Novel regulators of cell migration

Angela Oppelt

Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo
and
Department of Biochemistry, Institute for Cancer Research,
The Norwegian Radium Hospital, Oslo University Hospital
Oslo, Norway

UiO : Faculty of Medicine
University of Oslo

Centre for Cancer Biomedicine

Norwegian Cancer Society
Kreft foreningen

norway grants
Polish-Norwegian Research Fund

Oslo University Hospital
Norwegian Radium Hospital

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1534

ISBN 978-82-8264-437-2

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Akademika publishing.
The thesis is produced by Akademika publishing merely in connection with the thesis defence. Kindly direct all inquiries regarding the thesis to the copyright holder or the unit which grants the doctorate.

Science never solves a problem without creating ten more.

-George Bernard Shaw-

Table of Contents

Acknowledgements

The work presented in this thesis was carried out in Prof. Sjur Olsnes´/Antoni Więdłocha´s lab at the Department of Biochemistry and Centre for Cancer Biomedicine, Institute for Cancer Research at The Norwegian Radium Hospital in Oslo, Norway. Financial support was received from the Centre for Cancer Biomedicine, Norway Grants, The Norwegian Cancer Society and Radiumhospitalets Legater.

I want to dedicate my very first thanks to Prof. Sjur Olsnes and the German Academic Exchange Service DAAD, since they made my first steps in Norway possible!

A very deep gratitude goes to Jørgen Wesche, Antoni Więdłocha und Prof. Harald Stenmark, who supported me very much throughout all the years. They gave me the opportunity to work at the Department of Biochemistry – and stay for the whole period of a Ph.D. Thanks for great supervision, fruitful discussions and making this thesis possible! Furthermore I would like to thank the third group leader at the department, Prof. Kirsten Sandvig, for discussions and creating together with Antoni and Harald an amazing place to work and to grow up as a scientist. Well, I think I´m a bit spoiled now…

I am also very grateful to **all** the colleagues at the department – I would like to give warm thanks to all of you; especially to my great collaborators, Andreas, Ellen, Eva, Kaisa, Kay, Nina Marie, Tor Erik and Viola; to the girls of Antoni´s group, Yan, Vigdis, Torunn, Kaja and Beata, who accompanied me throughout the whole Ph.D. with a very friendly environment; to Chema: 1000 Petabytes of thanks for amazing and excellent help with all PC problems; to Anne Engen, Anne Mari and the other cell lab members for doing a great job - same to Anne Gro and Eva R.. Thanks to all of you who became besides a colleague also friends or even close friends and making the time in Norway brighter with “hytteturer”, sharing expensive Norwegian beers, with much laughter and PhD Survival Kits!! Thanks for listening and supporting words during the hard times, Eva, Nadja, Marina, Ellen and Yan.

Many many thanks also to my external collaborators; this was/is so much fun!; Ashley M. Mackey and Lucia E. Rameh; Prof. Knut Liestøl; Anita Sveen and Rolf I. Skotheim; Prof. Håvard Danielsen; Malgorzata Zakrzewska. Excellent work!

Last, but not least: my family – this work is dedicated to you! -, mom and dad, Philipp, aunts, uncles, grandparents and great-uncles, including Pieter, thanks for supporting me throughout my whole life, no matter where I chose to go or what I wanted to do. Pierre, my personal top chef, I am so glad to have found you, I think I wouldn´t had survived this without you. Thanks for your words, listening and your understanding. All my very close friends, whether in Germany, Sweden, USA, Spain, Italy or France, you pushed me so much during the hard times and showed me more than once how beautiful life is! Agnes, Ela, Ulli and Christian - this is also dedicated a little bit to you. Furthermore to all my friends from wonderful “Vogtland” at home, brightening up my life!

List of publications

I **Oppelt A**, Lobert VH, Haglund K, Mackey AM, Rameh LE, Liestøl K, Schink KO, Pedersen NM, Wenzel EM, Haugsten EM, Brech A, Rusten TE, Stenmark H, Wesche J. (2012) Production of phosphatidylinositol 5-phosphate via PIKfyve and MTMR3 regulates cell migration. *EMBO Reports* doi: 10.1038/embor.2012.183

II **Oppelt A**, Haugsten EM, Danielsen HE, Sveen A, Skotheim RI, Wesche J. (2012) Involvement of PIKfyve and MTMR3 in cancer cell migration and invasion. Manuscript.

III Zakrzewska M, Haugsten EM, Nadratowska-Wesolowska B, **Oppelt A**, Hausott B, Jin Y, Otlewski J, Wesche J, Wiedlocha A. (2012) ERK-mediated phosphorylation of FGF receptor 1 on Ser^{777} confers negative feedback on FGF signaling. *Science Signaling*, in press.

The publications are included at the end of the thesis and will be referred to in the text by their roman numerals.

Related publication not included in the thesis:

Lobert VH, Brech A, Pedersen NM, Wesche J, **Oppelt A**, Malerød L, Stenmark H. (2012) Ubiquitination of alpha5beta1 integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes. *Dev Cell*. 20;19(1):148-59.

Abbreviations

Cdc42	Cell division control protein 42
cDNA	complementary DNA
ECM	Extracellular matrix
EE	Early endosome
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
Fab	Formation of aploid and binucleate cells
FAK	Focal adhesion kinase
FGD	Faciogenital dysplasia 1 protein
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FRS2	Fibroblast growth factor receptor substrate 2
FYVE	present in Fab1, YOTB/ZK632.12, Vac1, EEA1
GAL4-UAS	GAL4 (Galactose-induced gene) - Upstream activation sequence
GEF	Guanine nucleotide exchange factor
GRAM	Domain found in Glucosyltransferases, Rab-like GTPase activators and myotubularins
Grb	Growth factor receptor-bound protein
GTPase	Guanosine triphosphate hydrolase
HPLC	High-pressure liquid chromatography
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
ING2	Inhibitor of growth protein 2
IpgD	Invasion plasmid gene D; virulence factor
LE	Late endosome
MAPK	Mitogen-activated protein kinase
MLCK	Myosin light chain kinase

MTM	Myotubular myopathy 1; Myotubularin 1
MTMR3	Myotubularin-related phosphatase 3
MVE	Multivesicular endosome
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PIKfyve	Phosphoinositide 5-kinase, FYVE finger containing
PI3K	Phosphatidylinositol 3-Kinase / Phosphoinositide 3-Kinase
PLC	Phospholipase C
PtdIns	Phosphatidylinositol
PtdIns3*P*	Phosphatidylinositol 3-phosphate
PtdIns4*P*	Phosphatidylinositol 4-phosphate
PtdIns5*P*	Phosphatidylinositol 5-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol 3,4-bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol 3,5-bisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
PTEN	Phosphatase and tensin homologue
PVR	PDGF/VEGF-receptor related
PX	Phox homology (phagocyte oxidase)
RhoA	Ras homolog gene family, member A
RNAi	RNA interference
RTK	Receptor tyrosine kinase
siRNA	small interfering RNA
slbo	slow border cells
SopB	Effector protein of *Salmonella typhimurium*
SOS	Son of Sevenless
STAT	Signal transducer and activator of transcription
VEGF	Vascular endothelial growth factor
VPS	Vacuolar protein sorting

1. Introduction

1.1 Cell migration

In 1863, Rudolf Virchow published his observations about motile cells, isolated from lymph fluid and cartilage tissue, and he already suggested that this is of importance for the concept of life processes (*1*). Today, we call this particular process cell migration and it has indeed an important role in several processes. Cell migration occurs due to different reasons, e.g. the need to feed in the amoeba *Dictyostelium discoideum (2)*, but also during embryogenesis, organogenesis and regeneration due to the generation of new structures, layers or organs (reviewed in (*3*)). The most active cells are, however, cells of the immune system, because of the necessity to be able to respond to pathogenic invaders and to keep the organism under immunological surveillance.

Depending on the cell type and the context, human cells exhibit different modes of cell migration. They can move collectively in a group, as it is the case in *Dictyostelium discoideum* aggregates or in *Drosophila melanogaster* oogenesis during border cell migration. Types of single cell migration can be amoeboid or mesenchymal. Amoeboid movement is characterized by a round or ellipsoid morphology and cells lack mature focal adhesions and stress fibers (reviewed in (*4*)). Movement is accomplished by rapid cycles of expansion and contraction, thereby enabling a primitive, but fast movement (reviewed in (*5*)). In contrast, mesenchymal migration shows an elongated shape, with cells having high levels of attachment and cytoskeletal contractibility. The movement is fibroblast-like and involves cell-matrix interactions including proteolytic degradation of the matrix, as well as cell polarity (reviewed in (*5,6*)). Mesenchymal migrating cells execute the complete migration cycle (described below), resulting in relatively slower migration velocities.

Different external stimuli can be the driving force for migration. Directed migration, which is triggered by a gradient of dissolved chemicals, is called chemotaxis. If the chemical is surface-attached, it is termed haptotaxis. Other modes are known, like phototaxis, galvanotaxis or geotaxis. If a confluent monolayer of cells in culture is

scratched, cells will move into the denuded area (mechanotaxis). This mimics to some extent migration of cells in wound healing and is therefore called a wound healing assay (*7,8*).

1.1.1 The Migration Cycle

Mesenchymal cell migration is a chemically and physically integrated molecular process, where the components work together as a dynamic and integrated system (reviewed in (*9*)). In general, it can be understood as a cyclic process (*10*) (Figure 1). The different steps are briefly presented below.

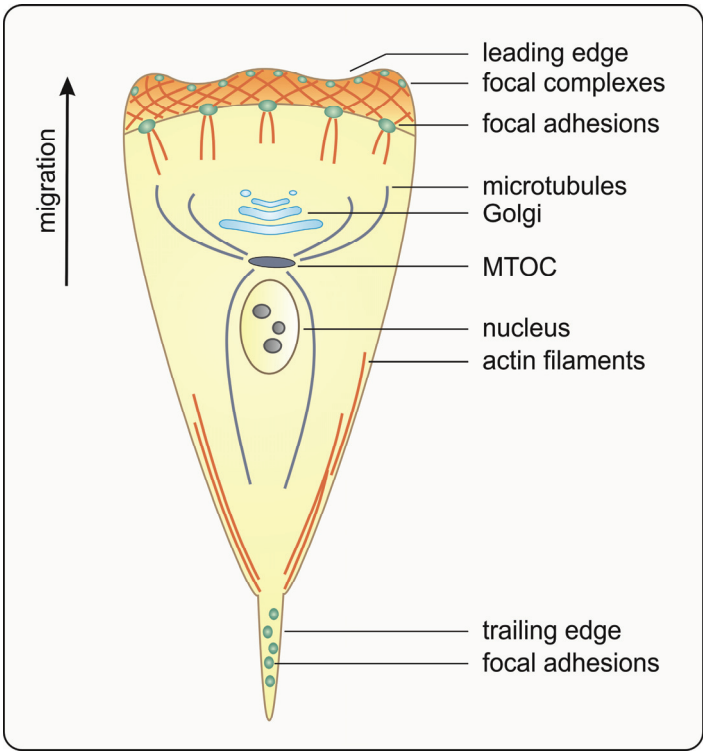


Figure 1. Schematic illustration of the migration cycle of a fibroblast. Protrusions form at the cell front, the leading edge, where actin filaments are organized in a branched network. Early adhesions, called focal complexes, form beneath. They mature into focal adhesions, which are connected to actin bundles. Golgi and microtubule-organizing center (MTOC) are in front of the nucleus, contributing to cell polarity. Focal adhesions are disassembled at the trailing edge, enabling the rear to retract.

Protrusion

The word protrusion originates from the Latin word *"protrusio"* and means pushing forward or away. During cell migration, membrane extensions form in the direction of migration, at the leading edge, which are then called protrusions. First, the plasma membrane expands, then, an underlying supporting backbone is formed and finally, contacts with the substratum are made. Membrane protrusion is driven by local polarized actin filaments and their polymerization (*11*). The organization of the filaments determinates the type of protrusion. With a branched actin network, forming a broad, flat and fan-like protrusion, the extensions are called lamellipodia, whereas when the polymerized actin filaments are organized into long parallel bundles, establishing a spike-like protrusion, they are termed filopodia. Filopodia act rather as mechanosensory devices to explore the environment or are involved in long-range cell-cell communications, while lamellipodia provide traction-surfaces and a basis for directional migration (reviewed in (*12*)). The Arp2/3 complexes and the Rho family GTPases play important roles (*13*), branching actin filaments into networks (*14*) and defining the type of protrusions, respectively. Microinjection of Rac induces dramatic lamellar ruffling, while injection of Cdc42 leads to filopodia-formation (*15-17*). In contrast, RhoA is degraded at protrusion sites through the activity of Smurf1, thereby preventing its signaling during dynamic membrane movements (*18*). However, a recent finding suggests a correlation of Rho activation with leading edge dynamics, which is restricted to the first 2 μm, whereas Cdc42 and Rac are activated behind (*19*).

Furthermore, several actin-binding proteins, like profilin, cortactin or the Ena/VASP proteins (reviewed in (*10*)), are involved in regulating the rate and organization of actin polymerization.

Polarity

For a cell to be able to move, it must be polarized, meaning that processes at the front and at the back must be distinct and lead to an asymmetric cell. Different positive feedback loops, including Rho GTPases, PI3Ks, integrins and microtubules contribute in establishing and maintaining cell polarity in response to stimuli.

One of the Rho GTPases, Cdc42, is active at the front of a migrating cell (*20*). Cdc42 influences polarity in several ways, e.g. by restricting where the leading edge forms and stabilizing it (*21*) or by localizing the microtubule-organizing centre (MTOC) and the Golgi apparatus in front of the nucleus (*22-29*). Local Cdc42 activation is accomplished by different feedback loops, among them a downstream target, PAK1 (*30*) or integrins (*31*). Another Rho GTPase, Rac, is also activated at the front (*21*). As for Cdc42 activity, several feedback loops help to define where Rac is active: microtubule polymerization activates Rac, which then stabilizes microtubules (*25,32*); a positive feedback loop with integrins was found (*33-35*), where also PtdIns(3,4,5)P_3 might be involved (*36*); Rac recruits and activates PI3K class I, which then produces PtdIns(3,4,5)P_3, which acts on Rac-GEFs (*21,37,38*). The latter feedback loop plays a special role in the accumulation of PtdIns(3,4,5)P_3 upon chemoattractant stimulation. The pathways of Rac and PI3K overlap, which is important for their intracellular functions and regulation of the responses (*39*). However, how Rac stimulates PI3K is not clear. The third best-characterized member of the Rho GTPase family, Rho, on the other hand, is active at the side and rear of the stimulated cell. It induces the formation of stress fibers, contributes to actomyosin contractility and microtubule stabilization (*23,40,41*).

The phosphoinositides PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 do not only act as gradient amplifiers in the feedback loops, they also contribute to asymmetry themselves. The accumulated PI3Ks produce PtdIns(3,4,5)P_3 at the cell front while the PTEN phosphatases act at the side and the rear to remove it (*42,43*).

Furthermore, the organization of the cytoskeleton is involved in polarity. As described above, actin is organized at the cell front as a network, whereas it is more filamentous and bundled in the rear (*44*). Myosin II has been shown to be important as well, especially in defining the rear (*45*).

Integrins and Adhesion

A cell does not only need to be polarized, it also must form adhesions at the leading edge and disassemble them at the trailing edge. Adhesion sites act as "molecular clutches", controlling the mechanical coupling between actin and the substrate and thus enabling the traction of the cell body and the retraction of the tail (reviewed in (*46*)). A large family of heterodimeric transmembrane receptors, the integrins, is the key family of migration-promoting receptors (reviewed in (*47,48*)). They connect the extracellular matrix (ECM) to the actin cytoskeleton, thereby acting as the "feet" of a migrating cell (*10*). Ligands of integrins are components of the ECM, for example fibronectin, vitronectin, collagen or laminin, or receptors of other cells, such as ICAMs (inter-cellular adhesion molecules) (reviewed in (*49,50*)). Characteristic for integrins is their activation through "inside-out" or "outside-in" signaling (reviewed in (*51*)). Activation can either be achieved by different cytoplasmic signals, resulting in a conformational change and thus an increase in ECM ligand affinity of the integrin ectodomain (inside-out). But integrins are also activated when they bind to an ECM ligand, leading to conformational changes and integrin clustering, resulting in intracellular changes (outside-in) (*52*). In both signaling processes, talin and also mechanical force play central roles. Integrins themselves are not catalytic active, but they transmit signals through direct or indirect binding of several interaction partners, e.g. talin, α-actinin, paxillin, FAK, vinculin, small GTPases and phospholipids (*52*).

Adhesion structures differ in their size, shape, localization and dynamics. Rapidly migrating cells such as leukocytes have rather submicroscopic adhesions, consisting of only a few integrin clusters. Small adhesions, the focal complexes, are also highly dynamic and occur at the leading edge, providing attachment to the ECM at the lamellipodium. Large integrin clusters are called focal adhesions and are matured focal complexes which did not disassemble. They are more stable and show a slower turnover of the adhesions, thus being typical for nonmigratory or slowly moving cells (*10,46*). Focal adhesions contain high levels of vinculin, talin, paxillin, FAK and integrins (reviewed in (*53*)).

Fibrillar adhesions, on the other hand, are elongated, centrally located structures and can arise from focal adhesions. They are only associated with thin actin cables and contain high levels of tensin, a capping protein negatively regulating actin assembly. Other unique structures are podosomes and invadopodia, rich in actin and matrix degradation properties. They are involved in physiological, but also pathological invasion processes and are formed by monocytic, endothelial and smooth muscle cells, but also carcinoma cells (reviewed in (*54,55*)).

However, not only adhesion structures towards the substrate are important. Cell-cell adhesions and subsequent initiated signaling is required to maintain the multicellular structure. Key components are cadherins and immunoglobulin-like cell-adhesion molecules (Ig-CAMs). Their involvement in cancer has also been shown, as reviewed by Cavallaro and Christofori (*56*).

Adhesion Disassembly and Detachment

Adhesion disassembly occurs both at the cell front and the back. At the front, the base of protrusions, adhesion structures must disassemble as new adhesions form near the leading edge (*57*). This process is termed adhesion turnover. It has been shown that FAK, Src, paxillin and ERK play a role in adhesion turnover at the cell front and that this is an important process for migration (*58*). Microtubules are involved, too, by bringing effectors in proximity with adhesion complexes and thereby promoting disassembly and remodeling (*59*). Several publications contribute to the model that the kinases FAK and Src play a role in the formation of a complex, which activates Rac and ERK locally, thereby leading to adhesion turnover at the leading edge (*10,58,60,61*).

Detachment at the rear of a cell is necessary for rapid migration. It is accomplished by adhesion disassembly as well as by mechanical contributions from cytoskeletal contraction and tension (*9*). The tension of strongly attached adhesions is sufficient to break integrin-actin cytoskeleton complexes, enabling migration while leaving membrane remnants behind (*62-64*). The disassembly is regulated by different

mechanisms. Myosin II, activated by its kinase MLCK, has been shown to localize at the rear and play a role (*65,66*). Proteolytic cleavage of proteins that link integrins to the actin cytoskeleton is mediated by the calcium-activated protease calpain (reviewed in (*67,68*)). Furthermore, endocytosis of integrins contributes to disassembly (*69,70*).

Together with the coordinated contraction of the actin cytoskeleton, the disassembly of adhesion sites promotes the retraction of the rear of a cell.

Contractile Forces and Traction

A migrating cell must at least generate two types of force: the protrusive force at the front and the contractile force, allowing the cell body to move forward. As described above, protrusion depends mainly on actin polymerization, whereas myosin-based motors provide contraction. These forces are not the only factors determining migration speed. Furthermore, the speed of a cell depends on the strength of its attachment. It is defined by the density of adhesive ligands, the density of adhesion receptors and finally, the affinity of the receptors for the ligands (*10*).

At adhesion sites, physical signals from outside, such as stretch and flow, are converted into intracellular (chemical) signaling, thereby creating a biological response. Since the stiffness and flexibility of the ECM are highly alterable, a cell needs to tightly regulate the forces created by the actomyosin system. Regulation of the myosin motor activity by the myosin light chain and heavy chain, and the microfilaments are some of the regulators of actomyosin contractility (reviewed in (*71*)). If defects in tension homeostasis occur, this can lead to tumorigenesis (described below).

1.1.2 Implications in Pathobiology

Alterations due to defects in the cell migration machinery are caused by either an impaired or an increased cell migration. In development, impaired migration leads to abnormalities in brain and heart, whereas in regeneration and in the immune system, wound healing and immune responses are affected. In contrast, an increase in cellular

motility of cells of the immune system can lead to infiltrations of improper sites, resulting in chronic inflammatory syndromes, such as arthritis and multiple sclerosis. Another negative aspect of increased cell migration is the development of atherosclerosis, where migration of vascular smooth muscle cells contributes to vessel thickening.

The most prominent pathological event of migration is occurring during cancer metastasis. Tumors resemble a wound that does not heal (*72*), because cells are able to hijack normal processes (*73*) and use them to spread into distant areas of a body.

Metastasis

Metastasis, the spread of malignant cells from the primary tumor to distant organs, is schematized as a multistep process (reviewed in (*74-76*)). First, tumor cells from the primary tumor must invade the surrounding tissue, then enter the bloodstream (intravasation) (*77*) or the lymphatics, survive the circulation until they arrest, followed by escaping the bloodstream (extravasation) and finally, colonize at distant organ site(s). At the new site, with a different microenvironment, the cells must initiate and maintain growth, and develop new blood vessels (angiogenesis). All of these steps must successfully be completed to lead to a metastatic disease. Studies have shown that especially the early steps are very efficient, whereas later steps are more inefficient (*74*). The infiltration of cancer cells into distant organs does not immediately lead to metastatic disease (reviewed in (*78*)). The time between primary tumor diagnosis and the colonization to detectable metastases can account up to decades and is called latency. Disseminated cells, unable to colonize, are termed dormant. A whole tumor mass can also be dormant, characterized by a balanced state of proliferation and apoptosis (reviewed in (*78*)).

Another aspect of metastasis is the organ-specific pattern of spread. Already in 1889, Stephen Paget published the “seed and soil” hypothesis (*79*). He documented a non-random pattern of metastasis and hypothesized that certain tumor cells (“seed”) have a special affinity for certain organs (“soil”). Forty years later, James Ewing proposed

that organ-specific metastasis occurs due to mechanical forces and circulatory patterns (*80-82*). Later, detailed analyses (*83,84*) showed that both seed-soil compatibility and mechanical factors contribute to these observations. Today, focus is also on the tumor microenvironment, which is not a passive bystander, but rather an active participant (*85*).

Invasion

Normally, cells in epithelial sheets are tightly bound to their neighbors and the underlying basement membrane. This immobilization is accomplished by adherens junctions, tight junctions, desmosomes and hemi-desmosomes. During tumor progression, cancer cells escape these associations and the first step is to dissolve the basement membrane. Then, they invade tumor-surrounding stroma and migrate to blood vessels or lymphatics (reviewed in (*86*)). Different modes of cancer cell migration are known, they move either as single cells or collectively. This depends on cell-specific as well as on microenvironment mechanisms (reviewed in (*87*)).

Single-cell movement is observed when cell-cell-adhesions are absent, and cells invade either in a mesenchymal or in an amoeboid manner.

Mesenchymal invasion is characterized by elongated cells, attaching to and pulling on the matrix, which show high levels of cell-matrix adhesions, Rac activity and proteolysis (*88-90*).

In contrast, amoeboid invasion, where cells protrude through spaces by changing shape and pushing off the matrix, is independent of the above mentioned requirements and the matrix is left intact behind. Cells require high actomyosin contractibility and Rho activation to squeeze and pull (*87,88,91-94*).

Collective cancer cell invasion is the most frequent type of invasion. Cells express cell-cell junctions and adhesion receptors and can therefore move in a multicellular unit (reviewed in (*56*)). Depending on the morphology, the cell-cell adhesions and the supracellular coupling of cell-cell signaling, collective invasion can vary. Cell groups can form small strands, bigger masses, where some cells do not have contact to the ECM, but also luminal structures (*95*). Within these broad masses, the

structure of the front varies, too. Depending on the invaded tissue, the front must combine proteolytic, protrusive and expanding functions. The underlying mechanisms still remain elusive, but collective invasion has initiated a debate on EMT (Epithelial-to-Mesenchymal Transition). EMT, a signaling program which weakens the cell-cell adhesions, thus enabling a higher motility of the cells, is thought to be an important factor during invasion. Tumors with intact adhesions and circulating grouped tumor cells demonstrate that metastasis can also occur without EMT. However, the possibility of EMT at the front of a moving cell mass may not be excluded (discussed in (*87*)).

Additionally, cancer cells are able to switch between the different types of migration (reviewed in (*6,89,94,96*)), making them more flexible. This has especially an impact on therapy resistance, e.g. when protease inhibitors are applied.

During invasion, the adherence to the ECM is mediated for example through integrins, whereas the cell-cell adhesions are mainly formed through cadherins. Recruitment of proteases, as mentioned above, is not only important to degrade the matrix, but also to liberate embedded growth factors and chemokines, and may activate cell surface proteins. Besides that, cancer cell invasion can also be ameliorated by the recruitment of inflammatory cells, which then produce matrix-degrading enzymes (reviewed in (*85*)).

Remodeling of the actin cytoskeleton plays of course, also an important role in cancer cell migration. Factors for actin polymerization have been found to be implicated in cancer (reviewed in (*97,98*)). As described above, phosphoinositides are involved in cell migration, and they clearly have a role in cancer progression, too (reviewed in (*99,100*)). Exemplary, the presently well studied phosphatase PTEN was found to be a tumor suppressor (reviewed in (*101,102*)) and mutations and amplifications of PI3Ks were identified (*103-105*), both being brought into cancer therapy as potential therapeutic candidates.

1.2 Phosphoinositides

Phosphoinositides (PIPs) are phosphorylated derivates of phosphatidylinositol (PtdIns) and belong to the group of glycerophospholipids. They consist of a glycerol-backbone, which is esterified by two fatty acids and a phosphate group substituted with an inositol polar head group, facing the cytosolic surface of membranes. The fatty acids form the lipid tail, which is mostly membrane-bound. Among the lipids, phosphoinositides together with their precursor phosphatidylinositol account for less than 10% of the total cellular phospholipids in eukaryotic membranes. However, they play a central role in a number of cellular processes; regulating membrane trafficking, cell signaling and cytoskeleton dynamics. They can act as components of membranes and can even define them.

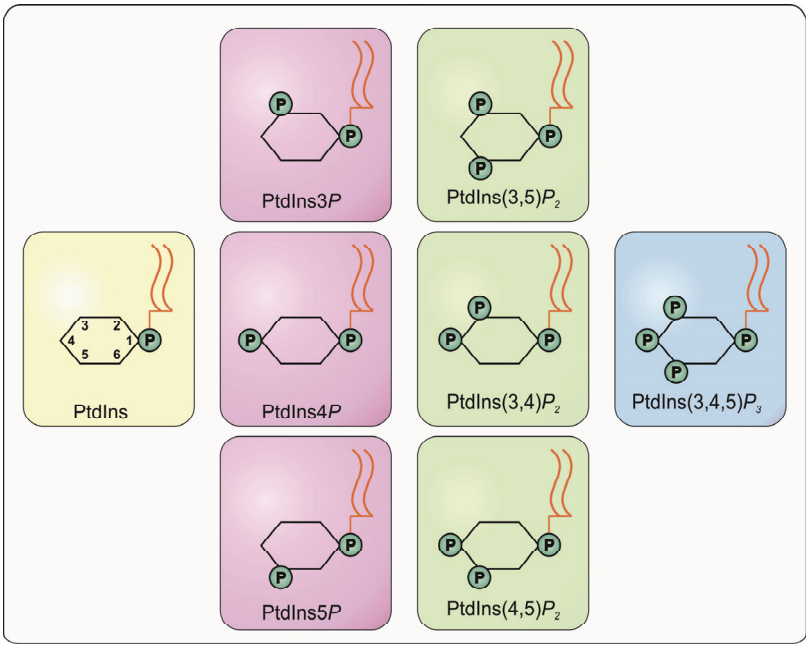


Figure 2. Overview phosphoinositides. To the left (yellow), phosphatidylinositol. Phosphorylated derivates of phosphatidylinositol are called phosphoinositides. They can either be monophosphorylated (purple), bisphosphorylated (green) or trisphosphorylated (blue). The glycerol-backbone esterified with two fatty acids is illustrated by red lines. The P indicates a phosphate group.

PtdIns is synthesized primarily in the endoplasmic reticulum, delivered then to other membranes either by vesicular transport or via cytosolic transfer proteins (reviewed in (*106*)). The inositol headgroup includes five stereochemically unique hydroxyls, where only three can reversibly be phosphorylated: the D3-, D4- and D5-position. Hence, this results in seven distinct, but interconvertible phosphoinositides. They can either be monophosphorylated, bis- or even trisphosphorylated (Figure 2). Phosphorylation is achieved by different kinases, whereas phosphatases hydrolyze the phosphate group. These phosphorylation/dephosphorylation cycles are highly regulated and build up a special spatio-temporal distribution of the phosphoinositides throughout a cell and its organelles (Figure 3). Therefore, the actual steady-state concentration of a phosphoinositide varies throughout the organelles. At the same time, it also defines them and their function.

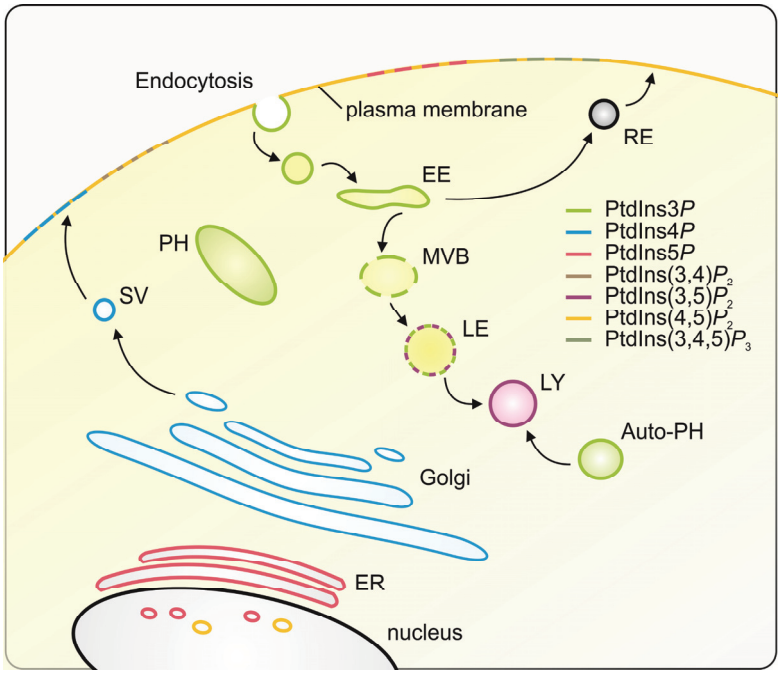


Figure 3. Localization of phosphoinositides (*107-110*). Phosphoinositides are distributed differently throughout the organelles of a cell. PtdIns(4,5)P_2 is highly enriched at the plasma membrane. Other phosphoinositides of the membrane are PtdIns(3,4,5)P_3 (at the leading edge), PtdIns3*P*, PtdIns4*P*, PtdIns5*P* and PtdIns(3,4)P_2. PtdIns3*P* and PtdIns(3,5)P_2 are

enriched at vesicles of the endocytic pathway. PtdIns3*P* is also enriched on phagosomes and autophagosomes. The major phosphoinositide at the Golgi is PtdIns4*P*. PtdIns5*P* is also found at the ER. Nuclear phosphoinositides are PtdIns5*P* and PtdIns(4,5)P_2. Auto-PH, autophagosome; EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; LY, lysosome; MVB, multivesicular bodies; PH, phagosomes; RE, recycling endosome; SV, secretory vesicle.

Together with the high turnover through the activity of kinases and phosphatases, the different subcellular distributions make the phosphoinositides to optimal signaling mediators.

1.2.1 Binding domains

In addition to the well-studied role of phosphoinositides in generating second messengers, the regulation of different signaling effects can also be achieved by binding to cytosolic proteins or cytosolic domains of proteins. This occurs via special binding domains. Binding to these domains regulates the localization of the target protein and can activate it through conformational changes or through its recruitment to special membranes/organelles. At present, there are 15 modules considered to be phosphoinositide-binding domains, and the most common are briefly introduced here.

PH domains

This protein module of around 100 amino acids was the first domain found to associate with phosphoinositides (*111-113*). The name reflects the sequence homology to the protein pleckstrin, which contains two PH domains. PH domains can be very different in their primary structure, but the tertiary structure is similar: they have a 7-stranded β-sandwich structure, where the loop between the first two β-strands functions as a "platform" for the interaction with the phosphoinositide (reviewed in (*114*)). Depending on the protein, PH domains bind PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. More than 200 human proteins containing a PH domain are known, with a varying specificity and affinity among them (reviewed in (*115*)).

FYVE domains

These domains are zinc finger modules and contain about 60-70 amino acids. The abbreviation evolved from the four proteins in which it has initially been found: Fab1p (formation of aploid and binucleate cells), YOTB, Vac1p (vacuolar segregation protein) and EEA1 (early endosome antigen 1) (*116*). The domain consists of two double-stranded antiparallel β-sheets and a small α-helix, which are held together by two Zn^{2+} ions (*117*). Even though zinc fingers are known to function in DNA-binding, the FYVE domain has to be distinguished because of the presence of other motifs, which form the actual binding site for PtdIns3*P*. The conserved sequences WxxD, RR/KHHCR and RVC form a highly positively charged binding site (*118*), thus enabling a high specificity and affinity for PtdIns3*P*. The 26 known FYVE-domain containing proteins have diverse functions and one major is the regulation of endocytic trafficking and fusion of endosomal membranes with transport vesicles and other organelles (*119,120*). Even though the FYVE domain is believed to be specific for only PtdIns3*P* (*121-124*), there is evidence that some atypical FYVE domains can bind the structural similar PtdIns5*P* as well (*125*) or even PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (*126*).

PX domains

First identified in two phagocyte NADPH oxidase (phox) subunits, $p40^{phox}$ and $p47^{phox}$ (*127*), this domain of 130 amino acids has been found in more than 30 human proteins. Like the PH domains, despite little sequence similarity, the PX domains show a highly conserved 3D structure consisting of a three-stranded β-sheet, packed against a helical subdomain composed of three or four α-helices (*128*). The preferred phosphoinositide is PtdIns3*P*, although there are examples in mammals where PtdIns(3,4)P_2 or PtdIns(4,5)P_2 are bound by PX domains. The binding of PtdIns3*P* is, like in the case of the FYVE domain, based on headgroup binding as well as on electrostatic attraction via hydrogen bonds (*129*). PX domains can also bind to other modules, such as the SH3 domain (*130*), which may contribute to phosphoinositide binding and function.

ENTH domains

The Epsin N-terminal homology domain is a region of approximately 150 amino acids (*131*) and is forming a well defined pocket to bind PtdIns(4,5)P_2. Proteins containing an ENTH domain are involved in endocytosis and regulation of cytoskeletal organization.

GRAM domains

This domain is found in Glucosyltransferases, Rab-like GTPase activators and Myotubularins, where the acronym comes from (*132*). It consists of 70 amino acids and can bind or contribute to binding to PtdIns(3,5)P_2 (*133,134*). There has also been reported a binding to PtdIns4*P* (*135*). Interestingly, the crystal structure of one of the myotubularin phosphatases, MTMR2, revealed that the GRAM domain in it forms β-strands, which are part of a PH domain (*136*). Hence, the GRAM domain in the myotubularin family is referred to as the PH-GRAM domain and became a hallmark of this family. It was shown that the PH-GRAM domain enables a positive feedback loop through binding to the allosteric activator PtdIns5*P* (*137,138*).

1.2.2 Phosphoinositide-metabolizing Enzymes

As mentioned above, the spatio-temporal distribution of phosphoinositides plays a very important role. Highly specific kinases and phosphatases with a balanced activity are necessary, since the phosphoinositides are highly interconvertible. These enzymes are distributed unequally in the cell, so that each organelle is equipped with different enzymes (reviewed in (*107*)). They are divided into different classes according to their catalytic reactions and substrate specificity.

Kinases

3-Kinases

Phosphoinositide 3-Kinases (PI3Ks) phosphorylate the inositol ring of three species of substrates at the D3-position. The 3-Kinases are divided into three classes, IA and B, II and III, showing different selective substrate specificity and subunit organization (reviewed in (*139-142*)).

The class I enzymes use PtdIns(4,5)P_2 as a substrate, thereby generating PtdIns(3,4,5)P_3. In mammals, they are present in all cell types and were shown to localize at the plasma membrane and in the nucleus (reviewed in (*107*)).

The PI3Ks class II prefer the substrates PtdIns4*P* and PtdIns, producing PtdIns(3,4)P_2 and PtdIns3*P*, respectively (*143,144*). The three mammal isoforms PI3K C2α, β and γ are monomeric and have different localizations, such as the Golgi complex, the plasma membrane or endosomes.

Finally, the class III, which consists of the sole catalytic subunit VPS34 (vacuolar protein sorting), is specific for the phosphorylation of PtdIns only (*145,146*). Therefore, the correct name for this enzyme should be phosphatidylinositol 3-kinase, as discussed in (*147*). VPS34 localizes to endosomal membranes and can form distinct protein complexes which then define VPS34´s biological role. Known functions in mammals relate to the regulation of endocytosis, in autophagy, mTOR signaling and phagocytosis.

Phosphatidylinositol 4-Kinases

These kinases are specific for the phosphorylation at the D4-position of the inositol ring, thereby producing PtdIns4*P*. Two classes of phosphatidylinositol 4-Kinases exist, the type II PI4Ks, PI4KIIα and PI4KIIβ, and the type III PI4Ks, PI4KIIIα and PI4KIIIβ. All four isoforms use only PtdIns as substrate, but they differ in their localization (reviewed in (*148*)).

Phosphatidylinositol phosphate kinases

According to their substrate specificity, this family is subdivided into three classes. The type I phosphatidylinositol phosphate kinases are responsible for the bulk of PtdIns(4,5)P_2 production. They use PtdIns4*P* as a substrate and phosphorylate it at the D5-position, hence called phosphatidylinositol phosphate 5-Kinases (PIP5Ks). Three isoforms exist (α, β and γ), not being redundant, with different localizations and different mechanisms of regulation (reviewed in (*149*)). Furthermore, it has been

suggested that they can also phosphorylate other phosphoinositides *in vitro* (*150-152*).

The second subclass, phosphatidylinositol phosphate 4-Kinases (PIP4Ks), uses PtdIns5*P* as a major and PtdIns3*P* as a minor substrate (*153,154*). Three isoforms with different localization are known, PI5P4Kα, PI5P4Kβ and PI5P4Kγ, with striking differences in activity, PI5K4α having the highest (reviewed in (*155*)). Since it is unlikely that PIP4Ks contribute largely to the production of PtdIns(4,5)P_2, their main function is suggested to be the regulation of PtdIns5*P* levels in the cell (*155-157*).

The third class of these kinases produces PtdIns(3,5)P_2, using PtdIns3*P* as a substrate (*158*) and thus being 5-Kinases. They are found in almost all eukaryotic cells (*159*). The mammalian enzyme is PIKfyve, a homolog of the yeast Fab1 PtdIns3*P* 5-kinase, which localizes to early and late endosomes and lysosomes (*160-164*). The production of PtdIns5*P* through PIKfyve has been suggested (*165-169*), but is controversial and could be indirectly via PtdIns(3,5)P_2 (*170*). Knockdown or knock out of the gene, or inhibition of the kinase activity results in enlarged vacuoles (*169,171-174*), suggesting a role in membrane and protein recycling. Other roles, like in Glucose transport, are reviewed by Shisheva (*175,176*).

Phosphatases

In mammalian cells, 35 phosphoinositide phosphatases have been identified (*177*). These phosphatases can be classified in different ways, e.g. by dividing them into two superfamilies according to their catalytic mechanisms (protein tyrosine phosphatase superfamily and inositide polyphosphate phosphatases) or by subdividing them on the basis of the position of the removed phosphate group. By giving a brief overview, the latter classification is used here.

Phosphoinositide 3-phosphate phosphatases

The probably most famous 3-phosphate phosphatase is PTEN, originally identified as a candidate tumor suppressor gene (*178-181*) and later found to be mutated in a

large number of cancers. It dephosphorylates PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, with the latter one being the primary substrate *in vivo* (*180,182*).

Another group are the myotubularins, a family of currently 14 members (reviewed in (*183-186*). The first member, MTM1, was found to be mutated in myotubular myopathy, leading then to the name for the whole family. Substrate preferences are restricted to PtdIns3*P* and PtdIns(3,5)P_2, resulting in the production of PtdIns and PtdIns5*P* (*136,137,187-199*). Interestingly, nearly half of the family members are inactive, but play however an important role: via heterodimerization they regulate the active members (reviewed in (*183,186*)).

Phosphoinositide 4-phosphate phosphatases

These phosphatases preferentially hydrolyze the D4-position phosphate of a target phosphoinositide. The family contains the two inactive members P-REX1 and P-REX2 (*200-202*), which are thought to be critical for regulation, similar to inactive members of the myotubularin family.

Active members can be subdivided into phosphatases specific for PtdIns(3,4)P_2 or PtdIns(4,5)P_2. Both groups contain each 2 mammalian isozymes (*203*), but interestingly, the group of PtdIns(4,5)P_2 4-phosphatases includes also bacterial ones, e.g. IpgD (Invasion plasmid gene D). IpgD is directly injected by the pathogen *Shigella flexneri*, which is responsible for causing bacillary dysentery in humans. In consequence, host cell membrane PtdIns(4,5)P_2 is dephosphorylated at the cell membrane, leading to membrane blebbing and actin filament remodeling (*204-206*).

Phosphoinositide 5-phosphate phosphatases

This relatively large family consists of 10 mammalian and 4 yeast enzymes. They remove the D5-position phosphate of polyphosphate phosphoinositides and inositol phosphates. The SHIP family, Synaptojanins as well as the INPP (inositol polyphosphate phosphatase) family belong to this group, to name some examples (reviewed in (*177*)).

Sac domain phosphoinositide phosphatases

The Sac phosphatase domain, which was first identified in the yeast suppressor of actin (ySac1) (*207*), is characterizing this group. This domain exhibits a broad specificity, thus enabling the enzymes to hydrolyze PtdIns3*P*, PtdIns4*P*, PtdIns5*P* and even PtdIns(3,5)P_2 (*208*). A subgroup is known, which utilizes in addition PtdIns(4,5)P_2.

1.2.3 Phosphoinositides

Phosphatidylinositol monophosphates

PtdIns3*P* is generated through the action of class II and class III PI3Ks. It can be further metabolized to PtdIns, PtdIns(3,4)P_2 and PtdIns(3,5)P_2 by kinases and phosphatases, as described above. PtdIns3*P* is highly enriched in early endosomes, intralumenal vesicles of MVEs and yeast vacuoles (*209*), but has recently been found also to localize at smooth endoplasmic reticulum and/or the Golgi (*210*) and the midbody (*211*). During autophagosome formation, PtdIns3*P* is highly enriched at compartments forming the autophagosome (*212-214*). The recruitment of intracellular proteins via PtdIns3*P*-binding domains, e.g. the FYVE or PX domains, is tightly linked to its functions. Roles of PtdIns3*P* are in endocytic membrane traffic, exocytosis and autophagy. Local pools of PtdIns3*P* control autophagy initiation and the regulation of autophagosome size (*215*). Furthermore, it has been suggested that this lipid can also act as an intracellular second messenger (reviewed in (*216,217*)).

PtdIns4*P* is the most abundant phosphoinositide in mammalian cells with about 10% of total phosphoinositides (*218*). The so called “canonical pathway” describes this lipid mainly as a precursor for PtdIns(4,5)P_2, however, it has its own direct effects (reviewed in (*219*)). Prevalent localization is at the Golgi complex, where it is crucial for function (reviewed in (*157,218,220,221*)). Interestingly, 2 studies showed that it was most abundant in the plasma membrane (*210,222*), where it fulfills its own functions (*223*).

PtdIns5*P* is the last member of the phosphoinositide family to be discovered (*153*). It was found to be constitutively present in many cell types, and to localize at the plasma membrane and at the smooth endoplasmic reticulum and/or Golgi (*210*). Several extracellular stimuli lead to an increase in PtdIns5*P* levels (*154,166,206,224-233*). A nuclear role in regulating apoptosis has been proposed (*225*). The main route of PtdIns5*P* production involves the dephosphorylation of another phosphoinositide, PtdIns(3,5)P_2 (*170*). A direct phosphorylation of PtdIns through PIKfyve has been suggested (*165*), but is controversial. It has been shown that 4-phosphatases are able to generate PtdIns5*P*, by using PtdIns(4,5)P_2 as a substrate (*203,206,229*). PtdIns5*P* can be further phosphorylated by the type II PIPKs to PtdIns(4,5)P_2 (*153*).

Phosphatidylinositol bisphosphates

PtdIns(3,4)P_2 is a minor phosphoinositide. This lipid has mainly been considered to be the precursor of PtdIns(3,4,5)P_3. However, through interaction with PH and PX domains it can also act independently (reviewed in (*157*)).

The entire pool of PtdIns(3,5)P_2 is synthesized through phosphorylation of its precursor, PtdIns3*P*, by the class III PIPkinase PIKfyve in mammals (*170,234*), or by Fab1 in yeast and plants (*171,235*). It is predominantly found on late endosomes and major roles are in endo-lysosome function, membrane trafficking, autophagy and Glucose transport (*236-241*). It can be further metabolized by the myotubularin phosphatases to PtdIns5*P* or by 5-phosphatases to PtdIns3*P*. The dephosphorylation through the myotubularins is very important for PtdIns5*P* synthesis, since it has been shown that most of the PtdIns5*P* pool is generated directly from PtdIns(3,5)P_2 (*170*).

PtdIns(4,5)P_2 is enriched on the inner surface of the plasma membrane. The major route of its synthesis occurs via phosphorylation of PtdIns4*P* by PIP5Kinases. A minor pool is generated through the action of PIP4Kinases, which phosphorylate PtdIns5*P*. Both 5- and 4-phosphatases can act on PtdIns(4,5)P_2, thus generating PtdIns4*P* or PtdIns5*P*, respectively. Initially, PtdIns(4,5)P_2 was known for its role as precursor to the second messengers diacylglycerol (DAG), inositol (1,4,5)-

trisphosphate and PtdIns(3,4,5)P_3 (reviewed in (*242*)). But since then, PtdIns(4,5)P_2 became to an important regulator of the actin cytoskeleton (*99,243,244*), being involved in cell shape regulation, motility and cytokinesis. Other functions are in endo- and exocytosis (reviewed in (*106*)).

Phosphatidylinositol trisphosphate

Barely detectable in resting cells (*245*), the interest in PtdIns(3,4,5)P_3 has highly increased since the discovery of its implications in cancer. Today, it is part of one of the most studied pathways in signal transduction. The main pool derives from PtdIns(4,5)P_2 through phosphorylation by the class I PI3Ks. In yeast, the possibility of generating it from PtdIns(3,4)P_2 has been suggested, and it might be an alternative route in mammals, too (reviewed in (*157*)). The tumor suppressor PTEN is the major phosphatase acting on PtdIns(3,4,5)P_3, dephosphorylating it on the D3 position, thereby leading to PtdIns(4,5)P_2 accumulation. As its precursor, it is found at the plasma membrane, but mainly at the leading edge (*38,246-249*). Briefly, roles of PtdIns(3,4,5)P_3 are in insulin signaling, cell proliferation, survival and growth; and in cell migration (reviewed in (*157*)).

1.3 Intracellular signaling during cell migration

During cell movement, a chemoattractant signal is detected by binding of chemokines or growth factors to specific cell membrane receptors. Chemokines, a large family of small secreted peptides, bind to G-protein-coupled receptors (GPCRs), whereas growth factors stimulate migration through the action of receptor tyrosine kinases (RTKs) (*75*). Signal transduction pathways are then activated to regulate the cytoskeleton and motility, but also invasion during cancer cell migration. GPCR stimulation results in the dissociation of a heterotrimeric G-protein into its subunits, which then initiate downstream signaling events. RTK activation, on the other hand, leads to receptor dimerization and subsequently to transphosphorylation of specific tyrosine residues (reviewed in (*250-252*)). This stabilizes catalytic activity and creates binding sites for adaptor and signaling proteins, such as SH2-domain containing proteins (reviewed in (*253*)). The binding of other phosphotyrosine-binding domain containing proteins leads to the activation of PI3Ks, which have, as discussed below, important functions in phosphoinositide regulation, which are in turn, key regulators of cell migration. Adaptor and scaffold proteins, lacking enzymatic activity, may act to improve assembly of signaling proteins to the RTKs. For example, interactions of Src with growth factor receptors and FAK with (catalytic inactive) integrins stimulate the formation of a FAK-Src complex, which then can trigger invasion (reviewed in (*254-256*)).

A well-known growth factor involved in motility is the hepatocyte growth factor (HGF), which binds to the c-met growth factor RTK. HGF is also called scatter factor, because dysregulation can lead to invasion (reviewed in (*257,258*)).

Downregulation of the signaling can be achieved in different ways. Receptor dephosphorylation by protein tyrosine phosphatases, as well as downregulation through endocytosis and degradation are mechanism to modulate and terminate signaling (*252*).

There is increasing evidence that RTKs are also regulated due to their membrane distribution (reviewed in (*259*)). Locally high levels of RTKs can promote the formation of dimers or clusters even in the absence of their ligand, offering a "primed" state for ligand-activation. Surface abundance of the RTKs could be controlled through different membrane domains or the actin cytoskeleton. This compartimentalization can also have an impact on receptor trafficking and endocytosis.

1.3.1 Fibroblast Growth Factor Receptors

One of the subfamilies of the RTKs are the fibroblast growth factor receptors (FGFRs). This family consists of 4 structurally similar receptors, encoded as FGFR1-4 (reviewed in (*260*)). A prototypical FGFR is composed of three extracellular immunoglobulin (Ig)-like domains (D1-D3), an acid box (within the D1-D2 linker), a single-pass transmembrane domain and a cytoplasmic tyrosine kinase domain (Figure 4). It has been shown that only the D2 and the D3 Ig-like domains, including their linker, participate in ligand binding, whereas the D1 and the acid box regulate receptor autoinhibition (*261*).

Ligands of the FGFRs are the fibroblast growth factors (FGFs) (described below), discovered at first as mitogens for cultured fibroblasts (*262*). To date, 18 FGFs are known which bind with high affinity to their FGFRs, but also with low affinity to heparin sulfate proteoglycans (HSPG). In contrast to many other growth factors, FGFs are not able to induce receptor dimerization themselves. However, through binding to the low-affinity receptors, a dimeric 2:2:2 ternary complex, including FGF, FGFR and HSPG, is formed (*263*). Receptor dimerization enables then, in turn, transphosphorylation of tyrosine residues in the activation loop in the kinase domain. This autophosphorylation occurs in three stages (*264,265*). At the first stage, tyrosine^{653} in the activation loop is phosphorylated. The inactive or low activity state of the tyrosine kinase is thereby turned into an active state by removing a "molecular brake" of hydrogen bonds at the kinase hinge region (*266*). The kinase activity is increased 50- to 100-fold. During the second stage, another tyrosine, tyrosine^{583} at

the kinase insert region is autophosphorylated, followed by tyrosine^{463} in the juxtamembrane region, tyrosine^{766} in the C-terminal tail and tyrosine^{585} in the kinase insert region. The third stage phosphorylation takes again place in the activation loop, on the other tyrosine (tyrosine^{654}), leading to an additional 10-fold stimulation. A seventh tyrosine is phosphorylated (tyrosine^{730}), but with much lower stoichiometry, presumably due to its location at the end of an α-helix (*267*). Thus, autophosphorylation in FGFR is a strictly ordered reaction. It was found that this sequential phosphorylation is kinetically controlled and limited by the rate of the phosphoryl transfer (*265*). Altogether, the activation of the receptors leads to the initiation of different signaling cascades and biological responses (reviewed in (*260,268-271*)).

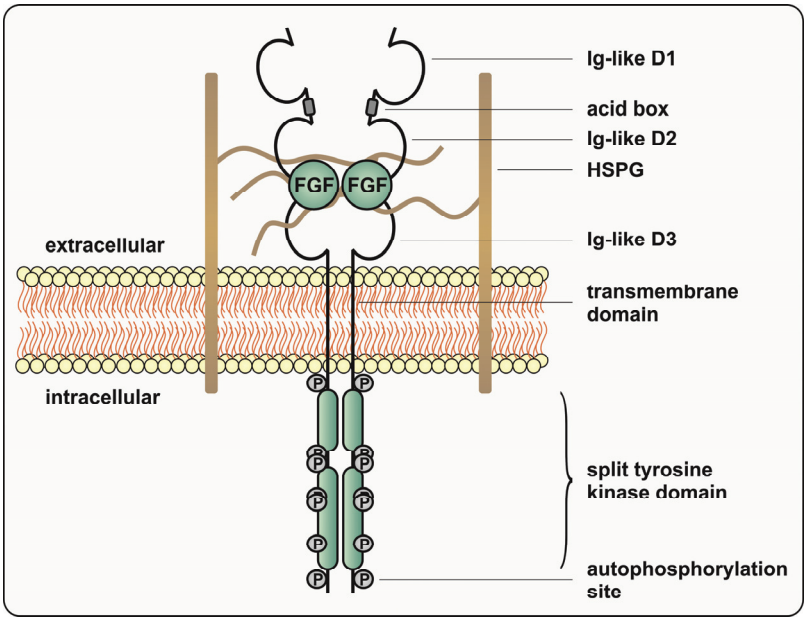


Figure 4. Illustration of the FGFR structure (activated). Prototypical FGFRs consist of three extracellular immunoglobulin (Ig)-like domains, an acid box, a single-pass transmembrane domain and an intracellular split tyrosine kinase domain. Ligand binding occurs between the second and the third Ig-like domain. The FGF-FGFR complex comprises two receptor molecules, two FGFs and two heparin sulfate proteoglycans (HSPG), which is known as the symmetric model. Complex formation leads to receptor autophosphorylation and activation. Major autophosphorylation sites are indicated (P).

Cellular signaling via FGFRs

Among the signaling proteins, which are activated by a phosphorylated FGFR, are phospholipase Cγ (PLCγ) and FRS2 (FGFR substrate 2) (*272-274*) (Figure 5).

The C-terminal part of the receptor comprises a tyrosine residue (Tyrosine^{766} in FGFR1), which serves as a binding site for PLCγ via its SH2 domain (*272*). Subsequently, PLCγ is phosphorylated and activated, thus leading to PtdIns(4,5)P_2 hydrolysis. The second messengers diacylglycerol and Inositol(1,4,5)trisphosphate are generated, which activate protein kinase C (PKC) via released calcium storages.

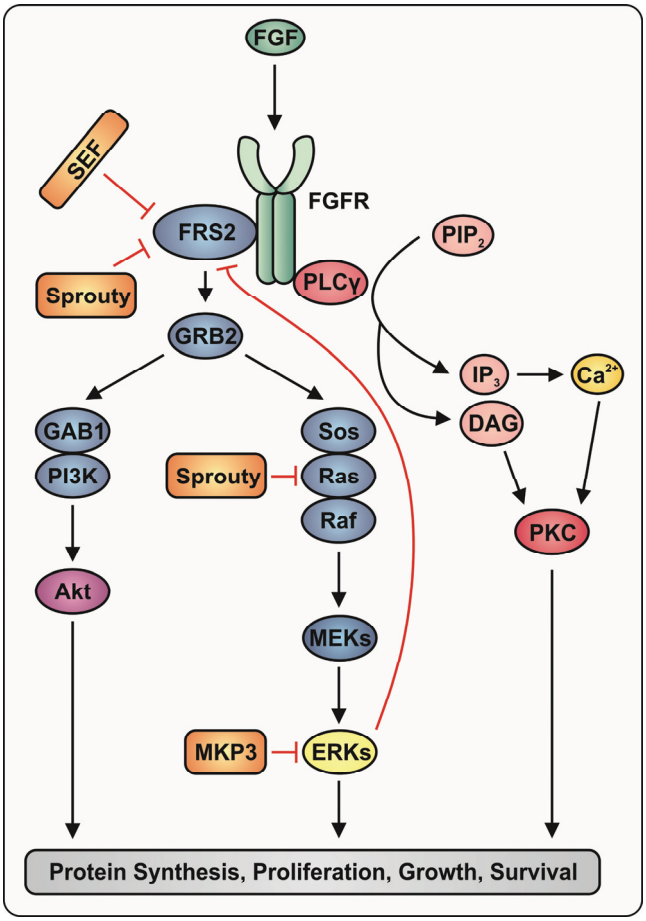


Figure 5. Signal transduction network downstream of FGFRs. Schematic overview of the three main pathways: PI3K-Akt, RAS-RAF-MAPK and PLCγ. Negative signaling and negative feedback loop are indicated in red. FRS2, FGFR substrate 2; SEF, similar

expression to *fgf* genes; GRB2, growth factor receptor-bound protein 2; GAB1, GRB2-associated binding protein 1; PI3K, phosphoinositide 3-kinase; Sos, son of sevenless; MEK, MAP/ERK kinase; ERK, extracellular-signal-regulated kinase; MKP3, MAPK phosphatase 3; PLCγ, phospholipase Cγ; PIP_2, PtdIns(4,5)P_2; IP_3, inositol(1,4,5)trisphosphate; DAG, diacylglycerol; PKC, protein kinase C.

The two members of the FRS2 family, FRS2α and FRS2β, contain phosphotyrosine binding (PTB) domains, which bind to the juxtamembrane domain of FGFRs (*273,274*). Interestingly, FRS2 is constitutively, independent of ligand stimulation and tyrosine phosphorylation, associated with FGFR. It becomes phosphorylated upon FGF-stimulation and serves then itself as docking site for other signaling complexes, which activate Ras/MAPK and PI3K/Akt signaling pathways (reviewed in (*270*)).

Another interesting aspect is the different routing of the receptors upon binding of distinct ligands. For example, depending on which FGF is bound to FGFR2, it is either degraded or recycled to prolong signaling (*275*).

Deactivation mechanisms

Mechanisms for signal attenuation of the FGFRs to prevent excessive intracellular signaling are not well understood at present. Two mechanisms have been shown to terminate the signals generated by activated FGFRs. Deactivation can be achieved on one hand through degradation of the receptor, via endocytosis and subsequent lysosomal degradation (*276*). On the other hand, negative regulators and negative feedback loops act to downregulate, such as ERKs, MAPK phosphatase 3, Sprouty proteins or Sef (similar expression to *fgf* genes) (*271,277-279*) (Figure 5).

1.3.2 Fibroblast Growth Factors

Fibroblast growth factors (FGFs) build a large family of total 22 structurally related polypeptides. They induce mitogenic, chemotactic and angiogenic activity in cells, which have mesodermal and neuroectodermal origin (reviewed in (*280*)). Most FGFs bind to specific receptor tyrosine kinase receptors, the FGFRs, with high affinity and

with lower affinity to HSPGs. Four of the growth factors do not bind to FGFRs and rather act intracellularly (FGF11-14; FGF homologous factors, FHFs), indicating a remarkable adaptability during evolution (reviewed in (*281*)). Depending on the status of a cell, different FGFRs and HSPGs are expressed on the surface, and FGF signaling can be modulated by the specific HSPGs. They can amplify, but also inhibit receptor activation. Furthermore, the presence of other growth factors can influence the effects of FGF signaling.

Most of the FGFs are secreted through the classical polypeptide secretion pathway, however, three family members, FGF1, FGF2 and FGF9 lack classical leader sequences (reviewed in (*269*)). These three growth factors use a non-classical pathway and evade the ER and Golgi, by a mechanism which is still elusive. After secretion, most FGFs are stored in the ECM through binding to HSPGs. Thereby, the growth factors are protected from proteolysis and a biologic reservoir is created. They can then either act short-range directly on target cells or are released due to ECM digestion.

As already mentioned, FGF signaling is important in embryonic development (reviewed in (*282*)) and wound healing processes. Some of the growth factors, FGF1-5 and FGF7, have angiogenic potential (*269*). FGFs might also play a role in apoptosis although this is not clear. Nuclear roles of FGFs have been identified, thus demonstrating downstream effects different from those initiated by the receptors (reviewed in (*283-285*)). Below, the role of FGF signaling in carcinogenesis will be described.

1.3.3 Involvement of FGFR signaling in Cancer

Since FGFRs are important regulators of fundamental processes, like organogenesis, tissue repair, angiogenesis and inflammation, it is not surprising that imbalances can cause severe diseases like cancer. There, increased FGFR signaling can be oncogenic, but might also be in some cases tumor suppressing.

Several mechanisms are known to increase the signaling of the FGFR. Upregulated expression of the receptor due to amplification or aberrant transcriptional regulation

can lead to overactivation (reviewed in (*286-288*)). Alterations in FGFR expression are discussed whether being a "driver" or a "passenger" in carcinogenesis. One of the most amplified chromosomal regions in breast cancer, 8p11-12, contains the *FGFR1* gene. This region is amplified in approximately 10% of human breast cancers and associated with poor prognosis for the patients. Since this region is gene rich, and FGFR1 seems to be not always overexpressed even though amplified, its contribution is not clear. However, *in vitro* studies indicate an involvement of FGFR1 in breast cancer progression and FGFR1 might be a therapeutic target. Human prostate cancer patients showed overexpressed FGFR1 and *in vitro* studies in mice revealed that FGFR1 activation is inducing EMT. However, not only FGFR1 is known to be associated with carcinogenesis. Overexpression of FGFR2 has also been associated with breast cancer, due to constitutive activation or an altered binding of transcription factors. FGFR3 has been found overexpressed in Multiple Myelomas and could be a therapeutic drug target.

Another mechanism which leads to altered signaling is the switch between alternatively spliced isoforms (reviewed in (*286-288*)). The shift to a more oncogenic isoform and/or modified ligand binding capacity can lead to excessive signaling of the receptors.

Furthermore, different mutations of all four receptors in various cancer types have been found (reviewed in (*286-288*)). The mutations can lead to more active, or even to constitutively active receptors. Enhanced ligand binding capabilities have been described, as well as modified ligand specificities (mutations in the extracellular domain). Both constitutive dimerization and disruption of the autoinhibitory mechanisms are accountable for constitutive activation. Autophosphorylation promoting mutations were found in the case of FGFR4 in rhabdomyosarcoma. Interestingly, FGF signaling was found to be one the most commonly mutated systems amongst 1000 somatic mutations in a human cancer genome screen (*289*). Chromosomal translocations resulting in a fusion protein can also be potentially oncogenic. Examples from haematological malignancies show constitutive

dimerization and consequently constitutive activation after fusion of FGFR with various intracellular partners.

Not only alterations at the receptor level influence carcinogenesis. Upregulated expression or increased release of the ligands, FGFs, resulting in increased receptor activation, can contribute to cancer development (reviewed in (*286-288*)). Increased expression of several FGFs has been identified in a number of cancers, such as melanoma, liver, colon and lung carcinomas. Not only the tumor cells themselves, but also the surrounding tissue has been found to overexpress FGFs which then can transform epithelial tissue. Another way of increasing the local levels of the ligand is via release of the reservoirs in the extracellular matrix. Proteases and heparanases secreted by tumor cells can enzymatically cleave the ECM and release ligands.

In addition to increased signaling, impaired termination could play a role in carcinogenesis (reviewed in (*286-288*)). The decrease of a regulatory protein, Sef, leads to a weak negative-feedback loop action and thereby sustained receptor signaling in e.g. prostate cancer. FGFR mutations resulting in an escape into recycling pathways or causing the loss of an endocytic signal sequence, thereby leading to inefficient degradation and signal termination are also known in breast, ovary, prostate or bladder cancer.

As already mentioned, FGFR has been suggested to act as a tumor suppressor gene (reviewed in (*286-288*)). FGFR2 seems to be downregulated in some cancers, leading to the assumption that it also functions as tumor suppressor. However, the detailed mechanisms are still unclear and might be context-dependent.

2. Aims of the studies

This work aimed to gain new insights into mechanisms of normal cell motility and cancer cell migration. Furthermore, we wanted to elucidate negative-feedback mechanisms on activated growth factor receptors. All the studies could provide new potential drug targets for cancer therapy.

PAPER I: Identification of novel effectors in cell migration

The main aim of this project was to identify new molecules involved in cell migration. Starting point was the observation that depletion of the PI3K class III catalytic subunit VPS34 resulted in decreased motility. Subsequently, we screened for PX- and FYVE-domain containing proteins and wanted to validate and closely investigate possible hits.

PAPER II: Evaluation of PIKfyve and MTMR3 in cancer cell migration and invasion

In this follow-up study of paper I we wanted to investigate, if PIKfyve and MTMR3 are involved in cancer cell migration. We also intended to study their invasive potential. To improve and simplify the manual tracking, we sought to develop new software to track cell migration in phase contrast settings.

PAPER III: Investigation of negative regulation mechanisms in FGFR signaling

Since deactivation mechanisms of FGF receptors are not well understood, we wanted to find out how the signaling of an activated receptor can be downregulated. In combination with that, we wanted to elucidate the role of the C-terminal tail of the receptor, since its function is not clear.

3. Summary of the included papers

PAPER I: Production of phosphatidylinositol 5-phosphate via PIKfyve and MTMR3 regulates cell migration

The depletion of the catalytic subunit VPS34 of the class III PI3K (PI3KIII) resulted in decreased cell velocity of fibroblasts in a wound healing scratch assay. Subsequently, an siRNA cell migration screen for effectors of PtdIns3*P*, the product of PI3KIII, was performed. Possible candidates were validated by different methods, both *in vitro* and *in vivo*. *In vitro* studies included time-lapse live-cell imaging, rescue experiments and perfusion assays with an inhibitor. Border cell migration in *Drosophila* egg chambers was performed in order to investigate migration *in vivo*. MTMR3 and PIKfyve were among the positive validated hits and interestingly, both enzymes are involved in the biogenesis of a phosphoinositide, PtdIns5*P* (Figure 6). Because cells have to be polarized in order to be able to migrate, we then monitored Golgi positioning and actin fiber orientation/organization upon depletion of MTMR3. Indeed, knockdown of MTMR3 caused impaired cell polarization and impaired actin remodeling, whereas integrin trafficking and focal adhesions were not affected. Since PtdIns5*P* is the product of MTMR3 and its level was elevated upon migratory stimulation, we tested if PtdIns5*P* could directly influence cell migration. Both exogenously and endogenously added PtdIns5*P* was able to stimulate cell migration upon MTMR3 or PIKfyve knockdown.

Thus, this study identified a novel role for a phosphoinositide pathway consisting of PI3KIII, PIKfyve and MTMR3, together with its product PtdIns5*P* in cell migration.

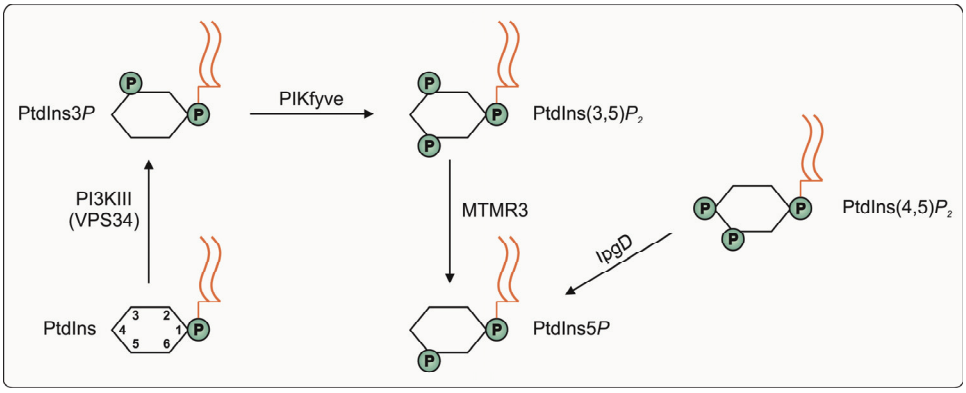


Figure 6. Schematic representation of pathways for PtdIns5*P* synthesis. First, PtdIns3*P* is produced by phosphorylation of PtdIns through PI3K class III with its catalytic subunit VPS34. Then, PtdIns3*P* is further phosphorylated by PIKfyve resulting in the generation of PtdIns(3,5)P_2. Through the following dephosphorylation by MTMR3, PtdIns5*P* is finally generated. PtdIns5*P* can also be produced by the bacterial phosphatase IpgD, which is indicated to the right.

PAPER II: Involvement of PIKfyve and MTMR3 in cancer cell migration and invasion

In paper I we showed that both PIKfyve and MTMR3 are involved in migration of human normal fibroblasts. Here, we wanted to test if they are also important for cancer cell migration. First, we found that gene and protein expression of both enzymes are relatively unchanged in cancerous tissues and cell lines compared to healthy tissues / normal cell lines. We continued our studies on three different cancer cell lines of different origin. Overexpression of MTMR3 did not change cell migration velocity, whereas the depletion of both PIKfyve and MTMR3 resulted in decreased migration. The fact that this was concordant in all three cell lines indicates that PIKfyve and MTMR3 are also involved in cancer cell migration. Toxic effects upon siRNA treatment were ruled out using different assays. Our time-lapse imaging is performed in phase contrast settings to reduce phototoxicity as much as possible. Manual tracking had to be applied earlier, but to improve and simplify cell tracking under these settings, we developed a new computer software, called TrackCell. In an invasion assay we could furthermore show that invasion into matrigel was inhibited

upon PIKfyve or MTMR3 knockdown, revealing their role in also 3D migration. Importantly, since both are enzymes, they provide possible drug targets for metastasis therapy.

PAPER III: ERK-mediated phosphorylation of FGF receptor 1 on Ser^{777} confers negative feedback on FGF signaling

FGF receptors are activated by 18 different fibroblast growth factors (FGFs). These activation mechanisms are well-studied, in contrast to the deactivation mechanisms. In this paper, we identified a novel regulatory mechanism to attenuate receptor signaling (Figure 7). First, it was tested whether a serine within a consensus sequence in the receptor could be phosphorylated *in vitro* by the MAP kinases (MAPKs) ERK1 and ERK2. Here we could show by several assays, that the receptor is phosphorylated directly and exclusively at serine^{777} by ERK1 and ERK2. Concordantly, ERK1 was identified to bind directly to FGFR1. Blocking MAPK activity by inhibitors or dominant-negative mutants abolished this serine phosphorylation and led to augmented and extended tyrosine phosphorylation of FGFR1. Similar effects were seen in FGFR1 serine^{777} mutants. Substituting serine^{777} with alanine (S777A) resulted in increased FGFR1 signaling, whereas substitution with aspartate (S777D) caused decreased signaling. Thus, the phosphorylation status of serine^{777} influences FGF signaling. We tested effects on several biological responses, such as proliferation and cell migration. We found that cells stable transfected with an FGFR1 S777D mutant migrated slower than wildtype receptor transfected cells, whereas an S777A mutation resulted in increased cell migration. FGF-independent MAPK activation also induces serine^{777} phosphorylation, which provides an additional protection against excessive intracellular signaling. Thus, a novel mechanism, regulating FGFR signaling through both FGF-dependent and -independent MAPK activation, was uncovered.

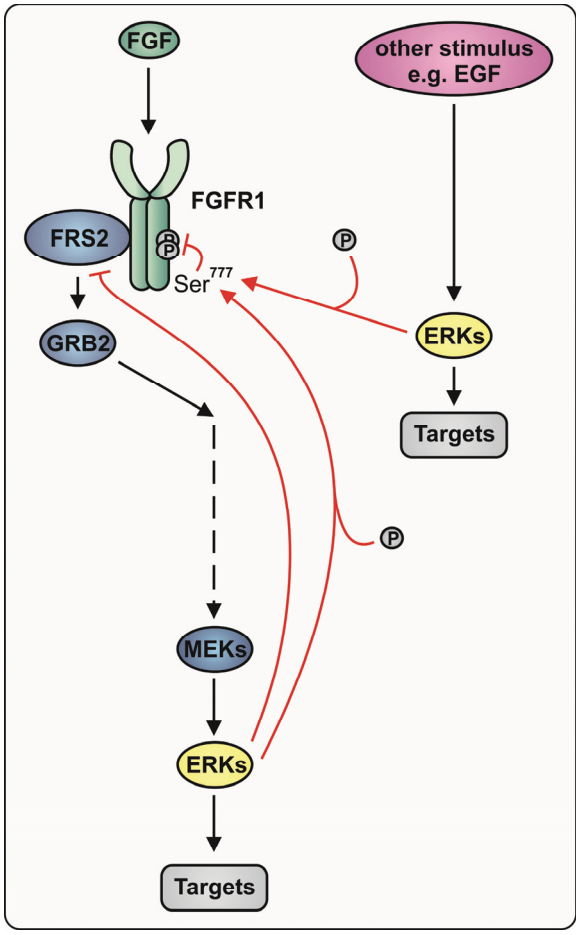


Figure 7. Schematic representation of negative feedback signaling on FGFR1. Following FGF1-induced FGFR activation, FRS2 is activated and further activates the MAPK pathway, including ERKs. ERKs can also be activated by other stimuli, e.g. serum or EGF. Activated ERKs facilitate negative feedback signaling via phosphorylation of FRS2, but also via direct phosphorylation of FGFR1 on serine^{777}. This in turn results in reduced tyrosine phosphorylation of the FGFR1 and attenuates FGFR1 signaling. Negative feedback loops are indicated in red. Phosphorylation and phosphorylation sites are indicated (P). FRS2, FGFR substrate 2; GRB2, growth factor receptor-bound protein 2; MEK, MAP/ERK kinase; ERK, extracellular-signal-regulated kinase; EGF, epidermal growth factor.

4. Discussion

The work presented in this thesis gave new insights into the regulation of cell migration. We proposed a novel role for the phosphoinositide PtdIns5*P* in cell migration. We have shown that the enzymes PIKfyve and MTMR3 are both involved in normal and cancer cell migration as well as in cancer cell invasion. Furthermore, we identified a novel negative feedback mechanism in FGF signaling, which also has an impact on cell migration. Since migration plays a critical role in development of metastasis, our results contribute to improve the understanding of the metastatic process.

4.1 Hits of the cell migration screen – MTMR3 and PIKfyve

Two of the best hits from the cell migration screen in paper I were MTMR3 and PIKfyve. Interestingly, both are involved in phosphoinositide metabolism, more precisely, they are the enzymes proposed to be important for the biogenesis of PtdIns5*P* (*166,170,190*). This intrigued us to validate these hits further and indeed they turned out to be involved in normal and cancer cell migration.

4.1.1 MTMR3

MTMR3 is a catalytically active member of the myotubularin family. It is a 3-phosphatase specific for PtdIn3*P* and PtdIns(3,5)P_2 (*190*), thereby generating PtdIns and PtdIns5*P*, respectively. Although it contains a FYVE domain, its localization is largely cytosolic, but is also found in a reticular staining pattern (suggested to be on the ER) and on membranous structures (*190,290*). Interestingly, the FYVE domain is atypical, since MTMR3 neither localizes to early endosomes, nor binds the FYVE domain its typical ligand PtdIns3*P* (*138*). Another domain of MTMR3, the PH-GRAM domain, has been shown to be required for enzymatic activity (*138*), and is the binding site for PtdIns5*P*, which then creates a positive feedback-loop (*137*). Even though MTMR3 is ubiquitously expressed (*290*), there are not many roles known to date. One important and well-studied function is connected to autophagy-initiation. Briefly, autophagy is induced by nutrient deprivation. In a multi-step

process, cytoplasm and organelles are sequestered, followed by their degradation. This leads to the recycling of cellular molecules and promotes therefore cell survival. A local increase of PtdIns3*P* on membranes plays a crucial role, produced by a special PI3K complex. Recently, it was reported that MTMR3 is involved in autophagosome formation and maintenance (*215*). MTMR3 suppresses autophagy under nutrient-rich conditions, by dephosphorylating PtdIns3*P*. This demonstrates a direct involvement of MTMR3 in autophagy regulation (*291-293*).

It raised the question, whether the role of MTMR3 in autophagy is connected to its role in migration we found in paper I and II. Since we could not detect any changes in PtdIns3*P* levels (paper I), these processes seem to be independent. According to our data, the dephosphorylation of PtdIns3*P* does then only interfere with autophagy, but not with migration. Probably, depending on its localization, MTMR3 possesses different substrate specificity and thus, functions.

The role of MTMR3 in cell migration would then be to accumulate PtdIns5*P*, facilitated via the positive feedback-loop. This could in turn recruit PtdIns5*P* binding effector proteins, which act on cell migration.

The myotubularins

Further investigation of the myotubularin family could also provide additional insight. MTMR3 is not the only active member; there are 7 more, where 5 of them are implicated in PtdIns5*P* production (reviewed in (*186*)). Therefore, other active members could potentially also be involved and combined knockdowns might reduce migration even more. Our screen in paper I was restricted to PX- and FYVE-domain containing proteins and aimed initially to find new PtdIns3*P* effectors. The involvement of other myotubularins is therefore not excluded. We could only see maximal 50% reduction in velocity, indicating that other molecules could be implicated in this process. Though MTMR4 was part of our screen, it did not appear to be a hit. However, we cannot exclude that this is due to poor knockdown

efficiency. It would still be a good candidate, since it is, besides MTMR3 itself, the only dimerization partner of MTMR3 (*198*).

4.1.2 PIKfyve

The evolutionary conserved protein PIKfyve is a phosphoinositide kinase, which is generating PtdIns(3,5)P_2 (*165,170*). The suggested production of PtdIns5*P* is controversial and might be indirect via dephosphorylation of PIKfyve-produced PtdIns(3,5)P_2 (discussed below). PIKfyve harbors, as the name indicates, a FYVE domain which binds to its substrate PtdIns3*P* (*294*). Colocalization of PIKfyve appears to be with markers of early and late endosomes as well as lysosomes (*160-164*). Since disruption of its function causes enlarged vacuoles (*171,173,174,295*), main functions of PIKfyve are thought to be in maintaining cell morphology and endomembrane homeostasis (*161,236*) (and reviewed in (*176*)). PIKfyve is implicated in endosome trafficking to lysosomes, shown by e.g. a failure of fusion of MVBs with lysosomes after acute PIKfyve inhibition (*296-299*). Thereby, tyrosine kinase receptor downregulation is blocked, and also autophagy is affected due to a decreased rate of fusion with lysosomes. The regulation of early-endosome-to-TGN retrograde trafficking has also been shown to be one of the functions of PIKfyve (*164*).

The follow-up studies in paper II showed that both enzymes are involved in cancer cell migration and also in invasion. The machinery controlled by MTMR3 and PIKfyve is thus not only maintaining normal cell migration, it also contributes to migratory and invasive phenotypes of cancer cells. It is known, that cancer cells “hijack” normal cellular functions to use them during carcinogenesis. MTMR3 and PIKfyve seem to belong to these basic mechanisms which are utilized by cancer cells. Subsequently, we also propose that the end product of the phosphoinositide pathway, consisting of VPS34, PIKfyve and MTMR3, PtdIns5*P* itself plays a role in cell migration.

4.2 Production of PtdIns5*P*

Since PtdIns5*P* was discovered in 1997 (*153*), the debate about its biosynthesis is highly controversial. The same group suggested one year later, that the class I PIP kinase phosphorylates PtdIns *in vitro* and produces thereby PtdIns5*P* (*150*). Shortly after, another group had proof that it was actually the class III PIP kinase PIKfyve, which is specifically phosphorylating PtdIns at the D-5 position (*160,165,166*). Evidence, that PtdIns5*P* might not be generated through direct phosphorylation on the D-5 position of PtdIns came in 2001, when it was discovered that one myotubularin member, MTMR3, dephosphorylates PtdIns(3,5)P_2 specifically on the D-3 position. This provided for the first time a clearly defined route for PtdIns5*P* synthesis (*190*). Other following studies revealed that this is a characteristic of all the active myotubularin members (*183-186,195*). Later it was also shown that PtdIns5*P* could be produced via dephosphorylation of PtdIns(4,5)P_2 (*203,206,229*).

Since then, it is a controversial issue, whether PIKfyve is able to directly produce PtdIns5*P* or not (*175,176,300-302*). To date it is not clear, if the effect of the kinase might be indirect, since PIKfyve produces the intermediate PtdIns(3,5)P_2. Myotubularins might associate tightly with PIKfyve *in vivo* and could interfere with research on PIKfyve. Furthermore, assays showing a direct PtdIns5*P* production *in vitro* might reflect catalytic artifacts. Tolias et al. (*150*), for example, used the bacterial PIP5K, whereas Sbrissa et al. worked with an overexpressed recombinant PIKfyve (*165,166*). An *in vivo* functional study of PIKfyve actually showed that it does not prefer PtdIns as a substrate, even when PtdIns3*P* was only present at contaminant-like concentrations (*158*). Recently, a gene-trap mouse study showed that PIKfyve is indeed responsible for nearly all the PtdInd5*P*, but via generating the entire PtdIns(3,5)P_2 pool (*170*). This demonstrated clearly *in vivo*, that PIKfyve is involved in PtdIns5*P* production, but as discussed earlier by different groups, only indirectly through its catalytic activity towards PtdIns3*P*. Interestingly, an article identified PI3K class II and its product PtdIns3*P* as important players in cell migration (*303*). Whether this effect is mediated through PtdIns(3,5)P_2 and

subsequently PtdIns5*P*, or if PtdIns3*P* itself recruits effectors in this case, remains open.

On the other hand, PtdIns5*P* levels are much higher than PtdIns(3,5)P_2 levels, questioning the role of the latter one as a major precursor. Some studies were made in yeast where PtdIns5*P* has not been detected (discussed in (*176*)). A recent study with the PIKfyve inhibitor YM201636 supported the direct PtdIns5*P* production through PIKfyve (*295*). However, the observed effects on PtdIns5*P* levels could be indirect, since a constant PtdIns(3,5)P_2 level is not a sufficient and satisfactory argument.

Unfortunately, our own studies do not help to clarify the controversy. On one hand, PtdIns5*P* levels are slightly more reduced upon PIKfyve knockdown, than in MTMR3 knockdown cells (paper I). It is probably depending on the lipid turnover, but one might have expected to see PtdIns5*P* levels most reduced in MTMR3 knockdown. On the other hand, we currently don´t know the redundancy in the myotubularin family and their interference. Another interesting aspect of paper I was, that we could rescue the PIKfyve knockdown phenotype with either exogenously added PtdIns(3,5)P_2 or PtdIns5*P*. This supports the suggestion, that PtdIns5*P* is generated through dephosphorylation of PtdIns(3,5)P_2.

Altogether, most of the evidence so far indicates that the majority of PtdIns5*P* is produced via dephosphorylation of PtdIns(3,5)P_2. However, we cannot exclude that a minor pool is generated by PIKfyve directly. Whether it is produced through dephosphorylation of PtdIns(3,5)P_2 or if a minor contribution is made from direct phosphorylation of PtdIns, does nevertheless not affect our conclusion that PtdIns5*P* is involved in cell migration.

4.3 A novel role for the phospholipid PtdIns5*P*

In paper I we propose a novel role for PtdIns5*P* in cell migration. Specifically, the production of PtdIns5*P* is important for normal cell migration *in vitro* and *in vivo* and for cancer cell migration *in vitro*. Endogenously or exogenously added PtdIns5*P* is able to stimulate migration. Furthermore, elevated levels of this phosphoinositide

were found upon stimulation of migration, whereas no other detectable phosphoinositide was found to be increased.

The specific roles of PtdIns5*P* are not completely clear yet. Due to its low abundance in cells (1-2% of PtdIns4*P* levels) and the lack of specific probes, it is still difficult to investigate its functions. Attempts to create probes were made with the PH-GRAM domain of MTMR3 (*138*) and the ING2 PHD domain (*225*). By constructing a tandem repeat PH-GRAM domain, it was found that PtdIns5*P* is indeed the preferred binding partner, but, unfortunately, not the sole one. The domain showed also binding activity for the other monophosphate phosphoinositides and to a lesser extent for PtdIns(3,5)P_2. In case of ING2, a three tandem repeat of the PHD domain (PHDX3) was used and found to bind PtdIns5*P*, but again, it was not completely specific. Probably due to the structural similarity of PHD to FYVE domains, PtdIns3*P* functions as a minor ligand, too. It can therefore not be excluded that localizations or phenotypes caused by the use of these probes are not exclusively due to their binding to PtdIns5*P*.

An alternative is offered by a novel and sensitive HPLC-based approach, which raised recently the possibility to detect cellular PtdIns5*P* directly via subcellular fractionation (*210*). Applying this assay, PtdIns5*P* was found mainly in the plasma membrane, but it was also enriched in the SER (smooth ER) and the Golgi. Interesting and new findings about the localization of several other phosphoinositides were made possible, e.g. that PtdIns4*P* was most abundant in the plasma membrane or that the majority of PtdIns3*P* was detected in the SER and Golgi. We used this assay to measure phosphoinositides directly in stimulated and knockdown cells (paper I). However, one should not forget that the approach is based on subcellular fractionation and possible errors can occur due to contaminations.

So far, several other studies showed increased levels of PtdIns5*P* after diverse stimuli. Elevated levels have been reported after acute thrombin stimulation of human platelets (*154*), during cell cycle progression (*224*), upon osmotic stress (*166*) (even in plants (*304*)), after T-cell receptor stimulation (*231*) and upon insulin

stimulation (*226,232*). A completely different role of PtdIns5*P* is known during infection with *Shigella flexneri* (*206,228*) or *Salmonella typhimurium* (*229*). The injection of bacterial phosphoinositide 4 phosphatases (IpgD and SopB, respectively), producing PtdIns5*P* from PtdIns(4,5)P_2 facilitates infection (*233*). Artificial increases in PtdIns5*P* levels, caused by ectopic expression of the IpgD, resulted in Akt phosphorylation (*228,233,305*). Thereby, PI3K/Akt and thus survival signaling is stimulated and bacterial colonization is promoted via delaying the onset of host cell apoptosis.

How these stimuli, including our observations upon FGF stimulation, cause increases in PtdIns5*P* levels remains elusive. Since we observed reduced migration also without FGF stimulation in knockdown cells (paper I), and upon HGF (hepatocyte growth factor) stimulation (paper II), the involved signaling cascade must rather be part of a more general, basal machinery. If this includes only the PI3K/Akt pathway or others pathways in addition, needs to be addressed in the future.

Furthermore, a nuclear role for PtdIns5P in regulating apoptosis by binding to ING2 has been proposed (*225,227,306*). UV irradiation or DNA damaging agents induce phosphorylation of a PIP4-kinase through the p38 stress-activated protein kinase, thus inhibiting the activity of the lipid kinase and leading to increased PtdIns5*P* accumulation. Nuclear changes of PtdIns5*P* in turn, regulate the chromatin association of ING2. ING2 was reported to stimulate p53 acetylation and to induce apoptosis (*225,307*).

Another interesting study demonstrated a link to cancer. In cells expressing an oncogenic tyrosine kinase, NPM-ALK (Nucleophosmin anaplastic lymphoma kinase), high levels of PtdIns5*P* were observed and it was suggested that this could be a general hallmark of transformed cells with deregulated tyrosine kinase activities (*230*). Wilcox et al. (*308*) also described the involvement of a tyrosine kinase in elevated PtdIns5*P* levels. Interestingly, Coronas et al. (*230*) found PIKfyve to localize at cell extensions, caused by cytoskeleton rearrangements. Their proposed mechanism that a pool of PtdIns5*P* can regulate actin remodeling, fits to our observations of altered actin organization upon lack of PtdIns5*P*. Furthermore, it is

consistent with data from Niebuhr et al., showing that expression of IpgD leads to actin filament remodeling (*206*). In a follow-up study of the NPM-ALK oncogene, it was shown that PIKfyve controls specifically the invasiveness and ECM degradation of cells expressing the oncogenic kinase (*309*). In paper II, we demonstrate similar effects upon PIKfyve knockdown, but we could also in addition show that MTMR3 knockdown inhibits invasion in the same manner, thereby pinpointing even more towards an involvement of the product PtdIns5*P*. Since we could not find any evidence that the adhesion machinery is affected (paper I), and together with the findings of other studies, this let us to hypothesize that actin cytoskeleton regulation/organization is one important function of PtdIns5*P*. Additionally, a role of PtdIns5*P* in membrane trafficking and exocytosis has been discussed in the literature (*300*), which is in agreement with the above findings on ECM degradation. PtdIns5*P* could thereby control the exocytosis of factors needed for matrix degradation and thus promotes invasiveness, if produced excessively.

It seems, as for other phosphoinositides, that the spatial distribution of PtdIns5*P* is of very high importance. Most likely, distinct pools localized at the plasma membrane, the nucleus and possibly on vesicular intermediates (between late endosomes and the membrane) fulfill different functions. If the system is disturbed due to oncogenic alterations, all the pools can contribute in their way to promote migration, survival and invasiveness.

However, the relatively minor contribution to the PtdIns(4,5)P_2 pool by phosphorylation of PtdIns5*P* should be kept in mind. The question, if the regulation of migration is really through membrane-localized, MTMR3-generated pools of PtdIns5*P*, arises. Would rather a PIP4-kinase act on PtdIns5*P* and the resulting PtdIns(4,5)P_2 be the main regulator, since PtdIns(4,5)P_2 is an important regulator of the actin cytoskeleton and focal adhesions? Whether PtdIns5*P* is the effector on cell migration or only a precursor, remains so far unclear and needs to be addressed in the future.

4.4 Actin remodeling during migration

During cell migration, remodeling of the actin cytoskeleton is essential. Among the molecules which regulate these processes are phosphoinositides and Rho GTPases. Members of the Rho GTPase family, Cdc42, Rac and Rho, have been shown to be involved in regulation of the actin cytoskeleton, including polymerization and crosslinking (reviewed in (*310,311*)). Rac was found to be a key regulator in migration (*312*), especially because it is able to stimulate lamellipodia extensions. Since phosphoinositides (PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2) are already known to play a role in Rac-signaling, it might be possible that also PtdIns5*P* interferes here. Whether Rac-activators bind PtdIns5*P* and if as a consequence their localization is affected, are points which could be addressed in future studies. Interacting molecules could for example be identified by a screen for PtdIns5*P*-binding proteins, even though a specific binding domain is not yet clear. A proper validation is then highly necessary.

The possibility that PtdIns5*P* is an intermediate to generate PtdIns(4,5)P_2 should also be considered. Maybe PtdIns5*P* is just a precursor and the final effector on actin cytoskeleton is actually PtdIns(4,5)P_2. Actually, PtdIns(4,5)P_2 is so far the best-characterized phosphoinositide regarding the actin cytoskeleton regulation (*99*). It interacts with several different actin-binding proteins, leading to enhanced plasma membrane interactions, increased adhesion to the ECM, enhanced actin assembly and reduced disassembly (reviwed in (*313-315*)). Studies have shown that most of PtdIns(4,5)P_2 is generated via 5-kinases, but whether there is a special pool derived from PtdIns5*P* that is responsible for cell migration cannot be excluded.

How phosphoinositides, Rho GTPases and actin cooperate together shows the example of *Shigella flexneri* entry into host cells. The bacterial phosphatase IpgD dephosphorylates PtdIns(4,5)P_2 and generates thereby attachment sites so that the pathogen can enter the host cell. The adhesion of the cytoskeleton to the membrane was found to be locally decreased, resulting in detachments. In addition, it was demonstrated that the actin remodeling effects of Cdc42 and Rac agonists were

enhanced by IpgD expression, showing that decreased levels of PtdIns(4,5)P_2 could directly increase Cdc42/Rac activity (*206*). However, here in turn, one cannot exclude that effects are not due to increases in PtdIns5*P* levels, caused by IpgD. The authors speculate if PtdIns5*P* is the acting phosphoinositide and a role of IpgD is actually to generate PtdIns5*P*, besides reducing membrane tether force.

In paper I, we expressed IpgD to restore PtdIn5*P* levels upon MTMR3 knockdown. This resulted in increased migration, even though PtdIns(4,5)P_2 levels were presumably decreased. Whether PtdIns5*P* is actually responsible to create a special localized PtdIns(4,5)P_2 pool, important for migration, remains to be tested.

Another suggestion that PtdIns5*P* could play a role in actin remodeling was made when a recombinant putative PtdIns5*P* binding domain (tandem PHD of ING2) localized at extensions of transformed cells (*230*). These structures are highly dynamic and thought to result from Rac1 activity (*316,317*). Here, again, a relationship between PtdIns5*P* and a Rho GTPase could be of important function.

Our results from paper I visibly show a connection between PtdIns5*P* and actin. Whether this is directly through PtdIns5*P* or via PtdIns(4,5)P_2 and what the nature of the effectors is, is not clear.

4.5 Implications in cancer: PIKfyve, MTMR3 and PtdIns5*P*

Cancer has become a major cause of death, and rates are rising. Today, more than 90% of cancer morbidity and mortality is associated with metastasis. The term "metastasis", meaning displacement, arose in 1829 (*318,319*), when it became clear that malignant tumors invade to distant organs and tissues. Since more than a decade now, tumor invasion and metastasis are together considered as one of the hallmarks of cancer (*85*). Many attempts, to identify drivers of metastasis have been made (reviewed in (*320*)), but effective therapies are still missing. One important step of metastasis is invasion, where mechanisms are highly similar to those of normal migration. Since we identified PIKfyve and MTMR3 to be involved in normal cell migration (paper I), we thought to investigate their involvement in cancer cell

migration (paper II). Several studies already detected PIKfyve to be connected with cancer. It was suggested that PIKfyve plays a role in bladder oncogenesis, where it is implicated in EGFR trafficking to the nucleus and transcription function (*321*). Furthermore, in a microarray analysis, the PIKfyve gene *PIP5K3* was found to be upregulated in gastric cancer (*322*). Two other studies showed that activity of PIKfyve and levels of PtdIns5*P* are increased in NPM-ALK transformed cells (*230,309*). NPM-ALK (nucleophosmin anaplastic lymphoma kinase) is an oncogenic tyrosine kinase which is detected in most of anaplastic large cell lymphomas of a special phenotype. PIKfyve associates with NPM-ALK and is responsible for the invasive characteristics of the cell line (*309*). These studies also showed for the first time a possible involvement of PtdIns5*P* in cancer.

MTMR3 on the other hand, was indentified in a cancer mutation discovery screen to be among the candidate cancer genes (*CAN*) in breast cancers (*323*). This means that MTMR3 is mutated at significant frequency in the analyzed breast carcinomas. In another study of a breast cancer mouse model, MTMR3 was among the overexpressed genes in invasive carcinoma cells, compared to the general population of tumor cells in the primary tumors (*324*). A mutation of MTMR3 in gastric cancer was found in an analysis of a public genomic database (*102*). This frameshift mutation probably leads to a nonfunctional MTMR3. In a genome-wide association study of lung cancer cases in a Chinese population, MTMR3 was newly identified together with another susceptibility region (*325*).

This suggests that both enzymes could be involved in carcinogenesis. In paper II we show that their contribution could possibly be through enhancing cell motility and invasion. Cells depleted for PIKfyve or MTMR3 have a decreased migration velocity, whereas proliferation was not affected. To strengthen our data, we performed the knockdowns in three cancer cell lines of different origin (lung, rhabdomyosarcoma and osteosarcoma) and obtained similar results. Furthermore, we could demonstrate that knockdown of PIKfyve or MTMR3 reduces the invasive phenotype of the H1299 lung carcinoma cell line. Altogether, PIKfyve and MTMR3 have a role in cancer cell migration and invasion. They may provide new therapeutic

targets specifically aiming to inhibit cancer cell migration and thereby avoiding side effects.

Not only the enzymes, also the product can in addition contribute to carcinogenesis. Altered PtdIns5*P* levels could disturb other normal cellular functions, since it is published that the lipid interferes with the PI3K/Akt pathway (*228,233*). The PI3K/Akt signaling is one of the most targeted pathways in cancer therapy and currently in various stages of clinical development (reviewed in (*326*)). Elevated PtdIns5*P* levels due to increased activity of PIKfyve/MTMR3 could lead to hyperactivation of the PI3K/Akt pathway which then results in cancer progression.

Another interesting point of PtdIns5*P* is its interaction with the chromatin-associated protein ING2 (*225*) (described above). ING family proteins are presumably involved in cancer and might function as tumor suppressors (reviewed in (*327*)).

4.6 Regulation of FGFR signaling

In paper III, we identified a novel mechanism of FGFR1-signaling regulation. We found that activated MAPKs ERK1 and ERK2 phosphorylate a single serine residue in the C-terminal part of the FGF receptor, which reduces the tyrosine phosphorylation in the kinase domain. This results in decreased receptor signaling and thus defines a new negative feedback loop.

A number of negative feedback loops for FGF signaling have been identified previously. These include MAPK phosphatase 3 (MKP3), Sprouty proteins and SEF (similar expression to *fgf* genes) (*271,277-279*). Another negative mechanism involves the MAPKs ERK1 and ERK2 (Figure 5). After stimulation and tyrosine phosphorylation of FGFRs, ERK1 and ERK2 are among the activated downstream signaling molecules. In addition to further activate other downstream molecules, ERKs also phosphorylate FRS2α, a major mediator of FGF signaling (reviewed in (*270*)), on multiple threonine residues (*328*). Thus, recruitment of other factors to FRS2α is prevented and FGFR signaling attenuated.

We could show that ERKs participate in another negative feedback loop, which is the direct phosphorylation of the receptor (paper III). Activated ERKs bind and specifically phosphorylate serine^{777} in FGFR1. Possible resulting consequences could be that either the phosphorylation of serine^{777} functions as binding site for tyrosine phosphatases, which are responsible for receptor inactivation; or it induces conformational changes within the receptor which then turns the receptor into a better substrate for tyrosine dephosphorylation. Furthermore, a phosphorylated serine^{777} could prevent interaction with specific binding partners and thereby disrupt downstream signaling.

If FGFR signaling would not be attenuated, cells would receive sustained signals and signaling pathways would remain active. Since MAPK and PI3K/Akt are two of the major signaling pathways of the FGFR, continuous signaling could lead to increased proliferation, apoptosis inhibition and subsequently to cancer (*271,286-288*). Other functions of FGF signaling, such as its biological role in angiogenesis and wound healing (*269,271*), can contribute to cancer development, when the regulation of the signaling is perturbed. Altered FGF signaling could also be involved in migration and invasion steps. *In vitro* data show that FGFRs play a role in invasion of pancreatic and breast cancer cells (*329,330*). Another study demonstrates that constitutive FGF signaling leads to polarity loss and gain in matrix metalloproteases (*331*). During angiogenesis, FGF signaling is also required for endothelial migration and chemotaxis (*332,333*). Thus, FGFs promote cancer progression not only through mitogenic and angiogenic effects, they could additionally enhance metastasis via their migratory effects (*334*).

Our results in paper III show that the phosphorylation status of serine^{777} of FGFR1 is important for proper signaling and thus regulates proliferation and migration. Upon expression of a mutant receptor, which mimics the unphosphorylated state of serine777, proliferation and migration was increased. This demonstrates that the negative feedback loop is crucial for deactivating the receptor signaling. So far, the C-terminal tail, which harbors the serine^{777}, was relatively uncharacterized. Indications, that it might indeed be important, were provided by studies on FGFR2:

C-terminally shortened isoforms have been found to be overexpressed in cancers. In addition, it was shown that the deletion of the C-terminus in FGFR2 correlates with increasing transforming potency in human cancers (*335*). Possibly, the associated loss of the corresponding serine, which is important for the negative feedback loop, provides a mechanistic explanation for the observed transforming effect.

4.7 Conclusions and Future Perspectives

In the first part of this work we identified novel molecules implicated in normal and cancer cell migration. Previously not described to have a role in cell migration, we found the phosphoinositide metabolizing enzymes PI3K class III, PIKfyve and MTMR3 to be involved. Furthermore, a novel function of their product, PtdIns5*P*, in cell migration was revealed. We demonstrated their role in cancer cell migration as well, and their contribution to invasiveness. The knowledge of invasive signaling cascades is important for the design of new treatments, and PIKfyve and MTMR3 might provide new drug targets. The second part of this work elucidated a novel negative feedback loop in FGFR1 signaling. Altered signaling from FGF receptors is known to be involved in carcinogenesis, since they are regulating a range of biological signals, such as proliferation, survival and migration. A tight regulation of FGF signaling is therefore necessary to maintain normal cellular functions. For cancer therapy, a detailed understanding of the involved mechanisms and the complex signaling cascade system is crucial. This can contribute to the development of more efficient therapies.

Since the initial cell migration screen was limited to PX- and FYVE-domain containing proteins, the investigation of other myotubularins could give more insight whether the whole family might be involved in migration. The question, if PtdIns5*P* or PtdIns(4,5)P_2 is the final regulator of cell migration needs to be addressed. Effectors of PtdIns5P (or PtdIns(4,5)P_2) remain elusive and screens could help to identify them. Furthermore, work on other candidate genes from our screen can be done, especially on those which gave an increase in migration upon knockdown. Regarding FGF signaling, other FGF receptors might be tested if the corresponding serine in the C-terminal tail also possesses a negative feedback loop. Direct serine phosphorylation through ERKs could represent a common mechanism of all four FGF receptors.

4.8 Experimental and Methodological Considerations

The majority of the methods and model systems in this thesis are well-established and well-described in the literature. However, some considerations need to be taken and contribute to the learning process of a scientist. Therefore, some aspects and pitfalls will be discussed, without giving an introduction into the individual methods.

4.8.1 Cell lines as model system

Today, cell culture facilities are part of the basic equipment of a laboratory. Thousands of cell lines are commercially available at any time, and provide with their self-replicating abilities an almost unlimited source of biological material. Long-term studies can easily be performed, with quite similar base material. It is a powerful and versatile model system which often fits best for basic research or first steps in a project before continuing with animal models. However, it is obvious that cells in culture are not growing in their original environment and thus do not completely reflect conditions *in vivo*. Genotypic and phenotypic transformations can occur during culturing, as well as senescence-caused changes. These facts need to be always kept in mind when drawing conclusions and it is highly recommended to perform experiments in various cell lines and/or other model systems to strengthen the data. In paper I, human foreskin non-immortalized fibroblasts were used, which are less transformed compared to permanent cell lines. They were kept in low passages to prevent as much as possible alterations due to culturing. Since they are the prototype of mesenchymal cells, they are well-suited for cell migration studies. Among the disadvantages are the difficulties in plasmid transfection, leading to a lower number of cells available for analysis in rescue studies in paper I. However, they are bridging artificial systems and animal experiments due to their relatively normal properties. In contrast, permanent cell lines, like tumor lines used in paper II, show an altered morphology and might not possess all of their original characteristics. Artifacts due to chromosomal aberrations and newly occurring mutations during culturing demand controls. Even though they provide an excellent

model system for invasion studies, since fibroblasts are only suited for normal migration, they do not always represent cancerous behavior *in vivo*. Ideally another organism, but also several different cancer cell lines can serve as controls. In paper II, 3 different cancer cell lines, from different origins were used and similar results can indicate a general mechanism, independent of special properties of one cell line. For paper III, a stable transfected cell line was prepared, which is advantageous in the way of working with relatively similar base material throughout the experiments. Furthermore, cells were selected for low overexpression levels and several clones were tested in most cases.

4.8.2 Depletion of proteins with siRNAs

Even though it may sound paradoxical, to investigate the function of a protein we first switch it off by the use of small interfering RNAs. This "knockdown" of a protein is a simple and fast assay, with normally high efficiency and specificity and provides a much easier alternative to knock out studies in animals. However, caution is advised regarding off-target effects giving false phenotypes (*336*). These effects arise when sequence similarities to other mRNAs are given and they are obviously not related to the target protein. To exclude this, one can apply and compare several different siRNA oligos targeting the same gene and perform in addition rescue experiments (restoring the phenotype by reintroducing the target protein via cDNA). Another alternative are specific inhibitors, especially when working with druggable enzymes. All these approaches were applied in paper I and to some extent in paper II. In paper III, a well-established siRNA was used to complement the inhibitor experiments.

To avoid unspecific effects due to the transfection procedure (*337*), a so called Control-siRNA experiment is always performed in parallel. The control siRNA nucleotide sequence does not match with any other sequence and interferes therefore not with the expression of any gene. However, cells go through the same procedure, including transfection reagent and siRNA treatment.

4.8.3 Confocal microscopy, Structured illumination microscopy and Electron microscopy

Compared to wide-field microscopy, confocal microscopy offers an improved optical resolution and contrast by eliminating out-of-focus light. It is an important, fast and powerful tool with many applications, such as localization-, colocalization- and live-studies. Quantitative information about differences in cell-staining can be gained, as done in paper III. Reconstruction of 3D-images makes it possible to depict for example invading cells in a matrix, as done in paper II.

Structured illumination microscopy (SIM) can however, increase the spatial resolution of wide-field fluorescence microscopy (*338,339*). It is using spatially structured illumination light, thereby illuminating objects with multiple interfering beams of light. Normally unreachable high-resolution information is gained and via computational extraction and three-dimensional reconstruction, an improved resolution in all three dimensions is achieved. We performed this technique in paper I and obtained detailed information about actin fibers.

Electron microscopy uses a beam of electrons to illuminate the specimen and gives an even greater magnification and resolution than optical microscopes.

But in all microscopic applications, care has to be taken and a lot of experience is needed. In confocal and SIM studies, results must reflect the whole population of cells and other assays must be performed to strengthen the findings. To avoid biased results, randomization during quantifications is necessary. Choosing the right microscope settings is very important and training is needed. Artifacts in electron microscopy can be caused by ultrathin sectioning and staining procedures and therefore, even more experience is necessary.

4.8.4 Cell migration studies

The analysis of cell migration represents the key method in this thesis. The BioStation IM (Nikon) provides an easy and stable system to monitor cell migration

over longer periods of time. A large number of cells can easily be analyzed with tracking software, giving different parameters as output. Fluorescence microscopy enables the tracking of labeled cells, which was essentially necessary in the rescue experiments, due to low transfection efficiency. Wound healing, random migration and perfusion assays were performed. Due to the fact that proliferation can affect these experiments, as well as the varying size of the "wound" during manual scratching led us to analyze cell velocity and not differences in wound closure.

Another assay, the Oris Cell Migration Assay, was used to perform the initial siRNA screen of paper I. This assay is advantageous in the way that it enables a high-throughput screen for migration in a 96-well format. Furthermore, due to the stoppers in each well, the artificial "wound" has a defined size, thus giving the same conditions to every well. However, only the endpoint was measured and since we do not know the efficiency of the individual knockdowns, validation is strictly required. In paper I, the hits of the siRNA screen were validated by different methods, followed by investigation in different cancer cell lines in paper II.

Both assays, however, do not resemble proper *in vivo* conditions. The microenvironment, consisting of cells, secreted factors and signaling molecules, extracellular matrix (ECM) and basement membranes can of course not completely be simulated *in vitro*. Normally, migration appears to be directed due to extracellular signals, but in tissue culture, cells can migrate randomly, since they are able to aquire transient polarized morphologies. Furthermore, a cell is not moving in 2D naturally. It was recently published, that many of the 2D migration data do not correlate with 3D migration data (*340*). Thus, data gained from 2D *in vitro* studies must always be confirmed with 3D assays. In paper I, we used *Drosophila melanogaster* as a model organism and studied border cell migration (*341*), whereas in paper II, we worked with an invasion assay (*342*). The matrigel in the latter should thereby reflect the composition of the ECM. However, even though invasion is only one step of metastasis, and 2D migration is far from *in vivo* 3D migration, the executed experiments provide relatively cheap, efficient and easy-to-perform methods with a high throughput.

In paper III, cell migration analysis was one of three different assays to prove the biological significance of the phosphorylation status of the FGF Receptor. Here, cell proliferation assays, axonal-growth and -morphology analysis of neurons were carried out in addition to strengthen the findings.

4.8.5 *In vivo* studies in *Drosophila melanogaster*

Border cell migration was studied in the fruit fly *Drosophila melanogaster*. This is an excellent tool to study migration *in vivo*, since *Drosophila* is one of the genetically best known eukaryotic organisms and therefore, a lot of genetic tools and markers are available (for instance GFP-labeled border cells, RNAi). *Drosophila* breeds quickly, lays many eggs and the whole life cycle is short, resulting in relatively short expenditure of time and low expenses. It is widely used for biological research, and mutation analyses in *Drosophila* are often a good starting point for research of many diseases. Although the border cell migration assay allows us to investigate migration in 3D, it does not resemble invasion, where a basal membrane has to be degraded and cells loose contacts. Here, border cell migration occurs naturally within the egg chamber, with cells in close contact and moving together in a cluster.

4.8.6 Detection of PtdIns5*P*

The first method to detect PtdIns5*P* was based on the enzymatic reaction of the phosphatidylinositol phosphate 4-kinase (type II PIPKs), which generates PtdIns(4,5)P_2 (*154*), followed by HPLC-measurements of PtdIns(4,5)P_2. PtdIns5*P* itself was not measurable with conventional HPLC, due to its poor separation from PtdIns4*P*. In addition, so far, mass spectrometry is not able to distinguish the monophosphorylated phosphoinositides (*343*). Fluorescent probes to detect specifically PtdIns5*P* are unfortunately lacking. However recently, a new, optimized HPLC method, which is able to resolve PtdIns5*P* from PtdIns4*P*, was described (*210*). This made it possible to directly detect the levels of PtdIns5*P* independent of enzymatic reactions, which actually are sensitive to kinase inhibitors (such as the

product itself). Furthermore, information about its localization was gained by subcellular fractionation prior to the HPLC analysis.

4.8.7 Use of FGF, chemical inhibitors and PIP Shuttle Kits

When cells were stimulated with FGF, heparin was added in addition, to stabilize the growth factor and to facilitate its binding to receptors, but also to prevent sequestering to heparin sulfate on the cell surface. To exclude that observed effects were due to heparin only, it was added to control cells during the experiments.

Chemical inhibitors facilitate fast and simple assays. Today, a wide range of inhibitors are available, but nevertheless, they do only provide a limited specificity. Concentrations have to be kept as low as possible to prevent side effects. Solvents, such as DMSO, have to be added to control experiments.

The Phosphoinositide (PIP) Shuttle Kits enabled us to provide cells directly with various PIPs. As mentioned in the introduction, the spatio-temporal distribution of phosphoinositides plays a very important role. A suboptimal distribution could therefore be the reason that exogenously added PtdIns5*P* is not able to rescue MTMR3/PIKfyve knockdown completely in paper I. The applied carriers were added to knockdown cells in control experiments. To show that PtdIns5*P* is not enhancing cell migration in general and that it is specifically rescuing the knockdown, it was also added to control siRNA treated cells (paper I).

References

1. R Virchow, "Ueber Bewegliche Thierische Zellen," (Virchows Archiv, 1863), pp.237-240.
2. Manahan, C. L., Iglesias, P. A., Long, Y., and Devreotes, P. N. Chemoattractant signaling in dictyostelium discoideum. *Annu. Rev. Cell Dev. Biol.* **20**, 223-253 (2004).
3. Ridley, A. J.*, et al.* Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709 (2003).
4. Lammermann, T. and Sixt, M. Mechanical modes of 'amoeboid' cell migration. *Curr. Opin. Cell Biol.* **21**, 636-644 (2009).
5. Friedl, P. Prespecification and plasticity: shifting mechanisms of cell migration. *Curr. Opin. Cell Biol.* **16**, 14-23 (2004).
6. Friedl, P. and Wolf, K. Plasticity of cell migration: a multiscale tuning model. *J. Cell Biol.* **188**, 11-19 (2010).
7. Liang, C. C., Park, A. Y., and Guan, J. L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc.* **2**, 329-333 (2007).
8. Wong, M. K. and Gotlieb, A. I. The reorganization of microfilaments, centrosomes, and microtubules during in vitro small wound reendothelialization. *J. Cell Biol.* **107**, 1777-1783 (1988).
9. Lauffenburger, D. A. and Horwitz, A. F. Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369 (1996).
10. Ridley, A. J.*, et al.* Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709 (2003).
11. Pollard, T. D., Blanchoin, L., and Mullins, R. D. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 545-576 (2000).
12. Welch, M. D. and Mullins, R. D. Cellular control of actin nucleation. *Annu. Rev. Cell Dev. Biol.* **18**, 247-288 (2002).
13. Small, J. V., Stradal, T., Vignal, E., and Rottner, K. The lamellipodium: where motility begins. *Trends Cell Biol.* **12**, 112-120 (2002).
14. Condeelis, J. How is actin polymerization nucleated in vivo? *Trends Cell Biol.* **11**, 288-293 (2001).
15. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410 (1992).
16. Kozma, R., Ahmed, S., Best, A., and Lim, L. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell Biol.* **15**, 1942-1952 (1995).
17. Nobes, C. D. and Hall, A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62 (1995).
18. Wang, H. R.*, et al.* Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. *Science* **302**, 1775-1779 (2003).

19. Machacek, M.*, et al.* Coordination of Rho GTPase activities during cell protrusion. *Nature* **461**, 99-103 (2009).

20. Itoh, R. E.*, et al.* Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol. Cell Biol.* **22**, 6582-6591 (2002).

21. Srinivasan, S.*, et al.* Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. *J. Cell Biol.* **160**, 375-385 (2003).

22. Kupfer, A., Louvard, D., and Singer, S. J. Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. *Proc. Natl. Acad. Sci. U. S. A* **79**, 2603-2607 (1982).

23. Etienne-Manneville, S. and Hall, A. Rho GTPases in cell biology. *Nature* **420**, 629-635 (2002).

24. Etienne-Manneville, S. Cdc42--the centre of polarity. *J. Cell Sci.* **117**, 1291-1300 (2004).

25. Rodriguez, O. C.*, et al.* Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat. Cell Biol.* **5**, 599-609 (2003).

26. Levy, J. R. and Holzbaur, E. L. Dynein drives nuclear rotation during forward progression of motile fibroblasts. *J. Cell Sci.* **121**, 3187-3195 (2008).

27. Dupin, I., Sakamoto, Y., and Etienne-Manneville, S. Cytoplasmic intermediate filaments mediate actin-driven positioning of the nucleus. *J. Cell Sci.* **124**, 865-872 (2011).

28. Dupin, I. and Etienne-Manneville, S. Nuclear positioning: mechanisms and functions. *Int. J. Biochem. Cell Biol.* **43**, 1698-1707 (2011).

29. Millarte, V. and Farhan, H. The Golgi in cell migration: regulation by signal transduction and its implications for cancer cell metastasis. *ScientificWorldJournal.* **2012**, 498278 (2012).

30. Li, Z.*, et al.* Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* **114**, 215-227 (2003).

31. Etienne-Manneville, S. and Hall, A. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106**, 489-498 (2001).

32. Waterman-Storer, C. M., Worthylake, R. A., Liu, B. P., Burridge, K., and Salmon, E. D. Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts. *Nat. Cell Biol.* **1**, 45-50 (1999).

33. del Pozo, M. A., Price, L. S., Alderson, N. B., Ren, X. D., and Schwartz, M. A. Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J.* **19**, 2008-2014 (2000).

34. Kiosses, W. B., Shattil, S. J., Pampori, N., and Schwartz, M. A. Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration. *Nat. Cell Biol.* **3**, 316-320 (2001).

35. Schwartz, M. A. and Shattil, S. J. Signaling networks linking integrins and rho family GTPases. *Trends Biochem. Sci.* **25**, 388-391 (2000).

36. Shattil, S. J. Function and regulation of the beta 3 integrins in hemostasis and vascular biology. *Thromb. Haemost.* **74**, 149-155 (1995).

37. Wang, F.*, et al.* Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat. Cell Biol.* **4**, 513-518 (2002).

38. Weiner, O. D.*, et al.* A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat. Cell Biol.* **4**, 509-513 (2002).

39. Welch, H. C., Coadwell, W. J., Stephens, L. R., and Hawkins, P. T. Phosphoinositide 3-kinase-dependent activation of Rac. *FEBS Lett.* **546**, 93-97 (2003).

40. Kolega, J. Asymmetric distribution of myosin IIB in migrating endothelial cells is regulated by a rho-dependent kinase and contributes to tail retraction. *Mol. Biol. Cell* **14**, 4745-4757 (2003).

41. Iwanicki, M. P.*, et al.* FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts. *J. Cell Sci.* **121**, 895-905 (2008).

42. Merlot, S. and Firtel, R. A. Leading the way: Directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J. Cell Sci.* **116**, 3471-3478 (2003).

43. Devreotes, P. and Janetopoulos, C. Eukaryotic chemotaxis: distinctions between directional sensing and polarization. *J. Biol. Chem.* **278**, 20445-20448 (2003).

44. Verkhovsky, A. B., Svitkina, T. M., and Borisy, G. G. Network contraction model for cell translocation and retrograde flow. *Biochem. Soc. Symp.* **65**, 207-222 (1999).

45. Vicente-Manzanares, M., Koach, M. A., Whitmore, L., Lamers, M. L., and Horwitz, A. F. Segregation and activation of myosin IIB creates a rear in migrating cells. *J. Cell Biol.* **183**, 543-554 (2008).

46. Le, C. C. and Carlier, M. F. Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol Rev.* **88**, 489-513 (2008).

47. Hynes, R. O. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25 (1992).

48. Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687 (2002).

49. Humphries, J. D., Byron, A., and Humphries, M. J. Integrin ligands at a glance. *J. Cell Sci.* **119**, 3901-3903 (2006).

50. Luo, B. H., Carman, C. V., and Springer, T. A. Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* **25**, 619-647 (2007).

51. Anthis, N. J. and Campbell, I. D. The tail of integrin activation. *Trends Biochem. Sci.* **36**, 191-198 (2011).

52. Legate, K. R. and Fassler, R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *J. Cell Sci.* **122**, 187-198 (2009).

53. Wehrle-Haller, B. Structure and function of focal adhesions. *Curr. Opin. Cell Biol.* **24**, 116-124 (2012).

54. Buccione, R., Orth, J. D., and McNiven, M. A. Foot and mouth: podosomes, invadopodia and circular dorsal ruffles. *Nat. Rev. Mol. Cell Biol.* **5**, 647-657 (2004).

55. Linder, S. The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends Cell Biol.* **17**, 107-117 (2007).

56. Cavallaro, U. and Christofori, G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat. Rev. Cancer* **4**, 118-132 (2004).

57. Laukaitis, C. M., Webb, D. J., Donais, K., and Horwitz, A. F. Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. *J. Cell Biol.* **153**, 1427-1440 (2001).

58. Webb, D. J.*, et al.* FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell Biol.* **6**, 154-161 (2004).

59. Small, J. V. and Kaverina, I. Microtubules meet substrate adhesions to arrange cell polarity. *Curr. Opin. Cell Biol.* **15**, 40-47 (2003).

60. Turner, C. E. and Brown, M. C. Cell motility: ARNOand ARF6 at the cutting edge. *Curr. Biol.* **11**, R875-R877 (2001).

61. Brahmbhatt, A. A. and Klemke, R. L. ERK and RhoA differentially regulate pseudopodia growth and retraction during chemotaxis. *J. Biol. Chem.* **278**, 13016-13025 (2003).

62. Regen, C. M. and Horwitz, A. F. Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. *J. Cell Biol.* **119**, 1347-1359 (1992).

63. Bard, J. B. and Hay, E. D. The behavior of fibroblasts from the developing avian cornea. Morphology and movement in situ and in vitro. *J. Cell Biol.* **67**, 400-418 (1975).

64. Chen, W. T. Mechanism of retraction of the trailing edge during fibroblast movement. *J. Cell Biol.* **90**, 187-200 (1981).

65. Post, P. L., DeBiasio, R. L., and Taylor, D. L. A fluorescent protein biosensor of myosin II regulatory light chain phosphorylation reports a gradient of phosphorylated myosin II in migrating cells. *Mol. Biol. Cell* **6**, 1755-1768 (1995).

66. Totsukawa, G.*, et al.* Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. *J. Cell Biol.* **164**, 427-439 (2004).

67. Franco, S. J. and Huttenlocher, A. Regulating cell migration: calpains make the cut. *J. Cell Sci.* **118**, 3829-3838 (2005).

68. Wells, A., Huttenlocher, A., and Lauffenburger, D. A. Calpain proteases in cell adhesion and motility. *Int. Rev. Cytol.* **245**, 1-16 (2005).

69. Chao, W. T. and Kunz, J. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. *FEBS Lett.* **583**, 1337-1343 (2009).

70. Ezratty, E. J., Bertaux, C., Marcantonio, E. E., and Gundersen, G. G. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. *J. Cell Biol.* **187**, 733-747 (2009).

71. Gimona, M. The microfilament system in the formation of invasive adhesions. *Semin. Cancer Biol.* **18**, 23-34 (2008).

72. Dvorak, H. F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* **315**, 1650-1659 (1986).

73. Condeelis, J., Singer, R. H., and Segall, J. E. The great escape: when cancer cells hijack the genes for chemotaxis and motility. *Annu. Rev. Cell Dev. Biol.* **21**, 695-718 (2005).

74. Chambers, A. F., Groom, A. C., and MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* **2**, 563-572 (2002).

75. Steeg, P. S. Tumor metastasis: mechanistic insights and clinical challenges. *Nat. Med.* **12**, 895-904 (2006).

76. Gupta, G. P. and Massague, J. Cancer metastasis: building a framework. *Cell* **127**, 679-695 (2006).

77. Wyckoff, J. B., Jones, J. G., Condeelis, J. S., and Segall, J. E. A critical step in metastasis: in vivo analysis of intravasation at the primary tumor. *Cancer Res.* **60**, 2504-2511 (2000).

78. Nguyen, D. X., Bos, P. D., and Massague, J. Metastasis: from dissemination to organ-specific colonization. *Nat. Rev. Cancer* **9**, 274-284 (2009).

79. Paget, S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev.* **8**, 98-101 (1889).

80. J Ewing, (W. B. Saunders, Philadelphia, Neoplastic Diseases edn 6, 1928).

81. Fidler, I. J. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat. Rev. Cancer* **3**, 453-458 (2003).

82. Langley, R. R. and Fidler, I. J. The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs. *Int. J. Cancer* **128**, 2527-2535 (2011).

83. Weiss, L. Comments on hematogenous metastatic patterns in humans as revealed by autopsy. *Clin. Exp. Metastasis* **10**, 191-199 (1992).

84. Weiss, L. Metastasis of cancer: a conceptual history from antiquity to the 1990s. *Cancer Metastasis Rev.* **19**, I-383 (2000).

85. Hanahan, D. and Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

86. Chaffer, C. L. and Weinberg, R. A. A perspective on cancer cell metastasis. *Science* **331**, 1559-1564 (2011).

87. Friedl, P., Locker, J., Sahai, E., and Segall, J. E. Classifying collective cancer cell invasion. *Nat. Cell Biol.* **14**, 777-783 (2012).

88. Wolf, K.*, et al.* Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J. Cell Biol.* **160**, 267-277 (2003).

89. Wolf, K.*, et al.* Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat. Cell Biol.* **9**, 893-904 (2007).

90. Sanz-Moreno, V.*, et al.* Rac activation and inactivation control plasticity of tumor cell movement. *Cell* **135**, 510-523 (2008).

91. Wilkinson, S., Paterson, H. F., and Marshall, C. J. Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nat. Cell Biol.* **7**, 255-261 (2005).

92. Wyckoff, J. B., Pinner, S. E., Gschmeissner, S., Condeelis, J. S., and Sahai, E. ROCK- and Myosin-Dependent Matrix Deformation Enables Protease-Independent Tumor-Cell Invasion In Vivo. *Curr. Biol.* **16**, 1515-1523 (2006).

93. Grinnell, F. and Petroll, W. M. Cell motility and mechanics in three-dimensional collagen matrices. *Annu. Rev. Cell Dev. Biol.* **26**, 335-361 (2010).

94. Sanz-Moreno, V. and Marshall, C. J. The plasticity of cytoskeletal dynamics underlying neoplastic cell migration. *Curr. Opin. Cell Biol.* **22**, 690-696 (2010).

95. Ewald, A. J., Brenot, A., Duong, M., Chan, B. S., and Werb, Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Dev. Cell* **14**, 570-581 (2008).

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

97. Kedrin, D., van, R. J., Hernandez, L., Condeelis, J., and Segall, J. E. Cell motility and cytoskeletal regulation in invasion and metastasis. *J. Mammary. Gland. Biol. Neoplasia.* **12**, 143-152 (2007).

98. Bravo-Cordero, J. J., Hodgson, L., and Condeelis, J. Directed cell invasion and migration during metastasis. *Curr. Opin. Cell Biol.* **24**, 277-283 (2012).

99. Saarikangas, J., Zhao, H., and Lappalainen, P. Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. *Physiol Rev.* **90**, 259-289 (2010).

100. Barber, M. A. and Welch, H. C. PI3K and RAC signalling in leukocyte and cancer cell migration. *Bull. Cancer* **93**, E44-E52 (2006).

101. Salmena, L., Carracedo, A., and Pandolfi, P. P. Tenets of PTEN tumor suppression. *Cell* **133**, 403-414 (2008).

102. Song, S. Y., Kang, M. R., Yoo, N. J., and Lee, S. H. Mutational analysis of mononucleotide repeats in dual specificity tyrosine phosphatase genes in gastric and colon carcinomas with microsatellite instability. *APMIS* **118**, 389-393 (2010).

103. Kang, S., Bader, A. G., and Vogt, P. K. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc. Natl. Acad. Sci. U. S. A* **102**, 802-807 (2005).

104. Yuan, T. L. and Cantley, L. C. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497-5510 (2008).

105. Sadeghi, N. and Gerber, D. E. Targeting the PI3K pathway for cancer therapy. *Future. Med. Chem.* **4**, 1153-1169 (2012).

106. Di Paolo, G. and De Camilli, P. Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651-657 (2006).

107. De Matteis, M. A. and Godi, A. PI-loting membrane traffic. *Nat. Cell Biol.* **6**, 487-492 (2004).

108. McCrea, H. J. and De, C. P. Mutations in phosphoinositide metabolizing enzymes and human disease. *Physiology. (Bethesda.)* **24**, 8-16 (2009).

109. Fiume, R.*, et al.* Nuclear phosphoinositides: location, regulation and function. *Subcell. Biochem.* **59**, 335-361 (2012).

110. Jean, S. and Kiger, A. A. Coordination between RAB GTPase and phosphoinositide regulation and functions. *Nat. Rev. Mol. Cell Biol.* **13**, 463-470 (2012).

111. Haslam, R. J., Koide, H. B., and Hemmings, B. A. Pleckstrin domain homology. *Nature* **363**, 309-310 (1993).

112. Mayer, B. J., Ren, R., Clark, K. L., and Baltimore, D. A putative modular domain present in diverse signaling proteins. *Cell* **73**, 629-630 (1993).

113. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature* **371**, 168-170 (1994).

114. Lemmon, M. A. Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* **9**, 99-111 (2008).

115. Maffucci, T. and Falasca, M. Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett.* **506**, 173-179 (2001).

116. Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A. Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J. Biol. Chem.* **271**, 24048-24054 (1996).

117. Misra, S. and Hurley, J. H. Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p. *Cell* **97**, 657-666 (1999).

118. Dumas, J. J., *et al.* Multivalent endosome targeting by homodimeric EEA1. *Mol. Cell* **8**, 947-958 (2001).

119. Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., and Stenmark, H. FYVE finger proteins as effectors of phosphatidylinositol 3-phosphate. *Chem. Phys. Lipids* **98**, 87-94 (1999).

120. Gillooly, D. J., Simonsen, A., and Stenmark, H. Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins. *Biochem. J.* **355**, 249-258 (2001).

121. Burd, C. G. and Emr, S. D. Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol. Cell* **2**, 157-162 (1998).

122. Gaullier, J. M., *et al.* FYVE fingers bind PtdIns(3)P. *Nature* **394**, 432-433 (1998).

123. Kutateladze, T. G., *et al.* Phosphatidylinositol 3-phosphate recognition by the FYVE domain. *Mol. Cell* **3**, 805-811 (1999).

124. Stenmark, H. and Aasland, R. FYVE-finger proteins--effectors of an inositol lipid. *J. Cell Sci.* **112 (Pt 23)**, 4175-4183 (1999).

125. Sankaran, V. G., Klein, D. E., Sachdeva, M. M., and Lemmon, M. A. High-affinity binding of a FYVE domain to phosphatidylinositol 3-phosphate requires intact phospholipid but not FYVE domain oligomerization. *Biochemistry* **40**, 8581-8587 (2001).

126. Gil, J. E., *et al.* Phosphoinositides Differentially Regulate Protrudin Localization through the FYVE Domain. *J. Biol. Chem.* **287**, 41268-41276 (2012).

127. Ponting, C. P. Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: binding partners of SH3 domains? *Protein Sci.* **5**, 2353-2357 (1996).

128. Kutateladze, T. G. Mechanistic similarities in docking of the FYVE and PX domains to phosphatidylinositol 3-phosphate containing membranes. *Prog. Lipid Res.* **46**, 315-327 (2007).

129. Bravo, J., *et al.* The crystal structure of the PX domain from p40(phox) bound to phosphatidylinositol 3-phosphate. *Mol. Cell* **8**, 829-839 (2001).

130. Hiroaki, H., Ago, T., Ito, T., Sumimoto, H., and Kohda, D. Solution structure of the PX domain, a target of the SH3 domain. *Nat. Struct. Biol.* **8**, 526-530 (2001).

131. Kay, B. K., Yamabhai, M., Wendland, B., and Emr, S. D. Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery. *Protein Sci.* **8**, 435-438 (1999).

132. Doerks, T., Strauss, M., Brendel, M., and Bork, P. GRAM, a novel domain in glucosyltransferases, myotubularins and other putative membrane-associated proteins. *Trends Biochem. Sci.* **25**, 483-485 (2000).

133. Berger, P., Schaffitzel, C., Berger, I., Ban, N., and Suter, U. Membrane association of myotubularin-related protein 2 is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module. *Proc. Natl. Acad. Sci. U. S. A* **100**, 12177-12182 (2003).

134. Tsujita, K., *et al.* Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction. *J. Biol. Chem.* **279**, 13817-13824 (2004).

135. Yamashita, S., Oku, M., Wasada, Y., Ano, Y., and Sakai, Y. PI4P-signaling pathway for the synthesis of a nascent membrane structure in selective autophagy. *J. Cell Biol.* **173**, 709-717 (2006).

136. Begley, M. J.*, et al.* Crystal structure of a phosphoinositide phosphatase, MTMR2: insights into myotubular myopathy and Charcot-Marie-Tooth syndrome. *Mol. Cell* **12**, 1391-1402 (2003).

137. Schaletzky, J.*, et al.* Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. *Curr. Biol.* **13**, 504-509 (2003).

138. Lorenzo, O., Urbe, S., and Clague, M. J. Analysis of phosphoinositide binding domain properties within the myotubularin-related protein MTMR3. *J. Cell Sci.* **118**, 2005-2012 (2005).

139. Fruman, D. A., Meyers, R. E., and Cantley, L. C. Phosphoinositide kinases. *Annu. Rev. Biochem.* **67**, 481-507 (1998).

140. Wymann, M. P. and Pirola, L. Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta* **1436**, 127-150 (1998).

141. Vanhaesebroeck, B.*, et al.* Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535-602 (2001).

142. Backer, J. M. The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem. J.* **410**, 1-17 (2008).

143. Arcaro, A.*, et al.* Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. *EMBO J.* **21**, 5097-5108 (2002).

144. Falasca, M.*, et al.* The role of phosphoinositide 3-kinase C2alpha in insulin signaling. *J. Biol. Chem.* **282**, 28226-28236 (2007).

145. Schu, P. V.*, et al.* Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science* **260**, 88-91 (1993).

146. Shin, H. W.*, et al.* An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. *J. Cell Biol.* **170**, 607-618 (2005).

147. Meijer, A. J. and Klionsky, D. J. Vps34 is a phosphatidylinositol 3-kinase, not a phosphoinositide 3-kinase. *Autophagy.* **7**, 563-564 (2011).

148. Balla, A. and Balla, T. Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol.* **16**, 351-361 (2006).

149. van, d. B., I and Divecha, N. PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions. *J. Cell Sci.* **122**, 3837-3850 (2009).

150. Tolias, K. F.*, et al.* Type I phosphatidylinositol-4-phosphate 5-kinases synthesize the novel lipids phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 5-phosphate. *J. Biol. Chem.* **273**, 18040-18046 (1998).

151. Zhang, X.*, et al.* Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. *J. Biol. Chem.* **272**, 17756-17761 (1997).

152. Halstead, J. R.*, et al.* A novel pathway of cellular phosphatidylinositol(3,4,5)-trisphosphate synthesis is regulated by oxidative stress. *Curr. Biol.* **11**, 386-395 (2001).

153. Rameh, L. E., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature* **390**, 192-196 (1997).

154. Morris, J. B., Hinchliffe, K. A., Ciruela, A., Letcher, A. J., and Irvine, R. F. Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay. *FEBS Lett.* **475**, 57-60 (2000).

155. Clarke, J. H. and Irvine, R. F. The activity, evolution and association of phosphatidylinositol 5-phosphate 4-kinases. *Adv. Enzyme Regul.* (2011).

156. Hinchliffe, K. A., Giudici, M. L., Letcher, A. J., and Irvine, R. F. Type IIalpha phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms. *Biochem. J.* **363**, 563-570 (2002).

157. Maffucci, T. An introduction to phosphoinositides. *Curr. Top. Microbiol. Immunol.* **362**, 1-42 (2012).

158. McEwen, R. K.*, et al.* Complementation analysis in PtdInsP kinase-deficient yeast mutants demonstrates that Schizosaccharomyces pombe and murine Fab1p homologues are phosphatidylinositol 3-phosphate 5-kinases. *J. Biol. Chem.* **274**, 33905-33912 (1999).

159. Michell, R. H., Heath, V. L., Lemmon, M. A., and Dove, S. K. Phosphatidylinositol 3,5-bisphosphate: metabolism and cellular functions. *Trends Biochem. Sci.* **31**, 52-63 (2006).

160. Shisheva, A., Sbrissa, D., and Ikonomov, O. Cloning, characterization, and expression of a novel Zn2+-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. *Mol. Cell Biol.* **19**, 623-634 (1999).

161. Ikonomov, O. C., Sbrissa, D., and Shisheva, A. Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. *J. Biol. Chem.* **276**, 26141-26147 (2001).

162. Zhang, Y.*, et al.* Modulation of synaptic function by VAC14, a protein that regulates the phosphoinositides PI(3,5)P(2) and PI(5)P. *EMBO J.* **31**, 3442-3456 (2012).

163. Cabezas, A., Pattni, K., and Stenmark, H. Cloning and subcellular localization of a human phosphatidylinositol 3-phosphate 5-kinase, PIKfyve/Fab1. *Gene* **371**, 34-41 (2006).

164. Rutherford, A. C.*, et al.* The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. *J. Cell Sci.* **119**, 3944-3957 (2006).

165. Sbrissa, D., Ikonomov, O. C., and Shisheva, A. PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. *J. Biol. Chem.* **274**, 21589-21597 (1999).

166. Sbrissa, D., Ikonomov, O. C., Deeb, R., and Shisheva, A. Phosphatidylinositol 5-phosphate biosynthesis is linked to PIKfyve and is involved in osmotic response pathway in mammalian cells. *J. Biol. Chem.* **277**, 47276-47284 (2002).

167. Shisheva, A., Rusin, B., Ikonomov, O. C., DeMarco, C., and Sbrissa, D. Localization and insulin-regulated relocation of phosphoinositide 5-kinase PIKfyve in 3T3-L1 adipocytes. *J. Biol. Chem.* **276**, 11859-11869 (2001).

168. Shisheva, A. PIKfyve: Partners, significance, debates and paradoxes. *Cell Biol. Int.* **32**, 591-604 (2008).

169. Sbrissa, D., Ikonomov, O. C., Filios, C., Delvecchio, K., and Shisheva, A. Functional dissociation between PIKfyve-synthesized PtdIns5P and PtdIns(3,5)P2 by means of the PIKfyve inhibitor YM201636. *Am. J. Physiol Cell Physiol* **303**, C436-C446 (2012).

170. Zolov, S. N.*, et al.* In vivo, Pikfyve generates PI(3,5)P2, which serves as both a signaling lipid and the major precursor for PI5P. *Proc. Natl. Acad. Sci. U. S. A* **109**, 17472-17477 (2012).

171. Gary, J. D., Wurmser, A. E., Bonangelino, C. J., Weisman, L. S., and Emr, S. D. Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell Biol.* **143**, 65-79 (1998).

172. Jefferies, H. B.*, et al.* A selective PIKfyve inhibitor blocks PtdIns(3,5)P(2) production and disrupts endomembrane transport and retroviral budding. *EMBO Rep.* **9**, 164-170 (2008).

173. Whitley, P., Hinz, S., and Doughty, J. Arabidopsis FAB1/PIKfyve proteins are essential for development of viable pollen. *Plant Physiol* **151**, 1812-1822 (2009).

174. Hirano, T., Matsuzawa, T., Takegawa, K., and Sato, M. H. Loss-of-function and gain-of-function mutations in FAB1A/B impair endomembrane homeostasis, conferring pleiotropic developmental abnormalities in Arabidopsis. *Plant Physiol* **155**, 797-807 (2011).

175. Shisheva, A. PIKfyve: Partners, significance, debates and paradoxes. *Cell Biol. Int.* **32**, 591-604 (2008).

176. Shisheva, A. PIKfyve and its Lipid Products in Health and in Sickness. *Curr. Top. Microbiol. Immunol.* **362**, 127-162 (2012).

177. Dyson, J. M., Fedele, C. G., Davies, E. M., Becanovic, J., and Mitchell, C. A. Phosphoinositide phosphatases: just as important as the kinases. *Subcell. Biochem.* **58**, 215-279 (2012).

178. Steck, P. A.*, et al.* Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15**, 356-362 (1997).

179. Li, J.*, et al.* PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947 (1997).

180. Myers, M. P.*, et al.* P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. U. S. A* **94**, 9052-9057 (1997).

181. Ali, I. U., Schriml, L. M., and Dean, M. Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J. Natl. Cancer Inst.* **91**, 1922-1932 (1999).

182. Maehama, T. and Dixon, J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 13375-13378 (1998).

183. Laporte, J., Bedez, F., Bolino, A., and Mandel, J. L. Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases. *Hum. Mol. Genet.* **12 Spec No 2**, R285-R292 (2003).

184. Clague, M. J. and Lorenzo, O. The myotubularin family of lipid phosphatases. *Traffic.* **6**, 1063-1069 (2005).

185. Robinson, F. L. and Dixon, J. E. Myotubularin phosphatases: policing 3-phosphoinositides. *Trends Cell Biol.* **16**, 403-412 (2006).

186. Hnia, K., Vaccari, I., Bolino, A., and Laporte, J. Myotubularin phosphoinositide phosphatases: cellular functions and disease pathophysiology. *Trends Mol. Med.* **18**, 317-327 (2012).

187. Taylor, G. S., Maehama, T., and Dixon, J. E. Myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. *Proc. Natl. Acad. Sci. U. S. A* **97**, 8910-8915 (2000).

188. Blondeau, F.*, et al.* Myotubularin, a phosphatase deficient in myotubular myopathy, acts on phosphatidylinositol 3-kinase and phosphatidylinositol 3-phosphate pathway. *Hum. Mol. Genet.* **9**, 2223-2229 (2000).

189. Zhao, R., Qi, Y., Chen, J., and Zhao, Z. J. FYVE-DSP2, a FYVE domain-containing dual specificity protein phosphatase that dephosphorylates phosphotidylinositol 3-phosphate. *Exp. Cell Res.* **265**, 329-338 (2001).

190. Walker, D. M.*, et al.* Characterization of MTMR3. an inositol lipid 3-phosphatase with novel substrate specificity. *Curr. Biol.* **11**, 1600-1605 (2001).

191. Kim, S. A., Taylor, G. S., Torgersen, K. M., and Dixon, J. E. Myotubularin and MTMR2, phosphatidylinositol 3-phosphatases mutated in myotubular myopathy and type 4B Charcot-Marie-Tooth disease. *J. Biol. Chem.* **277**, 4526-4531 (2002).

192. Laporte, J.*, et al.* Functional redundancy in the myotubularin family. *Biochem. Biophys. Res. Commun.* **291**, 305-312 (2002).

193. Berger, P., Bonneick, S., Willi, S., Wymann, M., and Suter, U. Loss of phosphatase activity in myotubularin-related protein 2 is associated with Charcot-Marie-Tooth disease type 4B1. *Hum. Mol. Genet.* **11**, 1569-1579 (2002).

194. Mochizuki, Y. and Majerus, P. W. Characterization of myotubularin-related protein 7 and its binding partner, myotubularin-related protein 9. *Proc. Natl. Acad. Sci. U. S. A* **100**, 9768-9773 (2003).

195. Tronchere, H.*, et al.* Production of phosphatidylinositol 5-phosphate by the phosphoinositide 3-phosphatase myotubularin in mammalian cells. *J. Biol. Chem.* **279**, 7304-7312 (2004).

196. Begley, M. J. and Dixon, J. E. The structure and regulation of myotubularin phosphatases. *Curr. Opin. Struct. Biol.* **15**, 614-620 (2005).

197. Begley, M. J.*, et al.* Molecular basis for substrate recognition by MTMR2, a myotubularin family phosphoinositide phosphatase. *Proc. Natl. Acad. Sci. U. S. A* **103**, 927-932 (2006).

198. Lorenzo, O., Urbe, S., and Clague, M. J. Systematic analysis of myotubularins: heteromeric interactions, subcellular localisation and endosome related functions. *J. Cell Sci.* **119**, 2953-2959 (2006).

199. Tosch, V.*, et al.* A novel PtdIns3P and PtdIns(3,5)P2 phosphatase with an inactivating variant in centronuclear myopathy. *Hum. Mol. Genet.* **15**, 3098-3106 (2006).

200. Welch, H. C.*, et al.* P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell* **108**, 809-821 (2002).

201. Donald, S.*, et al.* P-Rex2, a new guanine-nucleotide exchange factor for Rac. *FEBS Lett.* **572**, 172-176 (2004).

202. Rosenfeldt, H., Vazquez-Prado, J., and Gutkind, J. S. P-REX2, a novel PI-3-kinase sensitive Rac exchange factor. *FEBS Lett.* **572**, 167-171 (2004).

203. Ungewickell, A.*, et al.* The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases. *Proc. Natl. Acad. Sci. U. S. A* **102**, 18854-18859 (2005).

204. Allaoui, A., Menard, R., Sansonetti, P. J., and Parsot, C. Characterization of the Shigella flexneri ipgD and ipgF genes, which are located in the proximal part of the mxi locus. *Infect. Immun.* **61**, 1707-1714 (1993).

205. Niebuhr, K.*, et al.* IpgD, a protein secreted by the type III secretion machinery of Shigella flexneri, is chaperoned by IpgE and implicated in entry focus formation. *Mol. Microbiol.* **38**, 8-19 (2000).

206. Niebuhr, K.*, et al.* Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the S.flexneri effector IpgD reorganizes host cell morphology. *EMBO J.* **21**, 5069-5078 (2002).

207. Novick, P., Osmond, B. C., and Botstein, D. Suppressors of yeast actin mutations. *Genetics* **121**, 659-674 (1989).

208. Guo, S., Stolz, L. E., Lemrow, S. M., and York, J. D. SAC1-like domains of yeast SAC1, INP52, and INP53 and of human synaptojanin encode polyphosphoinositide phosphatases. *J. Biol. Chem.* **274**, 12990-12995 (1999).

209. Gillooly, D. J.*, et al.* Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* **19**, 4577-4588 (2000).

210. Sarkes, D. and Rameh, L. E. A novel HPLC-based approach makes possible the spatial characterization of cellular PtdIns5P and other phosphoinositides. *Biochem. J.* **428**, 375-384 (2010).

211. Sagona, A. P.*, et al.* PtdIns(3)P controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody. *Nat. Cell Biol.* **12**, 362-371 (2010).

212. Lindmo, K. and Stenmark, H. Regulation of membrane traffic by phosphoinositide 3-kinases. *J. Cell Sci.* **119**, 605-614 (2006).

213. Obara, K., Noda, T., Niimi, K., and Ohsumi, Y. Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in Saccharomyces cerevisiae. *Genes Cells* **13**, 537-547 (2008).

214. Axe, E. L.*, et al.* Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J. Cell Biol.* **182**, 685-701 (2008).

215. Taguchi-Atarashi, N.*, et al.* Modulation of local PtdIns3P levels by the PI phosphatase MTMR3 regulates constitutive autophagy. *Traffic.* **11**, 468-478 (2010).

216. Falasca, M. and Maffucci, T. Emerging roles of phosphatidylinositol 3-monophosphate as a dynamic lipid second messenger. *Arch. Physiol Biochem.* **112**, 274-284 (2006).

217. Falasca, M. and Maffucci, T. Rethinking phosphatidylinositol 3-monophosphate. *Biochim. Biophys. Acta* **1793**, 1795-1803 (2009).

218. Pendaries, C.*, et al.* Emerging roles of phosphatidylinositol monophosphates in cellular signaling and trafficking. *Adv. Enzyme Regul.* **45**, 201-214 (2005).

219. D'Angelo, G., Vicinanza, M., Di, C. A., and De Matteis, M. A. The multiple roles of PtdIns(4)P -- not just the precursor of PtdIns(4,5)P2. *J. Cell Sci.* **121**, 1955-1963 (2008).

220. Graham, T. R. and Burd, C. G. Coordination of Golgi functions by phosphatidylinositol 4-kinases. *Trends Cell Biol.* **21**, 113-121 (2011).

221. Wang, Y. J.*, et al.* Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell* **114**, 299-310 (2003).

222. Hammond, G. R., Schiavo, G., and Irvine, R. F. Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P(2). *Biochem. J.* **422**, 23-35 (2009).

223. Hammond, G. R.*, et al.* PI4P and PI(4,5)P2 are essential but independent lipid determinants of membrane identity. *Science* **337**, 727-730 (2012).

224. Clarke, J. H.*, et al.* Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. *Biochem. J.* **357**, 905-910 (2001).

225. Gozani, O.*, et al.* The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* **114**, 99-111 (2003).

226. Sbrissa, D., Ikonomov, O. C., Strakova, J., and Shisheva, A. Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. *Endocrinology* **145**, 4853-4865 (2004).

227. Jones, D. R.*, et al.* Nuclear PtdIns5P as a transducer of stress signaling: an in vivo role for PIP4Kbeta. *Mol. Cell* **23**, 685-695 (2006).

228. Pendaries, C.*, et al.* PtdIns5P activates the host cell PI3-kinase/Akt pathway during Shigella flexneri infection. *EMBO J.* **25**, 1024-1034 (2006).

229. Mason, D.*, et al.* Alteration of epithelial structure and function associated with PtdIns(4,5)P2 degradation by a bacterial phosphatase. *J. Gen. Physiol* **129**, 267-283 (2007).

230. Coronas, S.*, et al.* Elevated levels of PtdIns5P in NPM-ALK transformed cells: implication of PIKfyve. *Biochem. Biophys. Res. Commun.* **372**, 351-355 (2008).

231. Guittard, G.*, et al.* Cutting edge: Dok-1 and Dok-2 adaptor molecules are regulated by phosphatidylinositol 5-phosphate production in T cells. *J. Immunol.* **182**, 3974-3978 (2009).

232. Grainger, D. L., Tavelis, C., Ryan, A. J., and Hinchliffe, K. A. Involvement of phosphatidylinositol 5-phosphate in insulin-stimulated glucose uptake in the L6 myotube model of skeletal muscle. *Pflugers Arch.* **462**, 723-732 (2011).

233. Ramel, D.*, et al.* Shigella flexneri infection generates the lipid PI5P to alter endocytosis and prevent termination of EGFR signaling. *Sci. Signal.* **4**, ra61 (2011).

234. Dove, S. K.*, et al.* Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature* **390**, 187-192 (1997).

235. Cooke, F. T.*, et al.* The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in S. cerevisiae. *Curr. Biol.* **8**, 1219-1222 (1998).

236. Ikonomov, O. C.*, et al.* Functional dissection of lipid and protein kinase signals of PIKfyve reveals the role of PtdIns 3,5-P2 production for endomembrane integrity. *J. Biol. Chem.* **277**, 9206-9211 (2002).

237. Ikonomov, O. C., Sbrissa, D., Dondapati, R., and Shisheva, A. ArPIKfyve-PIKfyve interaction and role in insulin-regulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes. *Exp. Cell Res.* **313**, 2404-2416 (2007).

238. Ikonomov, O. C., Sbrissa, D., and Shisheva, A. YM201636, an inhibitor of retroviral budding and PIKfyve-catalyzed PtdIns(3,5)P2 synthesis, halts glucose entry by insulin in adipocytes. *Biochem. Biophys. Res. Commun.* **382**, 566-570 (2009).

239. Dove, S. K., Dong, K., Kobayashi, T., Williams, F. K., and Michell, R. H. Phosphatidylinositol 3,5-bisphosphate and Fab1p/PIKfyve underPPIn endo-lysosome function. *Biochem. J.* **419**, 1-13 (2009).

240. Ferguson, C. J., Lenk, G. M., and Meisler, M. H. Defective autophagy in neurons and astrocytes from mice deficient in PI(3,5)P2. *Hum. Mol. Genet.* **18**, 4868-4878 (2009).

241. Ferguson, C. J., Lenk, G. M., and Meisler, M. H. PtdIns(3,5)P2 and autophagy in mouse models of neurodegeneration. *Autophagy.* **6**, 170-171 (2010).

242. Divecha, N. and Irvine, R. F. Phospholipid signaling. *Cell* **80**, 269-278 (1995).

243. Carpenter, C. L., Tolias, K. F., Van, V. A., and Hartwig, J. Lipid kinases are novel effectors of the GTPase Rac1. *Adv. Enzyme Regul.* **39**, 299-312 (1999).

244. Tolias, K. F.*, et al.* Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr. Biol.* **10**, 153-156 (2000).

245. Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**, 167-175 (1989).

246. Haugh, J. M., Codazzi, F., Teruel, M., and Meyer, T. Spatial sensing in fibroblasts mediated by 3' phosphoinositides. *J. Cell Biol.* **151**, 1269-1280 (2000).

247. Funamoto, S., Milan, K., Meili, R., and Firtel, R. A. Role of phosphatidylinositol 3' kinase and a downstream pleckstrin homology domain-containing protein in controlling chemotaxis in dictyostelium. *J. Cell Biol.* **153**, 795-810 (2001).

248. Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R. A. Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* **109**, 611-623 (2002).

249. Iijima, M. and Devreotes, P. Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* **109**, 599-610 (2002).

250. Ullrich, A. and Schlessinger, J. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212 (1990).

251. Hubbard, S. R. Structural analysis of receptor tyrosine kinases. *Prog. Biophys. Mol. Biol.* **71**, 343-358 (1999).

252. Lemmon, M. A. and Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134 (2010).

253. Pawson, T. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* **116**, 191-203 (2004).

254. Playford, M. P. and Schaller, M. D. The interplay between Src and integrins in normal and tumor biology. *Oncogene* **23**, 7928-7946 (2004).

255. McLean, G. W.*, et al.* The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nat. Rev. Cancer* **5**, 505-515 (2005).

256. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **6**, 56-68 (2005).

257. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. Met, metastasis, motility and more. *Nat. Rev. Mol. Cell Biol.* **4**, 915-925 (2003).

258. Jung, K. H., Park, B. H., and Hong, S. S. Progress in cancer therapy targeting c-Met signaling pathway. *Arch. Pharm. Res.* **35**, 595-604 (2012).

259. Casaletto, J. B. and McClatchey, A. I. Spatial regulation of receptor tyrosine kinases in development and cancer. *Nat. Rev. Cancer* **12**, 387-400 (2012).

260. Johnson, D. E. and Williams, L. T. Structural and functional diversity in the FGF receptor multigene family. *Adv. Cancer Res.* **60**, 1-41 (1993).

261. Olsen, S. K.*, et al.* Insights into the molecular basis for fibroblast growth factor receptor autoinhibition and ligand-binding promiscuity. *Proc. Natl. Acad. Sci. U. S. A* **101**, 935-940 (2004).

262. Gospodarowicz, D. and Moran, J. Effect of a fibroblast growth factor, insulin, dexamethasone, and serum on the morphology of BALB/c 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A* **71**, 4648-4652 (1974).

263. Schlessinger, J.*, et al.* Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* **6**, 743-750 (2000).

264. Furdui, C. M., Lew, E. D., Schlessinger, J., and Anderson, K. S. Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction. *Mol. Cell* **21**, 711-717 (2006).

265. Lew, E. D., Furdui, C. M., Anderson, K. S., and Schlessinger, J. The precise sequence of FGF receptor autophosphorylation is kinetically driven and is disrupted by oncogenic mutations. *Sci. Signal.* **2**, ra6 (2009).

266. Chen, H.*, et al.* A molecular brake in the kinase hinge region regulates the activity of receptor tyrosine kinases. *Mol. Cell* **27**, 717-730 (2007).

267. Mohammadi, M.*, et al.* Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. *Mol. Cell Biol.* **16**, 977-989 (1996).

268. Schlessinger, J., Lax, I., and Lemmon, M. Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* **83**, 357-360 (1995).

269. Powers, C. J., McLeskey, S. W., and Wellstein, A. Fibroblast growth factors, their receptors and signaling. *Endocr. Relat Cancer* **7**, 165-197 (2000).

270. Eswarakumar, V. P., Lax, I., and Schlessinger, J. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* **16**, 139-149 (2005).

271. Turner, N. and Grose, R. Fibroblast growth factor signalling: from development to cancer. *Nat. Rev. Cancer* **10**, 116-129 (2010).

272. Mohammadi, M.*, et al.* A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol. Cell Biol.* **11**, 5068-5078 (1991).

273. Ong, S. H.*, et al.* FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. *Mol. Cell Biol.* **20**, 979-989 (2000).

274. Dhalluin, C.*, et al.* Structural basis of SNT PTB domain interactions with distinct neurotrophic receptors. *Mol. Cell* **6**, 921-929 (2000).

275. Belleudi, F.*, et al.* Keratinocyte growth factor receptor ligands target the receptor to different intracellular pathways. *Traffic.* **8**, 1854-1872 (2007).

276. Haugsten, E. M., Malecki, J., Bjorklund, S. M., Olsnes, S., and Wesche, J. Ubiquitination of fibroblast growth factor receptor 1 is required for its intracellular sorting but not for its endocytosis. *Mol. Biol. Cell* **19**, 3390-3403 (2008).

277. Furthauer, M., Lin, W., Ang, S. L., Thisse, B., and Thisse, C. Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. *Nat. Cell Biol.* **4**, 170-174 (2002).

278. Tsang, M., Friesel, R., Kudoh, T., and Dawid, I. B. Identification of Sef, a novel modulator of FGF signalling. *Nat. Cell Biol.* **4**, 165-169 (2002).

279. Cabrita, M. A. and Christofori, G. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis.* **11**, 53-62 (2008).

280. Basilico, C. and Moscatelli, D. The FGF family of growth factors and oncogenes. *Adv. Cancer Res.* **59**, 115-165 (1992).

281. Goldfarb, M. Fibroblast growth factor homologous factors: evolution, structure, and function. *Cytokine Growth Factor Rev.* **16**, 215-220 (2005).

282. Bottcher, R. T. and Niehrs, C. Fibroblast growth factor signaling during early vertebrate development. *Endocr. Rev.* **26**, 63-77 (2005).

283. Wiedlocha, A. and Sorensen, V. Signaling, internalization, and intracellular activity of fibroblast growth factor. *Curr. Top. Microbiol. Immunol.* **286**, 45-79 (2004).

284. Bryant, D. M. and Stow, J. L. Nuclear translocation of cell-surface receptors: lessons from fibroblast growth factor. *Traffic.* **6**, 947-954 (2005).

285. Beenken, A. and Mohammadi, M. The FGF family: biology, pathophysiology and therapy. *Nat. Rev. Drug Discov.* **8**, 235-253 (2009).

286. Haugsten, E. M., Wiedlocha, A., Olsnes, S., and Wesche, J. Roles of fibroblast growth factor receptors in carcinogenesis. *Mol. Cancer Res.* **8**, 1439-1452 (2010).

287. Wesche, J., Haglund, K., and Haugsten, E. M. Fibroblast growth factors and their receptors in cancer. *Biochem. J.* **437**, 199-213 (2011).

288. Ahmad, I., Iwata, T., and Leung, H. Y. Mechanisms of FGFR-mediated carcinogenesis. *Biochim. Biophys. Acta* **1823**, 850-860 (2012).

289. Greenman, C*., et al.* Patterns of somatic mutation in human cancer genomes. *Nature* **446**, 153-158 (2007).

290. Zhao, R., Qi, Y., and Zhao, Z. J. FYVE-DSP1, a dual-specificity protein phosphatase containing an FYVE domain. *Biochem. Biophys. Res. Commun.* **270**, 222-229 (2000).

291. Hamasaki, M. and Yoshimori, T. Where do they come from? Insights into autophagosome formation. *FEBS Lett.* **584**, 1296-1301 (2010).

292. Noda, T., Matsunaga, K., Taguchi-Atarashi, N., and Yoshimori, T. Regulation of membrane biogenesis in autophagy via PI3P dynamics. *Semin. Cell Dev. Biol.* **21**, 671-676 (2010).

293. Vergne, I. and Deretic, V. The role of PI3P phosphatases in the regulation of autophagy. *FEBS Lett.* **584**, 1313-1318 (2010).

294. Sbrissa, D., Ikonomov, O. C., and Shisheva, A. Phosphatidylinositol 3-phosphate-interacting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomenbrane localization. *J. Biol. Chem.* **277**, 6073-6079 (2002).

295. Sbrissa, D., Ikonomov, O. C., Filios, C., Delvecchio, K., and Shisheva, A. Functional dissociation between PIKfyve-synthesized PtdIns5P and PtdIns(3,5)P2 by means of the PIKfyve inhibitor YM201636. *Am. J. Physiol Cell Physiol* **303**, C436-C446 (2012).

296. de Lartigue, J*., et al.* PIKfyve regulation of endosome-linked pathways. *Traffic.* **10**, 883-893 (2009).

297. Kerr, M. C*., et al.* Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of Salmonella. *EMBO J.* **29**, 1331-1347 (2010).

298. Rusten, T. E*., et al.* Fab1 phosphatidylinositol 3-phosphate 5-kinase controls trafficking but not silencing of endocytosed receptors. *Mol. Biol. Cell* **17**, 3989-4001 (2006).

299. Rusten, T. E*., et al.* ESCRTs and Fab1 regulate distinct steps of autophagy. *Curr. Biol.* **17**, 1817-1825 (2007).

300. Lecompte, O., Poch, O., and Laporte, J. PtdIns5P regulation through evolution: roles in membrane trafficking? *Trends Biochem. Sci.* **33**, 453-460 (2008).

301. Coronas, S*., et al.* PtdIns5P: a little phosphoinositide with big functions? *Biochem. Soc. Symp.* 117-128 (2007).

302. Grainger, D. L., Tavelis, C., Ryan, A. J., and Hinchliffe, K. A. The emerging role of PtdIns5P: another signalling phosphoinositide takes its place. *Biochem. Soc. Trans.* **40**, 257-261 (2012).

303. Maffucci, T.*, et al.* Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration. *J. Cell Biol.* **169**, 789-799 (2005).

304. Meijer, H. J.*, et al.* Identification of a new polyphosphoinositide in plants, phosphatidylinositol 5-monophosphate (PtdIns5P), and its accumulation upon osmotic stress. *Biochem. J.* **360**, 491-498 (2001).

305. Guittard, G.*, et al.* Evidence for a positive role of PtdIns5P in T-cell signal transduction pathways. *FEBS Lett.* **584**, 2455-2460 (2010).

306. Zou, J., Marjanovic, J., Kisseleva, M. V., Wilson, M., and Majerus, P. W. Type I phosphatidylinositol-4,5-bisphosphate 4-phosphatase regulates stress-induced apoptosis. *Proc. Natl. Acad. Sci. U. S. A* **104**, 16834-16839 (2007).

307. Nagashima, M.*, et al.* DNA damage-inducible gene p33ING2 negatively regulates cell proliferation through acetylation of p53. *Proc. Natl. Acad. Sci. U. S. A* **98**, 9671-9676 (2001).

308. Wilcox, A. and Hinchliffe, K. A. Regulation of extranuclear PtdIns5P production by phosphatidylinositol phosphate 4-kinase 2alpha. *FEBS Lett.* **582**, 1391-1394 (2008).

309. Dupuis-Coronas, S.*, et al.* The nucleophosmin-anaplastic lymphoma kinase oncogene interacts, activates, and uses the kinase PIKfyve to increase invasiveness. *J. Biol. Chem.* **286**, 32105-32114 (2011).

310. Hall, A. Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514 (1998).

311. Raftopoulou, M. and Hall, A. Cell migration: Rho GTPases lead the way. *Dev. Biol.* **265**, 23-32 (2004).

312. Ridley, A. J. Rho GTPases and cell migration. *J. Cell Sci.* **114**, 2713-2722 (2001).

313. Toker, A. The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Cell Biol.* **10**, 254-261 (1998).

314. Sechi, A. S. and Wehland, J. The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. *J. Cell Sci.* **113 Pt 21**, 3685-3695 (2000).

315. Payrastre, B.*, et al.* Phosphoinositides: key players in cell signalling, in time and space. *Cell Signal.* **13**, 377-387 (2001).

316. Colomba, A.*, et al.* Activation of Rac1 and the exchange factor Vav3 are involved in NPM-ALK signaling in anaplastic large cell lymphomas. *Oncogene* **27**, 2728-2736 (2008).

317. Colomba, A.*, et al.* Inhibition of Rac controls NPM-ALK-dependent lymphoma development and dissemination. *Blood Cancer J.* **1**, e21 (2011).

318. J. C. Recamier, "Recherches Sur Le Traitement Du Cancer Sur La Compression Methodique Simple Ou Combinee Et Sur L'Historie Generale De La Meme Maladie.," 2nd ed., 1829).

319. Talmadge, J. E. and Fidler, I. J. AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res.* **70**, 5649-5669 (2010).

320. Sethi, N. and Kang, Y. Unravelling the complexity of metastasis - molecular understanding and targeted therapies. *Nat. Rev. Cancer* **11**, 735-748 (2011).

321. Kim, J.*, et al.* The phosphoinositide kinase PIKfyve mediates epidermal growth factor receptor trafficking to the nucleus. *Cancer Res.* **67**, 9229-9237 (2007).

322. Bennett, G., Sadlier, D., Doran, P. P., Macmathuna, P., and Murray, D. W. A functional and transcriptomic analysis of NET1 bioactivity in gastric cancer. *BMC. Cancer* **11**, 50 (2011).

323. Sjoblom, T.*, et al.* The consensus coding sequences of human breast and colorectal cancers. *Science* **314**, 268-274 (2006).

324. Wang, W.*, et al.* Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. *Cancer Res.* **67**, 3505-3511 (2007).

325. Hu, Z.*, et al.* A genome-wide association study identifies two new lung cancer susceptibility loci at 13q12.12 and 22q12.2 in Han Chinese. *Nat. Genet.* **43**, 792-796 (2011).

326. Sheppard, K., Kinross, K. M., Solomon, B., Pearson, R. B., and Phillips, W. A. Targeting PI3 kinase/AKT/mTOR signaling in cancer. *Crit Rev. Oncog.* **17**, 69-95 (2012).

327. Gong, W., Suzuki, K., Russell, M., and Riabowol, K. Function of the ING family of PHD proteins in cancer. *Int. J. Biochem. Cell Biol.* **37**, 1054-1065 (2005).

328. Lax, I.*, et al.* The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. *Mol. Cell* **10**, 709-719 (2002).

329. Nomura, S.*, et al.* FGF10/FGFR2 signal induces cell migration and invasion in pancreatic cancer. *Br. J. Cancer* **99**, 305-313 (2008).

330. Welm, B. E.*, et al.* Inducible dimerization of FGFR1: development of a mouse model to analyze progressive transformation of the mammary gland. *J. Cell Biol.* **157**, 703-714 (2002).

331. Xian, W., Schwertfeger, K. L., Vargo-Gogola, T., and Rosen, J. M. Pleiotropic effects of FGFR1 on cell proliferation, survival, and migration in a 3D mammary epithelial cell model. *J. Cell Biol.* **171**, 663-673 (2005).

332. Daviet, I., Herbert, J. M., and Maffrand, J. P. Involvement of protein kinase C in the mitogenic and chemotaxis effects of basic fibroblast growth factor on bovine cerebral cortex capillary endothelial cells. *FEBS Lett.* **259**, 315-317 (1990).

333. Landgren, E., Klint, P., Yokote, K., and Claesson-Welsh, L. Fibroblast growth factor receptor-1 mediates chemotaxis independently of direct SH2-domain protein binding. *Oncogene* **17**, 283-291 (1998).

334. Boilly, B., Vercoutter-Edouart, A. S., Hondermarck, H., Nurcombe, V., and Le, B., X FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev.* **11**, 295-302 (2000).

335. Cha, J. Y., Maddileti, S., Mitin, N., Harden, T. K., and Der, C. J. Aberrant receptor internalization and enhanced FRS2-dependent signaling contribute to the transforming activity of the fibroblast growth factor receptor 2 IIIb C3 isoform. *J. Biol. Chem.* **284**, 6227-6240 (2009).

336. Birmingham, A.*, et al.* 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* **3**, 199-204 (2006).

337. Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H., and Williams, B. R. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**, 834-839 (2003).

338. Gustafsson, M. G.*, et al.* Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. *Biophys. J.* **94**, 4957-4970 (2008).

339. Schermelleh, L.*, et al.* Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy. *Science* **320**, 1332-1336 (2008).

340. Meyer, A. S.*, et al.* 2D protrusion but not motility predicts growth factor-induced cancer cell migration in 3D collagen. *J. Cell Biol.* **197**, 721-729 (2012).

341. Duchek, P. and Rorth, P. Guidance of cell migration by EGF receptor signaling during Drosophila oogenesis. *Science* **291**, 131-133 (2001).

342. Hennigan, R. F., Hawker, K. L., and Ozanne, B. W. Fos-transformation activates genes associated with invasion. *Oncogene* **9**, 3591-3600 (1994).

343. Wenk, M. R.*, et al.* Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat. Biotechnol.* **21**, 813-817 (2003).

I

II

III

ERK-mediated phosphorylation of FGF receptor 1 on Ser^{777} confers negative feedback on FGF signaling

Malgorzata Zakrzewska[1,2], Ellen Margrethe Haugsten[1], Beata Nadratowska-Wesolowska[1], Angela Oppelt[1], Barbara Hausott[3], Yixin Jin[1], Jacek Otlewski[2], Jørgen Wesche[1], Antoni Wiedlocha[1]

[1] Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, and Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Montebello, 0310 Oslo, Norway

[2] Faculty of Biotechnology, University of Wroclaw, Tamka 2, 50-137, Wroclaw, Poland

[3] Division of Neuroanatomy, Innsbruck Medical University, Muellerstrasse 59, 6020 Innsbruck, Austria

Corresponding author:

Antoni Wiedlocha

e-mail: Antoni.Wiedlocha@rr-research.no

One-sentence summary: The study reveals a novel mechanism for negative regulation of FGF signaling based on direct Ser^{777} phosphorylation of FGFR1 by ERK1/2.

Abstract

Fibroblast growth factor 1 (FGF1) controls cellular activities through activation of specific cell-surface FGF receptors (FGFRs). Trans-phosphorylation of tyrosine residues in the kinase domain of FGFRs leads to activation of intracellular signal cascades including mitogen-activated protein kinases (MAPKs). In addition to the kinase domain, FGFRs contain a serine rich C-terminal tail of unknown function. Here, we unravel a novel regulatory mechanism of FGFR signaling based on direct phosphorylation of a specific serine residue (Ser^{777}) in the C-terminal part of the receptor by the MAPKs ERK1/2. This serine phosphorylation substantially reduces the tyrosine phosphorylation in the kinase domain of the receptor. Prevention of FGFR1 Ser^{777} phosphorylation or S777A mutation results in enhanced receptor tyrosine phosphorylation, increased cell proliferation, cell migration and axonal growth, while a phosphomimetic mutation at Ser^{777} reduces FGFR1 signaling. Importantly, FGFR-independent activation of MAPKs also results in phosphorylation of Ser^{777} of FGFR1, thereby allowing cross-control of FGFR activity by other signaling receptors. Our data reveal a novel negative-feedback mechanism that controls FGF signaling and thereby protects the cell against excessive activation of FGFR.

Introduction

Detailed understanding of the regulatory mechanisms of receptor tyrosine kinases (RTKs), including fibroblast growth factor receptors (FGFRs), is an important goal of current biomedical sciences since these transmembrane proteins are commonly affected in many kinds of human cancers and other pathological conditions, including skeletal and olfactory syndromes, and metabolic disorders.

The FGFR family consists of four closely related receptors (FGFR1 to FGFR4) (1), that play important roles in diverse physiological processes such as cell proliferation, differentiation, migration and survival (2). They have a conserved structure composed of three extracellular Ig-like domains (D1 – D3), an acidic box, a transmembrane domain, and a well conserved intracellular tyrosine kinase domain. The second and the third Ig-like domains of the receptors are crucial for ligand binding, whereas the first Ig-like domain plays a role in receptor autoinhibition (3). Besides the fact that one of the tyrosine residues (Tyr^{766} in FGFR1) in the receptor C-tail serves as a binding site for PLCγ (4), very little is known about the biological function of the unordered C-terminal part of the FGFRs, which is composed of approximately 50-56 amino acids.

FGFRs are activated by 18 high affinity extracellular ligands – fibroblast growth factors (FGFs). The binding of FGF to FGFR results in dimerization of the receptor which, in turn, activates the receptor's tyrosine kinase domain by trans-phosphorylation. Phosphorylation of the receptor tyrosines is a precisely ordered sequential event that includes seven tyrosine residues (Tyr^{463}, Tyr^{583}, Tyr^{585}, Tyr^{653}, Tyr^{654}, Tyr^{730}, and Tyr^{766}, in the case of FGFR1) (5, 6). The activated receptor phosphorylates multiple intracellular proteins including FRS2 (FGFR substrate 2) and PLCγ (phospholipase Cγ) (7). Phosphorylated FRS2α forms two specific binding sites for SHP2 (SH2 domain-containing protein tyrosine phosphatase-2) and four binding sites for GRB2 (growth factor receptor-bound protein 2). GRB2 is constitutively bound via its SH3 domains to SOS (son of sevenless protein) and GAB1 (GRB2-associated-binding protein 1) and these proteins constitute a

signaling complex activating the RAS/MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase)/AKT signaling pathways (7). Several other pathways, including p38 MAPK and JNK (c-Jun N-terminal kinase) pathways, and STAT (signal transducer and activator of transcription) signaling are induced by activated FGFR (8, 9).

The precise regulation of FGFR signaling is crucial for the cell fate as it may influence the proliferation potential and in consequence carcinogenesis and tumor progression. However, in contrast to the well researched mechanisms of FGFR activation, those ensuring its deactivation are only sketchily understood (8). Attenuation of the signal generated by an activated receptor can be achieved by FGFR endocytosis followed by lysosomal degradation (10). In addition, a negative feedback leading to the silencing of FGF-induced signals is mediated by several proteins such as MAPK phosphatase 3 (MKP3), Sprouty proteins, and SEF (similar expression to FGF) family members that modulate receptor signaling at different steps of the signal transduction pathway (8, 11-13). Furthermore, FGF receptor deactivation is also based on MAPK signaling and threonine phosphorylation of FRS2α (14). FGF stimulation has been shown to induce MAPK ERK1/2 (extracellular signal-regulated kinases 1/2)-dependent phosphorylation on at least eight threonine residues of FRS2α. This threonine phosphorylation effectively reduces tyrosine phosphorylation of FRS2α, thereby decreasing the recruitment of GRB2 and attenuating FGFR signaling (14).

In this study we reveal that FGF1 stimulation induces direct MAPK-mediated phosphorylation of a specific serine residue (Ser^{777}) in the C-terminal part of the receptor. Phosphorylation of Ser^{777} reduces the tyrosine phosphorylation in the receptor kinase domain and in this way provides a negative feedback route. Our work demonstrates a novel mechanism of FGFR1 activity control, which can protect against excessive intracellular signaling.

Results

FGFR1 is directly phosphorylated on Ser^{777} by the MAP kinases ERK1 and ERK2

The C-terminal part of FGFR1 (residues 755-822) is rich in serines (Fig. 1A), and we therefore considered the possibility that the signaling potential of the FGFR could be regulated by specific serine phosphorylation. Analysis of FGFR1 by the Scansite server (http://scansite.mit.edu) revealed that there is a strong consensus sequence in the C-terminal part of the receptor for the MAPK ERK1 with a predicted phosphorylation site at Ser^{777} and a binding site at Pro^{781} (15). Interestingly, Ser^{777} is conserved throughout the human FGFR family and also in different species ranging from zebrafish and newt to frog, rat, mouse and human (Fig. 1A).

To examine whether ERK1 and ERK2 are able to directly phosphorylate FGFR1 at Ser^{777}, we carried out an in vitro phosphorylation assay using recombinant kinases, [γ-^{33}P]ATP, and recombinant GST-tagged C-tail of FGFR1 or its mutants as substrates. Wild-type FGFR1 could clearly be phosphorylated by active forms of ERK1 or ERK2, whereas two FGFR1 mutants at the critical serine, S777A and S777D, were not phosphorylated (Fig. 1B). We used recombinant p38α kinase which is known to phosphorylate FGFR1 (16), as a control. Using the same number of kinase units we observed that the phosphorylation of FGFR1 by the ERKs was much stronger than that by p38α (Fig. 1B). A radiolabeled band of p38α, could be detected only upon longer exposure (fig. S1A). For control we used an excess of GST alone, which was very weakly phosphorylated (Fig. 1B). In addition, we performed phosphorylation reactions using a kinase unrelated to the MAPK pathway, AKT, which was completely unable to phosphorylate the C-terminal tail of FGFR1 (fig. S1A). Since we intended to block the ERKs in cellular experiments using specific inhibitors of the MAPK's upstream kinase (MAPKK, MEK), we also investigated if that kinase could phosphorylate the C-tail of FGFR1. Using active MEK1 we did not observe any band derived from phosphorylated FGFR1 (fig. S1B).

To confirm that ERKs can phosphorylate full-length FGFR1 and that this phosphorylation occurs specifically on Ser^{777}, we performed in vitro phosphorylation assay using recombinant kinases and FGFR1 immunoprecipitated from U2OS cells stably expressing FGFR1 or its mutants. The phosphorylation reaction was carried out in the presence of the specific FGFR tyrosine kinase inhibitor, PD173084, to prevent receptor autophosphorylation. The data clearly show that both ERK1 and ERK2 are able to efficiently phosphorylate wild-type FGFR1, but not the Ser^{777} mutants (Fig. 1C).

The results unequivocally indicate that the MAPKs, ERK1 and ERK2, but not MEK, can directly and specifically phosphorylate FGFR1 at Ser^{777}, and furthermore, that Ser^{777} is the only site in FGFR1 that is phosphorylated by the MAPKs.

ERK1 binds to FGFR1

To test whether ERK1 binds directly to the C-tail of FGFR1, we performed a pull-down experiment with recombinant active His-tagged ERK1, recombinant GST-tagged C-terminal part of FGFR1 or its mutants, and Dynabeads® with immobilized anti-GST antibodies. Using anti-MAPK antibody we found that only the GST-tagged C-tail of wild-type FGFR1 was able to pull down ERK1, in clear contrast to the S777A and S777D mutants (Fig. 1D). As a control of unspecific binding of ERK1 to GST we used GST alone and GST-tagged FGF1. For neither protein did we observe any bands reacting with anti-MAPK antibody.

We also analyzed the binding of recombinant His-tagged ERK1 to full-length FGFR1 immunoprecipitated from U2OSR1 cells with anti-FGFR1 antibodies conjugated to Dynabeads®. Using anti-His antibody we detected ERK1 in the complex with FGFR1 from cells (Fig. 1E). For negative control we used unspecific antibody for immunoprecipitation.

Both binding experiments revealed that ERK1 can bind directly to FGFR1. Moreover, besides being the phosphorylation site for ERK1/2, Ser^{777} of FGFR1 is also necessary for MAPK binding.

Blocking MAPK activity inhibits Ser^{777} phosphorylation of FGFR1

To study the involvement of MAP kinases (ERK1/2) in the phosphorylation of Ser^{777} of FGFR1 in living cells, we generated a phospho-specific antibody against phosphorylated Ser^{777} (pS777-FGFR1) and used two different cell lines: U2OS cells stably transfected with FGFR1 (U2OSR1) and NIH3T3 naturally expressing FGFR1. Using the specific antibodies against phosphorylated Ser^{777} of FGFR1, we observed that upon FGF1 stimulation, Ser^{777} was effectively phosphorylated in cells. Moreover, when the MAPKs ERK1/2 were inactivated (inhibited by U0126 or another MEK1/2 inhibitor – SL327) there was no phosphorylation of Ser^{777} (Fig. 2A).

In addition, we performed confocal microscopy using the phospho-Ser^{777} antibody and the U2OSR1 cell line. The cells were starved for 4 hours and then stimulated with Cy3-labeled FGF1 (Cy3-FGF1) for 20 min. Subsequently, the cells were fixed and stained with the anti-phospho-Ser^{777}-FGFR1 antibody and anti-EEA1 antibody, an endosomal marker (17). We observed clear staining for phospho-Ser^{777} in the presence of FGF1 (Fig. 2B, upper panel) and no traces of staining in unstimulated cells (Fig. 2B, middle panel). Moreover, the phospho-Ser^{777} staining colocalized with both Cy3-FGF1 and with the endosomal marker (EEA1) indicating that the staining is specific and can be detected intracellularly. We also performed the same experiment in the presence of a specific MEK inhibitor, U0126. Upon U0126 treatment we could not detect any phospho-Ser^{777} staining (Fig. 2B, lower panel). There were no substantial differences in the colocalization patterns of Cy3-FGF1 and EEA1 in the presence and in the absence of the MEK inhibitor (Fig. 2B), indicating that cellular localization of the FGF1-FGFR1 complex was not affected.

To investigate further the role of MAPKs ERK1/2 in the phosphorylation of FGFR1 on Ser^{777}, we used expression constructs previously characterized by Pouyssegur and coworkers, which modulate either positively or negatively the activity of MAPKs (18, 19). They reported that a MAPK kinase (MAPKK, MEK1) construct mutated on its two phosphorylation sites Ser^{218} and

Ser^{222} to aspartic residues (MEK1-SS/DD) was able to activate MAPKs constitutively, in contrast to the dominant-negative form of MEK1 (MEK1-S222A) in which Ser^{222} was mutated to alanine. Upon expression of the dominant-negative mutant of MEK1 (MEK1-S222A) and stimulation of the cells with FGF1 no staining for Ser^{777}-phosphorylated FGFR1 was visible (Fig. 2C). Conversely, when the constitutively active variant of MEK1 (MEK1-SS/DD) was expressed in U2OSR1 cells in the absence of FGF1, we observed staining of phospho-Ser^{777}-FGFR1 which disappeared in the presence of U0126 (Fig. 2C), suggesting that MAPKs are likely to be directly involved in the serine phosphorylation of FGFR1.

Tyrosine phosphorylation of FGFR1 is enhanced by blocking the activity of MAPKs ERK1 and ERK2

To examine the potential impact of MAPKs (ERK1/2) on FGFR1 activity, we studied the effect of ERK inhibition on FGF1-induced signaling using NIH3T3 and U2OSR1 cells. In both cell lines, the FGF1-induced tyrosine phosphorylation of FGFR1 (Tyr^{653}/Tyr^{654}) as well as the phosphorylation of downstream signaling molecules were augmented and extended in the presence of different MEK1/2 inhibitors, either U0126 or SL327, as compared to untreated cells (Fig. 3A). Using antibodies against phosphorylated Ser^{777} of FGFR1, we observed that the kinetics of Ser^{777} phosphorylation is similar to that of the tyrosine phosphorylation of FGFR1. Inactivation of MAPKs abrogated Ser^{777} phosphorylation, as seen before. To ensure complete blocking of the ERK1/2 activity, 20 µM U0126 was used in all experiments, even though 5 µM U0126 was found sufficient to inhibit the phosphorylation of ERK1/2 (fig. S2). Experiments in U2OSR1 and NIH3T3 cells were performed in the presence of 10 µg/ml cycloheximide or 2 µg/ml brefeldin A to prevent the appearance of newly synthesized receptors. No substantial differences were observed between these two inhibitors as shown in fig. S3.

Treatment with the MEK inhibitors, U0126 or SL327, precluding the activation of ERK1/2 also brought about a shift in the electrophoretic mobility of phosphorylated FRS2α (phosho-Tyr^{196}) and enhanced the intensity of the signal (Fig. 3A). This effect is triggered by a lack of ERK-mediated FRS2α phosphorylation on eight threonine residues which then in turn reduces Tyr^{196} phosphorylation (14).

To test further if the inhibition of the MAPKs ERK1/2 is the direct cause of the observed stronger and more sustained tyrosine phosphorylation of FGFR1, we attempted to specifically knock down the expression of ERK1 and 2 in U2OSR1 cells using validated siRNA oligonucleotides. Unfortunately, despite an efficient reduction of both proteins (fig. S4A), trace amounts remained that were highly active upon phosphorylation by upstream kinases. Therefore, we decided to use specific siRNA against GRB2, an adaptor protein recruited by activated FRS2α that is responsible for RAS activation and further MAP kinase pathway activation (20). A scrambled siRNA pool was used as a control. As demonstrated in Fig. 3B, the specific siRNA efficiently knocked down expression of GRB2, resulting in an almost complete deactivation of ERK1/2. As expected, depletion of GRB2 had a similar outcome as the chemical inhibition of MEK. We observed that the tyrosine phosphorylation of FGFR1 was prolonged and slightly more intense (Fig. 3B and fig. S4B). However, due to the residual activity of the ERKs, the observed effects were less pronounced as those caused by the low molecular weight inhibitors. Interestingly, we found that when GRB2 was depleted the phosphorylation of FRS2α was reduced.

Blocking serine/threonine phosphatases reduces tyrosine phosphorylation of FGFR1

In order to elucidate further the role of Ser^{777} phosphorylation, serum-starved U2OSR1 and NIH3T3 cells, after overnight treatment with 150 nM okadaic acid (a protein serine/threonine phosphatase (1 and 2A) inhibitor), were stimulated with FGF1. In U2OSR1 cells, the activation and tyrosine phosphorylation of FGFR1 (probed by anti-phospho-FGFR (Tyr^{653}/Tyr^{654}) antibody) upon

FGF1 treatment were substantially reduced when Ser^{777} of FGFR1 was prephosphorylated due to phosphatase inhibition (Fig. 4A). In the case of NIH3T3 cells, we hardly detected any tyrosine phosphorylation and in consequence cellular response upon pretreatment of the cells with okadaic acid (Fig. 4A). Taken together, the findings indicate that the phosphorylation status of Ser^{777} influences the response to FGF1 treatment.

FGF1-induced tyrosine phosphorylation of FGFR1 is prolonged in U2OS cells expressing the FGFR1 S777A mutant

We showed that when the Ser^{777} phosphorylation is reduced or blocked by treatment with different MEK inhibitors, the tyrosine phosphorylation of FGFR1 induced by FGF1 is enhanced and prolonged. Conversely, inhibition of serine/threonine phosphatases by okadaic acid and the ensuing enhancement of Ser^{777} phosphorylation decrease the FGF1-induced tyrosine phosphorylation of FGFR1. To shed more light on the relationship between Ser^{777} and tyrosine phosphorylation we generated two stable U2OS cell lines expressing the S777A or S777D mutants of FGFR1 (U2OSR1 S777A and U2OSR1 S777D). The S777A mutant cannot be phosphorylated and the S777D variant mimics the constitutively phosphorylated receptor. This made it possible to compare the biological responses of FGFR1 at different phosphorylation states in the same cellular system and under similar conditions.

We found that in U2OSR1 S777A cells the duration of FGF1-induced tyrosine phosphorylation of FGFR was prolonged as compared to that in U2OSR1 wild-type cells (Fig. 4B). Conversely, in U2OSR1 S777D cells the signal from activated receptor was much weaker and lasted for a shorter time (Fig. 4B). As expected, MEK inhibitor U0126 did not substantially increase the tyrosine phosphorylation of FGFR1, neither in U2OSR1 S777A nor S777D cells. However, the tyrosine phosphorylation of FRS2α was elevated and prolonged in the presence of U0126 regardless of the

amino-acid at position 777 of FGFR1 (Fig. 4B). These results demonstrate that the phosphorylation state of Ser^{777} dictates the strength and the duration of the tyrosine phosphorylation of FGFR1.

U2OSR1 S777A cells demonstrate enhanced mitogenic response and increased cell migration

As shown above, we observed substantial differences in FGF1-induced signaling between the phosphorylation-incompetent and phosphomimetic Ser^{777} mutants of FGFR1. To investigate the biological consequences of Ser^{777} phosphorylation, we studied the ability of U2OS cells stably expressing the S777A or S777D variants of FGFR1 (in comparison to U2OSR1 WT cells) to proliferate in response to FGF1. The cells were incubated for up to 96 hours with 100 ng/ml of FGF1 in the presence of heparin. We found that the cells expressing the S777A mutant proliferated more efficiently than the cells with the wild-type receptor, whereas the proliferation potential of cells expressing the S777D variant was substantially reduced (Fig. 5A). Similar results were obtained by analyzing additional clones stably expressing wild-type FGFR1, the S777A or S777D variant (fig. S5A). This assay reveals that the Ser^{777} phosphorylation of FGFR1 negatively regulating its tyrosine phosphorylation is a crucial element in controlling of FGF1 signaling and cell proliferation.

To test another functional consequence of the phosphorylation status of FGFR1 Ser^{777} we examined the motility of untransfected U2OS cells and those stably transfected with wild-type FGFR1 or its mutants. Time-lapse live-cell imaging upon stimulation with FGF1 revealed that U2OS cells expressing wild-type FGFR1 were moving faster than untransfected U2OS cells, indicating the role of FGFR1 in cell migration (Fig. 5B). The cell velocity was significantly increased for U2OSR1 S777A cells, in contrast to U2OSR1 S777D cells which migrated much slower than U2OSR1 wild-type (Fig. 5B). As similar results were obtained by investigating independent cell clones stably expressing the FGFR1 mutants, we conclude that the results are not cell clone dependent (fig. S5B). The observed effect of the phosphomimetic mutation demonstrates

that phosphorylation on Ser^{777} of FGFR1 is important in switching off multiple signal transduction pathways activated by FGF1.

S777A mutation enhances axonal growth in DRG (dorsal root ganglion) neurons

FGF signaling has been implicated in several processes during development and regeneration of the nervous system, such as neural induction, patterning, axon guidance and synapse formation (21). To further elucidate the physiological relevance of phosphorylation of Ser^{777}, we examined axonal growth and branching in adult DRG neurons overexpressing wild-type FGFR1 or its mutants (S777A or S777D). DRG neuron culture is a well-characterized system to investigate the mechanisms of neuritogenesis and it has previously been reported that FGFR1 signaling in DRG neurons enhances elongative axon growth but not branching (22). Total axonal length of adult DRG neurons and the maximal distance of the longest axon (a parameter for elongative axon growth) were strongly increased in neurons overexpressing the S777A mutant compared to wild-type or the S777D mutant (Fig. 5C). In contrast, the number of branch points was not enhanced by the S777A variant (Fig. 5C). Thus, the elongating properties of FGFR1 S777A overexpressing neurons provide evidence for an enhanced regenerative capacity of adult neurons directed by phosphorylation status of Ser^{777}. These findings are in accordance with previous results demonstrating that enhanced FGFR1 signaling leads to increased axon growth (22, 23).

EGF or serum induce Ser^{777} phosphorylation of FGFR1

Since other stimuli can activate MAP kinases in the absence of FGF-induced signaling, we tested if FGFR1 could be phosphorylated on Ser^{777} even without activation by its cognate ligand. We stimulated cells with EGF (epidermal growth factor) or serum, as both stimuli activate MAP kinases in an FGF1-independent manner and do not induce FGFR1 tyrosine phosphorylation or tyrosine phosphorylation of FRS2α. By immunoblotting we found that both EGF and serum

efficiently induced phosphorylation of Ser^{777} in FGFR1 in U2OSR1 cells and in NIH3T3 cells (Fig. 6, A and B, lane 2). Addition of the MEK inhibitor U0126 duly abrogated FGFR1 Ser^{777} phosphorylation (Fig. 6, A and B). We also pretreated both cell lines with serum or EGF for 30 min before stimulation with FGF1 in the presence or absence of the MEK inhibitor U0126 or the FGFR inhibitor PD173074. The phosphorylation of Ser^{777} induced by EGF or serum was again completely blocked in the presence of U0126 (Fig. 6, A and B). Under such conditions we also observed a strong shift in the electrophoretic mobility of FRS2α as described by Lax and others (14). Treatment of cells with the FGFR inhibitor PD173074 without addition of EGF or serum completely inhibited Ser^{777} phosphorylation (Fig. 6, A and B). However, when the cells were stimulated with EGF or serum, we could see a band representing FGFR1 phosphorylated at Ser^{777} even when PD173074 was present. Although we could detect strong ERK activation upon EGF stimulation, we were not able to detect any phosphorylated EGF receptor on Western blotting in these cells. Since U2OS cells produce low amounts of the EGF receptor, we decided to repeat the experiment in U2OSR1 cells transiently transfected with EGF receptor (Fig. 6A, U2OSR1 cells, lanes 7-12). Again, we observed substantial Ser^{777} phosphorylation upon EGF stimulation (Fig. 6A, U2OSR1 cells, lane 8) and no bands for phospho-Ser^{777}-FGFR1 in the presence of the MEK inhibitor when cells were pretreated with EGF and then stimulated by FGF1 (Fig. 6A, U2OSR1 cells, line 11).

In addition, we analyzed the effect of EGF on FGFR1 Ser^{777} phosphorylation by confocal microscopy in serum-starved U2OSR1 cells. Similarly to the immunoblotting results, we observed clear staining of phospho-Ser^{777}-FGFR1 in cells stimulated with EGF in the absence of FGF1 (Fig. 6C).

We also monitored the kinetics of FGFR activation (tyrosine phosphorylation) and downstream signaling in response to FGF1 after EGF pretreatment. Under these conditions, FGFR1 Ser^{777} was already phosphorylated when FGF1 was added to the cells. We found that cells pretreated with EGF before addition of FGF1 responded weaker than did cells stimulated with FGF1 alone: the tyrosine

phosphorylation of FGFR1 and phosphorylation of downstream signaling molecules were reduced and less prolonged (Fig. 6D).

Similar results were also obtained by confocal microscopy analysis. Serum-starved U2OSR1 cells were treated either with EGF or FGF1 or with FGF1 after 30-min pretreatment with EGF and stained with anti-phospho-FGFR (Tyr^{653}/Tyr^{654}) antibody. In accordance with the immunoblotting data, we observed a significant decrease in phospho-tyrosine FGFR signal in response to FGF1 upon pretreatment of the cells with EGF (by about 25%) (Fig. 6E).

These experiments demonstrate that the phosphorylation of FGFR1 on Ser^{777} is regulated by MAP kinase activity and can occur independently of FGF induction and FGFR activation. Thus, both FGF1-dependent and independent MAP kinase activation inhibits FGFR signaling through its serine phosphorylation.

Discussion

Upregulation of FGFR signaling is of critical importance in many human cancers (2), it is therefore essential to understand the system modulating the receptor activity. In this report, we have described a novel mechanism for the regulation of FGFR1 signaling based on the activity of the MAPKs ERK1 and ERK2 (Fig. 7A).

As the C-terminal part of the FGFRs is very rich in serine residues of unknown function, we analyzed potential phosphorylation sites in this region, finding that Ser^{777} in the C- tail of FGFR1 is a specific phosphorylation site for ERK1 and ERK2. We also showed that this phosphorylation takes place in cells in response to FGF1-dependent and FGF1-independent activation of ERKs. Furthermore, the phosphorylation status of FGFR1 Ser^{777} influenced the tyrosine phosphorylation of the receptor and its downstream signaling. We found that phosphorylation of Ser^{777} decreases the FGFR1 activity and its ability to propagate mitogenic signals. Analyzing the migratory response, we observed a clear correlation between the phosphorylation state of Ser^{777} and the ability of cells to

migrate, which can be of significant importance in many types of cancer. In addition, we showed that the phosphorylation status of Ser^{777} influences long distance axon extension required during nerve regeneration. Our results indicate that the lack of Ser^{777} phosphorylation has a clear physiological implication.

It has been shown earlier that the MAPK pathway can function as a negative feedback amplifier (24). Several feedback loops for ERKs, involving RAF, SOS and FRS2α, have been implicated in the attenuation of FGF-induced signaling. Negative modulation of FRS2α activity is achieved by its phosphorylation by ERKs at eight threonine residues in response to different stimuli. An FRS2α mutant deficient in ERK-mediated phosphorylation displayed increased tyrosine phosphorylation of FRS2α (14). Here we show that the ERKs, in a similar manner, are able to regulate FGFR1 tyrosine phosphorylation and activity through direct phosphorylation of Ser^{777} in FGFR1. It is not clear how phosphorylation of threonines in FRS2α inhibits its tyrosine phosphorylation. It is possible that upon threonine phosphorylation, FRS2α becomes a poor substrate for the receptor kinase or that its association with subcellular compartments and/or subcellular components are altered. Similar mechanisms might apply for the negative regulation of FGFR1. We suggest that the phosphorylated Ser^{777} could act as a binding site for tyrosine phosphatases responsible for receptor inactivation; alternatively ERK-mediated phosphorylation can cause local conformational changes within the receptor which make FGFR1 a better substrate for Tyr-dephosphorylation. It is also possible that the phosphate group could disrupt interactions of FGFR1 with its binding partners, resulting in the attenuation of receptor signaling.

Since ERKs can play a dual role in FGFR1 signal transduction by inducing both mitogenic signaling and negative regulation, it seems that their spatial and temporal dynamics is crucial for FGF1-induced biological activities. We observed that the maximal stimulation of ERK1/2 is reached already 5 min after FGF1 stimulation, while Ser^{777} phosphorylation attains its maximum approximately 10 min later. ERKs are therefore early event signaling molecules that are able to

activate their downstream targets before the activity of FGFR1 is negatively regulated by phosphorylated Ser^{777}.

Interestingly, an alternatively spliced variant of FGFR2 (FGFR2 IIIb) with a shortened C-terminal tail has been shown to be expressed in certain human cancers. Cha and coworkers found that deletion of the carboxyl-terminal sequence of FGFR2 IIIb contributed to the transforming activity of the receptor (25). They suggested a dual mechanism of enhanced transformation caused by aberrant receptor recycling and persistent FRS2-dependent signaling. Furthermore, they correlated this effect with the deletion of specific Tyr^{770} and Leu^{773} in the C-terminal tail of FGFR2. However, it is possible that the transforming effect of truncated FGFR2 IIIb receptors could be due to the deletion of Ser^{781}, corresponding to Ser^{777} in FGFR1, resulting in evasion of the MAPK-mediated negative feedback here described.

We showed that even in the absence of FGF1, ERKs activated by EGF, serum, or a constitutively active variant of MEK1 were able to phosphorylate Ser^{777} of FGFR1. Interestingly, pretreatment of cells with EGF leading to phosphorylation of Ser^{777} reduced the cell's response to FGF1 stimulation. These data confirm multifarious interplay of different signaling pathways acting through a novel regulatory mechanism of FGFR1 signaling. Direct serine phosphorylation of FGFR1 controls the tyrosine phosphorylation within the kinase domain of the receptor and thereby it's downstream signaling events and biological functions. This mechanism operates in addition to other negative feedbacks controlling FGFR1 activity and ensures tight and accurate signaling. The phosphorylation of Ser^{777} in FGFR1 following activation of ERKs protects the cell not only from ligand-independent receptor activation but also from simultaneous activation by several different stimuli. It is possible that this type of regulation also applies to other signaling receptors and functions as a mechanism to control inaccurate signaling upon exposure to multiple growth factors. Prephosphorylation of Ser777 in the absence of FGFR1 ligand desensitizes the cells regulating the magnitude of the response to upcoming FGF. Moreover, in the absence of FGFR1 ligand

prephosphorylation of Ser^{777} by other stimuli may protect the cell against inadvertent activation of FGFR1 signaling cascades by ligand independent dimerization of receptor molecules (14, 26). This could also be relevant in cancer where FGFRs are overactive and phosphorylated Ser^{777} may play a tumor suppressive role.

Indeed, FGFR1 is not the only receptor phosphorylated by MAP kinases. ERKs have been shown to interact and phosphorylate other transmembrane proteins including EGFR and the netrin receptor DCC (27, 28). The cytoplasmic domain of DCC phosphorylated by ERK2 is unstructured, similarly to the C-tail of FGFR1. The exact role of the DCC phosphorylation by MAPKs is unknown, nevertheless the link between ERKs signaling and DCC signaling suggests physiological importance of that ERKs-mediated threonine/serine modification (28).

The fact that the unordered C-terminal part of FGFR1 is very rich in serines is likely to be of physiological importance, as most serine-threonine phosphorylation sites are located in unstructured regions and are clustered in the primary amino acid sequence (29, 30). We suggest that Ser^{777} and probably other serines in the C-tail of FGFR1 work as molecular switches and modulate the primary response upon receptor stimulation attained by tyrosine phosphorylation. It has elegantly been shown by Tan and coworkers that the number of genomically encoded tyrosine residues (and thus potential phosphorylated tyrosines) decreases with increasing species complexity in metazoan species (31). This seems to be a result of evolutionary selection to eliminate phosphorylation events that could lead to uncontrolled or unspecific signaling. We calculated the number of tyrosine, serine and threonine residues in the cytoplasmic part of FGFR1 in different species and observed that the number of tyrosines decreases with the number of different cell types, in contrast to serines (and serines + threonines), whose number correlates positively with the organism's complexity (fig. S6 and Fig. 7B). The reason for this could lie in the fact that conserved phospho-tyrosine sites tend to be located in ordered protein domains, in contrast to phospho-serines located in disordered regions, which evolve rapidly and are more tolerant to extensive serine phosphorylation (31). The increased

number of serines and serines + threonines in FGFR1s of higher organisms suggests that simultaneously with the gain of new cell types, additional mechanisms of signaling control were developed.

In summary, our study reveals a novel regulation mechanism of FGFR1 signaling involving ERK-dependent Ser^{777} phosphorylation. We discovered that the C-tail of FGFR1 is a direct substrate for the MAPKs ERK1/2 and that these powerful mitogens are able to regulate their own signaling by tuning down the activity of the receptor. This newly discovered negative feedback loop based on Ser^{777} phosphorylation in FGFR1 is part of a complex system controlling proliferation signals that in excess may lead to dramatic events including cancer. Better understanding of the regulatory mechanisms of FGFR1, which are often interrupted in oncogenic malignancies, should ultimately lead to more efficient drug discovery and design.

Materials and methods

Antibodies and reagents

The following primary antibodies were used: rabbit anti-MAPK (ERK1/2, p44/p42), mouse anti-phospho-MAPK (ERK1/2, p44/p42) (Thr^{202}/Tyr^{204}), rabbit anti-phospho-AKT (Ser^{473}), rabbit anti-FGFR1, mouse anti-phospho-FGFR (Tyr^{653}/Tyr^{654}), rabbit anti-phospho-FRS2α (Tyr^{196}), rabbit anti-GRB2 from Cell Signaling Technology, rabbit anti-phospho-PLC-γ (Tyr^{783}), rabbit anti-FRS2α, mouse anti-GST, rabbit anti-GST and rabbit anti-FLAG from Santa Cruz Biotechnology, mouse anti-phospho-p38 MAPK antibody (Thr^{180}/Tyr^{182}), mouse anti-HSP90, mouse anti-early endosomal antigen 1 (EEA1), mouse anti-EGFR (activated form) from BD Transduction Laboratory, mouse anti-FLAG M2 from Sigma-Aldrich, mouse anti-Myc from Upstate Biotechnology, mouse anti-HA.11 and mouse anti-6-His from Nordic Biosite. Specific anti-phospho-FGFR1 (Ser^{777}) (pS777-FGFR1) antibody was made by GenScript using the following phospho-specific peptide CSMPLDQYpSPSFPDTR. The antibody was purified using the phosphopeptide and by cross-

adsorption to the corresponding non-phosphopeptide. HRP-conjugated and fluorescent secondary antibodies were from Jackson Immuno-Research Laboratories.

Heparin-Sepharose CL-6B affinity resin was from Amersham. Mowiol, brefeldin A, okadaic acid, PD173074 and MEK1/2 inhibitor (SL327) were from Calbiochem. Cycloheximide, heparin and U0126 were from Sigma-Aldrich. Protease inhibitor cocktail tablets (EDTA-free, complete) were from Roche Diagnostics. FGF1 was labeled with Cy3-maleimide (GE Healthcare) following the manufacturer's procedures. Hoechst 33342, AlamarBlue®, Dynabeads® anti-mouse IgG and Dynabeads® Protein G were purchased from Invitrogen. All other chemicals were from Sigma-Aldrich.

Cell lines and bacterial strains

NIH3T3 cells were grown in Quantum 333 medium (PAA Laboratories) supplemented with 2% bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin. DOTAP liposomal transfection reagent (Roche Diagnostics) was used according to the manufacturer's protocol to obtain U2OS cells stably expressing S777A and S777D mutants of FGFR1. Clones were selected with 1 mg/ml geneticin (G-418) (Invitrogen). Clones presented in this paper were chosen based on their receptor expression level analyzed by immunofluorescence and immunoblotting. U2OS cells stably expressing FGFR1 have been described previously (10). The cells were propagated in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. In addition, 0.2-1 mg/ml geneticin was added to the growth media of stably transfected U2OS cells.

Adult rat dorsal root ganglia (DRG) were dissected, collected in ice-cold RPMI medium supplemented with antibiotic-antimycotic (100 U/ml penicillin, 100 µg/ml streptomycine, 0.25 µg/ml amphotericine B, Gibco) and cultured as described previously [Hausott et al., 2008]. DRG

cultures were maintained in TNB 100 medium (Biochrom) supplemented with protein-lipid-complex (Biochrom) and antibiotic-antimycotic.

For expression of FGF1 and GST-C-tail FGFR1 constructs *Escherichia coli* strain Bl21(DE3)pLysS from New England Biolabs was used.

Plasmids

pcDNA3-FGFR1, pcDNA3-FGFR1 S777A and pcDNA3-FGFR1 S777D have been described previously (16, 32). A construct encoding 68 C-terminal amino acids from FGFR1 (residues 755-822) in pGEX vector was used for expression of FGFR1 C-tail fused to the C-terminus of GST (16). The FGF1 construct comprised a truncated form (residues 21-154) of human FGF1 in the pET-3c vector (33). pECE-HA-MEK1-SS/DD and pECE-HA-MEK1-S222A plasmids were a generous gift from Professor Jacques Pouyssegur (Institute of Developmental Biology and Cancer Research, University of Nice) (18, 19). The pEGFR plasmid was kindly provided by Professor Harald Stenmark (Institute for Cancer Research, The Norwegian Radium Hospital) and was originally a gift from Professor Alexander Sorkin (Department of Pharmacology, School of Medicine, University of Colorado, Denver).

siRNA oligonucleotides and cell transfection

siRNA oligos targeting GRB2 (targeting sequence: 5- CAUGUUUCCCCGCAAUUAUTT-3) were purchased from Dharmacon RNA Technologies. siRNA targeting ERK1 (p44) (targeting sequence: 5-CTCCCTGACCCGTCTAATATA-3) and ERK2 (p42) (targeting sequence: 5-AATGACATTATTCGAGCACCA-3) were purchased from QIAGEN. ON-TARGETplus siCONTROL siRNA were purchased from Dharmacon RNA Technologies.

siRNA knockdown was performed using DharmaFECT transfection reagent according to the manufacturer's protocol. Experiments were performed 72 hours after transfection.

Transient expression of the different constructs was performed by transfecting cells with plasmid DNA using Fugene 6 transfection reagent (Roche Diagnostics) according to the manufacturer's protocol. Softened DRGs were transfected by electroporation (Amaxa Nucleofector™(Lonza, program O-003)) using Rat Neuron Nucleofector® solution (Lonza).

Recombinant proteins

Active human recombinant MAP kinases, ERK1 (p44) and ERK2 (p42), were purchased from Calbiochem. Active human MEK1 was from Chemicon International, inactive MEK1 was from Upstate and human recombinant p38α MAPK from R&D Systems. Recombinant FGF1 was produced in *E.coli* as described previously (33). Recombinant EGF and MBP was obtained from Sigma-Aldrich.Recombinant fusion protein of the C-terminal part of FGFR1 with GST (GST-C-tail-FGFR1) and its mutants were produced in *E.coli* and purified with Glutathion Sepharose (Amersham Biosciences) according to standard procedure.

Analysis of signaling cascades

Serum-starved cells were stimulated with 20 ng/ml FGF1 in the presence of 10 U/ml heparin or 20 ng/ml EGF or 10% serum in the presence or absence of indicated inhibitors for different times. Inhibitors were added 15 min prior to stimulation and kept throughout the experiments which were carried out in the presence or absence of 10 μg/ml cycloheximide or 2 μg/ml brefeldin A. The cells were lysed with SDS sample buffer, scraped and sonicated. Total cell lysates were separated by SDS-PAGE, transferred onto Immobilon-P membrane and subjected to immunoblot analysis. The membrane was stripped and re-probed with different antibodies. ImageQuant version 5 was used for quantification of the intensity of the bands of interest. In the case of quantification of the intensity of bands corresponding to phosphorylated FGFR1 from U2OSR1, U2OSR1 S777A and U2OSR1 S777D cells, the intensity of the bands corresponding to phosphorylated FGFR1 were divided by

the intensity of bands for total FGFR1 and then normalized towards the maximum response of U2OSR1 WT cells.

In vitro phosphorylation of recombinant C-tail of FGFR1

In vitro phosphorylation experiments were performed with recombinant proteins. 1 µg of a fusion protein was incubated with kinases and 40 µCi/ml [γ-^{33}P]ATP in reaction buffer (25 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 1 mM Na_2MO_3, 20 mM sodium β-glycerophosphate, 1 mM DTT, 5 mM EGTA) at 30°C for 30 min. As a control, 2 µg of GST was used. The reaction was stopped by TCA precipitation (30 min on ice). Then, the samples were centrifuged, washed twice with cold acetone and dissolved in sample buffer. The proteins were analyzed by SDS-PAGE, electroblotting and autoradiography and then the membrane was stained with Coomassie Blue.

In vitro coimmunoprecipitation of ERK1 with recombinant C-tail of FGFR1

Human recombinant His-tagged ERK1 (100 ng) was incubated for 2 hours at 4°C with 500 ng of recombinant fusion protein of C-tail of FGFR1 or its mutants with GST-Tag or with GST alone or GST-FGF1 (controls) in PBS buffer supplemented with 0.1% (w/v) BSA and protease inhibitors. Complexes were then pelleted using mouse anti-GST antibodies immobilized on Dynabeads® anti-mouse IgG. The Dynabeads® were washed six times with binding buffer and then proteins were eluted in sample buffer and analyzed by SDS-PAGE and immunoblotting.

FGFR1 immunoprecipitation

FGFR1 was immunoprecipitated from lysates of U2OS cells stably expressing FGFR1 or its mutants using anti-FGFR1 antibodies immobilized to Dynabeads® Protein G. The immunoprecipitates were washed three times with lysis buffer (100 mM NaCl, 10 mM Tris pH 7,4, 5 mM EDTA, 1% Trixton-100) and once with PBS buffer supplemented with 0.1% (w/v) Tween

(washing buffer). To analyze binding of ERK1 to full-length FGFR1, FGFR1 immunoprecipitates were incubated with 50 ng of active recombinant His-tagged ERK1 at 4°C for 90 min. Complexes were then washed with washing buffer, eluted in samples buffer and analyzed by SDS-PAGE and immunoblotting. To test the phoshorylation of full-length FGFR1, FGFR1 immunoprecipitates were washed with high salt buffer (washing buffer with 1 M NaCl) and incubated for 15 min with 100 nM PD17034 before performing the in vitro phosphorylation assay as described above for recombinant C-tail of FGFR1.

Laser scanning confocal microscopy

Cells grown on coverslips were starved for 4 hours and then incubated for 30 min at 37°C with 100 ng/ml Cy3-FGF1 in the presence of 20 U/ml heparin or with 100 ng/ml EGF. The cells were fixed in 4% formaldehyde solution and permeabilized with 0.1% Triton X-100. Next the cells were incubated with primary antibodies for 20 min, washed and then incubated with secondary antibodies coupled to a fluorophore for 20 min before mounting in mowiol. The cells were examined with a Zeiss LSM 510 META confocal microscope (Carl Zeiss). Images were prepared with Zeiss LSM Image Browser version 3.2 (Carl Zeiss) and CorelDRAW11. Quantifications were performed using Image J software.

Proliferation assay

Serum-starved U2OS cells stably expressing FGFR1 or its mutants were treated with FGF1 (100 ng/ml) in the presence of heparin (10 U/ml) for 48, 72 and 96 hours. At each time point AlamarBlue® reagent was added to cells and after 3 h the fluorescence of reduced form of the dye was measured using EnVision® multimode plate reader (PerkinElmer). The fluorescence signal reflecting the number of cells in each experiment was normalized to the number of U2OSR1 cells at time point 0.

Time-lapse live-cell imaging and cell migration tracking

Serum-starved U2OS cells and U2OS cells stably expressing FGFR1 or its mutants were plated on Hi-Q4 culture dishes (ibidi Integrated BioDiagnostics) and observed with a BioStation IM Live Cell Recorder (Nikon Instruments Inc.) with a 20x objective and phase contrast at 37°C and 5% CO_2 in humidified air. In all experiments, cells were stimulated with Cy3-labeled FGF1 (200 ng/ml) and heparin (20 U/ml). Image acquisition was performed every 10 min over a period of 8 hours. Images were analyzed with Image J software with Manual Tracking and Chemotaxis and Migration Tool (ibidi GmbH) plugins. The velocity of cells was calculated for the whole timeframe.

Analysis of axon growth

For measurement of axon growth, DRG neuron cultures transfected with pcDNA3-FGFR1, pcDNA3-FGFR1 S777A or pcDNA3-FGFR1 S777D plus EGFP were documented by inverted fluorescence microscopy (Zeiss Axiovert 100) equipped with a SPOT RT digital camera. Transfected neurons were documented 48 hours and 72 hours after transfection before and after a 24-hour treatment with 100 ng/ml of FGF1 in the presence of heparin (10 U/ml). MetaMorph® (Visitron Systems) morphometry software was applied to measure the maximal distance of the longest axon, the total axonal length and the number of branch points. All morphologically intact neurons per dish with the maximal distance of the longest axon ≥ 100 µm were analyzed.

Statistical analysis

For statistical analysis one-way ANOVA with Tukey´s post test was applied.

Supplementary Materials

fig. S1. In vitro phosphorylation assay with recombinant C-tail of FGFR1 and AKT kinase or MEK1.

fig. S2. Effect of U0126 inhibitor on ERK1/2 activity.

fig. S3. Effect of MEK inhibitors on FGFR1 activity in the presence or absence of brefeldin A or cycloheximide.

fig. S4. The effect of siRNA knockdown of MAPKs (ERK1/2) and GRB2 on FGFR1 activity.

fig. S5. Effect of the phosphorylation state of FGFR1 Ser^{777} on cell proliferation and migration in additional clones of stably transfected U2OS cells.

fig. S6. Correlation of tyrosine and serine/threonine content in the cytoplasmic part of FGFR1s with organism complexity.

References

1. D. E. Johnson, L. T. Williams, Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* **60**, 1-41 (1993).

2. E. M. Haugsten, A. Wiedlocha, S. Olsnes, J. Wesche, Roles of fibroblast growth factor receptors in carcinogenesis. *Mol Cancer Res* **8**, 1439-1452 (2010).

3. S. K. Olsen, O. A. Ibrahimi, A. Raucci, F. Zhang, A. V. Eliseenkova, A. Yayon, C. Basilico, R. J. Linhardt, J. Schlessinger, M. Mohammadi, Insights into the molecular basis for fibroblast growth factor receptor autoinhibition and ligand-binding promiscuity. *Proc Natl Acad Sci U S A* **101**, 935-940 (2004).

4. M. Mohammadi, A. M. Honegger, D. Rotin, R. Fischer, F. Bellot, W. Li, C. A. Dionne, M. Jaye, M. Rubinstein, J. Schlessinger, A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol Cell Biol* **11**, 5068-5078 (1991).

5. C. M. Furdui, E. D. Lew, J. Schlessinger, K. S. Anderson, Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction. *Mol Cell* **21**, 711-717 (2006).

6. E. D. Lew, C. M. Furdui, K. S. Anderson, J. Schlessinger, The precise sequence of FGF receptor autophosphorylation is kinetically driven and is disrupted by oncogenic mutations. *Sci Signal* **2**, ra6 (2009).

7. V. P. Eswarakumar, I. Lax, J. Schlessinger, Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* **16**, 139-149 (2005).

8. N. Turner, R. Grose, Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer* **10**, 116-129 (2010).

9. J. Wesche, K. Haglund, E. M. Haugsten, Fibroblast growth factors and their receptors in cancer. *Biochem J* **437**, 199-213 (2011).

10. E. M. Haugsten, J. Malecki, S. M. Bjorklund, S. Olsnes, J. Wesche, Ubiquitination of fibroblast growth factor receptor 1 is required for its intracellular sorting but not for its endocytosis. *Mol Biol Cell* **19**, 3390-3403 (2008).

11. M. Furthauer, W. Lin, S. L. Ang, B. Thisse, C. Thisse, Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. *Nat Cell Biol* **4**, 170-174 (2002).

12. M. Tsang, R. Friesel, T. Kudoh, I. B. Dawid, Identification of Sef, a novel modulator of FGF signalling. *Nat Cell Biol* **4**, 165-169 (2002).

13. M. A. Cabrita, G. Christofori, Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* **11**, 53-62 (2008).

14. I. Lax, A. Wong, B. Lamothe, A. Lee, A. Frost, J. Hawes, J. Schlessinger, The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. *Mol Cell* **10**, 709-719 (2002).

15. J. C. Obenauer, L. C. Cantley, M. B. Yaffe, Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res* **31**, 3635-3641 (2003).

16. V. Sorensen, Y. Zhen, M. Zakrzewska, E. M. Haugsten, S. Walchli, T. Nilsen, S. Olsnes, A. Wiedlocha, Phosphorylation of fibroblast growth factor (FGF) receptor 1 at Ser777 by p38 mitogen-activated protein kinase regulates translocation of exogenous FGF1 to the cytosol and nucleus. *Mol Cell Biol* **28**, 4129-4141 (2008).

17. F. T. Mu, J. M. Callaghan, O. Steele-Mortimer, H. Stenmark, R. G. Parton, P. L. Campbell, J. McCluskey, J. P. Yeo, E. P. Tock, B. H. Toh, EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. *J Biol Chem* **270**, 13503-13511 (1995).

18. A. Brunet, G. Pages, J. Pouyssegur, Constitutively active mutants of MAP kinase kinase (MEK1) induce growth factor-relaxation and oncogenicity when expressed in fibroblasts. *Oncogene* **9**, 3379-3387 (1994).

19. G. Pages, A. Brunet, G. L'Allemain, J. Pouyssegur, Constitutive mutant and putative regulatory serine phosphorylation site of mammalian MAP kinase kinase (MEK1). *EMBO J* **13**, 3003-3010 (1994).

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

21. I. Mason, Initiation to end point: the multiple roles of fibroblast growth factors in neural development. *Nat Rev Neurosci* **8**, 583-596 (2007).

22. B. Hausott, B. Schlick, N. Vallant, R. Dorn, L. Klimaschewski, Promotion of neurite outgrowth by fibroblast growth factor receptor 1 overexpression and lysosomal inhibition of receptor degradation in pheochromocytoma cells and adult sensory neurons. *Neuroscience* **153**, 461-473 (2008).

23. B. Hausott, N. Vallant, M. Hochfilzer, S. Mangger, R. Irschick, E. M. Haugsten, L. Klimaschewski, Leupeptin enhances cell surface localization of fibroblast growth factor receptor 1 in adult sensory neurons by increased recycling. *Eur J Cell Biol* **91**, 129-138 (2012).

24. O. E. Sturm, R. Orton, J. Grindlay, M. Birtwistle, V. Vyshemirsky, D. Gilbert, M. Calder, A. Pitt, B. Kholodenko, W. Kolch, The mammalian MAPK/ERK pathway exhibits properties of a negative feedback amplifier. *Sci Signal* **3**, ra90 (2010).

25. J. Y. Cha, S. Maddileti, N. Mitin, T. K. Harden, C. J. Der, Aberrant receptor internalization and enhanced FRS2-dependent signaling contribute to the transforming activity of the fibroblast growth factor receptor 2 IIIb C3 isoform. *J Biol Chem* **284**, 6227-6240 (2009).

26. J. Schlessinger, Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225 (2000).

27. X. Li, Y. Huang, J. Jiang, S. J. Frank, ERK-dependent threonine phosphorylation of EGF receptor modulates receptor downregulation and signaling. *Cell Signal* **20**, 2145-2155 (2008).

28. W. Ma, Y. Shang, Z. Wei, W. Wen, W. Wang, M. Zhang, Phosphorylation of DCC by ERK2 is facilitated by direct docking of the receptor P1 domain to the kinase. *Structure* **18**, 1502-1511 (2010).

29. M. O. Collins, L. Yu, I. Campuzano, S. G. Grant, J. S. Choudhary, Phosphoproteomic analysis of the mouse brain cytosol reveals a predominance of protein phosphorylation in regions of intrinsic sequence disorder. *Mol Cell Proteomics* **7**, 1331-1348 (2008).

30. M. O. Collins, Cell biology. Evolving cell signals. *Science* **325**, 1635-1636 (2009).

31. C. S. Tan, A. Pasculescu, W. A. Lim, T. Pawson, G. D. Bader, R. Linding, Positive selection of tyrosine loss in metazoan evolution. *Science* **325**, 1686-1688 (2009).

32. E. M. Haugsten, V. Sorensen, A. Brech, S. Olsnes, J. Wesche, Different intracellular trafficking of FGF1 endocytosed by the four homologous FGF receptors. *J Cell Sci* **118**, 3869-3881 (2005).

33. M. Zakrzewska, D. Krowarsch, A. Wiedlocha, J. Otlewski, Design of fully active FGF-1 variants with increased stability. *Protein Eng Des Sel* **17**, 603-611 (2004).

34. **Acknowledgments:** We thank Prof. Harald Stenmark for critical reading of the manuscript. The skilful technical assistance of Anne Engen, Anne-Mari G. Pedersen and Anne Gro Bergersen and Agnieszka Kubiak is gratefully acknowledged.

Funding: M.Z. was a fellow of The Research Council of Norway and E.M.H. was a fellow of The Norwegian Cancer Society. J.W. is supported by South-Eastern Norway Regional Health Authority Fellowships. This work was supported by the Polish Ministry of Science and Higher Education (grant 0627/IP1/2011/71).

Author contribution: M.Z. designed the study, carried out in vitro phosphorylation experiments, all signaling experiments and cell proliferation study. E.M.H. and J.W. performed laser scanning confocal microscopy. B.N.W conducted immunoprecipitation and in vitro kinase assays. A.O.

performed cell migration experiments. B.H. carried out the analysis of axon growth. E.M.H and Y.J. prepared the stable cell lines. A.W. contributed to design of the study and supervised the project. M.Z., A.W., J.W. and J.O. contributed to analysis of obtained data. M.Z. wrote the manuscript. A.W., J.W., E.M.H., A.O and J.O edited the manuscript.

Competing interests: The authors declare that they have no competing interests.

Figure legends

Fig. 1

Phosphorylation of FGFR1 by MAPKs.

(A) ClustalX2 alignment of C-terminal regions of FGFRs from various species. Numbers refers to amino acid numbering used for human FGFR1. Ser^{777} of FGFR1 and corresponding serines of other FGFRs are indicated by *.

(B) In vitro phosphorylation of recombinant FGFR1 C-tail by MAPKs. GST-tagged C-terminal part of wild type FGFR1 (Ct-FGFR1-WT), its S777A mutant (Ct-FGFR1-S777A), S777D mutant (Ct-FGFR1-S777D) or GST-tag alone were subjected to phosphorylation reaction with recombinant active kinase ERK1 or ERK2. Shown are autoradiography (upper panel) and Coomassie Blue staining (lower panel).

(C) In vitro phosphorylation of full-length FGFR1 immunoprecipitated from U2OSR1, U2OSR1 S777A or U2OSR1 S777D cells by MAPKs. Immunoprecipitated receptors were subjected to phosphorylation reaction with recombinant active kinase ERK1 or ERK2. Shown are autoradiography (upper panel) and immunodetection using anti-FGFR1 antibody (lower panel).

(D) In vitro coimmunoprecipitation of recombinant FGFR1 C-tail and ERK1. GST-tagged Ct-FGFR1-WT, its mutants or GST-tag alone were incubated with recombinant ERK1. Protein complexes were immunoprecipitated with anti-GST antibody and analyzed by Western blotting.

(E) Binding of recombinant ERK1 to full-length FGFR1 immunoprecipitated from U2OSR1 cells. FGFR1 immunoprecipitates were incubated with active His-tagged ERK1 and complexes were analyzed by Western blotting.

Fig. 2

FGFR1 Ser^{777} phosphorylation depends on activity of MAPKs.

(A) Western blot analysis of serum-starved U2OSR1 or NIH3T3 cells pretreated with or without MEK1/2 inhibitors (20 μM U0126 or 1 μM SL327) and then stimulated with FGF1 for indicated times.

(B) Confocal microscopy images of serum-starved U2OSR1 cells incubated with or without Cy3-FGF1 for 30 min and stained with anti-pS777-FGFR1 and anti-EEA1 antibodies. As indicated, some cells were also incubated with U0126 before addition of FGF1 and throughout the experiment. Bar, 5 μm.

(C) Confocal microscopy images of U2OSR1 cells transfected with indicated constructs, starved and incubated with or without Cy3-FGF1 for 30 min and stained with anti-pS777-FGFR1 and anti-HA antibodies. As indicated, some cells were also incubated with U0126. d.n.MAPKK, dominant negative MAPKK and c.a.MAPKK, constitutively active MAPKK. Bar, 5 μm.

Fig. 3

Effect of blocking MAPK (ERK1/2) activity on FGFR1 signaling.

(A) Western blot analysis of serum-starved U2OSR1 or NIH3T3 cells pretreated as indicated with U0126 (20 μM) or SL327 (1 μM) and then stimulated with FGF1 in the presence of brefeldin A (2 μg/ml) for indicated times. Quantification of the bands representing phosphorylated FGFR1 (Tyr^{653}/Tyr^{654}) is presented in the graphs and expressed as a fraction of maximum response in the absence of inhibitor. Graphs represent the mean ± SD of three independent experiments.

(B) Western blot analysis of U2OSR1 cells transfected with GRB2 siRNA or a non-targeting siRNA control (scr), then serum-starved and stimulated with FGF1 for indicated times. The efficiency of knockdown was assessed in every experiment using anti-GRB2 antibody. Quantification of the bands representing phosphorylated FGFR1 (Tyr^{653}/Tyr^{654}) is presented in the graph and expressed as a fraction of the maximum response in cells transfected with control siRNA (scr). The graph represents the mean ± SD of three independent experiments.

Fig. 4

Interplay between Ser^{777} phosphorylation and tyrosine phosphorylation of FGFR1.

(A) Effect of blocking serine/threonine phosphatases on tyrosine phosphorylation of FGFR1. Western blot analysis of serum-starved U2OSR1 and NIH3T3 cells incubated overnight with or without 150 nM okadaic acid and then stimulated with FGF1 in the presence of cycloheximide (10 μg/ml) for indicated times. Quantification of the bands representing phosphorylated FGFR1 (Tyr^{653}/Tyr^{654}) is presented in the graphs and expressed as a fraction of maximum response in the absence of okadaic acid. The graphs represent the mean ± SD of three independent experiments.

(B) Effect of Ser^{777} mutation on tyrosine phosphorylation of FGFR1. Western blot analysis of serum-starved U2OSR1, U2OSR1 S777A or U2OSR1 S777D cells pretreated with or without U0126 (20 μM) and then stimulated with FGF1 in the presence of brefeldin A (2 μg/ml) for indicated times. Quantification of the bands representing phosphorylated FGFR1 (Tyr^{653}/Tyr^{654}) divided by the intensity of total FGFR1 is presented in the graphs and expressed as a fraction of maximum response in U2OSR1 WT cells. Graphs represent the mean ± SD of three independent experiments.

Fig. 5

Biological significance of the phosphorylation state of FGFR1 Ser^{777}.

(A) FGF1-induced cell proliferation of U2OSR1, U2OSR1 S777A and U2OSR1 S777D cells. AlamarBlue® reagent was added to the serum-starved cells stimulated with FGF1 for indicated times and the fluorescence corresponding to number of cells was measured. The graph represents the mean ± SEM of three independent experiments.

(B) Effect of Ser^{777} mutants on cell migration. FGF1-stimulated U2OS, U2OSR1, U2OSR1 S777A and U2OSR1 S777D cells were subjected to time-lapse live-cell imaging. Values are given as means ± SEM of three independent experiments, where 30-50 cells per experiment were analyzed.

(C) Effect of Ser^{777} mutants on axon growth and neuronal morphology of adult DRG neurons. Total axonal length, maximal distance of the longest axon and the number of branch points per cell were measured in FGF1-stimulated DRG neurons cotransfected with EGFP vector and indicated constructs. The graphs represent the mean ± SEM of three independent experiments with a total number of > 46 neurons per group. Representative images of neuronal morphology of adult DRG neurons before and after FGF1 treatment (24 hours) are shown.

Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 6

EGF and serum induce Ser^{777} phosphorylation of FGFR1.

(A, B) Western blot analysis of serum-starved U2OSR1, U2OSR1 transiently transfected with EGFR and NIH3T3 cells stimulated with FGF1 or EGF (A) or serum (B) for 15 min. When both stimuli are indicated cells were pretreated with serum or EGF for 30 min and subsequently treated with FGF1 for 15 min. U0126 (20 μM) and PD173074 inhibitor (100 nM) was added as indicated.

(C) Confocal microscopy images of serum-starved U2OSR1 stimulated with Cy3-FGF1 or EGF for 30 min. Bar, 5 μm.

(D) Western blot analysis of serum-starved U2OSR1 and NIH3T3 cells pretreated for 30 min with EGF and then stimulated with FGF1 for indicated times. Quantification of the bands representing phosphorylated FGFR1 (Tyr^{653}/Tyr^{654}), FRS2α and ERK1/2 is presented in the graphs and expressed as a fraction of maximum response in cells untreated with EGF. The graphs represent the mean ± range of data of two independent experiments.

(E) Confocal microscopy images of serum-starved U2OSR1 cells incubated either with EGF or FGF1 alone or after 30-min pretreatment with EGF and stained with anti-pTyr-FGFR. Bar, 5μm. Quantifications of pTyr-FGFR staining is presented in the graph. 80-100 cells were quantified per experiment for each condition. Graphs represent the mean ± SEM of three independent experiments (*** $p < 0.001$).

Fig. 7

Mechanisms of ERK-mediated regulation of FGFR signaling and evolutionary analysis of serine content in the C-terminal tail of FGFR1.

(A) Schematic representation of negative feedback attenuating FGFR1 signaling. FGF1-induced tyrosine phosphorylation of FGFR1 leads to activation of FRS2α followed by GRB2/SOS-mediated activation of RAS and MAPK (ERK1/2). ERK1/2 can also be activated by different pathways independent of FGFR e.g. in response to serum or EGF stimulation. Activated ERKs phosphorylate FRS2α on threonines (14) and FGFR1 on Ser^{777} resulting in reduced tyrosine phosphorylation of both FRS2α and FGFR1 and consequent attenuation of FGFR signaling.

(B) Correlation of expansion of serines in the cytoplasmic part of FGFR1 with organism complexity. Number of different cell types correlates positively and significantly (R^2=0.817) with serine content of FGFR1.

Figure 1

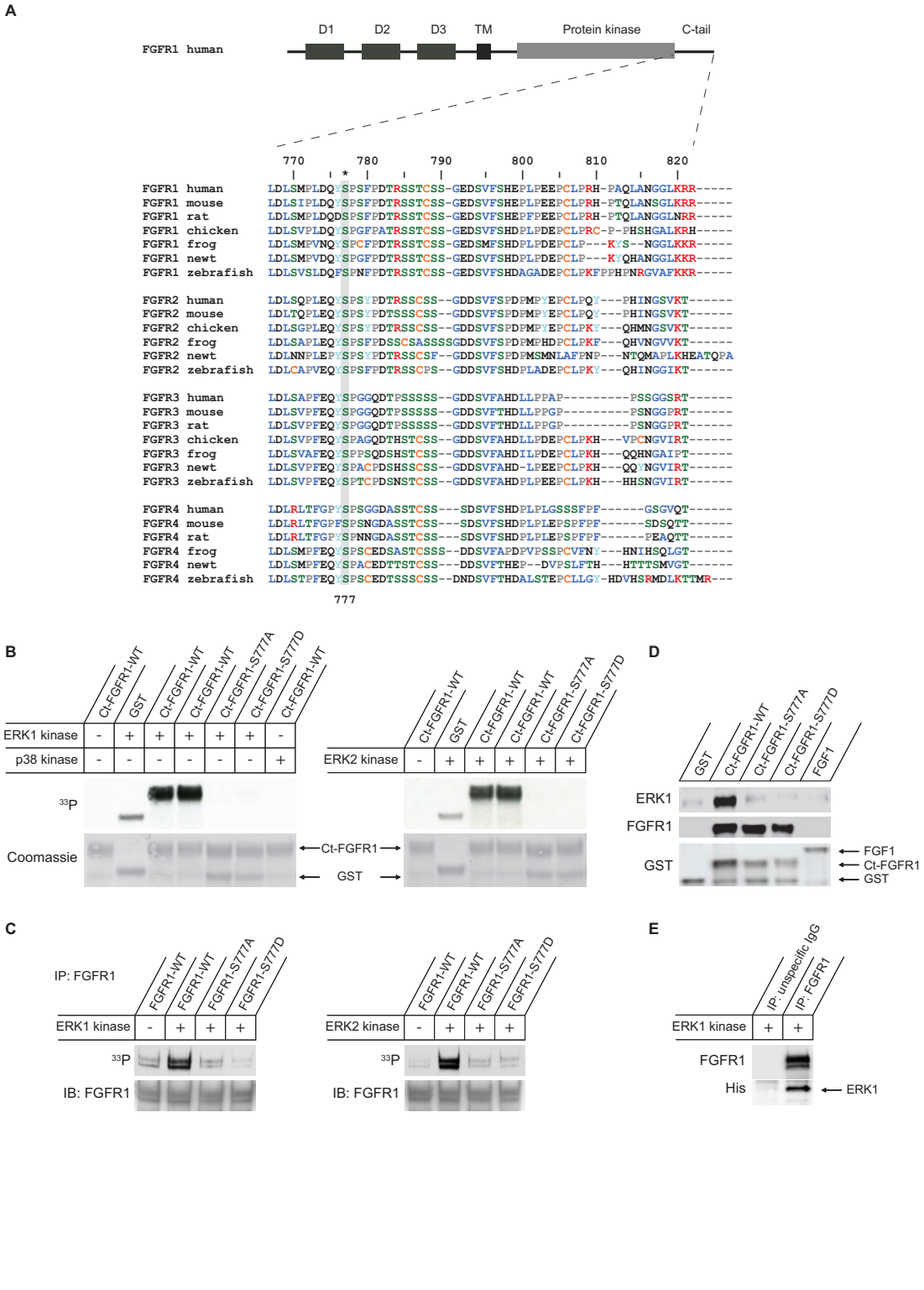

A
D1
D2
D3
TM
Protein kinase
C-tail
FGFR1 human
770
780
790
800
810
820
FGFR1 human LDLSMPLDQYSPSFPDTRSSTCSS-GEDSVFSHEPLPEEPCLPRH-PAQLANGGLKRR-----
FGFR1 mouse LDLSIPLDQYSPSFPDTRSSTCSS-GEDSVFSHEPLPEEPCLPRH-PTQLANSGLKRR-----
FGFR1 rat LDLSMPLDQDSPSFPDTRSSTCSS-GEDSVFSHEPFPEEPCLPRH-PTQLANGGLNRR-----
FGFR1 chicken LDLSVPLDQYSPGFPATRSSTCSS-GEDSVFSHDPLPDEPCLPRC-P-PHSHGALKRH-----
FGFR1 frog LDLSMPVNQYSPCFPDTRSSTCSS-GEDSMFSHDPLPDEPCLP---KYS--NGGLKKR-----
FGFR1 newt LDLSMPVDQYSPGFPDTRSSTCSS-GEDSVFSHDPLPDEPCLP---KYQHANGGLKKR-----
FGFR1 zebrafish LDLSVSLDQFSPNFPDTRSSTCSS-GEDSVFSHDAGADEPCLPKFPPHPNRGVAFKKR-----
FGFR2 human LDLSQPLEQYSPSYPDTRSSCSS--GDDSVFSPDPMPYEPCLPQY---PHINGSVKT------
FGFR2 mouse LDLTQPLEQYSPSYPDTSSSCSS--GDDSVFSPDPMPYEPCLPQY---PHINGSVKT------
FGFR2 chicken LDLSGPLEQYSPSYPDTRSSCSS--GDDSVFSPDPMPYEPCLPKY---QHMNGSVKT------
FGFR2 frog LDLSAPLEQYSPSFPDSSCSASSSSGDDSVFSPDPMPHDPCLPKF---QHVNGVVKT------
FGFR2 newt LDLNNPLEPYSPSYPDTRSSCSF--GDDSVFSPDPMSMNLAFPNP---NTQMAPLKHEATQPA
FGFR2 zebrafish LDLCAPVEQYSPSFPDTRSSCPS--GDDSVFSHDPLADEPCLPKY---QHINGGIKT------
FGFR3 human LDLSAPFEQYSPGGQDTPSSSSS--GDDSVFAHDLLPPAP---------PSSGGSRT------
FGFR3 mouse LDLSVPFEQYSPGGQDTPSSSSS--GDDSVFTHDLLPPGP---------PSNGGPRT------
FGFR3 rat LDLSVPFEQYSPGGQDTPSSSSS--GDDSVFTHDLLPPGP---------PSNGGPRT------
FGFR3 chicken LDLSVPFEQYSPAGQDTHSTCSS--GDDSVFAHDLLPDEPCLPKH---VPCNGVIRT------
FGFR3 frog LDLSVAFEQYSPPSQDSHSTCSS--GDDSVFAHDILPDEPCLPKH---QQHNGAIPT------
FGFR3 newt LDLSVPFEQYSPACPDSHSSCSS--GDDSVFAHD-LPEEPCLPKH---QQYNGVIRT------
FGFR3 zebrafish LDLSVPFEQYSPTCPDSNSTCSS--GDDSVFAHDPLPEEPCLPKH---HHSNGVIRT------
FGFR4 human LDLRLTFGPYSPSGGDASSTCSS---SDSVFSHDPLPLGSSSFPF------GSGVQT------
FGFR4 mouse LDLRLTFGPFSPSNGDASSTCSS---SDSVFSHDPLPLEPSPFPF------SDSQTT------
FGFR4 rat LDLRLTFGPYSPNNGDASSTCSS---SDSVFSHDPLPLEPSPFPF------PEAQTT------
FGFR4 frog LDLSMPFEQYSPSCEDSASTCSSS--DDSVFAPDPVPSSPCVFN---HNIHSQLGT------
FGFR4 newt LDLSMPFEQYSPACEDTTSTCSS---DDSVFTHEP--DVPSLFTH---HTTTSMVGT------
FGFR4 zebrafish LDLSTPFEQYSPSCEDTSSSCSS--DNDSVFTHDALSTEPCLLGY-HDVHSRMDLKTTMR---
777
B
Ct-FGFR1-WT
GST
Ct-FGFR1-WT
Ct-FGFR1-WT
Ct-FGFR1-S777A
Ct-FGFR1-S777D
Ct-FGFR1-WT
ERK1 kinase
p38 kinase
ERK2 kinase
33P
Coomassie
Ct-FGFR1
GST
D
GST
Ct-FGFR1-WT
Ct-FGFR1-S777A
Ct-FGFR1-S777D
FGF1
ERK1
FGFR1
GST
FGF1
Ct-FGFR1
GST
C
IP: FGFR1
FGFR1-WT
FGFR1-WT
FGFR1-S777A
FGFR1-S777D
ERK1 kinase
ERK2 kinase
33P
IB: FGFR1
E
IP: unspecific IgG
IP: FGFR1
ERK1 kinase
FGFR1
His
ERK1

Figure 2

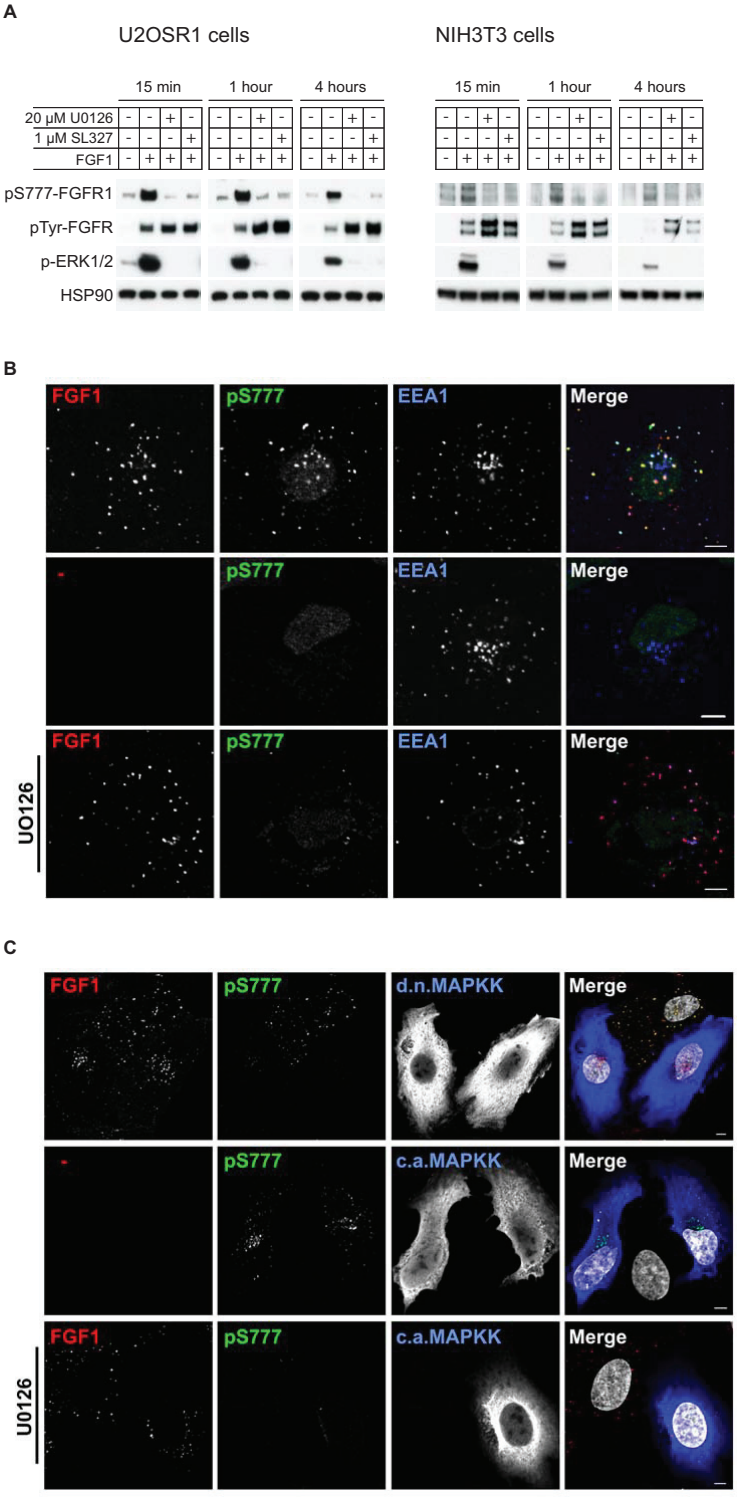
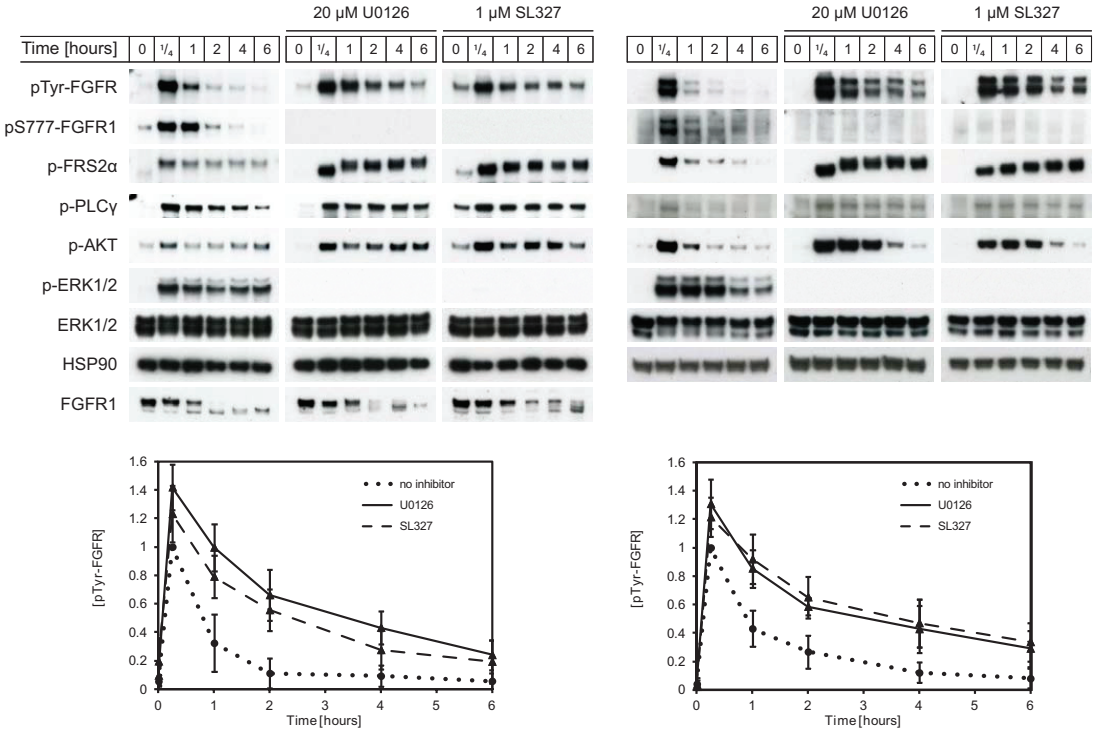

A
U2OSR1 cells
NIH3T3 cells
15 min
1 hour
4 hours
20 μM U0126
1 μM SL327
FGF1
pS777-FGFR1
pTyr-FGFR
p-ERK1/2
HSP90
B
FGF1
pS777
EEA1
Merge
UO126
C
d.n.MAPKK
c.a.MAPKK
U0126

Figure 3

A


U2OSR1 cells
NIH3T3 cells
20 μM U0126
1 μM SL327
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
pS777-FGFR1
p-FRS2α
p-PLCγ
p-AKT
p-ERK1/2
ERK1/2
HSP90
FGFR1
[pTyr-FGFR]
Time [hours]
no inhibitor
U0126
SL327

B

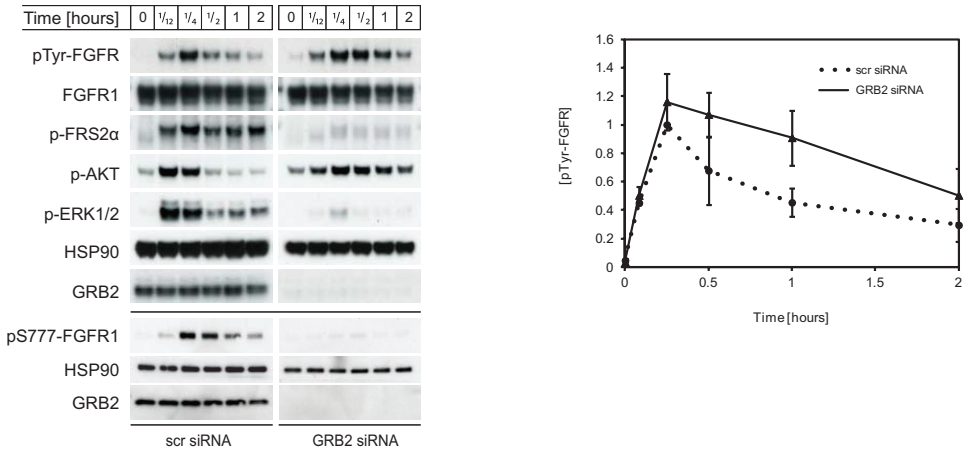
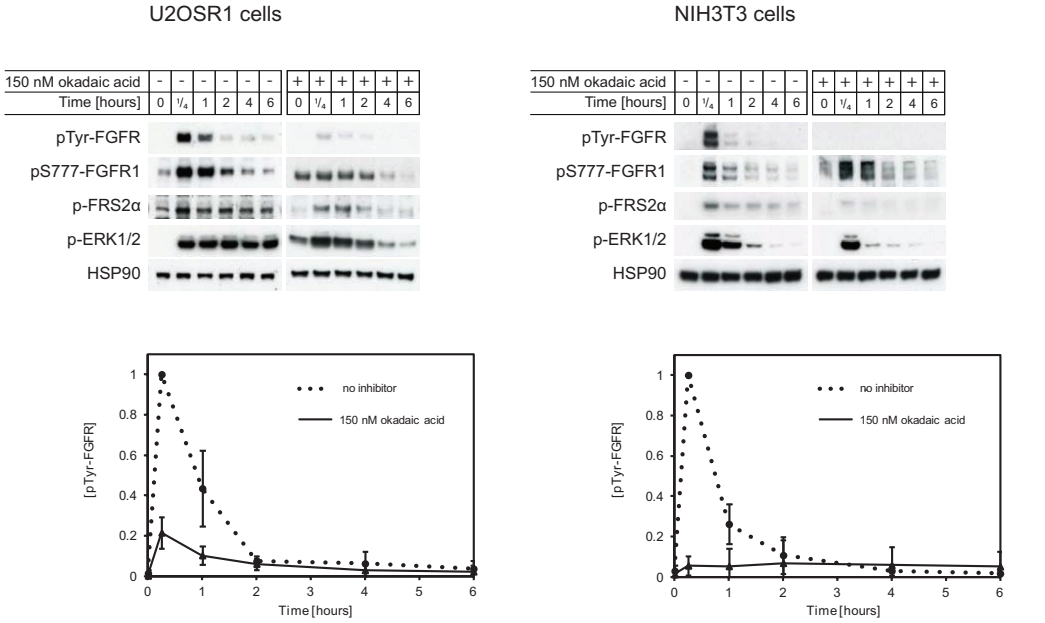

Time [hours]
0 1/12 ¼ ½ 1 2
pTyr-FGFR
FGFR1
p-FRS2α
p-AKT
p-ERK1/2
HSP90
GRB2
pS777-FGFR1
HSP90
GRB2
scr siRNA
GRB2 siRNA
[pTyr-FGFR]
Time [hours]

Figure 4

A


U2OSR1 cells
NIH3T3 cells
150 nM okadaic acid
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
pS777-FGFR1
p-FRS2α
p-ERK1/2
HSP90
no inhibitor
150 nM okadaic acid
[pTyr-FGFR]
Time [hours]

B

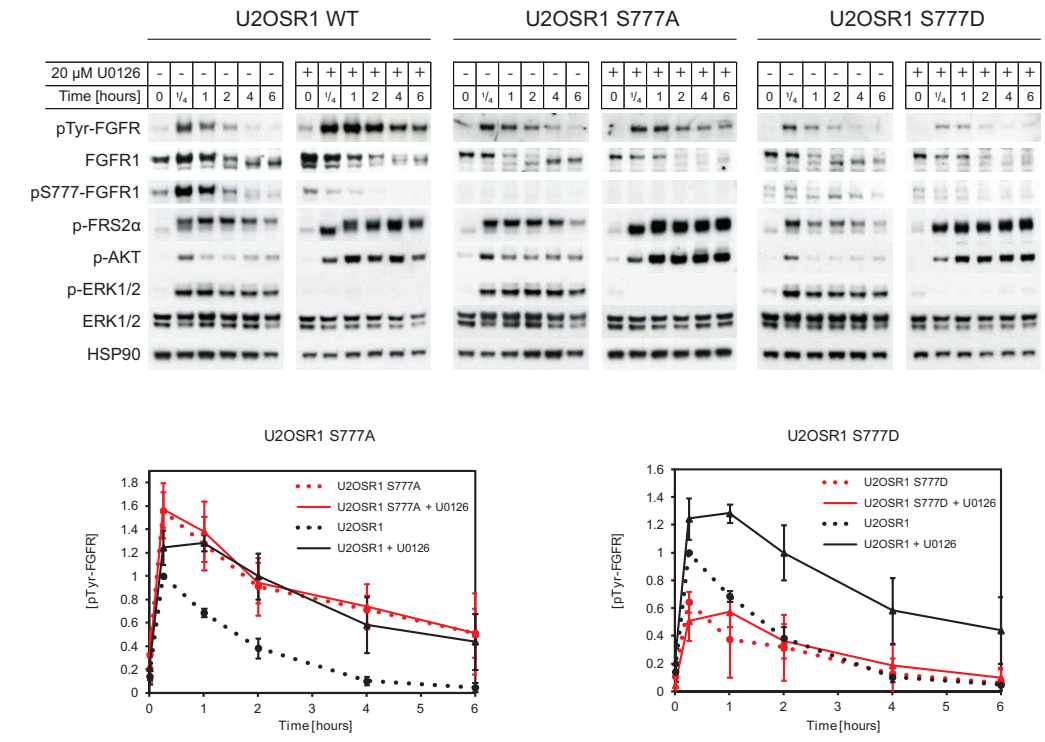

U2OSR1 WT
U2OSR1 S777A
U2OSR1 S777D
20 μM U0126
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
FGFR1
pS777-FGFR1
p-FRS2α
p-AKT
p-ERK1/2
ERK1/2
HSP90
U2OSR1 S777A
U2OSR1 S777A + U0126
U2OSR1
U2OSR1 + U0126
U2OSR1 S777D
U2OSR1 S777D + U0126
[pTyr-FGFR]
Time [hours]

Figure 5

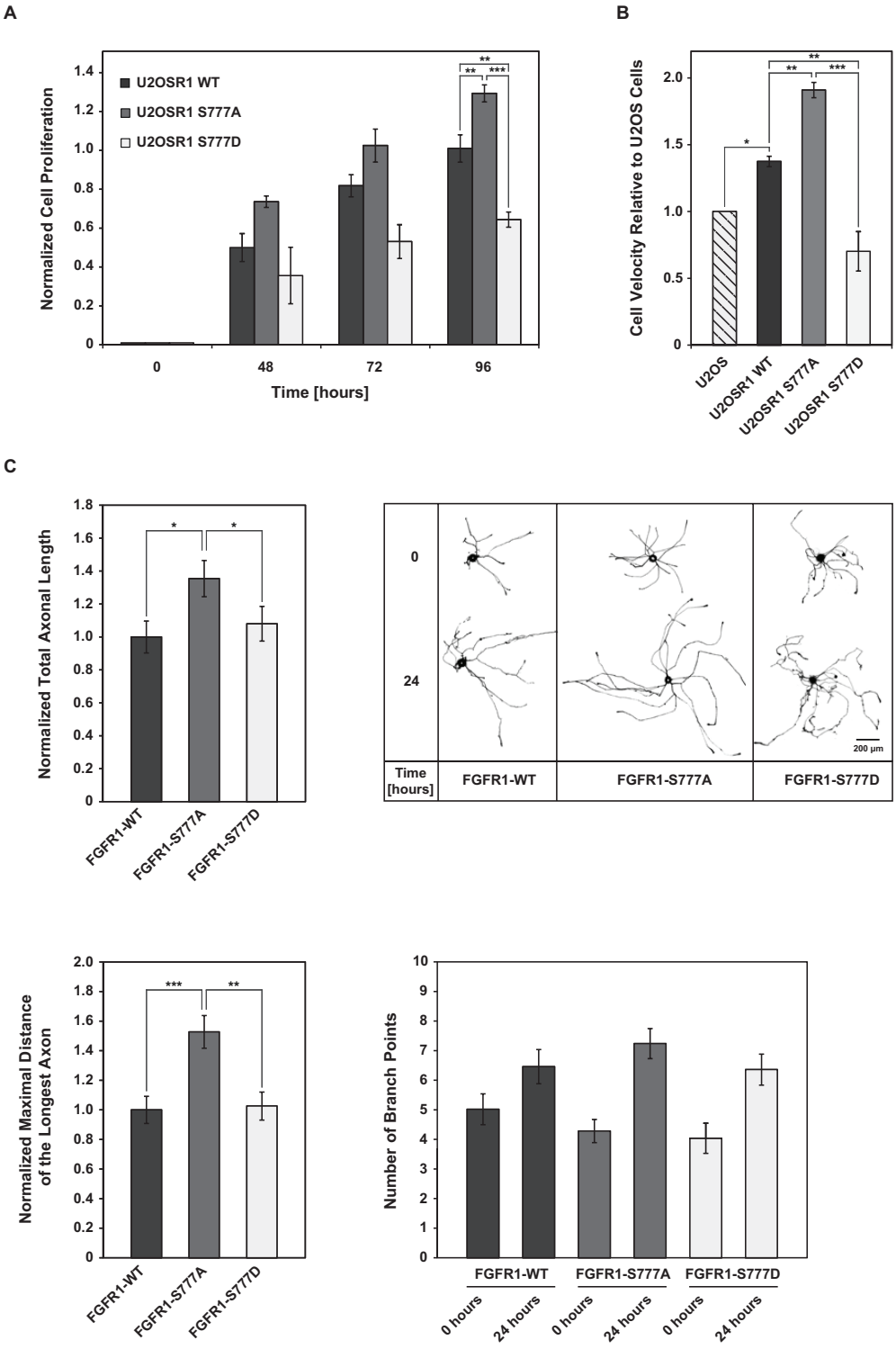
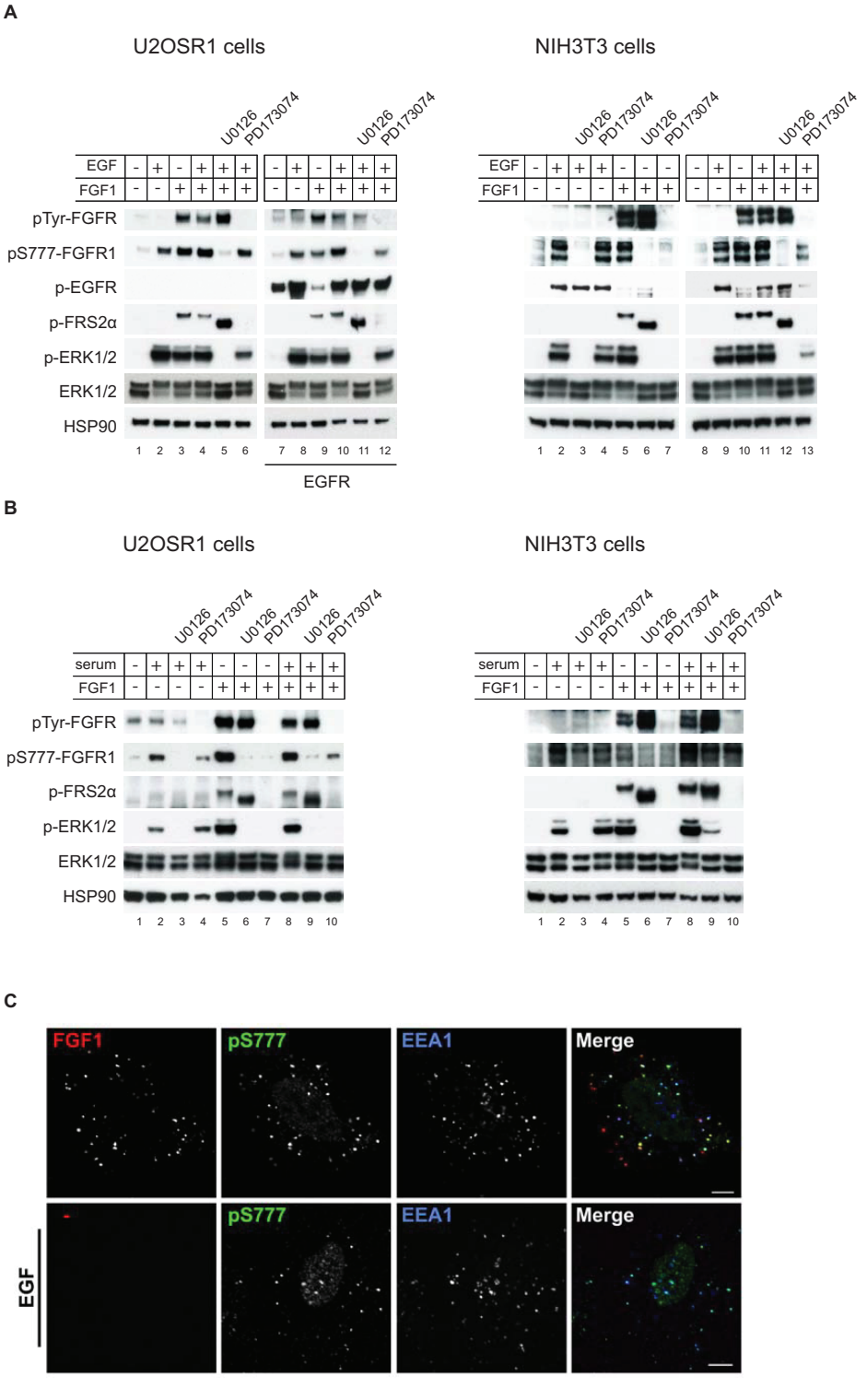
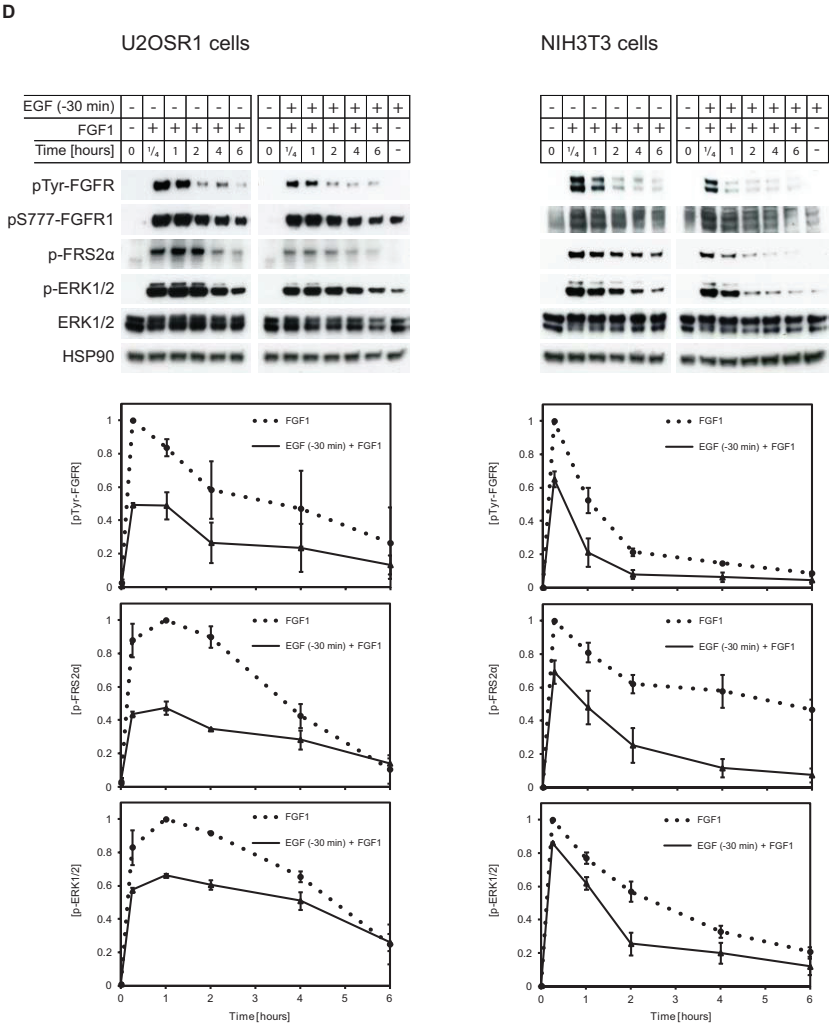

A
Normalized Cell Proliferation
U2OSR1 WT
U2OSR1 S777A
U2OSR1 S777D
Time [hours]
0
48
72
96
B
Cell Velocity Relative to U2OS Cells
U2OS
U2OSR1 WT
U2OSR1 S777A
U2OSR1 S777D
C
Normalized Total Axonal Length
FGFR1-WT
FGFR1-S777A
FGFR1-S777D
Time [hours]
0
24
200 µm
Normalized Maximal Distance of the Longest Axon
Number of Branch Points
0 hours
24 hours

Figure 6


A
U2OSR1 cells
NIH3T3 cells
U0126
PD173074
EGF
FGF1
pTyr-FGFR
pS777-FGFR1
p-EGFR
p-FRS2α
p-ERK1/2
ERK1/2
HSP90
EGFR
B
U2OSR1 cells
NIH3T3 cells
serum
FGF1
C
FGF1
pS777
EEA1
Merge
EGF

D


U2OSR1 cells
NIH3T3 cells
EGF (-30 min)
FGF1
Time [hours]
pTyr-FGFR
pS777-FGFR1
p-FRS2α
p-ERK1/2
ERK1/2
HSP90
FGF1
EGF (-30 min) + FGF1
[pTyr-FGFR]
[p-FRS2α]
[p-ERK1/2]
Time [hours]

E

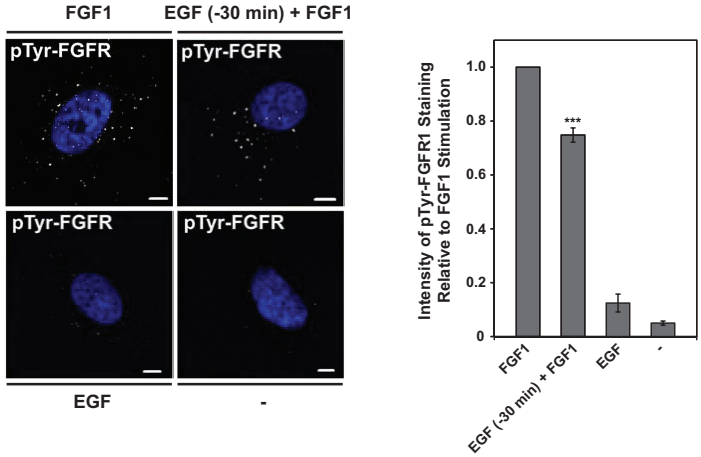
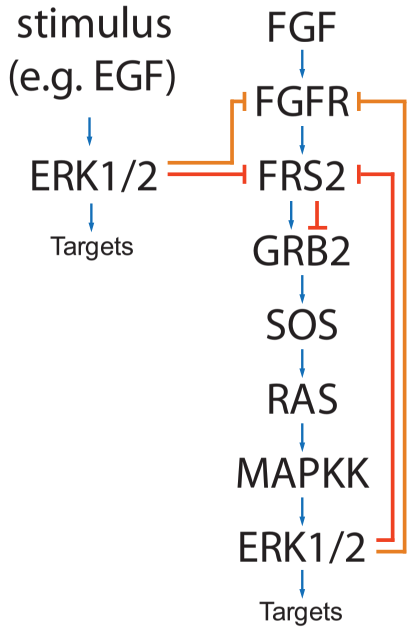
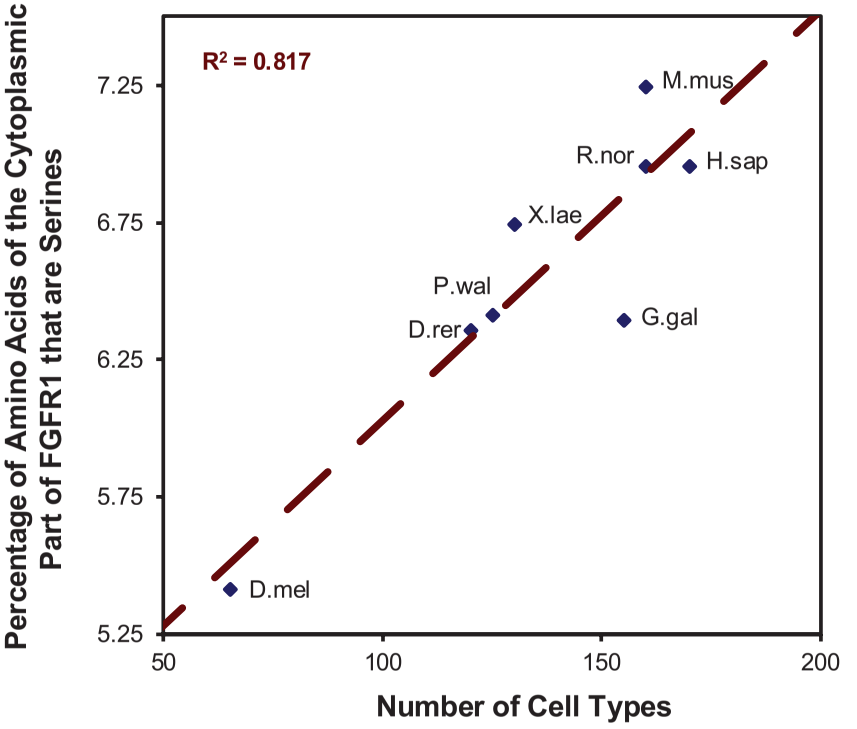

FGF1
EGF (-30 min) + FGF1
pTyr-FGFR
EGF
-
Intensity of pTyr-FGFR1 Staining Relative to FGF1 Stimulation
***
FGF1
EGF (-30 min) + FGF1
EGF
-

Figure 7

A


stimulus
(e.g. EGF)
ERK1/2
Targets
FGF
FGFR
FRS2
GRB2
SOS
RAS
MAPKK
ERK1/2
Targets

B


R² = 0.817
M.mus
R.nor
H.sap
X.lae
P.wal
D.rer
G.gal
D.mel
7.25
6.75
6.25
5.75
5.25
50
100
150
200
Percentage of Amino Acids of the Cytoplasmic Part of FGFR1 that are Serines
Number of Cell Types

Supplementary Materials

Supplementary Figure legends

fig. S1

In vitro phosphorylation assay with recombinant C-tail of FGFR1 and AKT kinase or MEK1.

Recombinant GST-tagged C-terminal wild type FGFR1 (Ct-FGFR1-WT), its S777A mutant (Ct-FGFR1-S777A), S777D mutant (Ct-FGFR1-S777D) or GST-tag alone were subjected to phosphorylation reaction with recombinant active AKT kinase (A) or MEK1 (B). p38 kinase, MBP (for AKT kinase), active ERK1/2 kinases and inactive ERK2 kinase (for MEK1) served as positive controls. "x" indicates inactive MEK1. U0126 (20 μM) was added as indicated. Shown are autoradiography (upper panels) and Coomassie Blue staining (lower panels).

fig. S2

Effect of U0126 inhibitor on ERK1/2 activity.

Western blot analysis of serum-starved U2OSR1 and NIH3T3 cells pretreated with indicated concentrations of U0126 and then stimulated with FGF1 for 15 min.

fig. S3

Effect of MEK inhibitors on FGFR1 activity in the presence or absence of brefeldin A or cycloheximide.

Western blot analysis of serum-starved U2OSR1 or NIH3T3 cells pretreated with or without U0126 or SL327 and then stimulated with FGF1 in the presence or absence of brefeldin A (2 μg/ml) or cycloheximide (CHX, 10 μg/ml) for indicated times.

fig. S4

The effect of siRNA knockdown of MAPKs (ERK1/2) and GRB2 on FGFR1 activity.

(A) Western blot analysis of U2OSR1 cells transfected with siRNA oligos targeting ERK1 and ERK2 or a non-targeting siRNA control (scr), then serum-starved and stimulated with FGF1 in the presence or absence of cycloheximide (10 μg/ml) for indicated times. The efficiency of knockdown was assessed by Western blotting using anti-ERK1/2 antibody.

(B) Western blot analysis of U2OSR1 cells transfected with siRNA oligos targeting GRB2 or a non-targeting siRNA control (scr), then serum-starved and stimulated with FGF1 in the presence of cycloheximide (10 μg/ml) for indicated times. The efficiency of knockdown was assessed by Western blotting using anti-GRB2 antibody. Quantification of the bands representing phosphorylated FGFR1 (Tyr^{653}/Tyr^{654}) is presented in the graph and expressed as a fraction of the maximum response in cells transfected with control siRNA (scr). The graph represents the mean ± SD of three independent experiments.

fig. S5

Effect of the phosphorylation state of FGFR1 Ser^{777} on cell proliferation and migration in additional clones of stably transfected U2OS cells.

(A) FGF1-induced cell proliferation of U2OSR1 cells (U2OSR1 WT #1*) and additional clones of stably transfected U2OS cells (U2OSR1 WT #2, U2OSR1 S777A #2 and U2OSR1 S777D #2). AlamarBlue® reagent was added to the serum-starved cells stimulated with FGF1 for indicated times and the fluorescence corresponding to number of cells was measured. The graph represents the mean ± SEM of three independent experiments.

(B) Effect of Ser^{777} mutants on cell migration. FGF1-stimulated U2OSR1 cells (U2OSR1 WT #1*) and additional clones of the stably transfected U2OS cells (U2OSR1 WT #2, U2OSR1 S777A #2 and U2OSR1 S777D #2) were subjected to time-lapse live-cell imaging. Values are given as means ± SEM from three independent experiments, where 30-50 cells per experiment were analyzed.

U2OSR1 WT #1* denotes U2OSR1 cells used in throughout the paper previously described by Haugsten *et al.* (10).

fig. S6

Correlation of tyrosine and serine/threonine content in the cytoplasmic part of FGFR1s with organism complexity.

Tyrosine content in the cytoplasmic part of FGFR1 correlates negatively (R^2=0.520) and serine/threonine content positively (R^2=0.660) with organism complexity as measured by number of different cell types.

Supplementary Figures

Figure S1

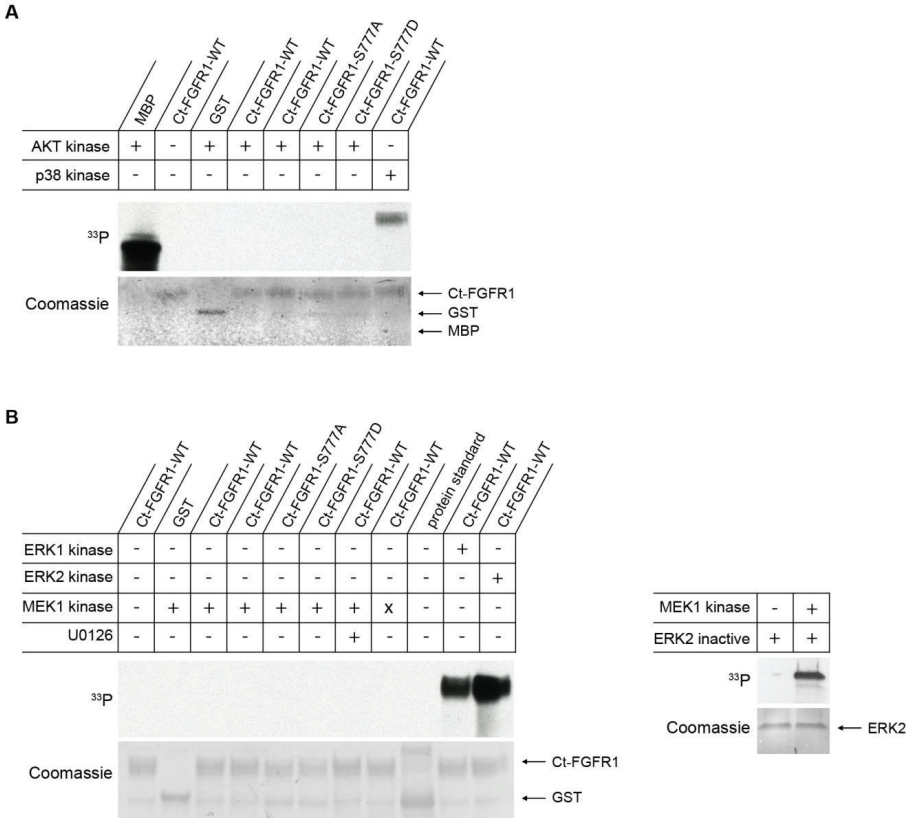


Figure S2

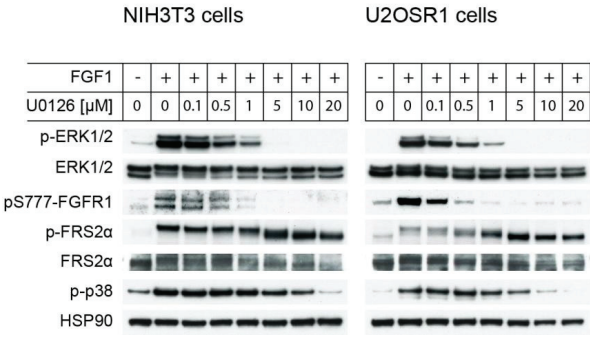
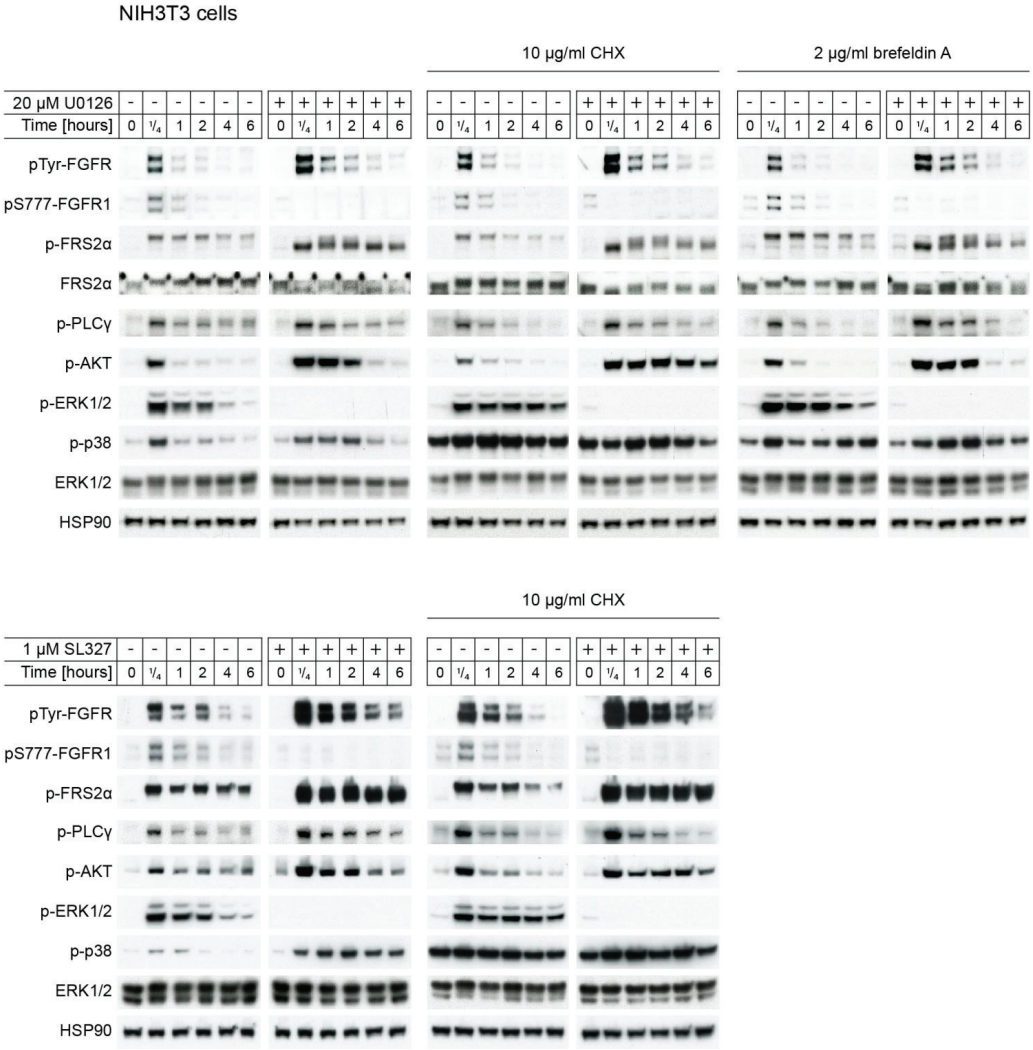

NIH3T3 cells
U2OSR1 cells
FGF1
U0126 [μM]
p-ERK1/2
ERK1/2
pS777-FGFR1
p-FRS2α
FRS2α
p-p38
HSP90

Figure S3


NIH3T3 cells
10 µg/ml CHX
2 µg/ml brefeldin A
20 µM U0126
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
pS777-FGFR1
p-FRS2α
FRS2α
p-PLCγ
p-AKT
p-ERK1/2
p-p38
ERK1/2
HSP90
10 µg/ml CHX
1 µM SL327
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
pS777-FGFR1
p-FRS2α
p-PLCγ
p-AKT
p-ERK1/2
p-p38
ERK1/2
HSP90

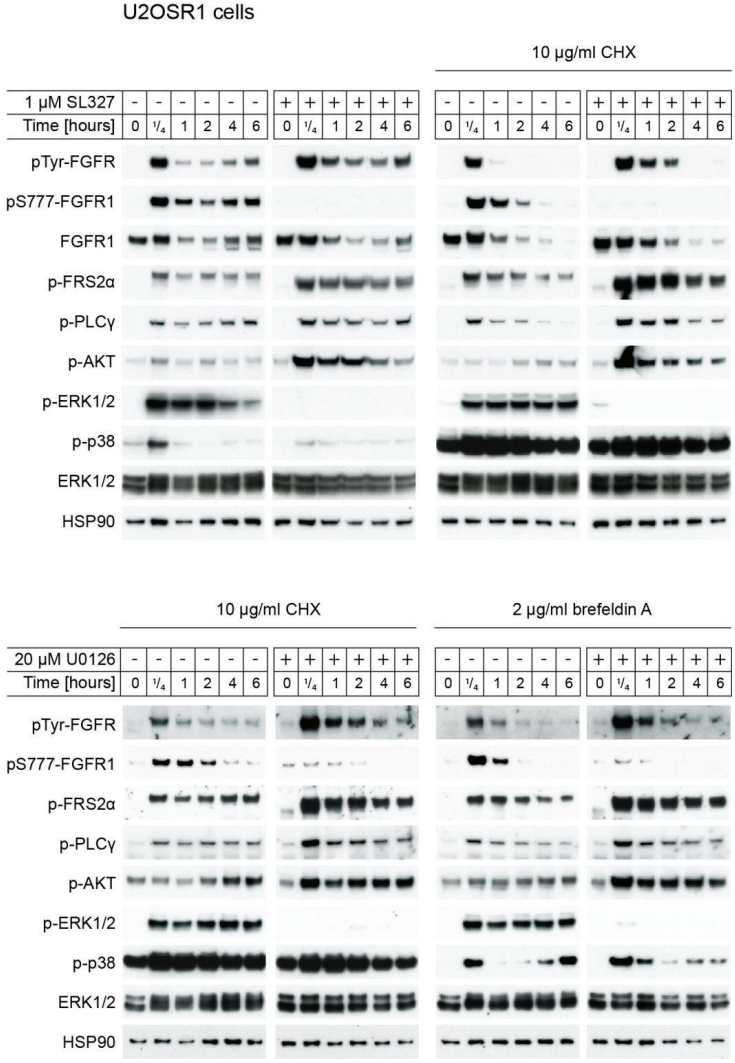
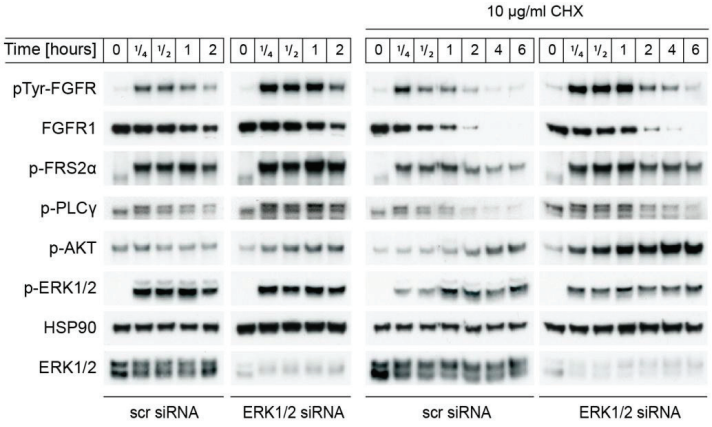
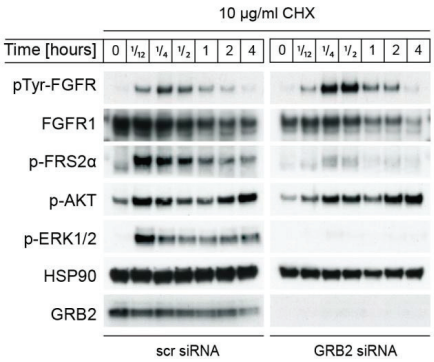

U2OSR1 cells
10 µg/ml CHX
1 µM SL327
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
pS777-FGFR1
FGFR1
p-FRS2α
p-PLCγ
p-AKT
p-ERK1/2
p-p38
ERK1/2
HSP90
10 µg/ml CHX
2 µg/ml brefeldin A
20 µM U0126
Time [hours]
0 ¼ 1 2 4 6
pTyr-FGFR
pS777-FGFR1
p-FRS2α
p-PLCγ
p-AKT
p-ERK1/2
p-p38
ERK1/2
HSP90

Figure S4

A


10 µg/ml CHX
Time [hours]
0 ¼ ½ 1 2
0 ¼ ½ 1 2
0 ¼ ½ 1 2 4 6
0 ¼ ½ 1 2 4 6
pTyr-FGFR
FGFR1
p-FRS2α
p-PLCγ
p-AKT
p-ERK1/2
HSP90
ERK1/2
scr siRNA
ERK1/2 siRNA
scr siRNA
ERK1/2 siRNA

B


10 µg/ml CHX
Time [hours]
0 1/12 ¼ ½ 1 2 4
0 1/12 ¼ ½ 1 2 4
pTyr-FGFR
FGFR1
p-FRS2α
p-AKT
p-ERK1/2
HSP90
GRB2
scr siRNA
GRB2 siRNA

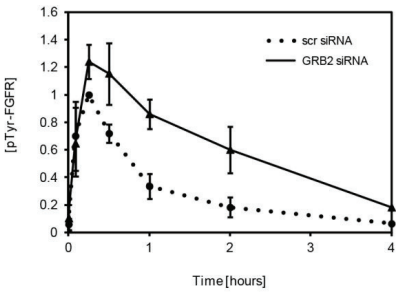
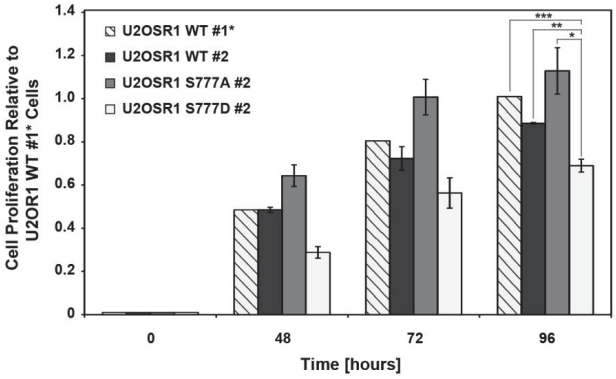

scr siRNA
GRB2 siRNA
[pTyr-FGFR]
Time [hours]
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
1
2
3
4

Figure S5

A


Cell Proliferation Relative to U2OR1 WT #1* Cells
U2OSR1 WT #1*
U2OSR1 WT #2
U2OSR1 S777A #2
U2OSR1 S777D #2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
***
**
*
0
48
72
96
Time [hours]

B

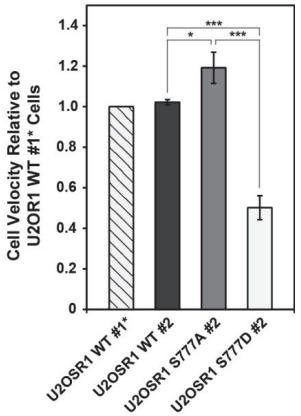
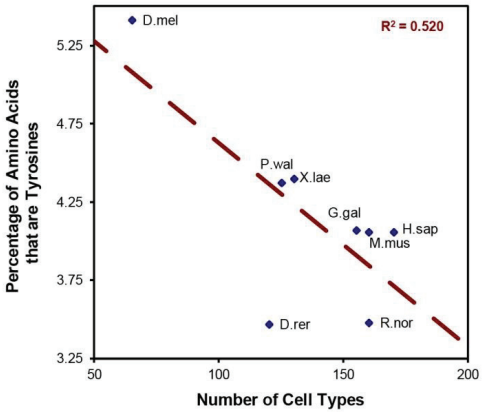
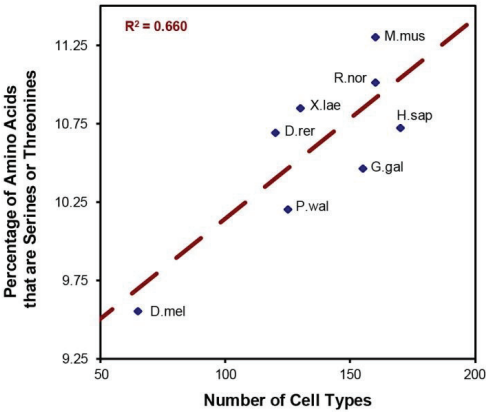

Cell Velocity Relative to U2OR1 WT #1* Cells
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
***
***
U2OSR1 WT #1*
U2OSR1 WT #2
U2OSR1 S777A #2
U2OSR1 S777D #2

Figure S6


D.mel
R² = 0.520
5.25
4.75
4.25
3.75
3.25
P.wal
X.lae
G.gal
H.sap
M.mus
D.rer
R.nor
50
100
150
200
Percentage of Amino Acids that are Tyrosines
Number of Cell Types


R² = 0.660
M.mus
11.25
R.nor
X.lae
H.sap
10.75
D.rer
G.gal
10.25
P.wal
9.75
D.mel
9.25
50
100
150
200
Percentage of Amino Acids that are Serines or Threonines
Number of Cell Types