding to integrin β-subunits and changing integrin conformation to the active, extended conformation. We demonstrated earlier that MDGI interacts with several integrin α-subunits. This led us to hypothesize that MDGI could modulate integrin function. To test this, we allowed stable GFP or GFP-MDGI expressing MDA-MB-468 and MDA-MB-231 cells to adhere to type I collagen for 60 minutes. This adhesion assay was developed in a 96-well plate format for the Acumen Explorer laser scanner and the number of attached green cells per well was counted at 488 nm. Indeed, GFP-MDGI expressing MDA-MB-468 and MDA-MB-231 cells adhered significantly less to type I collagen compared to control cells (III: Fig 3a). According to immunofluorescence studies with

stable GFP-MDGI expressing MDA-MB-468 cells, MDGI cells were also less spread on type I collagen after 60 minutes incubation compared to untransfected cells (III: Fig 4a). Next, we broadened our investigations with transient GFP or GFP-MDGI expressing MCF7 and SKBr3 cells. In agreement with our previous results, adhesion to type I collagen was again reduced in GFP-MDGI expressing cells (III: Fig 3a). The actual values of adherent cells vary due to different integrin expression profiles and, therefore, we measured cell surface integrin levels of different cell lines with FACS (III: Suppl 4). It is noteworthy that MDGI expression retained plasma membrane integrin distribution as unaltered (III: Suppl 1). In addition to type I collagen, adhesion of GFP-MDGI expressing cells to fibronectin was also reduced (III: Suppl 3). Next, we used two different integrin β1 antibody epitopes (9EG7 and HUTS-21), that recognize only active integrin conformation, to study integrin profiles further in FACS. Stable GFP-MDGI expressing MDA-MB-468 and MDA-MB-231 cells expressed significantly less active β1-integrin on their plasma membrane compared to GFP cells (III: Fig 3b). However, total integrin β1 levels on the cell surface were similar between GFP and GFP-MDGI cells when the pan-β1-integrin antibody, P5D2, was used for the measurements (III: Fig 3c). Treatment with divalent cation manganese (Mn^{2+}) alters integrin affinity and is sufficient to mediate outside-in integrin activation (Hynes, 2002). When stable GFP and GFP-MDGI expressing MDA-MB-468 and MDA-MB-231 cells were exposed to manganese stimulation in the presence of the Alexa-647-labeled fibronectin fragment, which contains the integrin-binding RGD motif, GFP-MDGI cells bound FN remarkably less (III: Fig 3d). These studies imply that when MDGI is present, integrin seems to adopt an inactive conformation. This was confirmed with PLA studies, where the PLA signal detected between total β1-integrin and MDGI was over threefold greater compared to the signal between the active β1-integrin epitope and MDGI (III: Fig 2a and Fig 3e). Moreover, the PLA signal detected between the active integrin β1 epitope and kindlin was significantly lower in GFP-MDGI expressing cells compared to GFP cells (III: Fig 3f). This suggests that MDGI binding to the integrin α-subunit could outcompete kindlin association with the β-subunit. MDGI-induced alteration in integrin activity and cell adhesion led us to investigate the role of MDGI expression in cell migration and invasion. Indeed, transiently transfected GFP-MDGI cells migrated significantly less on plastic compared to GFP cells (III: Fig 4b). Furthermore, stable GFP-MDGI expressing MDA-MB-468 cells showed reduced invasion capacity in the 3D invasion assay into reconstituted matrigel supplemented with fibronectin compared to GFP cells (III: Fig 4c). The above-mentioned *in vitro* findings strongly suggested MDGI to have a significant role in modulating integrin affinity, adhesion, and migration.

To examine the clinical role of MDGI *in vivo*, we stained a tissue microarray consisting of 1331 unilateral female invasive breast cancers with anti-MDGI antibody. Staining was scored according to intensity: 0 indicated no immunoreactivity (5.7%), 1 correlated with a faint immunopositivity (40.6%), 2 with moderate expression (36.5%), and 3 with strong expression (17.2%). Even a faint immunopositivity corresponded with an increased 10-year distant disease-free survival (DDFS) among patients (III: Fig 6a). When all immunopositive samples were grouped together and analyzed against

immunonegative samples, MDGI-positive patients had a more favorable 10-year prognosis compared to MDGI-negative ones: 72% versus 61% (P=0.047), respectively (III: Fig 6b). In subsequent subgroup analyses, MDGI immunopositivity correlated with increased DDFS in axillary node-positive disease (III: Fig 6c), HER2-negative disease (III: Fig 6d), and triple-negative disease (III: Fig 6e). P-values were 0.035, 0.008, and 0.031, respectively. Thus, retained MDGI expression in breast cancer patients predicts better overall survival and, surprisingly, this association is even stronger in subgroups that are normally linked to a lack of targeted therapies or poorer survival. Taken together, MDGI expression has a noteworthy role in breast cancer progression *in vivo*.

DISCUSSION

1. α1-integrin dictates TCPTP activity

Protein tyrosine phosphatases operate as counterparts to protein tyrosine kinases in various cellular signaling pathways linked to normal development and disease-related processes. An impressive number of studies have been carried out since 1988 when the first PTP was purified (Tonks et al., 1988), describing several mechanisms to regulate PTP activity. These include differential tissue expression patterns, subcellular localization, alternative splicing, limited proteolysis, phosphorylation of the catalytic domain, and reversible oxidation of the cysteine residue in the catalytic site (den Hertog et al., 2008). In addition, receptor-like PTPs are known to be regulated by dimerization and extracellular ligands (den Hertog et al., 2008). Our studies revealed a novel integrin-mediated activation mechanism for TCPTP where the cytoplasmic domain of integrin α1 specifically interacts with the N-terminus of TCPTP and catalyzes TCPTP phosphatase activity. A proposed mechanism of action is based on the allosteric structure modulation, where the integrin cytoplasmic domain association disrupts the autoinhibitory intramolecular interaction between the catalytic domain containing the N-terminus and the non-catalytic C-terminus. An analogous mechanism of action has been identified for maintaining SHP2 in its inactive conformation (Tartaglia et al., 2001) and *in vitro* studies have argued for the existence of such an intramolecular interface for TCPTP as well (Hao et al., 1997). Importantly, the short sequence of the cytoplasmic α1-integrin tail lacks tyrosine residues that would be able to act as direct substrates for TCPTP.

We demonstrate here for the first time that α1β1-integrin ligation activates TCPTP activity. This is in agreement with earlier studies by others where some PTPs have been shown to collaborate with integrins. Cytoplasmic PTP-PEST has been documented to localize to the tips of membrane protrusions in spreading fibroblasts, suggesting a finely controlled balance between the formation of a new adhesion site and adhesion disassembly (Sastry et al., 2002). Sastry and co-workers further demonstrated that integrin-mediated adhesion regulated PTP-PEST catalytic activity and PTP-PEST impaired cell migration through inhibition of Rac1 activity (Sastry et al., 2002). Moreover, non-receptor PTPB1 has been shown to coimmunoprecipitate with integrin β1 in nonionic detergent extracts and overexpression of a dominant negative mutant PTPB1 led to attenuated integrin-mediated adhesion and spreading (Arregui et al., 1998).

Cell adhesion is known to trigger phosphorylation of several downstream signaling molecules and the finely controlled balance between phosphorylation and dephosphorylation certainly requires simultaneous activation of phosphatases as well. Integrin α1 expression has been shown to be downregulated in carcinomas of the breast, ovary, and lung (Su et al., 2001). Thus, lack of an integrin-dependent stimulus on phosphatase activity may, in these situations, cause upregulated EGFR signaling

and assist tumor progression. In the future, small molecules targeted for TCPTP activation may prove to be a potent therapy mechanism.

2. Novel anti-EGFR therapy resistance mechanism mediated by MDGI

For three decades, various members of the fatty acid-binding protein family have been under intensive investigation due to their ability to transfer fatty acids and other hydrophobic molecules, but studies have mainly focused on measuring ligand affinity for different FABPs or cellular energy metabolism linked to metabolic syndromes and obesity. Cytoplasmic and nuclear FABPs have been proposed to be involved in the uptake and transport of fatty acids between various intracellular compartments, modulation of local concentrations of fatty acids for enzymatic pathways, and protection of enzymes or membrane structures against the toxic effects of high intracellular levels of hydrophobic fatty acids. However, it has remained elusive as to why nine different members exist, sharing a similar three-dimensional β-barrel structure for the same function.

Our results clearly demonstrated that MDGI (also known as H-FABP) overexpression caused extensive EGFR-specific receptor internalization from the plasma membrane of MDA-MB-468 cells. EGFR is required for mammary gland differentiation, which has been evidenced by grafting intact neonatal mammary rudiments of EGFR knockout mice under the renal capsule of adult female nude mice (Sebastian et al., 1998). *EGFR*$^{-/-}$ mice die latest soon after the birth, but studies with these transplanted glands showed that EGFR-null neonates developed fewer mammary branches and shorter ductal trees compared to wild type littermates (Sebastian et al., 1998). High levels of EGF mRNA were detected throughout ductal morphogenesis and in midpregnant or lactating mice (Snedeker et al., 1991). Furthermore, phosphorylated EGFR was absent at the prepubertal stage in mice (Sebastian et al., 1998), but increased protein levels were detected during pregnancy (Edery et al., 1985). In addition, MDGI expression has been linked to mammary development, where MDGI mRNA was absent in virgin mammary tissue, but present in midpregnant or lactating bovine tissue (Kurtz et al., 1990). Consistent results were obtained from virgin mouse mammary glands that were originally MDGI-negative, but could be hormonally induced in whole organ mammary gland cultures to express MDGI mRNA (Yang et al., 1994). When antisense oligonucleotides complementary to MDGI were added on these hormonally induced whole organ cultures, antisense oligonucleotide-treated glands showed suppressed alveolar differentiation and decreased β-casein expression (Yang et al., 1994). Thus, both EGFR and MDGI seem to play an essential role during mammary gland ductal and branching morphogenesis and they appear to have some kind of temporal and/or spatial relationship. When hormonally induced virgin mammary whole gland cultures were treated with EGF, mRNA levels of MDGI were suppressed, but this could be reverted by adding recombinant MDGI (Binas et al., 1992; Yang et al., 1994). As previously mentioned, the MDGI sequence lacks the signal sequence for extracellular

secretion, so the biological relevance of these studies is unclear. In our studies done with a MDGI overexpressing breast cancer cell line, we were not able to see any differences in EGFR protein levels between the control and MDGI overexpressing cells. A drastic disparity was only observed in the localization of the EGFR receptor, because MDGI overexpressing cells had 36% less EGFR on their cell surface when cells were cultured on plastic and analyzed with FACS.

Since the plasma membrane is mainly composed of lipids and proteins held together by non-covalent interactions, this led us to consider whether MDGI, which is known to bind to free fatty acids and hydrophobic molecules, could modulate plasma membrane composition via a collisional mechanism due to its ability to transfer long-chain fatty acids. Composition of the inner leaflet in the lipid bilayer plays a critical role for raft formation, but lipid flip-flop from one half of the lipid bilayer to the other is a slow process. Lipid rafts are small, heterogenous, and highly dynamic specialized structures on the plasma membrane rich in cholesterol and sphingolipids. Rafts organize membranes into microdomains being far from randomly organized, and thus influence the distribution of membrane proteins as well. Based on extensive proteomic analyses of rafts from various sources, lipid rafts can be roughly subdivided into two classes: cholesterol-enriched rafts are involved in normal cell signaling and contain caveolin-1, Src, and EGFR, but rafts rich in ceramide are thought to trigger apoptosis due to the high presence of FAS/Ezrin (Patra, 2008).

Long-chain (n-3) polyunsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid, are known to be incorporated in plasma membranes both *in vitro* and *in vivo* (Fan et al., 2003). EGFR prefers to be concentrated in lipid rafts and treatment of MDA-MB-231 breast cancer cell cultures with eicosapentaenoic acid and docosahexaenoic acid caused a marked decrease in EGFR levels in lipid rafts (Schley et al., 2007). However, the whole-cell EGFR protein levels remained unaltered (Schley et al., 2007). In addition, lipid raft composition was altered in eicosapentaenoic acid and docosahexaenoic acid treated cells, so that cholesterol levels decreased, but ceramide levels increased, and this led to diminished cell proliferation compared to non-treated cells (Schley et al., 2007). EGFR dissociation from lipid rafts has been shown to coincide with ligand binding and receptor kinase activity (Abulrob et al., 2004). Thus, EGFR association with caveolin-1 decreased in EGF-stimulated glioblastoma cells, when the activated receptor moved out from the lipid rafts (Abulrob et al., 2004). In addition to receptor activation by ligand binding, simple cholesterol depletion by methyl-β-cyclodextrin can trigger EGFR lateral diffusion along the plasma membrane and cause ligand-independent receptor activation (Chen and Resh, 2002). Cholesterol depletion also impaired EGFR internalization (Pike and Casey, 2002). In contrast, a dose-dependent correlation was observed in prostate cancer cells between cholesterol depletion and apoptosis induction (Oh et al., 2007). Interestingly, apoptosis could be reverted in these cells by cholesterol addition (Oh et al., 2007). Cholesterol deposition has been detected in cancer cells and it has been implicated in tumor aggressiveness, especially in prostate cancer, where enriched cholesterol can serve as a substrate for *de novo* androgen synthesis (Freeman and Solomon, 2004).

Very recently, lipid raft targeted edelfosine, which represents a synthetic alkyl-lysophospholipid analog, was shown to be a very potent inducer of apoptosis in multiple myeloma cells (Mollinedo et al., 2010). Edelfosine accumulated almost completely in myeloma cells, sparing normal cells, and cholesterol was required for edelfosine uptake (Mollinedo et al., 2010). Taken together, several studies have reported that the lipid composition of plasma membranes alters cell signaling and tumor cells possess a transformed lipid composition.

Our results in MDGI overexpressing MDA-MB-468 cells indicated that pure MDGI overexpression can cause a significant EGFR accumulation in cytoplasm. Closer immunofluorescence studies in MDGI expressing cells revealed that caveolin-1 colocalizes with EGFR in the limiting membrane of internalized structures, but also EGFR and the late endosomal marker LAMP1 overlapped partially. Percoll gradient subcellular fractionation analysis showed that in MDGI expressing cells EGFR was located in fractions that were positive either for the early endosome marker (EEA1) or late endosome marker (Rab7). This suggests that EGFR intracellular accumulation can occur both via clathrin-dependent and caveolae-dependent pathways. However, we were not able to detect any changes in the caveolin-1 phosphorylation or in the downstream signaling pathways between the control and MDGI overexpressing cell lines. Since MDGI has been demonstrated to be capable of a collisional transfer mechanism of fatty acids to the acceptor membrane (Wootan and Storch, 1994), we suggest that MDGI alters the lipid order of the plasma membrane via modulation of the local polyunsaturated fatty acid/cholesterol ratio in the inner membrane leaflet and this leads to intracellular EGFR accumulation in MDGI overexpressing cells.

Because MDA-MB-468 breast cancer cells carry high EGFR amplification, it was a stroke of good fortune that this cell line was selected for the analysis of MDGI function. In the early stages of these experiments, we had no idea of the link between MDGI and EGFR, and we might have missed the EGFR cytoplasmic accumulation in cells with lower EGFR protein levels. MDA-MB-468 cells also harbor wild type KRAS and PI3K that makes them sensitive to monoclonal antibody therapy against the EGFR ectodomain (cetuximab). Potential mechanisms of resistance to EGFR-targeted therapy include EGFR amplification, receptor mutations, or activation of other signaling mechanisms either laterally or downstream from the receptors. Known resistance mechanisms to cetuximab therapy are KRAS mutation status (Lievre et al., 2006), mutations in PI3K (Sartore-Bianchi et al., 2009), mutations in BRAF (Di Nicolantonio et al., 2008), mutations in the Fc receptor expressed in immune cells (Kurai et al., 2007), increased VEGF production (Viloria-Petit et al., 2001), increased Src signaling (Lu et al., 2007b; Wheeler et al., 2009), increased ubiquitination and further degradation of EGFR (Lu et al., 2007b), dysregulation of EGFR internalization/degradation (Wheeler et al., 2008), and nuclear localization of EGFR (Li et al., 2009). Activated Src can cause acquired resistance to cetuximab therapy via mediating the nuclear translocation of the EGFR (Li et al., 2009) or enhancing activation of HER3 and PI3K (Wheeler et al., 2009). The EGFR variant III (EGFRvIII) lacks the ligand-binding domain and is documented to be expressed in 42% of

squamous cell carcinomas of the head and neck, thereby contributing to the cetuximab resistance (Sok et al., 2006). Thus, we describe here a novel mechanism for cetuximab resistance, where MDGI overexpression causes a potent EGFR internalization from the plasma membrane. Even more importantly, MDGI might be exploited to identify patients who achieve a response to a targeted EGFR therapy.

MDGI has been associated with EGFR TKI gefitinib response in proteomic signature analysis between responders and nonresponders in lung adenocarcinoma (Okano et al., 2007). Authors have validated these results by measuring MDGI levels with enzyme-linked immunosorbent assays (ELISAs) from tumors of 55 lung adenocarcinoma patients and, indeed, patients with stable or progressive disease had lower levels of MDGI compared to patients showing complete or partial response (Okano et al., 2007). These results are in agreement with our studies, because MDGI overexpressing cells remained sensitive to tyrosine kinase inhibitor AG1478 and, in addition, retained MDGI expression in breast cancer patients correlated with better survival. In contrast, elevated levels of E-FABP could be found in gefitinib-resistant colorectal cancer cell lines according to proteome profiling (Loeffler-Ragg et al., 2005). Increased E-FABP levels were also detected in chemoresistant pancreatic adenocarcinoma cell lines (Sinha et al., 1999). This suggests that relative expression levels of various FABP family members may vary during tumor progression and that other FABPs may also contribute to drug sensitivity.

3. Retained MDGI expression promotes distant disease-free survival in breast cancer

Despite the fact that FABPs have been demonstrated to interact electrostatically with membranes, characterized protein–protein interactions are diminutive. Only physical association has been described between A-FABP and the hormone-sensitive lipase (HSL) (Smith et al., 2004). Our results show that MDGI associates with several integrin cytoplasmic α-subunits maintaining integrin in its inactive conformation, and thereby provoking diminished integrin-mediated adhesion, migration, and invasion. In addition, our results indicated that sustained MDGI expression in breast cancer patients correlated with increased 10-year distant disease-free survival. This is in agreement with our MDGI mRNA expression data, where MDGI mRNA levels are inversely correlated with the histological grade of lung cancer (II: Fig 1e), and colon cancer tissue expresses lower MDGI mRNA levels compared to normal colon tissue (II: Suppl 6). Thus, these results argue for MDGI's function as a tumor suppressor that had been speculated to be the case after the MDGI identification from lactating mammary tissue (Huynh et al., 1995).

Knockout models of various FABPs have shown that the loss of a particular FABP protein is often compensated for by the overexpression of another member of this gene family. As mentioned earlier, MDGI and E-FABP are the most ubiquitously expressed FABP members. The mammary gland is reported to express A-FABP and B-FABP as

well, but to a lesser extent. Because our results suggest that expression of MDGI is diminished in several cancer types including breast cancer, levels of other FABP members might be increased. Enhanced B-FABP levels are associated with neuronal migration during brain development, but some glioblastomas and astrocytomas express high B-FABP as well. Immunohistochemical staining of 123 glioblastomas showed that high cytoplasmic B-FABP expression had a borderline negative correlation with survival (Kaloshi et al., 2007). Interestingly, nuclear B-FABP staining was strongly associated with EGFR amplification and more invasive tumors when compared to tumors showing pure cytoplasmic staining (Kaloshi et al., 2007). Conversely, depletion of B-FABP expression in malignant glioma cells resulted in decreased cell motility, migration, and invasion (Mita et al., 2007). B-FABP-depleted cells showed a lack of extended protrusions that were typical for the parental cell line (Mita et al., 2007). In addition, immunofluorescence studies with the U251 glioma cell line, which shows high B-FABP endogenous expression, revealed concentrated B-FABP levels at the leading edge (Mita et al., 2007). This was further confirmed with immunohistochemical staining of grade IV astrocytomas, where strong B-FABP expression was observed in highly infiltrative regions of the tumor (Mita et al., 2007). Very recently, immunohistochemical studies showed that B-FABP is strongly overexpressed in triple-negative (negative for receptors of estrogen, progesterone, and HER2) breast cancers (Tang et al., 2010). Because our stainings demonstrated a significant correlation between retained MDGI expression and survival in triple-negative cases, one might speculate that MDGI is able to outcompete the B-FABP function at least partially, and their relative expression levels define the final outcome. Thus, MDGI and B-FABP might mechanistically regulate each other. Triple-negative cases are clinically challenging due to the limited number of available targets for therapies, because anti-estrogen therapy and HER2-based therapy are ruled out. A corresponding situation has been described in renal cell carcinoma where B-FABP has been shown to be very low in normal tissue, but expression is significantly upregulated in cancer and L-FABP shows an inverse relationship, having its strongest expression in normal tissue (Tölle et al., 2009). In addition to B-FABP, elevated E-FABP mRNA and protein levels have been identified both in prostate and breast carcinoma cell lines (Jing et al., 2000). When the nonmetastatic rat mammary epithelial cell line, Rama 37, was transfected with an E-FABP expression construct and cells were inoculated into female rats, 23% of E-FABP expressing tumors developed metastases compared to fully nonmetastatic parental cells (Jing et al., 2000). Subsequent studies revealed that E-FABP induced metastasis formation through stimulating endogenous VEGF levels (Jing et al., 2001).

The above-mentioned results are in agreement with our hypothesis that the expression levels of other FABP family members might change due to primary alterations in MDGI levels. The strongest candidates for mammary tissue tumor progression are upregulated B-FABP and E-FABP levels, which have been linked to poor prognosis. However, our hypothesis needs more experimental support and future work will involve systematic characterization of the expression profiles of different FABPs in various tissues originating both from normal and neoplastic sources.

4. The role of TCPTP and MDGI in therapy

The tumor microenvironment plays a critical role in cancer progression. Very recently, activation of β1-integrin signaling has been shown to induce metastatic growth of solitary dormant tumor cells (Barkan et al., 2010). Type I collagen enrichment in the microenvironment triggered a switch from the quiescent state to proliferative disease and caused upregulated downstream signaling through integrin-mediated phosphorylation of Src and ERK (Barkan et al., 2010). Additionally, gene expression profiling of the stromal microenvironment has shown that upregulated fibronectin and collagen levels correlate with disease progression in breast cancer patients (Ma et al., 2009). Thus, inhibition of integrin-mediated survival signals seems to be a very potent target for clinical therapies. Synergistic coregulation of EGFR and integrin signaling pathways has been documented earlier (Moro et al., 1998). Furthermore, EGFR has been shown to form a complex with β1- and β3-integrins through Src (Moro et al., 2002). However, the detailed molecular mechanism of synergism in downstream signaling has remained speculative. Here we have described a novel mechanism where the α1-integrin cytoplasmic tail is capable of stimulating the catalytic activity of TCPTP, thereby attenuating EGFR signaling. In the future, specific TCPTP activators could show their potency in clinical settings. A localized, controlled balance between phosphorylation and dephosphorylation plays a critical role in cellular signaling, where even minor local perturbations might cause major overall effects.

Based on epidemiological studies, abnormal energy regulation may significantly contribute to the pathogenesis of obesity, diabetes mellitus, cardiovascular disease, and cancer (Biro and Wien, 2010). Furthermore, cholesterol-lowering drugs called statins, which inhibit the rate-limiting enzyme HMG-CoA reductase in cholesterol synthesis, have for years been under intensive speculation due to the lowered cancer incidence and tumor progression exhibited in some studies (Murtola et al., 2010). Interestingly, simvastatin has been shown to induce L-FABP levels in a dose-dependent manner in rat hepatocytes, suggesting that statins have additional elusive mechanisms besides cholesterol synthesis inhibition (Landrier et al., 2004). High fat diets have been observed to increase A-FABP and L-FABP levels (Veerkamp and van Moerkerk, 1993). On the contrary, L-FABP-null mice showed increased age-dependent weight gain, fat tissue mass, and lean tissue mass (Martin et al., 2009). When serum A-FABP levels were measured from 200 patients attending for breast surgery, A-FABP levels were significantly higher in obese patients (Hancke et al., 2010). Additionally, A-FABP levels were significantly higher in breast cancer patients irrespective of obesity (Hancke et al., 2010). In nude mice, a diet supplemented with eicosapentaenoic acid and docosahexaenoic acid caused primary tumor retardation and a decrease in the occurrence of lung metastases in breast cancer models (Rose et al., 1995). Thus, fat content and type in diet have several mechanisms of action.

We have demonstrated here that overexpression of MDGI, a small fatty acid-binding protein, in breast cancer cells harboring EGFR amplification caused a novel resistance mechanism for EGFR therapy *in vivo* through enhanced EGFR cytoplasmic

accumulation. In addition, retained MDGI expression in breast cancer patients correlated with an increased 10-year distant disease-free survival. We speculate that, in addition to interacting directly with several cytoplasmic integrin α-tails, MDGI could modulate local distribution of lipids on the plasma membrane. Altered membrane fluidity may directly activate cell signaling events or modulate local distribution of plasma membrane receptors mediating signaling processes. A regulated balance between the expression levels of different FABP family members may prove to be critical for cancer progression. In the future, MDGI could be used as a predictive marker for responsiveness to anti-EGFR antibody therapy in clinical settings. It is noteworthy that the majority of studies concerning both integrins and FABPs have been carried out, for simplicity, in cell culture plastic that does not represent the natural microenvironment for either. Most breast cancer cell lines also show a lack of MDGI expression in culture conditions due to hypermethylation. A likely scenario is the systematic use of 3D cell culture models and *in vivo* models to improve our understanding of the complex cell signaling networks.

SUMMARY AND CONCLUSIONS

Membrane-traversing integrins are cell adhesion receptors pivotal for cell growth, differentiation, survival, and migration. Integrins are heterodimeric receptors consisting of an α- and β-subunit held together in a bent conformation, when integrin is said to be inactive, but adopting an extended conformation followed by separation of subunits during receptor activation. What makes integrins exceptional among other transmembrane receptors is their activation, that can be triggered either by extracellular ligand binding (outside-in signaling), or by intracellular molecule association (inside-out signaling). Because various extracellular ligands for integrins have already been identified, comprehensive studies have focused on recognizing intracellular molecules capable of modulating integrin activity. Despite a vast number of molecules identified in the integrin adhesome (Zaidel-Bar et al., 2007), characterized binding partners for integrin cytoplasmic α-domains have remained diminutive.

In this study, we have identified two novel binding partners for integrin cytoplasmic α-domains. TCPTP is a ubiquitously expressed non-receptor protein tyrosine phosphatase binding specifically to the non-conserved cytoplasmic sequence of α1-integrin, whereas a small fatty acid-binding protein, MDGI, prefers interaction with several α-subunits, suggesting an association via a conserved GFFKR sequence shared between integrin α-subunits. In addition, the biological consequences of these described interactions were studied. TCPTP binding to the α1-integrin cytoplasmic domain was sufficient to induce potent TCPTP phosphatase activity leading to attenuated EGFR phosphorylation and inhibition of malignant anchorage-independent growth. These results describe a novel mechanism as to how integrin-mediated adhesion can directly negatively regulate EGFR signaling via TCPTP activation. Thus, activators of TCPTP could be efficient drugs for clinical use. Pure MDGI overexpression in a breast cancer cell line caused significant EGFR accumulation into the cytoplasm, illustrating a novel resistance mechanism for anti-EGFR therapy. MDGI association with integrin cytoplasmic α-domains restrained the integrin in its inactive conformation and decreased integrin-mediated cell adhesion, migration, and invasion. In agreement with these results, retained MDGI expression in breast cancer patients correlated with an increased 10-year distant disease-free survival. Together, these results suggest that MDGI expression might be exploited to define potential responders for anti-EGFR therapy or to predict overall survival rates.

It is becoming increasingly apparent that the integrin α-subunit plays a coregulatory, active role in various cell signaling processes instead of just in passive pairing. In the future, this may help improve targeted therapeutic strategies against integrin heterodimers due to the fact that the currently known 24 integrin heterodimers comprise 18 different α-subunits and 8 β-subunits. More accurate therapies are needed to fulfill the needs of personalized medicine.

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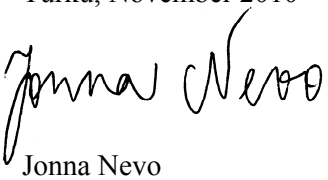

Jonna Nevo

REFERENCES

Abd El-Rehim DM, Pinder SE, Paish CE, Bell JA, Rampaul RS, Blamey RW *et al*. (2004). Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. *Br J Cancer* 91: 1532-1542.

Abulrob A, Giuseppin S, Andrade MF, McDermid A, Moreno M, Stanimirovic D. (2004). Interactions of EGFR and caveolin-1 in human glioblastoma cells: Evidence that tyrosine phosphorylation regulates EGFR association with caveolae. *Oncogene* 23: 6967-6979.

Adachi M, Taki T, Higashiyama M, Kohno N, Inufusa H, Miyake M. (2000). Significance of integrin alpha5 gene expression as a prognostic factor in node-negative non-small cell lung cancer. *Clin Cancer Res* 6: 96-101.

Adair BD, Xiong JP, Maddock C, Goodman SL, Arnaout MA, Yeager M. (2005). Three-dimensional EM structure of the ectodomain of integrin {alpha}V{beta}3 in a complex with fibronectin. *J Cell Biol* 168: 1109-1118.

Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI *et al*. (2002). Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* 2: 127-137.

Ahmed N, Riley C, Rice GE, Quinn MA, Baker MS. (2002). Alpha(v)beta(6) integrin-A marker for the malignant potential of epithelial ovarian cancer. *J Histochem Cytochem* 50: 1371-1380.

Aifa S, Aydin J, Nordvall G, Lundström I, Svensson SP, Hermanson O. (2005). A basic peptide within the juxtamembrane region is required for EGF receptor dimerization. *Exp Cell Res* 302: 108-114.

Albert JM, Cao C, Geng L, Leavitt L, Hallahan DE, Lu B. (2006). Integrin alpha v beta 3 antagonist cilengitide enhances efficacy of radiotherapy in endothelial cell and non-small-cell lung cancer models. *Int J Radiat Oncol Biol Phys* 65: 1536-1543.

Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A *et al*. (2004). Protein tyrosine phosphatases in the human genome. *Cell* 117: 699-711.

Amri EZ, Bertrand B, Ailhaud G, Grimaldi P. (1991). Regulation of adipose cell differentiation. I. fatty acids are inducers of the aP2 gene expression. *J Lipid Res* 32: 1449-1456.

Andersen JN, Jansen PG, Echwald SM, Mortensen OH, Fukada T, Del Vecchio R *et al*. (2004). A genomic perspective on protein tyrosine phosphatases: Gene structure, pseudogenes, and genetic disease linkage. *FASEB J* 18: 8-30.

Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH *et al*. (2001). Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol Cell Biol* 21: 7117-7136.

Anthis NJ, Haling JR, Oxley CL, Memo M, Wegener KL, Lim CJ *et al*. (2009). Beta integrin tyrosine phosphorylation is a conserved mechanism for regulating talin-induced integrin activation. *J Biol Chem* 284: 36700-36710.

Aoki N, Matsuda T. (2002). A nuclear protein tyrosine phosphatase TC-PTP is a potential negative regulator of the PRL-mediated signaling pathway: Dephosphorylation and deactivation of signal transducer and activator of transcription 5a and 5b by TC-PTP in nucleus. *Mol Endocrinol* 16: 58-69.

Aqeilan RI, Donati V, Gaudio E, Nicoloso MS, Sundvall M, Korhonen A *et al*. (2007). Association of wwox with ErbB4 in breast cancer. *Cancer Res* 67: 9330-9336.

Ardini E, Agresti R, Tagliabue E, Greco M, Aiello P, Yang LT *et al*. (2000). Expression of protein tyrosine phosphatase alpha (RPTPalpha) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth in vitro and in vivo. *Oncogene* 19: 4979-4987.

Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ. (2003). Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A* 100: 13298-13302.

Arregui CO, Balsamo J, Lilien J. (1998). Impaired integrin-mediated adhesion and signaling in fibroblasts expressing a dominant-negative mutant PTP1B. *J Cell Biol* 143: 861-873.

Baiocchi G, Lopes A, Coudry RA, Rossi BM, Soares FA, Aguiar S *et al*. (2009). ErbB family immunohistochemical expression in colorectal cancer patients with higher risk of recurrence after radical surgery. *Int J Colorectal Dis* 24: 1059-1068.

Barczyk M, Carracedo S, Gullberg D. (2010). Integrins. *Cell Tissue Res* 339: 269-280.

Bargmann CI, Weinberg RA. (1988). Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. *Proc Natl Acad Sci U S A* 85: 5394-5398.

Barkan D, El Touny LH, Michalowski AM, Smith JA, Chu I, Davis AS *et al*. (2010). Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. *Cancer Res* 70: 5706-5716.

Baronas-Lowell D, Lauer-Fields JL, Borgia JA, Sferrazza GF, Al-Ghoul M, Minond D *et al*. (2004). Differential modulation of human melanoma cell metalloproteinase expression by alpha2beta1 integrin and CD44 triple-helical ligands derived from type IV collagen. *J Biol Chem* 279: 43503-43513.

Baselga J, Arteaga CL. (2005). Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* 23: 2445-2459.

Bates RC, Bellovin DI, Brown C, Maynard E, Wu B, Kawakatsu H *et al*. (2005). Transcriptional activation of integrin beta6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *J Clin Invest* 115: 339-347.

Bathia DP, Carless DR, Viswanathan K, Hall AS, Barth JH. (2009). Serum 99th centile values for two heart-type fatty acid binding protein assays. *Ann Clin Biochem* 46: 464-467.

Bauknecht T, Kohler M, Janz I, Pfleiderer A. (1989). The occurrence of epidermal growth factor receptors and the characterization of EGF-like factors in human ovarian, endometrial, cervical and breast cancer. EGF receptors and factors in gynecological carcinomas. *J Cancer Res Clin Oncol* 115: 193-199.

Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G. (1996). All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem* 271: 5251-5257.

Bello L, Francolini M, Marthyn P, Zhang J, Carroll RS, Nikas DC *et al*. (2001). Alpha(v)beta3 and alpha(v)beta5 integrin expression in glioma periphery. *Neurosurgery* 49: 380-9; discussion 390.

Bengtsson T, Aszodi A, Nicolae C, Hunziker EB, Lundgren-Akerlund E, Fässler R. (2005). Loss of alpha10beta1 integrin expression leads to moderate dysfunction of growth plate chondrocytes. *J Cell Sci* 118: 929-936.

Bennett JH, Shousha S, Puddle B, Athanasou NA. (1995). Immunohistochemical identification of tumours of adipocytic differentiation using an antibody to aP2 protein. *J Clin Pathol* 48: 950-954.

Bhaskar V, Fox M, Breinberg D, Wong MH, Wales PE, Rhodes S *et al*. (2008). Volociximab, a chimeric integrin alpha5beta1 antibody, inhibits the growth of VX2 tumors in rabbits. *Invest New Drugs* 26: 7-12.

Binas B, Danneberg H, McWhir J, Mullins L, Clark AJ. (1999). Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. *FASEB J* 13: 805-812.

Binas B, Spitzer E, Zschiesche W, Erdmann B, Kurtz A, Müller T *et al*. (1992). Hormonal induction of functional differentiation and mammary-derived growth inhibitor expression in cultured mouse mammary gland explants. *In Vitro Cell Dev Biol* 28A: 625-634.

Birge RB, Kalodimos C, Inagaki F, Tanaka S. (2009). Crk and CrkL adaptor proteins: Networks for physiological and pathological signaling. *Cell Commun Signal* 7: 13.

Biro FM, Wien M. (2010). Childhood obesity and adult morbidities. *Am J Clin Nutr* 91: 1499S-1505S.

Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M *et al*. (2010). Randomized study of lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol* 28: 1124-1130.

Blagoev B, Ong SE, Kratchmarova I, Mann M. (2004). Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat Biotechnol* 22: 1139-1145.

Blanchetot C, Tertoolen LG, Overvoorde J, den Hertog J. (2002). Intra- and intermolecular interactions between intracellular domains of receptor protein-tyrosine phosphatases. *J Biol Chem* 277: 47263-47269.

Boeri Erba E, Bergatto E, Cabodi S, Silengo L, Tarone G, Defilippi P *et al*. (2005). Systematic analysis of the epidermal growth factor receptor by mass spectrometry reveals stimulation-dependent multisite phosphorylation. *Mol Cell Proteomics* 4: 1107-1121.

Bose R, Zhang X. (2009). The ErbB kinase domain: Structural perspectives into kinase activation and inhibition. *Exp Cell Res* 315: 649-658.

Boukerche H, Benchaibi M, Berthier-Vergnes O, Lizard G, Bailly M, Bailly M *et al*. (1994). Two human melanoma cell-line variants with enhanced in vivo tumor growth and metastatic capacity do not express the beta 3 integrin subunit. *Eur J Biochem* 220: 485-491.

Bradley DA, Daignault S, Ryan CJ, Dipaola RS, Smith DC, Small E *et al*. (2010). Cilengitide (EMD 121974, NSC 707544) in asymptomatic metastatic castration resistant prostate cancer patients: A randomized phase II trial by the prostate cancer clinical trials consortium. *Invest New Drugs* (DOI: 10.1007/s10637-010-9420-8).

Bretscher MS. (1996). Getting membrane flow and the cytoskeleton to cooperate in moving cells. *Cell* 87: 601-606.

Brooks PC, Strömblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG *et al*. (1996). Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 85: 683-693.

Brouard MC, Saurat JH, Ghanem G, Siegenthaler G. (2002). Urinary excretion of epidermal-type fatty acid-binding protein and S100A7 protein in patients with cutaneous melanoma. *Melanoma Res* 12: 627-631.

Buck E, Eyzaguirre A, Rosenfeld-Franklin M, Thomson S, Mulvihill M, Barr S *et al*. (2008). Feedback mechanisms promote cooperativity for small molecule inhibitors of epidermal and insulin-like growth factor receptors. *Cancer Res* 68: 8322-8332.

Burstein HJ, Sun Y, Dirix LY, Jiang Z, Paridaens R, Tan AR *et al*. (2010). Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer. *J Clin Oncol* 28: 1301-1307.

Butler B, Gao C, Mersich AT, Blystone SD. (2006). Purified integrin adhesion complexes exhibit actin-polymerization activity. *Curr Biol* 16: 242-251.

Böhmer FD, Kraft R, Otto A, Wernstedt C, Hellman U, Kurtz A *et al*. (1987). Identification of a polypeptide growth inhibitor from bovine mammary

gland. sequence homology to fatty acid- and retinoid-binding proteins. *J Biol Chem* 262: 15137-15143.

Calderwood DA, Huttenlocher A, Kiosses WB, Rose DM, Woodside DG, Schwartz MA *et al*. (2001). Increased filamin binding to beta-integrin cytoplasmic domains inhibits cell migration. *Nat Cell Biol* 3: 1060-1068.

Calderwood DA, Yan B, de Pereda JM, Alvarez BG, Fujioka Y, Liddington RC *et al*. (2002). The phosphotyrosine binding-like domain of talin activates integrins. *J Biol Chem* 277: 21749-21758.

Carpenter G, Lembach KJ, Morrison MM, Cohen S. (1975). Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. *J Biol Chem* 250: 4297-4304.

Carrell NA, Fitzgerald LA, Steiner B, Erickson HP, Phillips DR. (1985). Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. *J Biol Chem* 260: 1743-1749.

Carroll SL, Roth KA, Gordon JI. (1990). Liver fatty acid-binding protein: A marker for studying cellular differentiation in gut epithelial neoplasms. *Gastroenterology* 99: 1727-1735.

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL *et al*. (1992). Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A* 89: 4285-4289.

Caswell PT, Chan M, Lindsay AJ, McCaffrey MW, Boettiger D, Norman JC. (2008). Rab-coupling protein coordinates recycling of alpha5beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. *J Cell Biol* 183: 143-155.

Celis JE, Ostergaard M, Basse B, Celis A, Lauridsen JB, Ratz GP *et al*. (1996). Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. *Cancer Res* 56: 4782-4790.

Chang HR. (2010). Trastuzumab-based neoadjuvant therapy in patients with HER2-positive breast cancer. *Cancer* 116: 2856-2867.

Chattopadhyay A, Vecchi M, Ji Q, Mernaugh R, Carpenter G. (1999). The role of individual SH2 domains in mediating association of phospholipase C-gamma1 with the activated EGF receptor. *J Biol Chem* 274: 26091-26097.

Chau NM, Ashcroft M. (2004). Akt2: A role in breast cancer metastasis. *Breast Cancer Res* 6: 55-57.

Chen J, Diacovo TG, Grenache DG, Santoro SA, Zutter MM. (2002). The alpha(2) integrin subunit-deficient mouse: A multifaceted phenotype including defects of branching morphogenesis and hemostasis. *Am J Pathol* 161: 337-344.

Chen J, Salas A, Springer TA. (2003). Bistable regulation of integrin adhesiveness by a bipolar metal ion cluster. *Nat Struct Biol* 10: 995-1001.

Chen J, Yang W, Kim M, Carman CV, Springer TA. (2006). Regulation of outside-in signaling and affinity by the beta2 I domain of integrin alphaLbeta2. *Proc Natl Acad Sci U S A* 103: 13062-13067.

Chen LM, Bailey D, Fernandez-Valle C. (2000). Association of beta 1 integrin with focal adhesion kinase and paxillin in differentiating schwann cells. *J Neurosci* 20: 3776-3784.

Chen X, Resh MD. (2002). Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor. *J Biol Chem* 277: 49631-49637.

Cheon MS, Kim SH, Fountoulakis M, Lubec G. (2003). Heart type fatty acid binding protein (H-FABP) is decreased in brains of patients with down syndrome and alzheimer's disease. *J Neural Transm Suppl* (67): 225-234.

Choi CK, Vicente-Manzanares M, Zareno J, Whitmore LA, Mogilner A, Horwitz AR. (2008). Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol* 10: 1039-1050.

Cianfrocca ME, Kimmel KA, Gallo J, Cardoso T, Brown MM, Hudes G *et al*. (2006). Phase 1 trial of the antiangiogenic peptide ATN-161 (ac-PHSCN-NH(2)), a beta integrin antagonist, in patients with solid tumours. *Br J Cancer* 94: 1621-1626.

Ciardiello F, Bianco R, Caputo R, Caputo R, Damiano V, Troiani T *et al*. (2004). Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin Cancer Res* 10: 784-793.

Cistola DP, Sacchettini JC, Banaszak LJ, Walsh MT, Gordon JI. (1989). Fatty acid interactions with rat intestinal and liver fatty acid-binding proteins expressed in escherichia coli. A comparative 13C NMR study. *J Biol Chem* 264: 2700-2710.

Citri A, Skaria KB, Yarden Y. (2003). The deaf and the dumb: The biology of ErbB-2 and ErbB-3. *Exp Cell Res* 284: 54-65.

Clark AJ, Neil C, Gusterson B, McWhir J, Binas B. (2000). Deletion of the gene encoding H-FABP/MDGI has no overt effects in the mammary gland. *Transgenic Res* 9: 439-444.

Cohen M. (2009). Antiplatelet therapy in percutaneous coronary intervention: A critical review of the 2007 AHA/ACC/SCAI guidelines and beyond. *Catheter Cardiovasc Interv* 74: 579-597.

Cohen P. (2001). The role of protein phosphorylation in human health and disease. the sir hans krebs medal lecture. *Eur J Biochem* 268: 5001-5010.

Cohen S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 237: 1555-1562.

Cohen S, Carpenter G. (1975). Human epidermal growth factor: Isolation and chemical and biological

properties. *Proc Natl Acad Sci U S A* 72: 1317-1321.

Cohen S, Levi-Montalcini R. (1957). Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180. *Cancer Res* 17: 15-20.

Cool DE, Tonks NK, Charbonneau H, Walsh KA, Fischer EH, Krebs EG. (1989). cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. *Proc Natl Acad Sci U S A* 86: 5257-5261.

Coppolino M, Leung-Hagesteijn C, Dedhar S, Wilkins J. (1995). Inducible interaction of integrin alpha 2 beta 1 with calreticulin. dependence on the activation state of the integrin. *J Biol Chem* 270: 23132-23138.

Coppolino MG, Woodside MJ, Demaurex N, Grinstein S, St-Arnaud R, Dedhar S. (1997). Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* 386: 843-847.

Dadke SS, Li HC, Kusari AB, Begum N, Kusari J. (2000). Elevated expression and activity of protein-tyrosine phosphatase 1B in skeletal muscle of insulin-resistant type II diabetic goto-kakizaki rats. *Biochem Biophys Res Commun* 274: 583-589.

Davidson NO, Ifkovits CA, Skarosi SF, Hausman AM, Llor X, Sitrin MD *et al*. (1993). Tissue and cell-specific patterns of expression of rat liver and intestinal fatty acid binding protein during development and in experimental colonic and small intestinal adenocarcinomas. *Lab Invest* 68: 663-675.

Dawson JP, Berger MB, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. (2005). Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. *Mol Cell Biol* 25: 7734-7742.

DeMali KA, Wennerberg K, Burridge K. (2003). Integrin signaling to the actin cytoskeleton. *Curr Opin Cell Biol* 15: 572-582.

den Hertog J, Östman A, Böhmer FD. (2008). Protein tyrosine phosphatases: Regulatory mechanisms. *FEBS J* 275: 831-847.

Di Guglielmo GM, Baass PC, Ou WJ, Posner BI, Bergeron JJ. (1994). Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of raf-1 by EGF but not insulin in liver parenchyma. *EMBO J* 13: 4269-4277.

Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P *et al*. (2008). Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 26: 5705-5712.

Di Paola R, Frittitta L, Miscio G, Bozzali M, Baratta R, Centra M *et al*. (2002). A variation in 3' UTR of hPTP1B increases specific gene expression and associates with insulin resistance. *Am J Hum Genet* 70: 806-812.

Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K *et al*. (2008). Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 455: 1069-1075.

Dumin JA, Dickeson SK, Stricker TP, Bhattacharyya-Pakrasi M, Roby JD, Santoro SA *et al*. (2001). Pro-collagenase-1 (matrix metalloproteinase-1) binds the alpha(2)beta(1) integrin upon release from keratinocytes migrating on type I collagen. *J Biol Chem* 276: 29368-29374.

Echwald SM, Bach H, Vestergaard H, Richelsen B, Kristensen K, Drivsholm T *et al*. (2002). A P387L variant in protein tyrosine phosphatase-1B (PTP-1B) is associated with type 2 diabetes and impaired serine phosphorylation of PTP-1B in vitro. *Diabetes* 51: 1-6.

Edelson BT, Li Z, Pappan LK, Zutter MM. (2004). Mast cell-mediated inflammatory responses require the alpha 2 beta 1 integrin. *Blood* 103: 2214-2220.

Eden ER, White IJ, Futter CE. (2009). Down-regulation of epidermal growth factor receptor signalling within multivesicular bodies. *Biochem Soc Trans* 37: 173-177.

Edery M, Pang K, Larson L, Colosi T, Nandi S. (1985). Epidermal growth factor receptor levels in mouse mammary glands in various physiological states. *Endocrinology* 117: 405-411.

Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL *et al*. (1999). Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 283: 1544-1548.

Elenius K, Choi CJ, Paul S, Santiestevan E, Nishi E, Klagsbrun M. (1999). Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase. *Oncogene* 18: 2607-2615.

Elenius K, Corfas G, Paul S, Choi CJ, Rio C, Plowman GD *et al*. (1997). A novel juxtamembrane domain isoform of HER4/ErbB4. isoform-specific tissue distribution and differential processing in response to phorbol ester. *J Biol Chem* 272: 26761-26768.

Engelman JA, Jänne PA. (2008). Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* 14: 2895-2899.

Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO *et al*. (2007). MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 316: 1039-1043.

Falls DL. (2003). Neuregulins: Functions, forms, and signaling strategies. *Exp Cell Res* 284: 14-30.

Fan YY, McMurray DN, Ly LH, Chapkin RS. (2003). Dietary (n-3) polyunsaturated fatty acids remodel mouse T-cell lipid rafts. *J Nutr* 133: 1913-1920.

Ferguson KM, Berger MB, Mendrola JM, Cho HS, Leahy DJ, Lemmon MA. (2003). EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Mol Cell* 11: 507-517.

Frampton JE, Plosker GL. (2009). Efalizumab: A review of its use in the management of chronic moderate-to-severe plaque psoriasis. *Am J Clin Dermatol* 10: 51-72.

Franco SJ, Rodgers MA, Perrin BJ, Han J, Bennin DA, Critchley DR *et al*. (2004). Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol* 6: 977-983.

Freeman MR, Solomon KR. (2004). Cholesterol and prostate cancer. *J Cell Biochem* 91: 54-69.

French AR, Tadaki DK, Niyogi SK, Lauffenburger DA. (1995). Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. *J Biol Chem* 270: 4334-4340.

Friedl P, Maaser K, Klein CE, Niggemann B, Krohne G, Zänker KS. (1997). Migration of highly aggressive MV3 melanoma cells in 3-dimensional collagen lattices results in local matrix reorganization and shedding of alpha2 and beta1 integrins and CD44. *Cancer Res* 57: 2061-2070.

Friedl P, Wolf K. (2003). Tumour-cell invasion and migration: Diversity and escape mechanisms. *Nat Rev Cancer* 3: 362-374.

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA. (1995). Definition of two angiogenic pathways by distinct alpha v integrins. *Science* 270: 1500-1502.

Friedrichs K, Ruiz P, Franke F, Gille I, Terpe HJ, Imhof BA. (1995). High expression level of alpha 6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res* 55: 901-906.

Fässler R, Meyer M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev* 9: 1896-1908.

Gahmberg CG, Fagerholm SC, Nurmi SM, Chavakis T, Marchesan S, Grönholm M. (2009). Regulation of integrin activity and signalling. *Biochim Biophys Acta* 1790: 431-444.

Gálvez BG, Matías-Román S, Yáñez-Mó M, Sánchez-Madrid F, Arroyo AG. (2002). ECM regulates MT1-MMP localization with beta1 or alphavbeta3 integrins at distinct cell compartments modulating its internalization and activity on human endothelial cells. *J Cell Biol* 159: 509-521.

Gardner H, Kreidberg J, Koteliansky V, Jaenisch R. (1996). Deletion of integrin alpha 1 by homologous recombination permits normal murine development but gives rise to a specific deficit in cell adhesion. *Dev Biol* 175: 301-313.

Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO *et al*. (2003). The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* 11: 495-505.

Gericke A, Smith ER, Moore DJ, Mendelsohn R, Storch J. (1997). Adipocyte fatty acid-binding protein: Interaction with phospholipid membranes and thermal stability studied by FTIR spectroscopy. *Biochemistry* 36: 8311-8317.

Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T *et al*. (2006). Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med* 355: 2733-2743.

Gilmore AP, Burridge K. (1996). Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. *Nature* 381: 531-535.

Gimbrone MA,Jr, Leapman SB, Cotran RS, Folkman J. (1972). Tumor dormancy in vivo by prevention of neovascularization. *J Exp Med* 136: 261-276.

Gimona M, Buccione R, Courtneidge SA, Linder S. (2008). Assembly and biological role of podosomes and invadopodia. *Curr Opin Cell Biol* 20: 235-241.

Goksoy E, Ma YQ, Wang X, Kong X, Perera D, Plow EF *et al*. (2008). Structural basis for the autoinhibition of talin in regulating integrin activation. *Mol Cell* 31: 124-133.

Gruber G, Hess J, Stiefel C, Aebersold DM, Zimmer Y, Greiner RH *et al*. (2005). Correlation between the tumoral expression of beta3-integrin and outcome in cervical cancer patients who had undergone radiotherapy. *Br J Cancer* 92: 41-46.

Guo W, Pylayeva Y, Pepe A, Yoshioka T, Muller WJ, Inghirami G *et al*. (2006). Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* 126: 489-502.

Gushiken FC, Patel V, Liu Y, Pradhan S, Bergeron AL, Peng Y *et al*. (2008). Protein phosphatase 2A negatively regulates integrin alpha(IIb)beta(3) signaling. *J Biol Chem* 283: 12862-12869.

Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL,3rd. (1994). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A* 91: 8132-8136.

Hambley TW, Hait WN. (2009). Is anticancer drug development heading in the right direction? *Cancer Res* 69: 1259-1262.

Hanada N, Lo HW, Day CP, Pan Y, Nakajima Y, Hung MC. (2006). Co-regulation of B-myb expression by E2F1 and EGF receptor. *Mol Carcinog* 45: 10-17.

Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. *Cell* 100: 57-70.

Hancke K, Grubeck D, Hauser N, Kreienberg R, Weiss JM. (2010). Adipocyte fatty acid-binding protein as a novel prognostic factor in obese breast cancer patients. *Breast Cancer Res Treat* 119: 367-367.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J *et al*. (1996). Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature* 379: 91-96.

Hao L, Tiganis T, Tonks NK, Charbonneau H. (1997). The noncatalytic C-terminal segment of the T cell protein tyrosine phosphatase regulates activity via an intramolecular mechanism. *J Biol Chem* 272: 29322-29329.

Harris RC, Chung E, Coffey RJ. (2003). EGF receptor ligands. *Exp Cell Res* 284: 2-13.

Haunerland NH, Spener F. (2004). Fatty acid-binding proteins--insights from genetic manipulations. *Prog Lipid Res* 43: 328-349.

Hazelbag S, Kenter GG, Gorter A, Dreef EJ, Koopman LA, Violette SM *et al*. (2007). Overexpression of the alpha v beta 6 integrin in cervical squamous cell carcinoma is a prognostic factor for decreased survival. *J Pathol* 212: 316-324.

He L, Pappan LK, Grenache DG, Li Z, Tollefsen DM, Santoro SA *et al*. (2003). The contributions of the alpha 2 beta 1 integrin to vascular thrombosis in vivo. *Blood* 102: 3652-3657.

Herr FM, Aronson J, Storch J. (1996). Role of portal region lysine residues in electrostatic interactions between heart fatty acid binding protein and phospholipid membranes. *Biochemistry* 35: 1296-1303.

Hertzel AV, Bernlohr DA. (2000). The mammalian fatty acid-binding protein multigene family: Molecular and genetic insights into function. *Trends Endocrinol Metab* 11: 175-180.

Hobbs SS, Coffing SL, Le AT, Cameron EM, Williams EE, Andrew M *et al*. (2002). Neuregulin isoforms exhibit distinct patterns of ErbB family receptor activation. *Oncogene* 21: 8442-8452.

Hogervorst F, Kuikman I, von dem Borne AE, Sonnenberg A. (1990). Cloning and sequence analysis of beta-4 cDNA: An integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J* 9: 765-770.

Honda S, Shirotani-Ikejima H, Tadokoro S, Maeda Y, Kinoshita T, Tomiyama Y *et al*. (2009). Integrin-linked kinase associated with integrin activation. *Blood* 113: 5304-5313.

Hood JD, Bednarski M, Frausto R, Guccione S, Reisfeld RA, Xiang R *et al*. (2002). Tumor regression by targeted gene delivery to the neovasculature. *Science* 296: 2404-2407.

Hosotani R, Kawaguchi M, Masui T, Koshiba T, Ida J, Fujimoto K *et al*. (2002). Expression of integrin alphaVbeta3 in pancreatic carcinoma: Relation to MMP-2 activation and lymph node metastasis. *Pancreas* 25: e30-5.

Hostetler HA, McIntosh AL, Atshaves BP, Storey SM, Payne HR, Kier AB *et al*. (2009). L-FABP directly interacts with PPARalpha in cultured primary hepatocytes. *J Lipid Res* 50: 1663-1675.

Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. (1996). Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 274: 1377-1379.

Hsu SC, Hung MC. (2007). Characterization of a novel tripartite nuclear localization sequence in the EGFR family. *J Biol Chem* 282: 10432-10440.

Huang F, Goh LK, Sorkin A. (2007). EGF receptor ubiquitination is not necessary for its internalization. *Proc Natl Acad Sci U S A* 104: 16904-16909.

Huang F, Khvorova A, Marshall W, Sorkin A. (2004). Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem* 279: 16657-16661.

Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ *et al*. (1996). Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *J Biol Chem* 271: 6571-6574.

Hung LY, Tseng JT, Lee YC, Xia W, Wang YN, Wu ML *et al*. (2008). Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating aurora-A gene expression. *Nucleic Acids Res* 36: 4337-4351.

Huveneers S, Danen EH. (2009). Adhesion signaling - crosstalk between integrins, src and rho. *J Cell Sci* 122: 1059-1069.

Huynh H, Alpert L, Pollak M. (1996). Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. *Cancer Res* 56: 4865-4870.

Huynh HT, Larsson C, Narod S, Pollak M. (1995). Tumor suppressor activity of the gene encoding mammary-derived growth inhibitor. *Cancer Res* 55: 2225-2231.

Hynes RO. (2002). Integrins: Bidirectional, allosteric signaling machines. *Cell* 110: 673-687.

Hynes RO. (1987). Integrins: A family of cell surface receptors. *Cell* 48: 549-554.

Hyun YM, Lefort CT, Kim M. (2009). Leukocyte integrins and their ligand interactions. *Immunol Res* 45: 195-208.

Ieguchi K, Fujita M, Ma Z, Davari P, Taniguchi Y, Sekiguchi K *et al*. (2010). Direct binding of the EGF-like domain of neuregulin-1 to integrins ({alpha}v{beta}3 and {alpha}6{beta}4) is involved in neuregulin-1/ErbB signaling. *J Biol Chem* 285: 31388-31398.

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N *et al*. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377: 539-544.

Ithychanda SS, Das M, Ma YQ, Ding K, Wang X, Gupta S *et al*. (2009). Migfilin, a molecular switch in regulation of integrin activation. *J Biol Chem* 284: 4713-4722.

Jia Y, Zeng ZZ, Markwart SM, Rockwood KF, Ignatoski KM, Ethier SP *et al*. (2004). Integrin fibronectin receptors in matrix metalloproteinase-1-dependent invasion by breast cancer and mammary epithelial cells. *Cancer Res* 64: 8674-8681.

Jiang X, Huang F, Marusyk A, Sorkin A. (2003). Grb2 regulates internalization of EGF receptors through clathrin-coated pits. *Mol Biol Cell* 14: 858-870.

Jing C, Beesley C, Foster CS, Chen H, Rudland PS, West DC *et al*. (2001). Human cutaneous fatty acid-binding protein induces metastasis by up-regulating the expression of vascular endothelial growth factor

gene in rat rama 37 model cells. *Cancer Res* 61: 4357-4364.

Jing C, Beesley C, Foster CS, Rudland PS, Fujii H, Ono T *et al*. (2000). Identification of the messenger RNA for human cutaneous fatty acid-binding protein as a metastasis inducer. *Cancer Res* 60: 2390-2398.

Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS *et al*. (2009). Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell* 15: 429-440.

Junttila TT, Sundvall M, Lundin M, Lundin J, Tanner M, Härkonen P *et al*. (2005). Cleavable ErbB4 isoform in estrogen receptor-regulated growth of breast cancer cells. *Cancer Res* 65: 1384-1393.

Juwana YB, Suryapranata H, Ottervanger JP, van 't Hof AW. (2010). Tirofiban for myocardial infarction. *Expert Opin Pharmacother* 11: 861-866.

Kaloshi G, Mokhtari K, Carpentier C, Taillibert S, Lejeune J, Marie Y *et al*. (2007). FABP7 expression in glioblastomas: Relation to prognosis, invasion and EGFR status. *J Neurooncol* 84: 245-248.

Kamatkar S, Radha V, Nambirajan S, Reddy RS, Swarup G. (1996). Two splice variants of a tyrosine phosphatase differ in substrate specificity, DNA binding, and subcellular location. *J Biol Chem* 271: 26755-26761.

Kamisaka, Maezawa H, Inagaki T, Okano K. (1981). A low molecular weight binding protein for organic anions (Z protein) from human hepatic cytosol: Purification and quantitation. *Hepatology* 1: 221-227.

Kasirer-Friede A, Kahn ML, Shattil SJ. (2007). Platelet integrins and immunoreceptors. *Immunol Rev* 218: 247-264.

Kaur S, Kenny HA, Jagadeeswaran S, Zillhardt MR, Montag AG, Kistner E *et al*. (2009). {Beta}3-integrin expression on tumor cells inhibits tumor progression, reduces metastasis, and is associated with a favorable prognosis in patients with ovarian cancer. *Am J Pathol* 175: 2184-2196.

Khalili P, Arakelian A, Chen G, Plunkett ML, Beck I, Parry GC *et al*. (2006). A non-RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo. *Mol Cancer Ther* 5: 2271-2280.

Kiema T, Lad Y, Jiang P, Oxley CL, Baldassarre M, Wegener KL *et al*. (2006). The molecular basis of filamin binding to integrins and competition with talin. *Mol Cell* 21: 337-347.

Kiewe P, Hasmuller S, Kahlert S, Heinrigs M, Rack B, Marme A *et al*. (2006). Phase I trial of the trifunctional anti-HER2 x anti-CD3 antibody ertumaxomab in metastatic breast cancer. *Clin Cancer Res* 12: 3085-3091.

Kil SJ, Hobert M, Carlin C. (1999). A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand-receptor complexes to lysosomes. *J Biol Chem* 274: 3141-3150.

Kim HP, Yoon YK, Kim JW, Han SW, Hur HS, Park J *et al*. (2009). Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2. *PLoS One* 4: e5933.

Kim M, Carman CV, Springer TA. (2003). Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* 301: 1720-1725.

Kim SM, Kwon MS, Park CS, Choi KR, Chun JS, Ahn J *et al*. (2004). Modulation of thr phosphorylation of integrin beta1 during muscle differentiation. *J Biol Chem* 279: 7082-7090.

Klein CE, Dressel D, Steinmayer T, Mauch C, Eckes B, Krieg T *et al*. (1991). Integrin alpha 2 beta 1 is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. *J Cell Biol* 115: 1427-1436.

Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P. (1999). Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J* 18: 2459-2471.

Klingler-Hoffmann M, Fodero-Tavoletti MT, Mishima K, Narita Y, Cavenee WK, Furnari FB *et al*. (2001). The protein tyrosine phosphatase TCPTP suppresses the tumorigenicity of glioblastoma cells expressing a mutant epidermal growth factor receptor. *J Biol Chem* 276: 46313-46318.

Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. (2000). The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* 275: 35-40.

Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M *et al*. (2005). EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 352: 786-792.

Koutras AK, Kalogeras KT, Dimopoulos MA, Wirtz RM, Dafni U, Briasoulis E *et al*. (2008). Evaluation of the prognostic and predictive value of HER family mRNA expression in high-risk early breast cancer: A hellenic cooperative oncology group (HeCOG) study. *Br J Cancer* 99: 1775-1785.

Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA. (1989). Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci U S A* 86: 9193-9197.

Krieg P, Feil S, Fürstenberger G, Bowden GT. (1993). Tumor-specific overexpression of a novel keratinocyte lipid-binding protein. identification and characterization of a cloned sequence activated during multistage carcinogenesis in mouse skin. *J Biol Chem* 268: 17362-17369.

Krop IE, Beeram M, Modi S, Jones SF, Holden SN, Yu W *et al*. (2010). Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J Clin Oncol* 28: 2698-2704.

Kurai J, Chikumi H, Hashimoto K, Yamaguchi K, Yamasaki A, Sako T *et al*. (2007). Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. *Clin Cancer Res* 13: 1552-1561.

Kurtz A, Vogel F, Funa K, Heldin CH, Grosse R. (1990). Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. *J Cell Biol* 110: 1779-1789.

Kurtz A, Zimmer A, Schnütgen F, Brüning G, Spener F, Müller T. (1994). The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. *Development* 120: 2637-2649.

Laidler P, Gil D, Pituch-Noworolska A, Ciolczyk D, Ksiazek D, Przybylo M *et al*. (2000). Expression of beta1-integrins and N-cadherin in bladder cancer and melanoma cell lines. *Acta Biochim Pol* 47: 1159-1170.

Lam MH, Michell BJ, Fodero-Tavoletti MT, Kemp BE, Tonks NK, Tiganis T. (2001). Cellular stress regulates the nucleocytoplasmic distribution of the protein-tyrosine phosphatase TCPTP. *J Biol Chem* 276: 37700-37707.

Landrier JF, Thomas C, Grober J, Duez H, Percevault F, Souidi M *et al*. (2004). Statin induction of liver fatty acid-binding protein (L-FABP) gene expression is peroxisome proliferator-activated receptor-alpha-dependent. *J Biol Chem* 279: 45512-45518.

Lange A, Wickström SA, Jakobson M, Zent R, Sainio K, Fässler R. (2009). Integrin-linked kinase is an adaptor with essential functions during mouse development. *Nature* 461: 1002-1006.

Lau TL, Kim C, Ginsberg MH, Ulmer TS. (2009). The structure of the integrin alphaIIbbeta3 transmembrane complex explains integrin transmembrane signalling. *EMBO J* 28: 1351-1361.

Lau TL, Partridge AW, Ginsberg MH, Ulmer TS. (2008). Structure of the integrin beta3 transmembrane segment in phospholipid bicelles and detergent micelles. *Biochemistry* 47: 4008-4016.

Lax I, Johnson A, Howk R, Sap J, Bellot F, Winkler M *et al*. (1988). Chicken epidermal growth factor (EGF) receptor: CDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. *Mol Cell Biol* 8: 1970-1978.

Lee D, Yu M, Lee E, Kim H, Yang Y, Kim K *et al*. (2009). Tumor-specific apoptosis caused by deletion of the ERBB3 pseudo-kinase in mouse intestinal epithelium. *J Clin Invest* 119: 2702-2713.

Lee JO, Rieu P, Arnaout MA, Liddington R. (1995). Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell* 80: 631-638.

Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP *et al*. (2008). A central role for HER3 in HER2-amplified breast cancer: Implications for targeted therapy. *Cancer Res* 68: 5878-5887.

Legate KR, Fässler R. (2009). Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *J Cell Sci* 122: 187-198.

Legate KR, Wickström SA, Fässler R. (2009). Genetic and cell biological analysis of integrin outside-in signaling. *Genes Dev* 23: 397-418.

Legler DF, Wiedle G, Ross FP, Imhof BA. (2001). Superactivation of integrin alphavbeta3 by low antagonist concentrations. *J Cell Sci* 114: 1545-1553.

Lehnert K, Ni J, Leung E, Gough S, Morris CM, Liu D *et al*. (1999a). The integrin alpha10 subunit: Expression pattern, partial gene structure, and chromosomal localization. *Cytogenet Cell Genet* 87: 238-244.

Lehnert K, Ni J, Leung E, Gough SM, Weaver A, Yao WP *et al*. (1999b). Cloning, sequence analysis, and chromosomal localization of the novel human integrin alpha11 subunit (ITGA11). *Genomics* 60: 179-187.

Lemmon MA. (2009). Ligand-induced ErbB receptor dimerization. *Exp Cell Res* 315: 638-648.

Lenferink AE, Pinkas-Kramarski R, van de Poll ML, van Vugt MJ, Klapper LN, Tzahar E *et al*. (1998). Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J* 17: 3385-3397.

Leung-Hagesteijn CY, Milankov K, Michalak M, Wilkins J, Dedhar S. (1994). Cell attachment to extracellular matrix substrates is inhibited upon downregulation of expression of calreticulin, an intracellular integrin alpha-subunit-binding protein. *J Cell Sci* 107: 589-600.

Li C, Iida M, Dunn EF, Ghia AJ, Wheeler DL. (2009). Nuclear EGFR contributes to acquired resistance to cetuximab. *Oncogene* 28: 3801-3813.

Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR *et al*. (2008). BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* 27: 4702-4711.

Li R, Babu CR, Lear JD, Wand AJ, Bennett JS, DeGrado WF. (2001). Oligomerization of the integrin alphaIIbbeta3: Roles of the transmembrane and cytoplasmic domains. *Proc Natl Acad Sci U S A* 98: 12462-12467.

Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. (2005). Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 7: 301-311.

Li Z, Huang C, Bai S, Pan X, Zhou R, Wei Y *et al*. (2008). Prognostic evaluation of epidermal fatty

acid-binding protein and calcyphosine, two proteins implicated in endometrial cancer using a proteomic approach. *Int J Cancer* 123: 2377-2383.

Liao HJ, Carpenter G. (2007). Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. *Mol Biol Cell* 18: 1064-1072.

Liapis H, Flath A, Kitazawa S. (1996). Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. *Diagn Mol Pathol* 5: 127-135.

Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H *et al*. (1985). Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* 313: 144-147.

Liddington R, Bankston L. (1998). The integrin I domain: Crystals, metals and related artefacts. *Structure* 6: 937-938.

Lieberman JM, Marks WH, Cohn S, Jaicks R, Woode L, Sacchettini J *et al*. (1998). Organ failure, infection, and the systemic inflammatory response syndrome are associated with elevated levels of urinary intestinal fatty acid binding protein: Study of 100 consecutive patients in a surgical intensive care unit. *J Trauma* 45: 900-906.

Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF *et al*. (2006). KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 66: 3992-3995.

Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY *et al*. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* 3: 802-808.

Liu S, Slepak M, Ginsberg MH. (2001). Binding of paxillin to the alpha 9 integrin cytoplasmic domain inhibits cell spreading. *J Biol Chem* 276: 37086-37092.

Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, Pfaff M *et al*. (1999). Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. *Nature* 402: 676-681.

Lo HW, Hsu SC, Ali-Seyed M, Gunduz M, Xia W, Wei Y *et al*. (2005). Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* 7: 575-589.

Loeffler-Ragg J, Skvortsov S, Sarg B, Skvortsova I, Witsch-Baumgartner M, Mueller D *et al*. (2005). Gefitinib-responsive EGFR-positive colorectal cancers have different proteome profiles from non-responsive cell lines. *Eur J Cancer* 41: 2338-2346.

Loftus JC, Smith JW, Ginsberg MH. (1994). Integrin-mediated cell adhesion: The extracellular face. *J Biol Chem* 269: 25235-25238.

Lorenzen JA, Dadabay CY, Fischer EH. (1995). COOH-terminal sequence motifs target the T cell protein tyrosine phosphatase to the ER and nucleus. *J Cell Biol* 131: 631-643.

Lu C, Takagi J, Springer TA. (2001). Association of the membrane proximal regions of the alpha and beta subunit cytoplasmic domains constrains an integrin in the inactive state. *J Biol Chem* 276: 14642-14648.

Lu X, Chen J, Sasmono RT, Hsi ED, Sarosiek KA, Tiganis T *et al*. (2007a). T-cell protein tyrosine phosphatase, distinctively expressed in activated-B-cell-like diffuse large B-cell lymphomas, is the nuclear phosphatase of STAT6. *Mol Cell Biol* 27: 2166-2179.

Lu Y, Li X, Liang K, Luwor R, Siddik ZH, Mills GB *et al*. (2007b). Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance escaping treatment by the anti-EGFR monoclonal antibody cetuximab. *Cancer Res* 67: 8240-8247.

Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA *et al*. (1994). The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev* 8: 399-413.

Luo BH, Springer TA, Takagi J. (2004). A specific interface between integrin transmembrane helices and affinity for ligand. *PLoS Biol* 2: e153.

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW *et al*. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129-2139.

Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC. (2009). Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res* 11: R7.

MacDonald TJ, Taga T, Shimada H, Tabrizi P, Zlokovic BV, Cheresh DA *et al*. (2001). Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. *Neurosurgery* 48: 151-157.

Madsen P, Rasmussen HH, Leffers H, Honoré B, Celis JE. (1992). Molecular cloning and expression of a novel keratinocyte protein (psoriasis-associated fatty acid-binding protein [PA-FABP]) that is highly up-regulated in psoriatic skin and that shares similarity to fatty acid-binding proteins. *J Invest Dermatol* 99: 299-305.

Major EO. (2010). Progressive multifocal leukoencephalopathy in patients on immunomodulatory therapies. *Annu Rev Med* 61: 35-47.

Martin GG, Atshaves BP, McIntosh AL, Payne HR, Mackie JT, Kier AB *et al*. (2009). Liver fatty acid binding protein gene ablation enhances age-dependent weight gain in male mice. *Mol Cell Biochem* 324: 101-115.

Martin GG, Danneberg H, Kumar LS, Atshaves BP, Erol E, Bader M *et al*. (2003). Decreased liver fatty acid binding capacity and altered liver lipid distribution in mice lacking the liver fatty acid-binding protein gene. *J Biol Chem* 278: 21429-21438.

Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z *et al*. (1995). Epithelial

immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* 376: 337-341.

Millon-Frémillon A, Bouvard D, Grichine A, Manet-Dupé S, Block MR, Albiges-Rizo C. (2008). Cell adaptive response to extracellular matrix density is controlled by ICAP-1-dependent beta1-integrin affinity. *J Cell Biol* 180: 427-441.

Mimura K, Kono K, Hanawa M, Mitsui F, Sugai H, Miyagawa N *et al*. (2005). Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma. *Br J Cancer* 92: 1253-1260.

Mita R, Coles JE, Glubrecht DD, Sung R, Sun X, Godbout R. (2007). B-FABP-expressing radial glial cells: The malignant glioma cell of origin? *Neoplasia* 9: 734-744.

Mitra SK, Schlaepfer DD. (2006). Integrin-regulated FAK-src signaling in normal and cancer cells. *Curr Opin Cell Biol* 18: 516-523.

Moasser MM. (2007). The oncogene HER2: Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene* 26: 6469-6487.

Mok A, Cao H, Zinman B, Hanley AJ, Harris SB, Kennedy BP *et al*. (2002). A single nucleotide polymorphism in protein tyrosine phosphatase PTP-1B is associated with protection from diabetes or impaired glucose tolerance in oji-cree. *J Clin Endocrinol Metab* 87: 724-727.

Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. (2001). Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* 61: 4744-4749.

Mollinedo F, de la Iglesia-Vicente J, Gajate C, Estella-Hermoso de Mendoza A, Villa-Pulgarin JA, Campanero MA *et al*. (2010). Lipid raft-targeted therapy in multiple myeloma. *Oncogene* 29: 3748-3757.

Montanez E, Ussar S, Schifferer M, Bösl M, Zent R, Moser M *et al*. (2008). Kindlin-2 controls bidirectional signaling of integrins. *Genes Dev* 22: 1325-1330.

Morgan EA, Forootan SS, Adamson J, Foster CS, Fujii H, Igarashi M *et al*. (2008). Expression of cutaneous fatty acid-binding protein (C-FABP) in prostate cancer: Potential prognostic marker and target for tumourigenicity-suppression. *Int J Oncol* 32: 767-775.

Moro L, Dolce L, Cabodi S, Bergatto E, Boeri Erba E, Smeriglio M *et al*. (2002). Integrin-induced epidermal growth factor (EGF) receptor activation requires c-src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *J Biol Chem* 277: 9405-9414.

Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L *et al*. (1998). Integrins induce activation of EGF receptor: Role in MAP kinase induction and adhesion-dependent cell survival. *EMBO J* 17: 6622-6632.

Morrison C, Zanagnolo V, Ramirez N, Cohn DE, Kelbick N, Copeland L *et al*. (2006). HER-2 is an independent prognostic factor in endometrial cancer: Association with outcome in a large cohort of surgically staged patients. *J Clin Oncol* 24: 2376-2385.

Moser M, Legate KR, Zent R, Fässler R. (2009). The tail of integrins, talin, and kindlins. *Science* 324: 895-899.

Mosinger B,Jr, Tillmann U, Westphal H, Tremblay ML. (1992). Cloning and characterization of a mouse cDNA encoding a cytoplasmic protein-tyrosine-phosphatase. *Proc Natl Acad Sci U S A* 89: 499-503.

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J *et al*. (1999). The integrin alpha v beta 6 binds and activates latent TGF beta 1: A mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96: 319-328.

Murray C, Alpert JS. (1994). Diagnosis of acute myocardial infarction. *Curr Opin Cardiol* 9: 465-470.

Murtola TJ, Tammela TL, Määttänen L, Huhtala H, Platz EA, Ala-Opas M *et al*. (2010). Prostate cancer and PSA among statin users in the finnish prostate cancer screening trial. *Int J Cancer* 127: 1650-1659.

Nguyen DX, Bos PD, Massagué J. (2009). Metastasis: From dissemination to organ-specific colonization. *Nat Rev Cancer* 9: 274-284.

Ni CY, Murphy MP, Golde TE, Carpenter G. (2001). Gamma -secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* 294: 2179-2181.

Nip J, Shibata H, Loskutoff DJ, Cheresh DA, Brodt P. (1992). Human melanoma cells derived from lymphatic metastases use integrin alpha v beta 3 to adhere to lymph node vitronectin. *J Clin Invest* 90: 1406-1413.

Ockner RK, Manning JA, Poppenhausen RB, Ho WK. (1972). A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science* 177: 56-58.

Ogiso H, Ishitani R, Nureki O, Fukai S, Yamanaka M, Kim JH *et al*. (2002). Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell* 110: 775-787.

Oh HY, Lee EJ, Yoon S, Chung BH, Cho KS, Hong SJ. (2007). Cholesterol level of lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated akt and ERK signal transduction. *Prostate* 67: 1061-1069.

Okano T, Kondo T, Fujii K, Nishimura T, Takano T, Ohe Y *et al*. (2007). Proteomic signature corresponding to the response to gefitinib (iressa, ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor in lung adenocarcinoma. *Clin Cancer Res* 13: 799-805.

Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P *et al*. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127: 635-648.

Otey CA, Vasquez GB, Burridge K, Erickson BW. (1993). Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. *J Biol Chem* 268: 21193-21197.

O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V *et al*. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol* 124: 1047-1059.

Owada Y, Suzuki I, Noda T, Kondo H. (2002). Analysis on the phenotype of E-FABP-gene knockout mice. *Mol Cell Biochem* 239: 83-86.

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S *et al*. (2004). EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500.

Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I *et al*. (2004). EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 101: 13306-13311.

Patra SK. (2008). Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochim Biophys Acta* 1785: 182-206.

Pavalko FM, LaRoche SM. (1993). Activation of human neutrophils induces an interaction between the integrin beta 2-subunit (CD18) and the actin binding protein alpha-actinin. *J Immunol* 151: 3795-3807.

Pellinen T, Arjonen A, Vuoriluoto K, Kallio K, Fransen JA, Ivaska J. (2006). Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. *J Cell Biol* 173: 767-780.

Pellinen T, Ivaska J. (2006). Integrin traffic. *J Cell Sci* 119: 3723-3731.

Persson C, Sävenhed C, Bourdeau A, Tremblay ML, Markova B, Böhmer FD *et al*. (2004). Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase. *Mol Cell Biol* 24: 2190-2201.

Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc Natl Acad Sci U S A* 89: 9064-9068.

Pfaff M, Du X, Ginsberg MH. (1999). Calpain cleavage of integrin beta cytoplasmic domains. *FEBS Lett* 460: 17-22.

Phelan CM, Larsson C, Baird S, Futreal PA, Ruttledge MH, Morgan K *et al*. (1996). The human mammary-derived growth inhibitor (MDGI) gene: Genomic structure and mutation analysis in human breast tumors. *Genomics* 34: 63-68.

Pignatelli M, Smith ME, Bodmer WF. (1990). Low expression of collagen receptors in moderate and poorly differentiated colorectal adenocarcinomas. *Br J Cancer* 61: 636-638.

Pike LJ, Casey L. (2002). Cholesterol levels modulate EGF receptor-mediated signaling by altering receptor function and trafficking. *Biochemistry* 41: 10315-10322.

Plowman GD, Culouscou JM, Whitney GS, Green JM, Carlton GW, Foy L *et al*. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci U S A* 90: 1746-1750.

Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ *et al*. (1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci U S A* 87: 4905-4909.

Popova SN, Barczyk M, Tiger CF, Beertsen W, Zigrino P, Aszodi A *et al*. (2007). Alpha11 beta1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor. *Mol Cell Biol* 27: 4306-4316.

Pozzi A, Wary KK, Giancotti FG, Gardner HA. (1998). Integrin alpha1beta1 mediates a unique collagen-dependent proliferation pathway in vivo. *J Cell Biol* 142: 587-594.

Prickett TD, Agrawal NS, Wei X, Yates KE, Lin JC, Wunderlich JR *et al*. (2009). Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. *Nat Genet* 41: 1127-1132.

Ramakrishnan MS, Eswaraiah A, Crombet T, Piedra P, Saurez G, Iyer H *et al*. (2009). Nimotuzumab, a promising therapeutic monoclonal for treatment of tumors of epithelial origin. *MAbs* 1: 41-48.

Reschke M, Mihic-Probst D, van der Horst EH, Knyazev P, Wild PJ, Hutterer M *et al*. (2008). HER3 is a determinant for poor prognosis in melanoma. *Clin Cancer Res* 14: 5188-5197.

Rio C, Buxbaum JD, Peschon JJ, Corfas G. (2000). Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. *J Biol Chem* 275: 10379-10387.

Rivera F, Salcedo M, Vega N, Blanco Y, López C. (2009). Current situation of zalutumumab. *Expert Opin Biol Ther* 9: 667-674.

Roovers K, Davey G, Zhu X, Bottazzi ME, Assoian RK. (1999). Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol Biol Cell* 10: 3197-3204.

Rose DM, Alon R, Ginsberg MH. (2007). Integrin modulation and signaling in leukocyte adhesion and migration. *Immunol Rev* 218: 126-134.

Rose DP, Connolly JM, Rayburn J, Coleman M. (1995). Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. *J Natl Cancer Inst* 87: 587-592.

Ruoslahti E. (1996). RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* 12: 697-715.

Sacchettini JC, Scapin G, Gopaul D, Gordon JI. (1992). Refinement of the structure of escherichia coli-derived rat intestinal fatty acid binding protein with bound oleate to 1.75-A resolution. correlation with the structures of the apoprotein and the protein with bound palmitate. *J Biol Chem* 267: 23534-23545.

Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J *et al*. (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol* 164: 769-779.

Sainsbury JR, Farndon JR, Needham GK, Malcolm AJ, Harris AL. (1987). Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet* 1: 1398-1402.

Salmeen A, Andersen JN, Myers MP, Tonks NK, Barford D. (2000). Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. *Mol Cell* 6: 1401-1412.

Salomon DS, Brandt R, Ciardiello F, Normanno N. (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 19: 183-232.

Samson T, Smyth N, Janetzky S, Wendler O, Müller JM, Schüle R *et al*. (2004). The LIM-only proteins FHL2 and FHL3 interact with alpha- and beta-subunits of the muscle alpha7beta1 integrin receptor. *J Biol Chem* 279: 28641-28652.

Sánchez-Font MF, Bosch-Comas A, Gonzàlez-Duarte R, Marfany G. (2003). Overexpression of FABP7 in down syndrome fetal brains is associated with PKNOX1 gene-dosage imbalance. *Nucleic Acids Res* 31: 2769-2777.

Sangalli F, Moiola L, Bucello S, Annovazzi P, Rizzo A, Radaelli M *et al*. (2010). Efficacy and tolerability of natalizumab in relapsing-remitting multiple sclerosis patients: A post-marketing observational study. *Neurol Sci* (DOI: 10.1007/s10072-010-0344-z).

Sangwan V, Paliouras GN, Abella JV, Dube N, Monast A, Tremblay ML *et al*. (2008). Regulation of the met receptor-tyrosine kinase by the protein-tyrosine phosphatase 1B and T-cell phosphatase. *J Biol Chem* 283: 34374-34383.

Sartore-Bianchi A, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S *et al*. (2009). PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. *Cancer Res* 69: 1851-1857.

Sastry SK, Horwitz AF. (1993). Integrin cytoplasmic domains: Mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr Opin Cell Biol* 5: 819-831.

Sastry SK, Lyons PD, Schaller MD, Burridge K. (2002). PTP-PEST controls motility through regulation of Rac1. *J Cell Sci* 115: 4305-4316.

Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K *et al*. (2009). Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* 28: 803-814.

Schley PD, Brindley DN, Field CJ. (2007). (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J Nutr* 137: 548-553.

Schulze WX, Deng L, Mann M. (2005). Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* 1: 2005.0008.

Schwartz MA, Ginsberg MH. (2002). Networks and crosstalk: Integrin signalling spreads. *Nat Cell Biol* 4: E65-8.

Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, Derynck R *et al*. (1998). Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Differ* 9: 777-785.

Sebastian S, Settleman J, Reshkin SJ, Azzariti A, Bellizzi A, Paradiso A. (2006). The complexity of targeting EGFR signalling in cancer: From expression to turnover. *Biochim Biophys Acta* 1766: 120-139.

Sequist LV, Besse B, Lynch TJ, Miller VA, Wong KK, Gitlitz B *et al*. (2010). Neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor: Results of a phase II trial in patients with advanced non-small-cell lung cancer. *J Clin Oncol* 28: 3076-3083.

Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM *et al*. (2007). Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* 445: 437-441.

Shattil SJ, Kim C, Ginsberg MH. (2010). The final steps of integrin activation: The end game. *Nat Rev Mol Cell Biol* 11: 288-300.

She QB, Solit D, Basso A, Moasser MM. (2003). Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. *Clin Cancer Res* 9: 4340-4346.

Shen TL, Guan JL. (2001). Differential regulation of cell migration and cell cycle progression by FAK complexes with src, PI3K, Grb7 and Grb2 in focal contacts. *FEBS Lett* 499: 176-181.

Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. (2010). ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc Natl Acad Sci U S A* 107: 7692-7697.

Shields BJ, Court NW, Hauser C, Bukczynska PE, Tiganis T. (2008). Cell cycle-dependent regulation of SFK, JAK1 and STAT3 signalling by the protein tyrosine phosphatase TCPTP. *Cell Cycle* 7: 3405-3416.

Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF. (1998). A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *EMBO J* 17: 719-731.

Sibilia M, Wagner B, Hoebertz A, Elliott C, Marino S, Jochum W *et al*. (2003). Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart. *Development* 130: 4515-4525.

Sibilia M, Wagner EF. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269: 234-238.

Siegel DH, Ashton GH, Penagos HG, Lee JV, Feiler HS, Wilhelmsen KC *et al*. (2003). Loss of kindlin-1, a human homolog of the caenorhabditis elegans actin-extracellular-matrix linker protein UNC-112, causes kindler syndrome. *Am J Hum Genet* 73: 174-187.

Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P *et al*. (2005). Clathrin-independent endocytosis of ubiquitinated cargos. *Proc Natl Acad Sci U S A* 102: 2760-2765.

Simoncic PD, Bourdeau A, Lee-Loy A, Rohrschneider LR, Tremblay ML, Stanley ER *et al*. (2006). T-cell protein tyrosine phosphatase (tcptp) is a negative regulator of colony-stimulating factor 1 signaling and macrophage differentiation. *Mol Cell Biol* 26: 4149-4160.

Simoncic PD, Lee-Loy A, Barber DL, Tremblay ML, McGlade CJ. (2002). The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3. *Curr Biol* 12: 446-453.

Singh AB, Harris RC. (2005). Autocrine, paracrine and juxtacrine signaling by EGFR ligands. *Cell Signal* 17: 1183-1193.

Sinha P, Hütter G, Köttgen E, Dietel M, Schadendorf D, Lage H. (1999). Increased expression of epidermal fatty acid binding protein, cofilin, and 14-3-3-sigma (stratifin) detected by two-dimensional gel electrophoresis, mass spectrometry and microsequencing of drug-resistant human adenocarcinoma of the pancreas. *Electrophoresis* 20: 2952-2960.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. (1987). Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235: 177-182.

Smith AJ, Sanders MA, Thompson BR, Londos C, Kraemer FB, Bernlohr DA. (2004). Physical association between the adipocyte fatty acid-binding protein and hormone-sensitive lipase: A fluorescence resonance energy transfer analysis. *J Biol Chem* 279: 52399-52405.

Smith ER, Storch J. (1999). The adipocyte fatty acid-binding protein binds to membranes by electrostatic interactions. *J Biol Chem* 274: 35325-35330.

Snedeker SM, Brown CF, DiAugustine RP. (1991). Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc Natl Acad Sci U S A* 88: 276-280.

Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL *et al*. (2006). Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin Cancer Res* 12: 5064-5073.

Sorkin A, Carpenter G. (1991). Dimerization of internalized epidermal growth factor receptors. *J Biol Chem* 266: 23453-23460.

Sorkin A, Krolenko S, Kudrjavtceva N, Lazebnik J, Teslenko L, Soderquist AM *et al*. (1991). Recycling of epidermal growth factor-receptor complexes in A431 cells: Identification of dual pathways. *J Cell Biol* 112: 55-63.

Specht B, Bartetzko N, Hohoff C, Kuhl H, Franke R, Börchers T *et al*. (1996). Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. *J Biol Chem* 271: 19943-19949.

Stamos J, Sliwkowski MX, Eigenbrot C. (2002). Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. *J Biol Chem* 277: 46265-46272.

Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA *et al*. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev* 9: 1883-1895.

Stern DF, Heffernan PA, Weinberg RA. (1986). P185, a product of the neu proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. *Mol Cell Biol* 6: 1729-1740.

Stoeltzing O, Liu W, Reinmuth N, Fan F, Parry GC, Parikh AA *et al*. (2003). Inhibition of integrin alpha5beta1 function with a small peptide (ATN-161) plus continuous 5-FU infusion reduces colorectal liver metastases and improves survival in mice. *Int J Cancer* 104: 496-503.

Storch J, Thumser AE. (2010). Tissue-specific functions in the fatty acid-binding protein family. *J Biol Chem* 285: 32679-32683.

Stoscheck CM, Carpenter G. (1984a). Characterization of the metabolic turnover of epidermal growth factor receptor protein in A-431 cells. *J Cell Physiol* 120: 296-302.

Stoscheck CM, Carpenter G. (1984b). Down regulation of epidermal growth factor receptors: Direct demonstration of receptor degradation in human fibroblasts. *J Cell Biol* 98: 1048-1053.

Su AI, Welsh JB, Sapinoso LM, Kern SG, Dimitrov P, Lapp H *et al*. (2001). Molecular classification of human carcinomas by use of gene expression signatures. *Cancer Res* 61: 7388-7393.

Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Girard OM *et al*. (2009). Tissue-penetrating delivery of compounds and nanoparticles into tumors. *Cancer Cell* 16: 510-520.

Suzuki T, Watanabe K, Ono T. (1990). Immunohistochemical demonstration of liver fatty acid-binding protein in human hepatocellular malignancies. *J Pathol* 161: 79-83.

Sweetser DA, Hauft SM, Hoppe PC, Birkenmeier EH, Gordon JI. (1988). Transgenic mice containing

intestinal fatty acid-binding protein-human growth hormone fusion genes exhibit correct regional and cell-specific expression of the reporter gene in their small intestine. *Proc Natl Acad Sci U S A* 85: 9611-9615.

Söderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J *et al*. (2006). Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* 3: 995-1000.

Tabiti K, Smith DR, Goh HS, Pallen CJ. (1995). Increased mRNA expression of the receptor-like protein tyrosine phosphatase alpha in late stage colon carcinomas. *Cancer Lett* 93: 239-248.

Takagi J, Petre BM, Walz T, Springer TA. (2002). Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* 110: 599-511.

Takagi J, Strokovich K, Springer TA, Walz T. (2003). Structure of integrin alpha5beta1 in complex with fibronectin. *EMBO J* 22: 4607-4615.

Takala H, Nurminen E, Nurmi SM, Aatonen M, Strandin T, Takatalo M *et al*. (2008). Beta2 integrin phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. *Blood* 112: 1853-1862.

Tam EM, Wu YI, Butler GS, Stack MS, Overall CM. (2002). Collagen binding properties of the membrane type-1 matrix metalloproteinase (MT1-MMP) hemopexin C domain. the ectodomain of the 44-kDa autocatalytic product of MT1-MMP inhibits cell invasion by disrupting native type I collagen cleavage. *J Biol Chem* 277: 39005-39014.

Tamkun JW, DeSimone DW, Fonda D, Patel RS, Buck C, Horwitz AF *et al*. (1986). Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell* 46: 271-282.

Tan NS, Shaw NS, Vinckenbosch N, Liu P, Yasmin R, Desvergne B *et al*. (2002). Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription. *Mol Cell Biol* 22: 5114-5127.

Tang MK, Kindler PM, Cai DQ, Chow PH, Li M, Lee KK. (2004). Heart-type fatty acid binding proteins are upregulated during terminal differentiation of mouse cardiomyocytes, as revealed by proteomic analysis. *Cell Tissue Res* 316: 339-347.

Tang XY, Umemura S, Tsukamoto H, Kumaki N, Tokuda Y, Osamura RY. (2010). Overexpression of fatty acid binding protein-7 correlates with basal-like subtype of breast cancer. *Pathol Res Pract* 206: 98-101.

Tanner B, Hasenclever D, Stern K, Schormann W, Bezler M, Hermes M *et al*. (2006). ErbB-3 predicts survival in ovarian cancer. *J Clin Oncol* 24: 4317-4323.

Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H *et al*. (2001). Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause noonan syndrome. *Nat Genet* 29: 465-468.

Taverna D, Moher H, Crowley D, Borsig L, Varki A, Hynes RO. (2004). Increased primary tumor growth in mice null for beta3- or beta3/beta5-integrins or selectins. *Proc Natl Acad Sci U S A* 101: 763-768.

ten Hoeve J, de Jesus Ibarra-Sanchez M, Fu Y, Zhu W, Tremblay M, David M *et al*. (2002). Identification of a nuclear Stat1 protein tyrosine phosphatase. *Mol Cell Biol* 22: 5662-5668.

Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D *et al*. (1995). Targeted disruption of mouse EGF receptor: Effect of genetic background on mutant phenotype. *Science* 269: 230-234.

Thumser AE, Storch J. (2000). Liver and intestinal fatty acid-binding proteins obtain fatty acids from phospholipid membranes by different mechanisms. *J Lipid Res* 41: 647-656.

Tiganis T. (2002). Protein tyrosine phosphatases: Dephosphorylating the epidermal growth factor receptor. *IUBMB Life* 53: 3-14.

Tiganis T, Bennett AM. (2007). Protein tyrosine phosphatase function: The substrate perspective. *Biochem J* 402: 1-15.

Tiganis T, Bennett AM, Ravichandran KS, Tonks NK. (1998). Epidermal growth factor receptor and the adaptor protein p52Shc are specific substrates of T-cell protein tyrosine phosphatase. *Mol Cell Biol* 18: 1622-1634.

Tillmann U, Wagner J, Boerboom D, Westphal H, Tremblay ML. (1994). Nuclear localization and cell cycle regulation of a murine protein tyrosine phosphatase. *Mol Cell Biol* 14: 3030-3040.

Tong WM, Ellinger A, Sheinin Y, Cross HS. (1998). Epidermal growth factor receptor expression in primary cultured human colorectal carcinoma cells. *Br J Cancer* 77: 1792-1798.

Tonks NK. (2006). Protein tyrosine phosphatases: From genes, to function, to disease. *Nat Rev Mol Cell Biol* 7: 833-846.

Tonks NK, Diltz CD, Fischer EH. (1988). Purification of the major protein-tyrosine-phosphatases of human placenta. *J Biol Chem* 263: 6722-6730.

Tu Y, Wu S, Shi X, Chen K, Wu C. (2003). Migfilin and mig-2 link focal adhesions to filamin and the actin cytoskeleton and function in cell shape modulation. *Cell* 113: 37-47.

Tölle A, Jung M, Lein M, Johannsen M, Miller K, Moch H *et al*. (2009). Brain-type and liver-type fatty acid-binding proteins: New tumor markers for renal cancer? *BMC Cancer* 9: 248.

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW *et al*. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309: 418-425.

Ulmer TS, Yaspan B, Ginsberg MH, Campbell ID. (2001). NMR analysis of structure and dynamics of

the cytosolic tails of integrin alpha IIb beta 3 in aqueous solution. *Biochemistry* 40: 7498-7508.

Valdramidou D, Humphries MJ, Mould AP. (2008). Distinct roles of beta1 metal ion-dependent adhesion site (MIDAS), adjacent to MIDAS (ADMIDAS), and ligand-associated metal-binding site (LIMBS) cation-binding sites in ligand recognition by integrin alpha2beta1. *J Biol Chem* 283: 32704-32714.

van Vliet C, Bukczynska PE, Puryer MA, Sadek CM, Shields BJ, Tremblay ML *et al*. (2005). Selective regulation of tumor necrosis factor-induced erk signaling by src family kinases and the T cell protein tyrosine phosphatase. *Nat Immunol* 6: 253-260.

Wang HL, Kurtz A. (2000). Breast cancer growth inhibition by delivery of the MDGI-derived peptide P108. *Oncogene* 19: 2455-2460.

Wang SC, Hung MC. (2009). Nuclear translocation of the epidermal growth factor receptor family membrane tyrosine kinase receptors. *Clin Cancer Res* 15: 6484-6489.

Wang SC, Nakajima Y, Yu YL, Xia W, Chen CT, Yang CC *et al*. (2006). Tyrosine phosphorylation controls PCNA function through protein stability. *Nat Cell Biol* 8: 1359-1368.

Wang W, Goswami S, Lapidus K, Wells AL, Wyckoff JB, Sahai E *et al*. (2004). Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. *Cancer Res* 64: 8585-8594.

Wang W, Luo BH. (2010). Structural basis of integrin transmembrane activation. *J Cell Biochem* 109: 447-452.

Wang Y, Pennock S, Chen X, Wang Z. (2002). Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. *Mol Cell Biol* 22: 7279-7290.

Ward CW, Garrett TP. (2001). The relationship between the L1 and L2 domains of the insulin and epidermal growth factor receptors and leucine-rich repeat modules. *BMC Bioinformatics* 2: 4.

Ward CW, Hoyne PA, Flegg RH. (1995). Insulin and epidermal growth factor receptors contain the cysteine repeat motif found in the tumor necrosis factor receptor. *Proteins* 22: 141-153.

Vassileva G, Huwyler L, Poirier K, Agellon LB, Toth MJ. (2000). The intestinal fatty acid binding protein is not essential for dietary fat absorption in mice. *FASEB J* 14: 2040-2046.

Veale D, Ashcroft T, Marsh C, Gibson GJ, Harris AL. (1987). Epidermal growth factor receptors in non-small cell lung cancer. *Br J Cancer* 55: 513-516.

Veerkamp JH, van Moerkerk HT. (1993). Fatty acid-binding protein and its relation to fatty acid oxidation. *Mol Cell Biochem* 123: 101-106.

Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH *et al*. (2007). Structural basis of integrin activation by talin. *Cell* 128: 171-182.

Weinstein IB, Joe A. (2008). Oncogene addiction. *Cancer Res* 68: 3077-80.

Weisel JW, Nagaswami C, Vilaire G, Bennett JS. (1992). Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. *J Biol Chem* 267: 16637-16643.

Wheeler DL, Huang S, Kruser TJ, Nechrebecki MM, Armstrong EA, Benavente S *et al*. (2008). Mechanisms of acquired resistance to cetuximab: Role of HER (ErbB) family members. *Oncogene* 27: 3944-3956.

Wheeler DL, Iida M, Kruser TJ, Nechrebecki MM, Dunn EF, Armstrong EA *et al*. (2009). Epidermal growth factor receptor cooperates with src family kinases in acquired resistance to cetuximab. *Cancer Biol Ther* 8: 696-703.

White DE, Kurpios NA, Zuo D, Hassell JA, Blaess S, Mueller U *et al*. (2004). Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell* 6: 159-170.

Vicente-Manzanares M, Ma X, Adelstein RS, Horwitz AR. (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nat Rev Mol Cell Biol* 10: 778-790.

Viloria-Petit A, Crombet T, Jothy S, Hicklin D, Bohlen P, Schlaeppi JM *et al*. (2001). Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: A role for altered tumor angiogenesis. *Cancer Res* 61: 5090-5101.

Vinogradova O, Vaynberg J, Kong X, Haas TA, Plow EF, Qin J. (2004). Membrane-mediated structural transitions at the cytoplasmic face during integrin activation. *Proc Natl Acad Sci U S A* 101: 4094-4099.

Vinogradova O, Velyvis A, Velyviene A, Hu B, Haas T, Plow E *et al*. (2002). A structural mechanism of integrin alpha(IIb)beta(3) "inside-out" activation as regulated by its cytoplasmic face. *Cell* 110: 587-597.

Vlahakis NE, Young BA, Atakilit A, Hawkridge AE, Issaka RB, Boudreau N *et al*. (2007). Integrin alpha9beta1 directly binds to vascular endothelial growth factor (VEGF)-A and contributes to VEGF-A-induced angiogenesis. *J Biol Chem* 282: 15187-15196.

Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI *et al*. (2003). Compensation mechanism in tumor cell migration: Mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol* 160: 267-277.

Wootan MG, Storch J. (1994). Regulation of fluorescent fatty acid transfer from adipocyte and heart fatty acid binding proteins by acceptor membrane lipid composition and structure. *J Biol Chem* 269: 10517-10523.

Wu M, Rivkin A, Pham T. (2008). Panitumumab: Human monoclonal antibody against epidermal growth factor receptors for the treatment of metastatic colorectal cancer. *Clin Ther* 30: 14-30.

Xiao T, Takagi J, Coller BS, Wang JH, Springer TA. (2004). Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* 432: 59-67.

Xiong JP, Stehle T, Diefenbach B, Zhang R, Dunker R, Scott DL *et al*. (2001). Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science* 294: 339-345.

Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL *et al*. (2002). Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an arg-gly-asp ligand. *Science* 296: 151-155.

Xu S, Kitayama J, Yamashita H, Souma D, Nagawa H. (2008). Nuclear translocation of HER-4/c-erbB-4 is significantly correlated with prognosis of esophageal squamous cell carcinoma. *J Surg Oncol* 97: 44-50.

Yamada KM, Even-Ram S. (2002). Integrin regulation of growth factor receptors. *Nat Cell Biol* 4: E75-6.

Yamamoto T, Sekine Y, Kashima K, Kubota A, Sato N, Aoki N *et al*. (2002). The nuclear isoform of protein-tyrosine phosphatase TC-PTP regulates interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. *Biochem Biophys Res Commun* 297: 811-817.

Yang XD, Jia XC, Corvalan JR, Wang P, Davis CG. (2001). Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. *Crit Rev Oncol Hematol* 38: 17-23.

Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A *et al*. (1994). Members of the fatty acid binding protein family are differentiation factors for the mammary gland. *J Cell Biol* 127: 1097-1109.

Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T *et al*. (2008). Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 68: 9479-9487.

Yano T, Doi T, Ohtsu A, Boku N, Hashizume K, Nakanishi M *et al*. (2006). Comparison of HER2 gene amplification assessed by fluorescence in situ hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer. *Oncol Rep* 15: 65-71.

Yao ES, Zhang H, Chen YY, Lee B, Chew K, Moore D *et al*. (2007). Increased beta1 integrin is associated with decreased survival in invasive breast cancer. *Cancer Res* 67: 659-664.

Yi ES, Harclerode D, Gondo M, Stephenson M, Brown RW, Younes M *et al*. (1997). High c-erbB-3 protein expression is associated with shorter survival in advanced non-small cell lung carcinomas. *Mod Pathol* 10: 142-148.

You-Ten KE, Muise ES, Itié A, Michaliszyn E, Wagner J, Jothy S *et al*. (1997). Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice. *J Exp Med* 186: 683-693.

Yuan W, Leisner TM, McFadden AW, Wang Z, Larson MK, Clark S *et al*. (2006). CIB1 is an endogenous inhibitor of agonist-induced integrin alphaIIbbeta3 activation. *J Cell Biol* 172: 169-175.

Zaidel-Bar R, Itzkovitz S, Ma'ayan A, Iyengar R, Geiger B. (2007). Functional atlas of the integrin adhesome. *Nat Cell Biol* 9: 858-867.

Zander NF, Cool DE, Diltz CD, Rohrschneider LR, Krebs EG, Fischer EH. (1993). Suppression of v-fms-induced transformation by overexpression of a truncated T-cell protein tyrosine phosphatase. *Oncogene* 8: 1175-1182.

Zhang H, Berg JS, Li Z, Wang Y, Lång P, Sousa AD *et al*. (2004). Myosin-X provides a motor-based link between integrins and the cytoskeleton. *Nat Cell Biol* 6: 523-531.

Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. (2006). An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125: 1137-1149.

Zheng DQ, Woodard AS, Fornaro M, Tallini G, Languino LR. (1999). Prostatic carcinoma cell migration via alpha(v)beta3 integrin is modulated by a focal adhesion kinase pathway. *Cancer Res* 59: 1655-1664.

Zhou BB, Peyton M, He B, Liu C, Girard L, Caudler E *et al*. (2006). Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. *Cancer Cell* 10: 39-50.

Zhou W, Carpenter G. (2000). Heregulin-dependent trafficking and cleavage of ErbB-4. *J Biol Chem* 275: 34737-34743.

Zhu J, Carman CV, Kim M, Shimaoka M, Springer TA, Luo BH. (2007). Requirement of alpha and beta subunit transmembrane helix separation for integrin outside-in signaling. *Blood* 110: 2475-2483.

Zhu J, Luo BH, Barth P, Schonbrun J, Baker D, Springer TA. (2009). The structure of a receptor with two associating transmembrane domains on the cell surface: Integrin alphaIIbbeta3. *Mol Cell* 34: 234-249.

Zimmerman AW, Veerkamp JH. (2002). New insights into the structure and function of fatty acid-binding proteins. *Cell Mol Life Sci* 59: 1096-1116.

Zutter MM, Santoro SA, Staatz WD, Tsung YL. (1995). Re-expression of the alpha 2 beta 1 integrin abrogates the malignant phenotype of breast carcinoma cells. *Proc Natl Acad Sci U S A* 92: 7411-7415.