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Regulation and Function of MAP Kinases in PDGF Signaling

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Abstract

Eger, G. 2016. Regulation and Function of MAP Kinases in PDGF Signaling. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1246. 51 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9660-9.

Platelet-derived growth factor (PDGF) is a family of signaling molecules that stimulates cell growth, survival and migration. PDGF is recognized by specific transmembrane proteins, the PDGF receptors, which relay the signals to the cell activating the Mitogen-activated protein (MAP) kinases and other signaling pathways. Aberrant activation of these pathways is frequently detected in cancer. Hence, the study of these processes is essential for identifying potential drug targets or diagnostic markers.

In paper I, we identified Receptor Subfamily 4 Group A Member 1 NR4A1 to be regulated by PDGF via MAP kinases, clarifying the role of Extracellular signal–regulated kinases (Erk) 1/2, Erk5 and Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in its regulation. NR4A1 was found to be important for the tumorigenic potential, measured as anchorage-independent growth, of glioblastoma cells.

Since the cellular responses elicited by PDGF result from the balance between phosphorylation and dephosphorylation events, we investigated the role of the dual specificity phosphatases DUSP4/MKP-2 and DUSP6/MKP-3. In paper II, we describe the crucial role of Erk1/2 and p53 in the expression of DUSP4/MKP2. Moreover, we observed that DUSP4/MKP-2 downregulation decreases Erk5 activation and accelerates PDGFRβ internalization and downregulation resulting in a specific inhibition of Signal transducers and activators of transcription (Stat) 3, Src and protein kinase C (PKC), and partially of p38, Stat1/5 and Phoshoplipase Cγ (PLCγ).

In paper III, we report that DUSP6/MKP-3 creates a negative cross-talk between Erk1/2 and Erk5 and an auto-inhibitory feedback loop on the PI3-kinase/Akt pathway. In paper IV, we identify a new regulative mechanism of the PDGF pathway. PDGF induces Erk5 expression and activation that modulates the PDGFRβ activity. After Erk5 downregulation, the receptor undergoes to a faster and stronger activation that results in a faster internalization and degradation.

In conclusion, we present a mechanism through which the PDGF/MAP kinases support tumor growth, and elucidate different regulatory pathways involved in PDGF signaling.

Keywords: PDGF, PDGFR, MAP kinase, Erk1/2, Erk5, Dusp/MKP, NR4A1, cancer

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Eger, G**., Papadopoulos, N., Lennartsson, J., Heldin, C.H. (2014) NR4A1 promotes PDGF-BB-induced cell colony formation in soft agar. *PLoS One*, 30;9(9):e109047

II **Eger, G**., Rorsman, C., Heldin, C.H., Lennartsson, J. (2016) Depletion of DUSP4 results in enhanced PDGFRβ cell surface clearance and suppresses PDGF-BB-induced activation of Erk5, Stat3, Src and PKC. *Manuscript*

III Razmara, M., **Eger, G**., Rorsman, C., Heldin, C.H., Lennartsson. J. (2012) MKP-3 negatively modulates PDGF-induced Akt and Erk5 phosphorylation as well as chemotaxis. *Cell Signal*, 24(3):635-40

IV Voytyuk, O., **Eger, G.**, Heldin, C.H., Lennartsson, J. (2016) Erk5 promotes prolonged PDGFRβ activation by limiting ligand-induced receptor internalization and degradation. *Manuscript*

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Contents

Abbreviations

AF-1	Activation Function 1
Alix	ALG-2-Interacting Protein-X
BCL-2	Converting B-Cell Lymphoma - 2
BMK1	Big MAP Kinase 1
Cbl	Casitas B-Lineage Lymphoma
CD	Catalytic Domain
CUB	Complement Subcomponents C1r/C1s, Urchin EGF-Like Protein, and Bone Morphogenetic Protein 1
DAG	Diacylglycerol
DMSO	Dimethyl Sulfoxide
Dusp	Dual Specific Phosphatase
Egr	Early Growth Response Protein
ERK	Extracellular-Signal-Regulated Kinase
GAP	Gtpase-Activating Protein
IKK	Iκb Kinase
IκBs	Inhibitor of κB
JNK	C-Jun Amino-Terminal Kinase
KIM	Kinase Interaction Motif
MAP	Mitogen-Activated Protein
MAPKK	MAP Kinase Kinase
MAPKKK	MAP Kinase Kinase Kinase
Mek	MAP Kinase/*ERK* Kinase
MKP	MAP Kinase Phosphatase
MMP-9	Matrix Metallopeptidase 9
mTOR	Mammalian Target of Rapamycin
NES	Nuclear Export Signal
NF-κB	Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells
NLS	Nuclear Localization Signal
NR4A1/2/3	Nuclear Receptor Subfamily 4 Group A Member1/2/3

Oct-4	Octamer-Binding Transcription Factor 4
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDK1	Phosphoinositide-Dependent Protein Kinase 1
PEST	Proline (P), Glutamic Acid (E), Serine (S), And Threonine (T)
PI3K	Phosphoinositide-3'-Kinase
PIP_2	Phosphatidylinositol (4,5)-Bisphosphate
PIP_3	Phosphatidylinositol (3,4,5) -Trisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLCγ	Phoshoplipase Cγ
PTP	Protein Tyrosine Phosphatase
Raf	Rapidly Accelerated Fibrosarcoma
RasGAP	Ras GTPase Activating Protein
RTK	Receptor Tyrosine Kinase
SAP	Stress-Activated Protein
SH2	Src Homology 2
SHP-2	SH-2 Tyrosine Phosphatase
Src	Rous Sarcoma Virus Cellular Oncogene
SSV	Simian Sarcoma Virus
STAT	Signal Transducers and Activators of Transcription
TGFβ	Transforming Growth Factor-β
VEGF	Vascular Endothelial Growth Factor

Introduction

The cell is the smallest unit of life. In multicellular organisms, cells are structured to perform highly specialized functions, cooperate in tissues and orchestrate processes essential for the life of the whole body.

As the members of a society, cells interact with each other and with the environment. Cells perceive and integrate information in order to coordinate different responses in a finely regulated process called cell signaling.

Signal transduction is initiated upon binding of extracellular signaling molecules to cellular receptors. One important class of receptors present on the cellular membrane is the receptor tyrosine kinase (RTK) family. Growth factors activate the RTKs inducing dimerization of the receptors. RTKs dimerize after ligand binding, allowing the receptors to interact with each other and promote autophosphorylation on specific tyrosine residues. Thus, the receptors change their conformation allowing interaction with proteins that transmit and amplify the signal into the cell. Among the several stimuli that can activate RTKs in humans, we focus on the platelet-derived growth factor (PDGF) which elicits cell proliferation, migration, differentiation and survival. These processes are essential during embryonic development and to maintain homeostasis in the adult organism but, if deregulated, they can promote tumorigenesis.

PDGF signal is transduced into the cell toward PDGF receptor activation and, as a cascade, various downstream signaling pathways are initiated, among them, we focus on the mitogen-activated protein (MAP) kinases pathways.

The aim of this thesis is to clarify the regulation and function of MAP kinases in PDGF signaling, with the hope that this knowledge can be used for improved therapeutic intervention for cancer patients.

PDGF

PDGF was discovered in the 1970s; it was one of the first growth factors purified and characterized, and it owes its name to the fact that it was originally isolated from platelets. PDGF is as potent mitogenic signal for cells of mesenchymal origin, including fibroblasts, smooth muscle cells nd glia cells (1–3).

PDGF isoforms are secreted by epithelial or endothelial cells and act in a paracrine manner on mesenchymal cells promoting proliferation, migration, and differentiation (4,5). In adults, PDGF stimulation is important in the wound healing process, recruiting e.g. fibroblasts, smooth muscle cells, neutrophils, and macrophages to the injured area. Moreover, PDGF promotes synthesis of different matrix molecules (6,7). One of the two PDGFRs, PDGFRβ also exerts a role in the control of the intestinal fluid pressure and preventing edema formation probably, inducing contraction of fibroblasts and myofibroblasts that, via their integrins, affect the extracellular components (8,9).

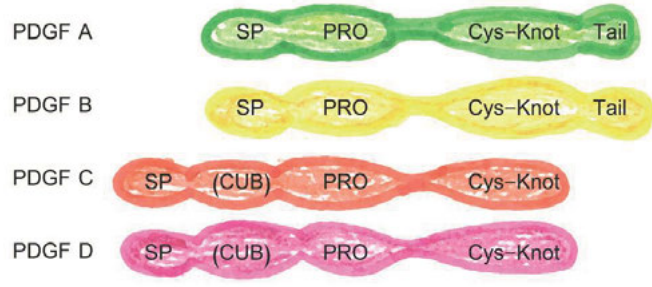

Figure 1. Domain structure of the PDGF isoforms. The protein structure comprises the signal peptide (SP); the pro-sequence (PRO) cleaved from mature peptide; the cysteine-knot growth factor domain (Cys-Knot) that is responsible for receptor recognition; the tails of the classic PDGF-A and -B, which contain in the a basic retention motif that bind to cell membranes; the PDGF-C and -D also contains the complement subcomponents C1r/C1s, urchin EGF-like protein, and bone morphogenetic protein 1-like domain (CUB).

The PDGF family is encoded by four genes that are translated into four polypeptide chains, PDGF-A, -B, -C and –D, that form five biologically active dimers, PDGF-AA, -AB, -BB, -CC, and -DD (10). PDGF isoforms are synthesized as precursor molecules and are activated by proteolytic processing (7,11). Each of the PDGF isoforms contains a growth factor domain, a signal sequence and a propeptide sequence

that in the PDGF-A and PDGF-B is cleaved from the mature growth factors by furin or other proprotein convertases. However, the domain organization between the classical PDGF isoforms (-A, -B) and the novel isoforms (-C and –D), shows some differences (*Figure 1*) (10,12). The classic PDGFs, contain in the tails a basic retention motif that bind to cell membranes (13,14) and undergo intracellular activation during transport in the exocytic pathway. In contrast, the novel PDGFs, that lack the tail sequences, are secreted as latent factors. PDGF-C and –D are characterized by the N-terminal CUB domain (complement subcomponents C1r/C1s, Urchin EGF-like protein, and Bone morphogenetic protein 1) that may regulate the extracellular distribution of latent forms by interacting with other proteins or carbohydrates (11,15).

PDGFR

The five PDGF isoforms are recognized by two types of PDGFRs, PDGFRα and PDGFRβ, which belong to the RTK superfamily. The two PDGFRs are structurally similar, but differ in their ligand-binding specificities, expression patterns and physiological roles (16). PDGFRα binds with high affinity and is activated by PDGF-AA, PDGF-BB, PDGF-CC and PDGF-AB. Whereas PDGFRβ can only bind and be activated by PDGF-BB and PDGF-DD (*Figure 2*) (7).

During embryogenesis, PDGFRα mediates crucial signals for the development of the facial skeleton, hair follicles, spermatogenesis, oligodendrocytes and astrocytes, as well as for the development of the lung and intestinal villi. PDGF-B and PDGFRβ signaling have a critical role in the establishment of functional blood vessels by recruiting pericytes. PDGF-B and PDGFRβ knockout mice die perinatally from vascular defects found in many organs (17–19). PDGFA and PDGFRα are more broadly required during embryogenesis and, as knockout models have shown, they have essential roles in numerous contexts, including the development of the central nervous system, neural crest and certain organs (20–24,24–26).

In the homeostasis of adult organisms, PDGFRβ activation controls interstitial fluid pressure (8,9) and wound healing. In the wound area, it recruits fibroblasts, smooth muscle cells, neutrophils, and macrophages (6) and stimulates the production of different matrix molecules (reviewed by (7).

A role for PDGFRα has been described in juvenile pancreatic islets where it promotes proliferation of β cells (12).

The functions of DGFRα and PDGFRβ are not interchangeable. Whereas the loss of the cytoplasmic part of PDGFRα could be rescued by the cytoplasmic part of PDGFRβ, the loss of the intracellular part of

PDGFRβ could be only partly rescued by the intracellular part of PDGFRα (27).

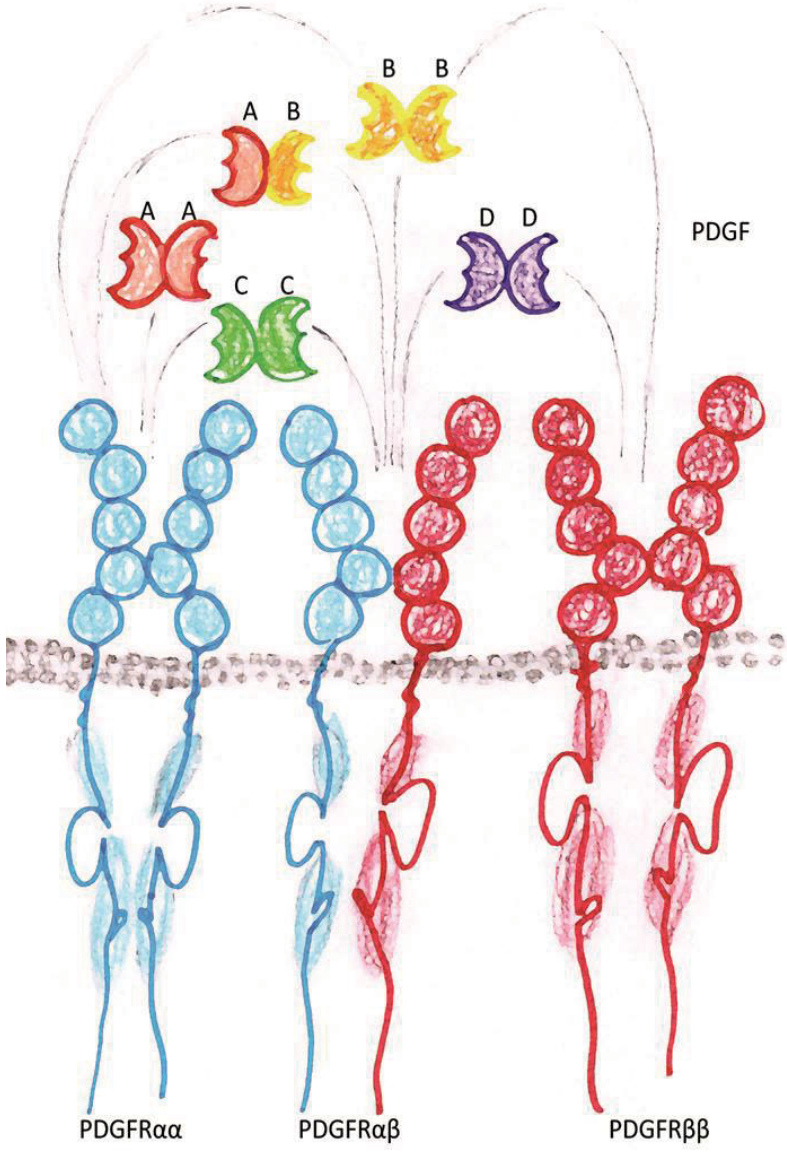

Figure 2. Schematic illustration of how the different PDGF isoforms bind to and dimerize PDGFRα and β with different specificities. Not in scale.

PDGFR activation and signaling

The PDGFR family has a modular architecture characteristic of the RTKs consisting of an extracellular domain, a single transmembrane helix, a juxtamembrane segment, a tyrosine kinase domain, and a carboxy-terminal tail (*Figure 3*).

The extracellular portion consists of five immunoglobulin-like domains which is characteristic for class III RTK, out of which domains 2 and 3 are the most important for interaction with the ligands, whereas domains 4 and 5 stabilize the receptor dimers (28–30). Intracellularly, each of the PDGFR contains a juxtamembrane segment that likely suppresses the basal activity of the tyrosine kinase domain (31). In addition, the activation loop and the C-terminal tail inhibit the kinase activity (32).

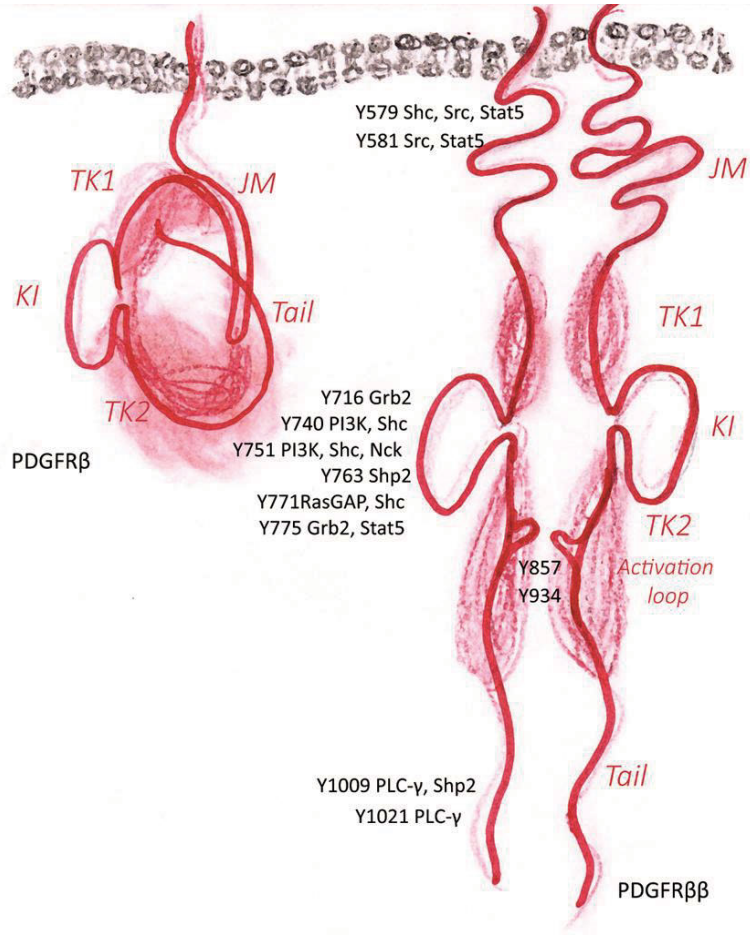

Figure 3. Schematic illustration of ligand-induced dimerization, transphosphorylatyion and conformational change of PDGFRββ. Not in scale.

Similarly to other tyrosine kinase receptors, PDGFRs are activated by ligand-induced dimerization (33). This process brings the intracellular kinase domains of two receptors close to each other allowing for transphosphorylation.

The first important step is the phosphorylation within the kinase domains (Tyr-849 in PDGFRα and Tyr-857 in PDGFRβ) that open the conformation of the receptor leading to increased catalytic efficiency. Upon activation, also the juxtamembrane segment and the C-terminal tail become phosphorylated, reliving the inhibition and allowing for increased kinase activity (34) (*Figure 3*).

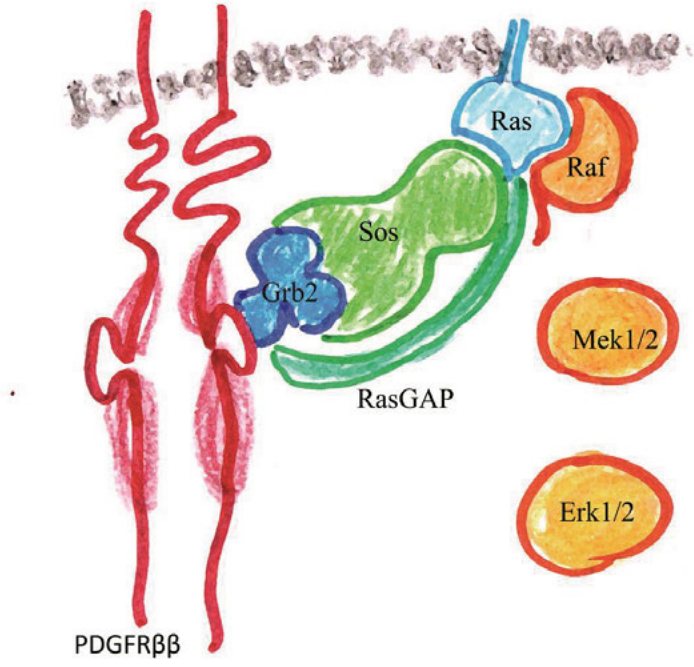

Figure 4. Representation of some proteins docking to the activated PDGFRββ and MAP kinase activation. Not in scale.

The phosphorylation of other tyrosine residues creates high affinity docking sites for about 10 different families of transduction molecules containing Rous sarcoma virus (Src) homology 2 (SH2) domains. Each of the three dimeric PDGF receptor complex, αα, αβ, and ββ, shows different autophosphorylation patterns. Consequently, they bind and activate different SH2 domain proteins (35). Proteins docked to the PDGFR become activated by receptor-mediated tyrosine phosphorylation or by binding-induced conformational changes (*Figure 4*). In other cases, translocation from the cytoplasm to the activated receptor at the plasma membrane is

sufficient for signaling initiation. Many of the signaling molecules binding to the PDGFR have intrinsic enzymatic activity, e.g. phosphatidylinositol 3′-kinase (PI3-kinase), phospholipase C (PLC)-γ, the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2 (alias PTPN11), and a GTPase activating protein (GAP) for Ras. In some cases the signal is relayed by adaptors, e.g. Grb2, Grb7, Nck, Shc, and Crk that couple the receptors to other signaling proteins that can't bind directly to the receptor.

Also the carboxy-terminal tail of the PDGF receptors participate in the interactions with certain signaling molecules independently of autophosphorylation e.g., the PDZ-domain protein NHERF enhances receptor signaling, and the adaptor molecule Alix/HP95 facilitates the binding of the ubiquitin ligase casitas B-lineage lymphoma (Cbl) (7,34).

MAP kinase pathways

MAP kinases pathways are important signal cascades activated by PDGF. The core module of these signaling pathways comprises a highly conserved cascade of three-tiered protein kinases. Sequential phosphorylation events culminate with the activation of MAP kinases that are phosphorylated on threonine and tyrosine residues within the activation loop containing the signature sequence T–X–Y (single letter code). There are four groups of classical MAP kinases (i.e. Erk1/2, Erk5, p38 and JNK) (*Figure 5*) and four atypical MAP kinases (i.e. Erk3, Erk4, NLK and Erk7); however, little is known about the latter.

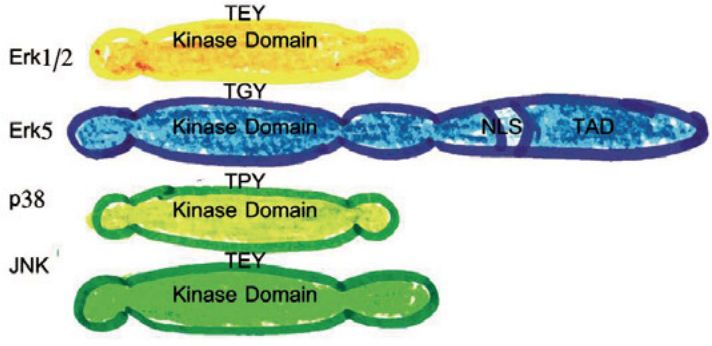

Figure 5. Structure of classical MAP kinases. The kinase domain contains the conserved threonine—amino acid—tyrosine sequence (T-X-Y) motif. Erk5 has an extended, unique C-terminal-region containing the transactivation domain (TAD) and Nuclear Localization Signal (NLS).

Among the classical MAP kinases, Extracellular signal–regulated kinases (Erk) 1 and 2 has been most studied, but the other pathways have a similar architecture. In brief, PDGFRs rapidly and transiently activate Erk1/2 by first recruiting the adaptor Grb2 in complex with the Ras nucleotide-exchanging factor Sos to the plasma membrane. Activated Ras interacts with and activate the MAP kinase kinase kinase (MAPKKK) Raf which phosphorylates and activates the MAP kinase kinase (MAPKK) Mek1/2, which in turn phosphorylates and activates the MAP kinase Erk1/2 (36) (*Figure 6*).

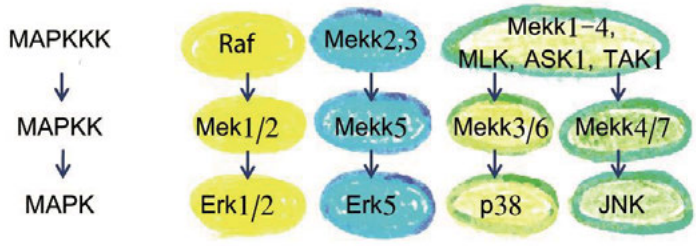

Figure 6. The three-tiered classical MAP kinase module.

Erk1/2 has been found to be widely expressed and able to phosphorylate hundreds of different substrates, including other protein kinases and transcription factors (37).

The kinetic of Erk1/2 activation is regulated by complex mechanisms involving interactions with scaffold proteins, phosphatases, feedback loops and cross-talk with other signaling pathways. Scaffold proteins, which form complexes with Erk1/2, determine with which upstream activators and downstream substrates it will interact (38,39). It has been demonstrated that, in porcine aortic endothelial cells, a rapid Erk1/2 activation requires Src and protein kinase C (PKC)/phospholipase C (PLC), whereas the magnitude of Erk1/2 activation is negatively regulated by the PI3 kinase/Akt pathway and RasGAP (40,41). Erk1/2 can generate a variety of different biological responses, including G1/S progression in the cell cycle by regulating the expression of genes such as the D-type cyclins (42). Erk1/2 can also promote cell survival by phosphorylating BCL-2 family members at the mitochondria (43), as well as, migration and differentiation; Erk1/2 also changes metabolism and adhesion of cells (37,41).

These key cellular processes are deregulated in cancer and represent some of the hallmarks and driving aspects of the cancer cell. Activating mutations in genes encoding RTKs, Ras, Raf, Mek1 or Mek2, act as drivers of tumorigenesis, Thus, hyper-activation of Erk1/2 signaling is fre-

quently exhibited in cancer (e.g. over 90% of melanomas) (43,44). Therefore, Raf, Mek1/2, and Erk1/2 provide rational therapeutic targets for the design of low molecular weight inhibitors. A plethora of new selective and potent drugs that inhibit Raf or Mek1/2 have recently been approved or are currently undergoing late-stage clinical trials (45,46).

Erk5, one of the least characterized classical MAP kinases, presents numerous similarities with Erk1/2 structure and activation mode, however, it has some distinctive features (47,48). Erk5 has N-terminal and kinase domains homologous to Erk1/2 but it contains an extended, unique C-terminal region; therefore, the molecule is also called big MAP kinase 1 (BMK1). This C-terminal portion contains a bipartite nuclear localization signal (NLS) (49) and a transcriptional activation domain (50), suggesting that Erk5 may function both as a kinase and as a transcription factor. It has been proposed that the large C-terminal domain regulates also the subcellular localization. Although Erk5 has a bipartite NLS, it has recently been proposed that Erk5 can assume a folded conformation that dampen the NLS or generate a nuclear export signal (NES). This may explain why, Erk5 localizes to different compartments depending on the cell line and type of stimulus that the cell receives (Buschbeck and Ullrich, 2005; Kondoh et al., 2006), and is likely to be important for the regulation of the Erk5 transcription factor function. The sequestration of Erk5 in a specific cellular compartment might be due to a differential expression of proteins that anchor Erk5 in a specific cellular compartment or to a specific post-translational modifications.

It is known that Erk5 is rapidly activated by PDGF-BB stimulation via Mek5 (51). However, our group has shown that PDGF-BB-induced Erk5 activation is dependent, in a way that is cell specific, also on Src (52) and on classical PKCs, PI3-kinase, Mek1/2, Mekk2, suggesting a tight co-regulation of Erk5 with phosphatidylinositol-3' (PI3) kinase / Akt and Erk1/2 pathways (48). Activated Erk5 phosphorylates and activates several transcription factors, including c-Myc, c-Fos, Sap1a, Fra-1, MEF2 and Smad1/5/8 (48,53).

Deregulation of Erk5 is connected with malignant properties of cells, for example increased metastatic potential, proliferation and angiogenesis, decreased cell death, as well as resistance to chemotherapy. Erk5 protein level is increased by amplification of chromosome locus 17p11 and by down-regulation of the microRNAs miR-143 and miR-145, and its activity is stimulated by several oncogenes (54,55).

PI3 kinase and PLCγ pathways

The phosphatidylinositol-3' (PI3) kinase signaling pathway can be activated by PDGF and plays an important role in regulating cell prolifera-

tion and maintaining the biological characteristics of malignant phenotype (56). PI3 kinase is a heterodimeric enzyme, consisting of two subunits, the regulatory p85 and the catalytic p110. In humans there are eight isoforms of the catalytic subunit and seven isoforms of the regulatory subunit, which have been grouped in three classes based on structure, regulation, and *in vitro* lipid substrate specificity (57).

In response to stimulation by many different growth factors, the regulatory subunit of class IA PI3 kinases is recruited to the membrane where it interacts with a consensus binding motif (pYXXM) in the receptor (58), and becomes phosphorylated at tyrosine 508 (59). PI3 kinase activation can also occur through the adaptor protein Grb2 that binds to the scaffolding protein Gab, which in turn recruits the regulatory subunit. A third way to activate PI3 kinase pathways is via Ras. Grb2 binds to and activates Sos, which in turn activates Ras, which interacts with and activates the catalytic subunit of PI3 kinase independently of the regulatory subunit (60,61).

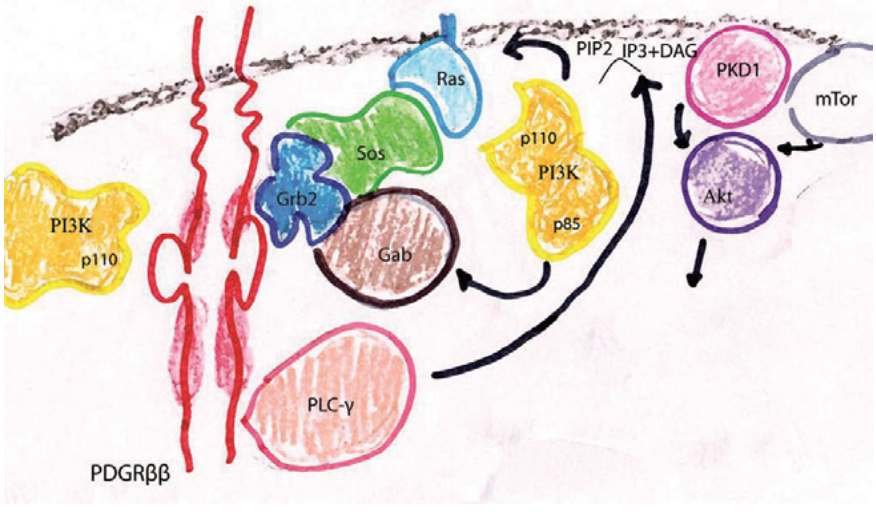

Figure 7. Schematic illustration of PI3 kinase and Akt activation. Not in scale.

Once activated, the catalytic subunit of PI3 kinase phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP_2) into phosphatidylinositol (3,4,5) -trisphosphate (PIP_3). PIP_3 binds the pleckstrin homology domain of phosphoinositide-dependent protein kinase 1 (PDK1) and Akt, translocating them to the plasma membrane. Akt, also known as Protein Kinase B (PKB), represents a central downstream target of PI3-kinase (62,63). At the plasma membrane, PKD1 thus phosphorylates Akt on Thr308 (64). In addition, Akt is phosphorylated on Ser473 by the mammalian target of rapamycin (mTOR) complex 2 which also promotes activation (65). Once

activated, Akt provides strong anti-apoptotic signals by phosphorylation and thereby inactivation of proapoptotic proteins and promoting Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling (*Figure 7*).

It has been reported by different research groups that PI3 kinase inhibition prevents Erk1/2 stimulation (66–69), indicating a possible cross-talk between PI3 kinase and Erk1/2 pathways.

There are two phoshoplipase Cγ (PLCγ) isoforms that are both activated by PDGF. PLCγ binds to the activated receptors at phosphorylated Tyr 988 and 1018 in PDGFRα (70) and at phosphorylated Tyr 1009 and 1021 in PDGFRβ (71–73). The catalytic activity of PLCγ is enhanced by PDGFR-mediated phosphorylation at Tyr 783 (74), leading to hydrolysis of PIP_3 into soluble inositol-(1,4,5)-trisphosphate (IP_3) and membrane associated diacylglycerol (DAG) (75). The increase in IP_3 results in an increased cytoplasmic Ca^{2+} accumulation, which together with DAG are required for protein kinase C (PKC) activation (76).

PDGF-induced cell cycle progression and chemotaxis requires PI3 kinase and PLCγ activation. Mice that bear a PDGFRβ in which the binding sites for PI_3 kinase, and PLCγ were mutated are born alive, however, they show defect neointima formation after vascular injury (77,78).

PI3-kinase and PLCγ pathways, being activated by PDGF, also promote the sorting to recycling of PDGFRβ , thereby slowing down its degradation (79,80).

NF-κB pathway

NF-κB was identified as transcription factor bound to the promoter of the kappa chain of immunoglobulins in B cells, but has subsequently been shown to be present in every cell type. A large number of stimuli coalesce on NF-κB activation, which can in turn mediate a broad range of biological processes, such as immune, inflammatory and stress responses, apoptosis and differentiation.

In mammals, the NF-κB family comprises five members: RelA (p65), RelB and c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively. All NF-κB proteins share a Rel homology domain responsible for DNA binding and dimerization. Dimeric NF-κB factors bind to κB sites in promoters and enhancers of a variety of genes.

In unstimulated cells, NF-κB localizes in an inactive state in the cytoplasm and interacts with inhibitory proteins known as IκBs (Inhibitor of κB) that masks the NLS of NF-κB.(81).

NF-κB activation requires degradation of IκB proteins that, in the canonical activation pathway, occurs primarily via activation of IκB kinase

(IKK). IKK is a heterodimer form by the catalytic subunits IKKα and IKKβ and a regulatory subunit called NEMO (NF-κB essential modulator) or IKK gamma.

PDGF, via the PI3 kinase/Akt pathway, activates IκK which phosphorylates two serine residues located in an IκB regulatory domain, leading to its ubiquitination and proteasomal degradation. Consequently, NF-κB can migrate to the nucleus and activate transcription of target genes (82) (*Figure 8*).

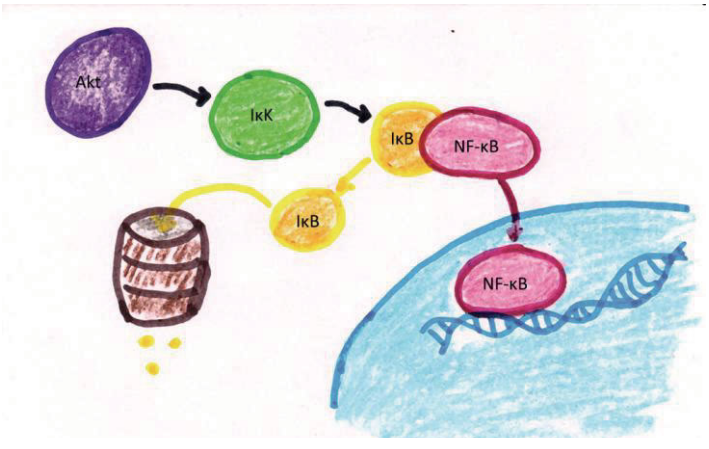

Figure 8. Schematic illustration of NF-κB activation. Not in scale.

NF-κB has been implicated in many hallmarks of cancer development and several reports have indicated that elevated NF-κB activation contributes to cancer cell survival and to reduce sensitivity to chemotherapeutic agents and ionizing radiation (83,84). NF-κB has often found overactivated in hematologic malignancies, in breast cancer (85) and glioblastoma where it is associated with poor prognosis (86). Moreover, NF-κB plays a central role in PDGF-induced transformation of mouse fibroblast cells (87).

NR4A1 pathway

The nuclear receptor subfamily 4 group A member 1 (NR4A1; alias Nur77, TR3, NGFIB) and its family members NR4A2 (Nurr1) and NR4A3 (Nor-1) function as ligand-activated transcription factors, however, no ligands have been identified. The three family members share high similarity in the DNA binding domain suggesting overlaps in responsive genes; however, the activation function 1 (AF-1) in the N-terminal region that regulates transactivation and co-factor recruitment, is very divergent suggesting the possibility of non-redundant functions (88) (*Figure 9*).

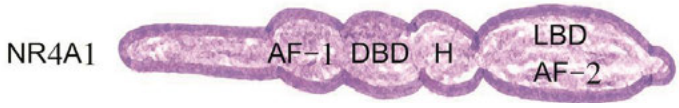

Figure 9. The NR4A1 is composed of the N-terminal activation function (AF)-1 domain, the DNA binding domain (DBD), the hinge region (H) and, at the C-terminal the ligand binding domain (LBD), also colled (AF)-2 domain.

NR4A1 orchestrates diverse and sometimes opposing cellular responses, such as, proliferation, apoptosis, metabolism, inflammation, energy balance and vascular remodeling (89). The basal expression of NR4A1 is low, but it can rapidly be induced by numerous signals, including PDGF. In the PDGF pathway, NR4A1 expression is promoted through activation of MAP kinases (90,91,89,92,93). Some publications point to NF-κB as mediator of NR4A1 expression in macrophages (94), and Leydig cells (95). Furthermore, cross-talk between NF-κB and NR4A1 has been proposed (96). NR4A1 overexpression in HEK293 cells leads to enhanced NF-κB transcriptional activity of primarily anti-apoptotic genes without significantly affecting the expression of pro-apoptotic factors induced by NF-κB (97). Thus, NR4A1 promotes the antiapoptotic response mediated by NF-κB while it inhibits the NF-κB inflammatory pathway competing with the NF-κB-binding sites in the promoter of IL-2 decreasing its transcription (96).

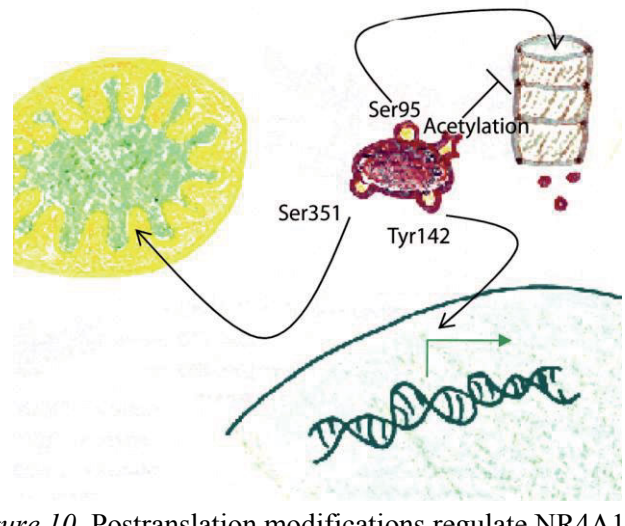

Figure 10. Postranslation modifications regulate NR4A1 localization and function. Not in scale.

The main part of the broad functions exerted by NR4A1 occurs through its ability to regulate the gene expression (98–100).

The transcriptional activity of NR4A1 is primarily regulated through expression and post-translational modifications (*Figure 10*). The phosphorylation state of NR4A1 has been suggested to control its subcellular localization (101–104), whereas acetylation stabilize the protein (105). Multiple functions of NR4A1 have been described in different cell types, which may be related to differences in subcellular localization; nuclear NR4A1 may regulate cell proliferation, whereas cytoplasmic NR4A1 may affect survival. A mitochondrial localization of NR4A1 was associated with an apoptotic effect by converting B-cell lymphoma (BCL)-2 from an anti- to a pro-apoptotic protein (106–108). NR4A1 in cancer has a dual role (109). It has been shown that, on one hand, NR4A1 acts as a tumor suppressor by inhibiting growth of pancreatic cancer cells (110), and a double knockout of NR4A1 and NR4A3 in mice was found to promote acute myeloid leukemia (111,112). On the other hand, NR4A1 is frequently overexpressed in lung cancer patients and correlates to poor prognosis (113), and it has been shown to confer a proliferative advantage to colon cancer cells as well as increasing the invasive behavior of breast cancer by enhancing transforming growth factor-β (TGFβ) signaling (114,115). Although, it is known that NR4A1 can exert a pro-apoptotic role, it has also been reported that its overexpression can protect cells from apoptosis (88). NR4A1 may contribute to tumor development also by promoting cell migration, invasion through inducing matrix metallopeptidase 9 (MMP-9) expression, inflammation, repair of DNA double-strand breaks, and by inducing vascular endothelial growth factor (VEGF)-dependent angiogenesis (89,94,95,116). Moreover, NR4A1 can also induce in gastric cancers cells the expression of stemness-related genes, i.e. Oct-4 (octamer-binding transcription factor 4) and Nanog, that are associated with an undifferentiated phenotype, self-renewal ability and epithelial to mesenchymal transition (95).

Modulation of PDGF signaling

Since PDGF signaling controls central cellular functions, such as proliferation and survival, it is not surprising that negative regulatory mechanisms operate at multiple levels in the pathway.

The number of cell-surface receptors available for ligand binding is regulated by ligand-induced endocytosis and degradation. After receptor activation, the PDGFR-ligand-complex is internalized and sorted toward lysosomal degradation or, driven by PI3 kinase and PLCγ over activation, recycled back to the cell surface (117–119,79,80). PDGFR downregulation is a ubiquitin-dependent process (120,121) that involves

E3 ubiquitin ligase Cbl (122–124). c-Cbl contains a SH2 domain through which it can bind activated PDGFR, facilitating receptor ubiquitination.

It has been proposed that dephosphorylation of receptors by phosphotyrosine phosphatases (PTPs) contributes to their inactivation (125,126). Despite that many PTPs (e.g. SHP-1, SHP-2, DEP-1, LMW-PTP and CD45) have been found to interact with PDGFRs, very little is known about their functions (127–131).

Down-regulation of the receptors is not the only mechanism to limit signaling; also the intracellular signaling pathways activated by the receptor are subjected to regulation. The MAP-kinases are subject to negative regulation at multiple levels, for example by Serine / Threonine phosphatases (Ser/ThrPP) or by PTP and, in some cases, by the compartmentalization of the signaling molecules. There is an apparent redundancy in the modulation of MAP kinases signaling. For example, at least 13 distinct phosphatases can directly dephosphorylate Erk1/2 alone (132) depending on the cell type and context.

The balance between activation and deactivation of MAP-kinases is of great importance since it has been found that the magnitude and duration of signaling plays a major role in determining the biological outcome (133).

MAP kinase phosphatases

Dual-specificity phosphatases (Dusp) represent a special group of PTPs that remove phosphate residues not only from tyrosine but also from threonine. Among these phosphatases, the MAP kinase phosphatases (MKP) catalyze the reaction within the activation loop of the MAP kinases thereby inactivating them (134,135). All the MKP family members have a kinase interaction motif (KIM) within the N-terminus, which is responsible for MAP kinase binding, in addition to the Dusp domain. Moreover, they contain either one or all of the following regions: NLS, NES, and a sequence reach in proline, glutamic acid, serine, and threonine (PEST). MKPs show different substrate specificities, tissue distribution, subcellular localization and regulation by extracellular stimuli. Based on these characteristics, MKPs have been traditionally classified into three groups. The first of these includes mitogen-and stress-inducible nuclear MKPs, the second group comprises cytoplasmic Erk-specific MKP and in the third are JNK/p38-specific phosphatases found both in nucleus and cytoplasm. Nevertheless, this classification is merely didactic and often the features are fluid and cell type dependent.

Transcriptional regulation is an important regulative mechanism. Many MKPs/Dusps are, in fact, expressed at low levels in unstimulated cells and induced as early response genes after activation of the MAPK

pathways establishing a negative-feedback mechanism (136,137). Other mechanisms of regulation are epigenetic modifications and the control of mRNA stability by protein and microRNA interaction (138–140) Post translational regulation is also a possible check-point of phosphatase activity. Protein stability can be controlled by ubiquitination and phosphorylation (134,141,142). Moreover, phosphorylation or acetylation of the KIM domain can change the charge profile modifying the MKP affinity and selectivity (143). Finally, also the catalytic activity in some cases can be regulated; some of these phosphatases require the binding of their substrate in order to assume the active conformation (e.i.Dusp1/MKP-1, Dusp2, DUSP6/MKP-3 and Dusp9/MKP-4) (144,145).

DUSP4/MKP-2

Among the different MKPs, we focus our interest on DUSP4/MKP-2 and DUSP6/MKP-3. DUSP4/MKP-2 is not well characterized; it has been described as an Erk1/2 selective phosphatase (146), mainly localized in the nucleus (147). The activity of DUSP4/MKP-2 seems to be mainly regulated by its expression which can be induced by stress and growth factors.

The role of DUSP4/MKP-2 in cancer is still poorly understood (148). DUSP4/MKP-2 expression has been connected to progression of pancreatic cancers (149,150), acute myeloid leukemia (151), familial medullary thyroid carcinoma, multiple endocrine neoplasia, and papillary thyroid carcinoma (149). DUSP4/MKP-2 expression correlate frequently with microsatellite instability in colorectal cancer (152) and it was increased in breast cancer (153). However, the DUSP4/MKP-2 gene is located in a chromosome area that commonly exhibits loss of heterozygosity in breast (154,155) and lung (156) cancer. In support of the idea that DUSP4/MKP-2 acts as a tumor suppressor, it has been found to be frequently epigenetically silenced in glioma; its overexpression inhibited glioblastoma cell growth (139). The expression levels of DUSP4/MKP-2 were also found to be higher in benign ovarian cancer compared with ovarian carcinomas (157). Furthermore, DUSP4/MKP-2 expression has been linked to cellular senescence (158,159) and increased sensitivity to chemotherapy in breast cancer (160,161). However, also on this topic there is not always accordance among studies, for example, it has been found that DUSP4/MKP-2 can rescue 293T cells from apoptosis upon UV or cisplatin exposure (162).

DUSP6/MKP-3

DUSP6/MKP-3 is a specific and potent regulator of Erk1/2 that also acts on p38 and possibly on Erk5 (163–167). DUSP6/MKP-3, besides its role in inactivation of Erk, may play a role in preventing the rapid nuclear translocation of Erk1/2 that normally follows mitogenic stimulation. It has been suggested that an DUSP6/MKP-3fragment, generated by activated caspase 3 cleavage, can sequester Erk1/2 in the cytoplasm without affecting Erk1/2 kinase activity since the fragment lacks a catalytic domain. The fragment, containing the KIM, competes with full-length DUSP6/MKP-3-3/DUSP6 for Erk1/2 (168).

DUSP6/MKP-3 has been found to be involved in both a positive feedforward mechanism, as well as a negative feedback mechanism, controlling Erk1/2 signaling. Upon activation of the Erk1/2 pathways, DUSP6/MKP-3is phosphorylated by Erk1/2 leading to DUSP6/MKP-3degradation, thus facilitating further Erk1/2 signaling. At a later phase, DUSP6/MKP-3transcription is stimulated in a Erk1/2-dependent manner and thus will eventually limit the extent of Erk1/2 signaling (169). Another study pointed out the involvement of the PI3 kinase/mTOR signaling pathway in phosphorylation and degradation of DUSP6/MKP-3(141).

Several studies suggest a role of DUSP6/MKP-3in cancer, but there is no consensus regarding its exact mechanism of action. For example, the DUSP6/MKP-3level was significantly reduced in many pancreatic cancer cell lines following hypermethylation of the promoter (170) or loss of heterozygosity (171). On the other hand, DUSP6/MKP-3expression has been found to be upregulated in several cancer types including glioblastoma (172), and papillary and poorly differentiated thyroid carcinoma (173); it has also been associated with tamoxifen resistance in breast cancer cell lines (174). The constitutive activation of Ras, common in many tumors, and the loss of expression of DUSP6/MKP-3may act synergistically to stimulate signaling though the Erk1/2 pahtway leading to uncontrolled cell growth. Furthermore, it was recently shown that high DUSP6/MKP-3expression can interfere with the metastatic process (175,176).

Cross-talk among signaling pathways

When a signaling pathway is activated, the effector proteins relay and amplify the signal interacting with several downstream players of the pathway. The complexity of the process arises not only from the great number of components but also by the fact that it is not merely linear and extensive cross-talks between different signaling pathways occur. In addition, signaling pathways are often regulated by positive feedback, that

allow to maintain the transmission of the signal active even when the initiating signaling molecule is removed, and negative feedback that generate a finely tuned response.

To extricate the pathways is even more difficult since the enormous variation existing among different cell types, a variation that may be larger than what observed between cells from different species. The cellular differentiation contributes to the diverse expression of enhancer or repressor of the transcription, of scaffold proteins, as well as other protein regulating the pathways leading to different response to the same stimulus. This accounts for the different localization and functions that the same protein can exert in different contexts and represents a further challenge in understanding the cell biology.

Role of PDGF in cancer

In adults PDGF-B signaling is primarily restricted to wound repair, tissues homeostasis and blood vessel formation and maintenance. Activation of the PDGF signaling activation is an important feature of different diseases involving excessive cell division for example, inflammation, fibrosis atherosclerosis and cancer (177).

The role of PDGF in malignant transformation was already illustrated when PDGF-B was characterized by amino acid sequencing revealing a homology to the simian sarcoma virus (SSV) protein $p28^{sis}$, encoded by the v-sis oncogene (178,179). Furthermore, it was possible to rescue the phenotype of SSV-transformed fibroblasts by inhibition of PDGF signaling (180).

Activating mutations in the PDGFR promote tumorigenesis; for example, some gastrointestinal stromal tumors are due to gain of function in PDGFRα (181).

PDGF signaling supports proliferation and survival of both tumor and stroma cells. The tumor microenvironment is not static and inert but, on the contrary, it contributes to tumor growth. In this context, PDGF stimulates pericytes, and smooth muscle cells, thereby promoting angiogenesis, and acts on stromal fibroblasts and myofibroblasts controlling the interstitial fluid pressure (181).

The autocrine stimulation is of great relevance in many different cancer types. It has been shown that in about 30% of the gliomas PDGF signaling is overactivated. Moreover, increased expression of PDGF or PDGFR has been observed in sarcomas, e.g. osteosarcoma, Kaposi's sarcoma, meningeomas, rhabdomyosarcoma, non-small-cell lung cancer and large granular lymphocyte leukemia of both T- and NK-cell origin. Moreover, translocations of the PDGFR gene that result in fusion proteins with different partners, have been found in chronic myelomonocytic leu-

kemia, in the hypereosinophilic syndrome and in systemic mastocytosis. The PDGFR gene is also amplified in some glioblastoma, anaplastic oligodendrogliomas, esophageal squamous cell carcinoma, and in pulmonary artery intimal sarcoma. This amplification increases the sensitivity to PDGF stimulation and it has been proposed that the high receptor density is responsible for ligand-independent receptor activation. Importantly, it has been shown that PDGFR expression correlates with invasiveness and unfavorable prognosis of human mammary carcinomas and lung carcinoma (34,181).

Considering the involvement of PDGF signaling in tumor initiation and progression, inhibition of this pathway is actively pursued in the development of anti-cancer drugs. Several types of PDGF antagonists have been produced, including neutralizing antibodies, receptor decoys, aptamers, low-molecular-weight inhibitors of the receptor kinases (182). In this context, PDGF and PDGFRs are validated therapeutic targets and several compounds, with different specificities, have been approved for clinical use, e.g. imatinib, sunitinib and sorafenib.

Present investigations

PDGF is a potent mitogenic factor and deregulation of its signaling is frequently associated with diseases including cancer. Understanding the process of PDGF signal transduction is essential to understand the development of cancer, and a prerequisite for identifying potential targets for therapeutic intervention or molecular markers to aid in determining diagnosis or prognosis.

The MAP kinases are important players of the PDGF cascade. In the first arm of this thesis, we focus on downstream targets of MAP kinases identifying NR4A1 as one of them.

The cellular response to PDGF results not only from the initiation of different downstream signaling pathways, but also from the modulation and termination of the signal. In the second arm of this work, we develop three projects where we investigated negative regulatory mechanisms of the PDGF pathway. We study the role of the dual specificity phosphatases DUSP4/MKP-2 and DUSP6/MKP-3 and we identify a new function for Erk5 that modulates PDGFR activation.

Our specific aims have been:

I	to investigate the functions and regulation of NR4A1 in PDGF pathways
II	to explore the role of DUSP4/MKP-2 in PDGF signaling
III	to explore the role of DUSP6/MKP-3 in PDGF signaling
IV	to elucidate Erk5 function in PDGF signaling regulation

Paper I

Eger, G., Papadopoulos, N., Lennartsson, J., Heldin, C.H. (2014) NR4A1 promotes PDGF-BB-induced cell colony formation in soft agar. *PLoS One*, 30;9(9):e109047

The orphan nuclear receptor NR4A1 is a transcription factor induced by e.g. PDGF and regulated by post-translational modifications. Consistently with other studies, we show that PDGF-BB induces a transient expression of NR4A1 in NIH3T3 via Erk1/2 and, to some extent, Erk5. The NR4A1 induction, promoted by Erk1/2, is very robust in fact, using the

chemical inhibitor CI-1040, NR4A1 expression was abolished whereas, interfering with Erk5, we did not observed change in the magnitude of the expression whereas the kinetics was delayed. A decrease of NR4A1 expression was also achieved by treatment with the NF-κB inhibitor BAY11-7082 or the proteasomal inhibitor MG132 that prevents IκB degradation and thus NF-kB activation.

It has been proposed that the complex biological function of NR4A1 depends on its subcellular localization. Therefore, we performed a cellular fractionation and found that the majority of NR4A1 localized to the cytoplasm and only a minor pool translocated to the nucleus after continued PDGF-BB treatment.

In NIH3T3 cells, NR4A1 only slightly increased the PDGF-BB-induced proliferation without affecting migration or survival. However, it was found to be important for the tumorigenic potential of glioblastoma cell lines U-105MG and U-251MG cell lines, where it promoted anchorage-independent growth, affecting both the number and diameter of the colonies.

In summary, we found that NR4A1 is expressed in response to PDGF-BB via Erk1/2 and NF-κB and have elucidated its important role for anchorage-independent growth in glioblastoma cells.

Paper II

Eger, G., Rorsman, C., Heldin, C.H., Lennartsson, J. (2016) Depletion of DUSP4 results in enhanced PDGFRβ cell surface clearance and suppresses PDGF-BB-induced activation of Erk5, Signal transducers and activators of transcription (Stat) 3, Src and PKC. *Manuscript*

All the components of the PDGF pathway are tightly regulated at multiple levels. MKPs represent one of the mechanisms that cells use to rapidly inactivate kinases. MKP family members have different substrate specificities, tissue distribution, subcellular localization, expression level and they respond in different way to extracellular stimuli.

We report here that, in primary human fibroblasts (AG1523), PDGF-induced DUSP4/MKP-2 expression was impaired using siRNA targeting p53 or using the Mek1/2 inhibitor CI-1040. Moreover, we observed that DUSP4/MKP-2 siRNA decreased activation of the Erk5 MAP kinase without affecting Erk1/2 MAP kinases. Furthermore, we found that PDGFRβ was internalized and downregulated faster in cells lacking DUSP4/MKP2. A decrease in the PDGFRβ activation was correlated with a specific reduced activation of Stat3, Src and PKC, and a partial decrease of p38, Stat1/5 and PLCγ activation, suggesting an important role of DUSP4/MKP-2 in PDGF signaling.

Paper III

Razmara, M., **Eger, G**., Rorsman, C., Heldin, C.H., Lennartsson. J. (2012) MKP-3 negatively modulates PDGF-induced Akt and Erk5 phosphorylation as well as chemotaxis. *Cell Signal*, 24(3):635-40

DUSP6/MKP-3 is a dual specificity phosphatase described to be selective for Erk1/2. In our study, we illustrate that, in NIH3T3 cells, PDGF activates a negative feedback loop that involves DUSP6/MKP-3 and decrease the migratory ability that it confers to the cells. DUSP6/MKP-3 expression is induced in response to PDGF-BB and this induction is dependent on the MAP kinase Erk1/2 and PI3 kinase, but is Erk5-independent. Interfering with Mek1/2 or PI3 kinase using the inhibitors CI-1040 or LY-294002, respectively, the PDGF-BB-induced DUSP6/MKP-3 expression was inhibited. In accordance with other reports (167,183), silencing of DUSP6/MKP-3 expression increased the basal level of Erk1/2 and p38 phosphorylation, but we did not observe changes in the PDGF-BB-induced phosphorylation level. However, we found an enhanced PDGF-BB-mediated activation of Erk5 and Akt in cells depleted of DUSP6/MKP-3.

Based on the premise that both Erk5 and PI3 kinase pathways are linked to increased migration, we investigated whether DUSP6/MKP-3 downregulation affects PDGF-BB-induced chemotaxis. Indeed, we observed a more pronounced chemotactic response when DUSP6/MKP-3 was silenced. Although Akt and Erk5 have been implied to cell survival, we were unable to detect any augment of the ability of PDGF-BB to either induce DNA synthesis or to protect cells from starvation-induced apoptosis in cells where the DUSP6/MKP-3 level was reduced compared to control cells. However, DUSP6/MKP-3 downregulation increased the level of apoptosis in unstimulated cells.

In conclusion, DUSP6/MKP-3 establishes a negative cross-talk between Erk1/2 and Erk5 and mediates an auto-limitating function of PI3 kinase that not only promotes Akt phosphorylation but also negatively modulates it (*Figure 11*). Our data thus support the notion that DUSP6/MKP-3 exerts a tumor suppressive function.

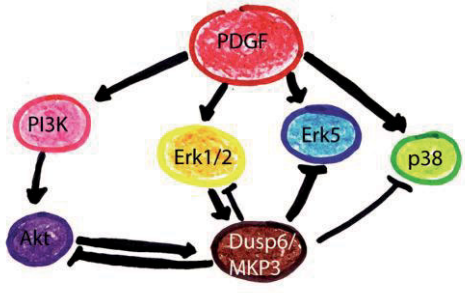

Figure 11. Proposed effect of DUSP6/MKP-3 in PDGF pathways.

Paper IV

Voytyuk, O., **Eger, G.**, Heldin, C.H., Lennartsson, J. (2016) Erk5 promotes prolonged PDGFRβ activation by limiting ligand-induced receptor internalization and degradation. *Manuscript*

PDGF signaling induces cell growth, survival and migration, by activation of MAP kinases and other signaling pathways. However, the function of the Erk5 MAP kinases is still not fully understood.

In this work, we have studied the role of Erk5 in the regulation of PDGF-BB-induced signal transduction in NIH3T3 cells.

PDGF-BB stimulation induces Erk5 expression at both mRNA and protein level, as well as its phosphorylation and activation. We found that Erk5, in turn, modulates the PDGFRβ activation. After Erk5 depletion, the receptor incurs in a more powerful activation that result in a faster internalization and degradation suggesting a regulative role of Erk5 in the PDGFRβ signaling.

Future Perspectives

Paper I

In this paper we showed that NR4A1 expression is controlled by both MAP kinases and NF-κB. To further characterize this mechanism, we would like to investigate if MAP kinases signaling converges on NF-κB thereby promoting NR4A1 expression. We will use NF-κB luciferase constructs and study the effect of Erk1/2 and Erk5 RNA interference on the reporter activity.

It has been described that phosphorylation of NR4A1 controls it subcellular localization. We found that NR4A1 can be phosphorylated by Akt at Ser351 and by other kinases in some undefined sites. Mutating the putative sites of these phosphorylations and studying the effect on subcellular localization could give information on important domains of the protein and help to determine regulatory kinases that act on it.

We will expand our finding that NR4A1 affects anchorage-independent growth of glioblastoma cells, including more cell lines. We will also try to establish the mechanism by which NR4A1 expression facilitates the growth in soft agar testing in this condition MAP kinase inhibitors.

The interest for NR4A1 aroused from the analysis of an Affymetrix gene expression array. We compared the ability of PDGF to influence gene expression of mouse embryonic fibroblasts in which the Erk5 gene has been deleted, with a clone of these cells in which the Erk5 level has been reconstituted (184). Beyond NR4A1, we also found other genes that were strongly induced in our control cells, but not in cells in which Erk5 has been deleted (e.g. Early growth response protein - Egr), and conversely genes that were suppressed by PDGF and whose expressions were enhanced in cells lacking Erk5 (e.g. Slingshot homolog). In the future, we want to focus on these genes in order to get a deeper knowledge of Erk5 downstream targets and thus of its functions.

Paper II and III

The importance of protein phosphatases has for a long time been quite neglected. We believe that they are important regulators of the signaling cascades thus they will continue to be one of the centers of our research.

We will identify the components of the PDGF signaling pathway that, via p53, induce DUSP4/MKP-2 expression. We will thus perform a luciferase reporter assay in the presence of signaling inhibitors.

An important step in our work will be the validation of the siRNA targetting DUSP4/MKP-2 using two alternative sequences in order to avoid possible off target effects.

In order to further elucidate the role of DUSP4/MKP2, we will carry out functional assays in cells depleted of DUSP4/MKP-2 and in control condition. PDGF-induced migration will be evaluated both with a chemotaxis assay measuring the cells migrated through microplate filters toward different PDGF concentrations and with wound healing assay in the presence or absence of PDGF in order to distinguish between chemotaxis and random migration. Proliferation will be measured with a EdU incorporation assay using different PDGF concentrations. Apoptosis and cell cycle will be analyzed by cytofluorometry. The ability to confer malignant properties will be investigated by recording colony number and size formed in soft agar in presence or absence of PDGF. This aspect is especially relevant since DUSP4/MKP-2 seems to affect Stat3 phosphorylation, that, we have previously demonstrated to be important for promoting anchorage-independent growth.

Paper IV

We are interested to explore the regulative role exerted by Erk5 on the PDGF signaling and understand the functional significance in terms of cellular migration, proliferation and apoptosis.

Furthermore, we will perform an in-vitro kinase assay where we will incubate recombinant Erk5 with immunoprecipitated PDGFRβ in order to investigate if Erk5 exert its regulative function by direct phosphorylation on the receptor. In case we will not observe direct phosphorylation, we will extend the investigation using as substrate the ubiquitin ligase Cbl that, as it has been previously demonstrated in our lab, it is able to bind the PDGFRβ and regulate its activity.

Long-term goals are to establish the importance of Erk5 in cancer development and to identify and validate the usefulness of Erk5 selective inhibitors in cancer therapy. An initial step towards understanding the importance of Erk5 in cancer will be to study, with immunohistochemical techniques, Erk5 expression in tumor material from patients with

glioblastoma, in which PDGF signaling is often overactive. This part of the work will be performed in collaboration with Dr. Simon Ekman the Oncology clinic at Karolinska Institute. This will enable to see whether Erk5 expression changes upon tumor formation and progression and if it correlates with disease aggressiveness and treatment outcome.

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Who thinks that Sweden is a cold place, clearly, has never been to the Ludwig Institute.

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Carl-Henrik (**Calle**) Heldin. I incredibly appreciate that you committed your life to science without forgetting be Human. Thank you for your brilliant suggestions at the numerous Calle meetings; you have passed on passion and enthusiasm to all of us. Despite your thousands of appointments, tasks and meetings, you are not hectic and you have always found time to help me and discuss. It is mainly because of your great personality that we have such a friendly atmosphere, here in Ludwig.

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(*continue...*)

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