UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1538

Studies of PDGF receptor signaling *in vitro* and *in vivo*

MARIA TSIOUMPEKOU

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2019

ISSN 1651-6206
ISBN 978-91-513-0571-4
urn:nbn:se:uu:diva-376248

Dissertation presented at Uppsala University to be publicly examined in B42, Biomedical Center, Husargatan 3, Uppsala, Friday, 22 March 2019 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Jean-Baptiste Demoulin (De Duve Institute, Université Catholique de Louvain, Brussels, Belgium).

Abstract
Tsioumpekou, M. 2019. Studies of PDGF receptor signaling *in vitro* and *in vivo*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1538. 67 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0571-4.

Platelet-derived growth factor receptor (PDGFR) signaling is essential for proliferation, migration and survival of cells of mesenchymal origin; however, its deregulation has been associated with various diseases, including cancer. The aim of this thesis was to clarify the molecular mechanisms of PDGFR signaling regulation. We have studied PDGFR downregulation, identified the E3 ligases and deubiquitinases (DUBs) acting on the receptor, characterized the role of the downstream effector Erk5, as well as elucidated the role of PDGFRβ isoform in tumor growth and angiogenesis.

As Erk5 activation has been associated with tumorigenesis, it is important to delineate the pathway from activated PDGFR to Erk5. Here, we demonstrate not only a complex mechanism for PDGF-induced Erk5 activation that involves Mek5, Mekk2, Mek1/2, PI3K and classical PKCs, but also a novel function for Erk5 by showing that PDGF-BB affects BMP-Smad signaling in an Erk5 pathway-dependent manner, indicating a crosstalk between tyrosine kinase receptor and serine/threonine receptor signaling.

By investigating PDGFRβ downregulation, we demonstrated that ubiquitination of PDGFRβ, mediated by Cbl-b and c-Cbl, is essential for the receptor internalization, signaling, as well as downstream biological responses. Additionally, as ubiquitination is a reversible post-translational modification, we identified USP4 as one of the DUBs acting on PDGFRβ and discovered that USP4 interacts with PDGFRβ, removing both K48- and K63-linked polyubiquitin chains, and increases its stability, in both normal and cancer cells.

Although several studies have highlighted the therapeutic benefit of PDGFR inhibition in cancer treatment, all available PDGFR kinase inhibitors have secondary targets; consequently, the details underlying the importance of PDGFR in tumorigenesis remain unknown. By targeting specifically PDGFRβ in the stroma of various tumor models, we showed that specific inhibition of PDGFRβ signaling suppresses growth of tumors with high levels of PDGF-BB, whereas the multi-target kinase inhibitor imatinib has less effect, indicating the significance of selective targeting of PDGFRβ.

Our data provide new insights into the molecular events underlying PDGFRβ signaling and downregulation, highlight its importance as cancer therapeutic target and lead the way for further discoveries.

Keywords: PDGF, signaling, cancer, Erk5, kinase, Cbl, ubiquitination, deubiquitinase, pericytes, tumor growth, angiogenesis, low molecular weight inhibitors

Maria Tsioumpekou, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-513-0571-4
urn:nbn:se:uu:diva-376248 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-376248)

Αφιερωμένο στους γονείς μου
Dedicated to my parents

List of Papers

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

I **Tsioumpekou, M**., Papadopoulos, N., Burovic F., Heldin C.-H., Lennartsson J. (2016). Platelet-derived growth factor (PDGF)-induced activation of Erk5 MAP kinase is dependent on Mekk2, Mek1/2, PKC and PI3-K, and affects BMP signaling. *Cellular Signaling*, 28:1422–1431

II Rorsman C., **Tsioumpekou M.**, Heldin C-.H., Lennartsson J. (2016). The ubiquitin ligases c-Cbl and Cbl-b negatively regulate PDGF-BB induced chemotaxis by affecting PDGFRβ internalization and signaling. *Journal of Biological Chemistry*, 27: 11608-18

III **Tsioumpekou M.**, Sarri N., Papadopoulos N., Lennartsson J., Heldin C.-H. (2018). The ubiquitin-specific protease 4 de-ubiquitinates and stabilizes PDGF receptor β. *Manuscript*

IV **Tsioumpekou M.***, Cunha S.*, Ma H., Åhgren A., Cedervall J., Olsson A.-K., Heldin C.-H., Lennartsson J. (2018). Specific targeting of PDGFRβ inhibits growth and angiogenesis of tumors with high PDGF-BB expression. *Under revision*

* Indicates equal contribution to the work

Reprints were made with permission from the respective publishers.

Other publications not included in the thesis:

I Reyhani V., **Tsioumpekou M.**, van Wieringen T., Rask L., Lennartsson J., Rubin K. (2017). PDGF-BB enhances collagen gel contraction through a PI3K-PLCγ-PKC-cofilin pathway. *Scientific reports*. 7: doi:10.1038/s41598-017-08411-1

II Delis C., Krokida A., Tomatsidou A., Tsikou D., Beta R., **Tsioumpekou M.**, Moustaka J., Balatsos N., Papadopoulou K. (2016). AtHesperin: A novel regulator of circadian rhythms with poly(A)-degrading activity in plants. *RNA Biology*. 13:68-82

Contents

Abbreviations

Abl	Abelson
Akt	RAC-alpha serine/threonine protein kinase
αSMA	α smooth muscle actin
BMP	Bone morphogenetic protein
BMDC	Bone marrow-derived cells
Bcr-Abl	Breakpoint cluster region-Abelson
CAF	Cancer-associated fibroblast
Cbl	Casitas B-lineage lymphoma
CML	Chronic myeloid leukemia
CXCR4	CXC-chemokine receptor 4
DAG	Diaglycerol
DFSP	Dermatofibrosarcoma protuberans
DUB	Deubiquitinase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
Erk	Extracellular regulated signal kinase
GAP	GTPase-activating protein
GBM	Glioblastoma multiforme
GIST	Gastrointestinal stromal tumor
Grb	Growth factor receptor bound protein
IGF	Insulin growth factor
IP3	Inositol 1,4,5-triphosphate
MAPK	Mitogen activated protein kinase
Mek	MAPK/Erk kinase
MVB	Multivesicular bodies
1-NaPP1	1-(1,1-dimethylethyl)-3-(1-naphthalenyl1)-1H-pyrazolo[3,4-d]pyrimidin-4-amine
NSCLC	Non-small-cell lung carcinoma
NG2	Neuron-glial antigen 2
NHERF	Na+/H+ exchange regulatory family
NLS	Nuclear localization signal
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	3-phosphoinositide-dependent protein kinase-1

PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3'-kinase
PIP2	Phosphatidylinositol-4,5-bisphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
Ras	Rat sarcoma protein
RTK	Receptor tyrosine kinase
RCC	Renal cell carcinoma
SH2	Src homology 2
SHP2	Src homology phosphatase 2
Smad	Small mothers against decapentaplegic
Src	Rous sarcoma oncogene cellular homolog
Stat	Signal transducer and activator of transcription
TGFβ	Transforming growth factor β
TKI	Tyrosine kinase inhibitor
Ub	Ubiquitin
USP	Ubiquitin-specific processing protease
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMCs	Vascular smooth muscle cells

Introduction

Cells, often referred to as the building blocks of life, rely on a highly complicated but ordered signaling network in order to communicate with each other and the environment. Cellular events, such as proliferation, migration and survival, are regulated by signals a cell receives by binding of ligands to various receptors at the cell surface. An important family of receptors is the receptor tyrosine kinases (RTKs), which are not only critical regulators of physiological cell functions, but also key drivers in pathological conditions. 58 genes encoding receptor tyrosine kinases exist among 90 tyrosine kinase genes identified in the human genome (Robinson *et al.*, 2000). Increased amount or activity of RTKs is often associated with the development of malignant diseases, highlighting the need to investigate in detail the signaling pathways downstream of these receptors as well as their roles in tumorigenesis (Lemmon and Schlessinger, 2010).

Cancer is the second leading cause of death globally with almost 10 million deaths accounted in 2018 (WHO, World Health Organization). Although great progress in understanding and treating cancer has taken place over the last decades, still the late-stage presentation of the disease, the various risk factors and most importantly, the extensive tumor heterogeneity observed even in the same cancer patient, hinder the efforts of finding a cure. Cancer is characterized by uncontrolled cell proliferation, dissemination of tumor cells and formation of metastases, with the latter being the main cause of death for cancer patients (WHO, World Health Organization). As described by Hanahan and Weinberg as the hallmarks of cancer, cancer cells acquire various traits during tumor progression, i.e. sustained proliferative signaling, evading growth arrest and avoiding immune destruction, inducing angiogenesis and inflammation, promoting metastasis, reprogramming energy metabolism, as well as cell death resistance (Hanahan and Weinberg, 2000, 2011). However, cancer cells are not "single players"; instead, they interact with various adjacent cell types as well as the extracellular matrix (ECM), resulting in a complex tumor microenvironment that resembles a battlefield (Polyak *et al.*, 2009; Hanahan and Coussens, 2012). The tumor microenvironment, first mentioned in Stephen Paget's "seed and soil" theory in the late 19th century, consists of immune cells, fibroblasts, bone marrow derived cells (BMDCs), pericytes, endothelial cells and ECM; importantly, it is known to shape therapeutic responses and resistance (Paget, 1889; Ribatti *et al.*, 2006).

This present thesis focuses on the platelet-derived growth factor (PDGF)/ platelet-derived growth factor receptor (PDGFR) signaling pathway and its involvement in tumorigenesis. Specifically, it aims to clarify the molecular mechanisms underlying PDGFR downregulation, its downstream effectors and its role in tumor development and angiogenesis.

PDGF ligands and receptors

PDGF, discovered more than four decades ago, is a potent mitogen that stimulates proliferation, survival and migration of cells of mesenchymal origin (Ross *et al.*, 1974; Westermark and Wasteson, 1976; Heldin *et al.*, 1979; Antoniades, 1981). The PDGF family consists of four polypeptide chains, which form five functional disulphide-bonded dimers that bind to PDGFRα and PDGFRβ on the cell surface. Ligand binding to the receptors results in receptor homo- or heterodimerization and autophosphorylation, which triggers the initiation of downstream signaling pathways (Heldin *et al.*, 1981, 1988, 1989, Claesson-Welsh *et al.*, 1988, 1989; Heldin, 2013).

All PDGF isoforms are synthesized as precursor molecules and their activation requires proteolytic cleavage (*Figure 1*). Specifically, PDGF-AA and PDGF-BB, known as the classical PDGF ligands, are cleaved and activated intracellularly, whereas the novel PDGF ligands, PDGF-CC and PDGF–DD, are secreted as latent factors and their activation depends on cleavage by tPA or plasmin and uPA or matripase, respectively (Li *et al.*, 2000; Bergsten *et al.*, 2001; Fredriksson *et al.*, 2004; Reigstad *et al.*, 2005; Chen *et al.*, 2013).

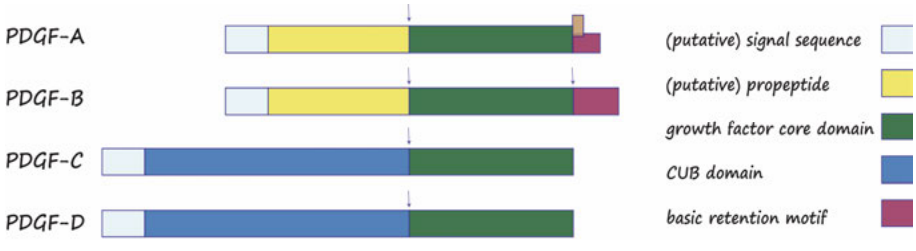

Figure 1. Structure of PDGF precursor isoforms. The classical PDGFs (PDGF-A and PDGF-B) and novel PDGFs (PDGF-C and PDGF-D) contain basic retention motifs and CUB domains, respectively. The arrows indicate proteolytic cleavage sites. Two alternatively spliced isoforms of PDGF-A exist, which differ in their C-terminal regions (Rorsman *et al.*, 1988).

The five PDGF dimers are recognized by the two PDGFRs, which are structurally similar, but have different ligand-binding specificities. Particularly, PDGFRs, members of the class III family of RTKs, are composed of an extracellular part with five immunoglobulin (Ig)-like domains, a single transmembrane helix, a juxtamembrane segment, a tyrosine kinase domain and a carboxy-terminal tail in the intracellular part. Ligand binding to the receptor is mediated via the immunoglobulin-like domains 2 and 3 triggering receptor dimerization, which is further stabilized by interactions between the immunoglobulin-like domains 4 and 5 (Omura *et al.*, 1997; Miyazawa *et al.*, 1998;

Heldin and Lennartsson, 2013). Various PDGF/PDGFR interactions have been proposed and observed over the years; however, only few have been characterized to be important *in vivo*, as illustrated in *Figure 2*.

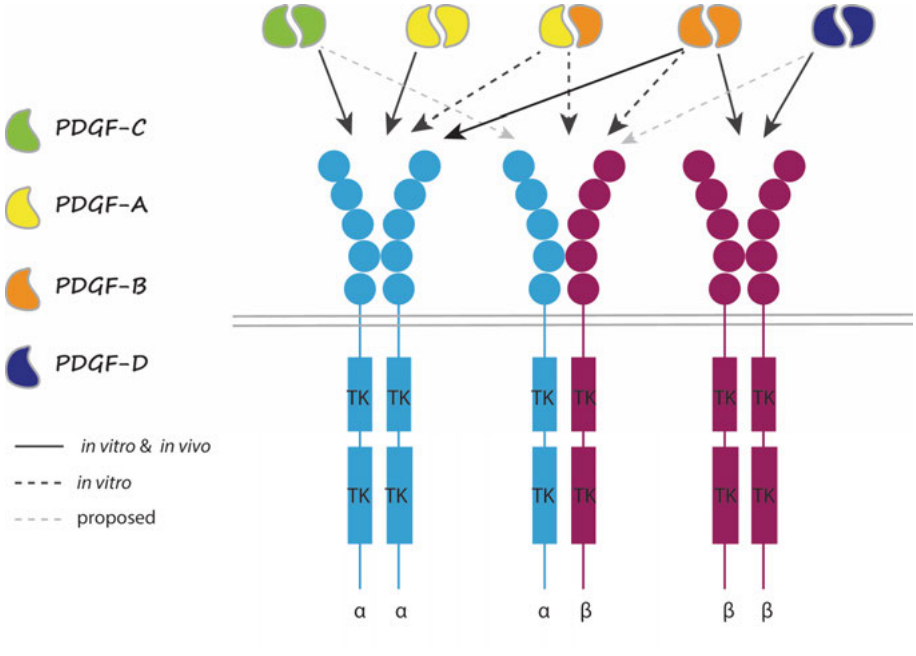

Figure 2. Binding of PDGF isoforms to the PDGFR α and β homodimers or heterodimer based on the receptor ligand-binding specificities. The four PDGF homodimers (PDGF-AA, -BB, -CC and -DD) and the heterodimer (-AB) have been shown to bind to the two PDGFRs with different affinity. Specifically, ligand binding illustrated by black arrows has been observed both *in vitro* and *in vivo*, whereas dashed black arrows indicate interactions only observed in cell culture experiments. Grayscale dashed arrows depict weak proposed interactions or conflicted results.

PDGF/PDGFR expression and biological function

The expression of PDGF and PDGFRs is spatiotemporally regulated *in vivo* during development and in certain physiological conditions. Furthermore, both PDGF and PDGFR expression is dynamic and responsive to a variety of stimuli, such as cytokines and growth factors (Andrae *et al.*, 2008; Heldin *et al.*, 2018).

PDGF ligands are secreted by epithelial and endothelial cells, macrophages and glial cells, as well as activated platelets. On the other hand, PDGFRs are expressed in cells of mesenchymal origin such as fibroblasts, vascular smooth

muscle cells, pericytes and mesenchymal as well as oligodendrocyte progenitors (Fredriksson *et al.*, 2004; Tallquist and Kazlauskas, 2004; Demoulin and Montano-Almendras, 2012).

Over the years, various knockout and transgenic mouse experiments have provided insights into the importance of PDGF signaling in both physiological and pathological conditions. Knockout of *pdgfb* and *pdgfrβ* genes exhibit similar phenotypes, as *pdgfb*- and *pdgfrβ*-null mice die perinatally showing severe hemorrhaging due to a significant lack of mural cells in their vasculature (Levéen *et al.*, 1994; Soriano, 1994; Betsholtz, 1995, 2003; Lindahl, 1997; Hellstrom *et al.*, 1999). These findings highlight the key role of PDGF-BB and PDGFRβ in angiogenesis, which will be discussed further on page 35. In contrast, knockout of the *pdgfd* gene resulted in a much milder phenotype, with mice being viable and fertile but with elevated blood pressure and morphological defects in pericytes of the cardiac vasculature (Gladh *et al.*, 2016; Heldin *et al.*, 2018).

Although *pdgfrβ*-null mice displayed vascular defects, knockout of *pdgfrα* revealed the importance of PDGFRα in oligodendrocyte and neural development in the central nervous system. Specifically, skeletal abnormalities as well as defects in the development of oligodendrocytes, hair follicles, teeth and spermatogenesis were a few of the symptoms that the *pdgfrα*-null mice exhibited; however, deletion of the genes coding for the ligands binding to PDGFRα resulted in different phenotypes (Soriano, 1997). Mice lacking PDGF-A presented defects in lung development as well as oligodendrocyte development, whereas the phenotype of *pdgfc*-null mice varied in different mouse strains and genetic backgrounds (Boström *et al.*, 1996; Ding *et al.*, 2004; Fredriksson *et al.*, 2012; Folestad *et al.*, 2018).

The differences in the phenotype of mice lacking PDGFRα and PDGFRβ, as well as the overall roles of the two receptors, can be explained by their distinct expression patterns and signaling properties. Particularly, the two receptors are usually expressed by different cell types, but even in cells where they coexist, their signaling outcome varies (Lindahl and Betsholtz, 1998). Knock-in mouse experiments swapping the intracellular signaling domains of the two receptors revealed that the two PDGFRs trigger the initiation of both overlapping and distinct signaling events (Klinghoffer *et al.*, 2001). Although PDGFRα expression is more critical during embryogenesis, PDGFRβ plays an important role also in adulthood, as it is involved in wound healing and angiogenesis, regulation of interstitial fluid pressure and blood-brain barrier integrity (Cooper *et al.*, 1994; Rodt *et al.*, 1996; Hoch and Soriano, 2003; Lindblom *et al.*, 2003; Lidén *et al.*, 2006; Heldin *et al.*, 2010).

PDGF signaling

PDGF stimulation induces homo- as well as heterodimerization of the two PDGFRs, which results in receptor autophosphorylation, and thereby, initiation of signaling pathways. In absence of ligand, three different mechanisms involving the activation loop, the juxtamembrane segment and the C-terminal tail seem to be responsible for PDGFR autoinhibition and inactive state (Chiara *et al.*, 2004; Demoulin and Essaghir, 2014). Upon ligand binding and subsequent receptor dimerization, conformational changes occur resulting in release of these autoinhibitory interactions and *in trans* autophosphorylation of certain tyrosine residues in the intracellular part of the receptor (Kazlauskas and Cooper, 1989; Baxter *et al.*, 1998). These autophosphorylation sites (10 sites in PDGFRα and 11 sites in PDGFRβ), when phosphorylated, act as docking sites for various Src homology 2 (SH2)-domain-containing signaling molecules leading to their activation and initiation of signaling pathways downstream of the receptor, which result in specific cellular responses (Kazlauskas *et al.*, 1991; Heldin and Lennartsson, 2013).

As depicted in *Figure 3*, several molecules have been shown to interact with PDGFR upon ligand stimulation; some of these molecules have an intrinsic enzymatic activity, i.e. Src, phospholipase C-γ (PLC-γ), Src homology phosphatase 2 (SHP2), Rat sarcoma protein-GTPase activating protein (Ras-GAP) and phosphatidylinositol-3'-kinase (PI3K), whereas others, such as growth factor receptor-bound protein 2/7 (Grb2/7), non-catalytic region of tyrosine kinase adaptor protein 1, and Src homology and collagen, are not enzymes themselves but can function as adaptor molecules (Basciani *et al.*, 2010; Heldin and Lennartsson, 2013). Binding specificity of signaling molecules to the different receptor dimeric complexes has also been observed and Ras-GAP and Crk are two characteristic examples, as they bind only to PDGFRβ and PDGFRα homodimers, respectively (Heldin *et al.*, 1999). Apart from SH2-domain-containing molecules, other binding partners for PDGFR have been identified; Na+/H+ exchange regulatory factors (NHERFs) adaptor proteins bind to the C-terminal tail of PDGFRβ via their PDZ domains, and Alix binds constitutively to PDGFRβ and regulates the receptor downregulation (Demoulin *et al.*, 2003; James *et al.*, 2004; Lennartsson *et al.*, 2006).

The signaling pathways of more importance and related to this thesis work are described below.

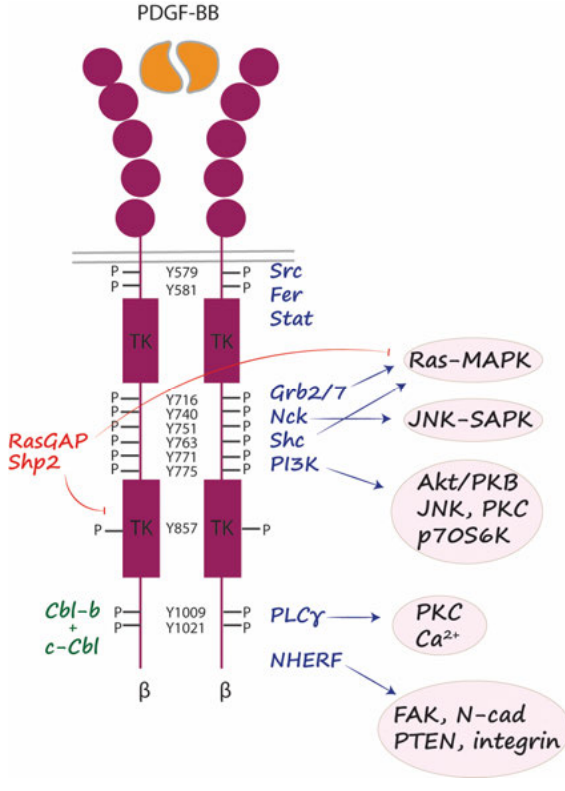

Figure 3. Signaling molecules and pathways activated upon binding of PDGF-BB to PDGFRβ homodimer. Ligand binding induces receptor homo/heterodimerization and autophosphorylation of various tyrosine residues in the intracellular part of the receptor. These phosphorylated tyrosine residues act as docking sites for several proteins that bind to the receptor, become activated and either trigger the initiation of signaling pathways or promote the receptor internalization and downregulation. Signaling is modulated both positively (blue arrows) and negatively (red arrows), ensuring a tight regulation of signaling molecules and perfect tuning of cellular responses. The two E3 ubiquitin ligases, that have been shown to bind to PDGFRβ, are illustrated in green.

MAP kinase signaling pathways

Much effort has been made over the years to elucidate the various signaling cascades downstream of PDGFR. The mitogen activated protein (MAP) kinase module, one of the most evolutionary conserved pathways in eukaryotes, plays an important role in the regulation of cellular processes such as proliferation and differentiation. This module consists of three members (MAPKKK, MAPKK and MAPK), whose activation is sequential and results in the transduction of the mitogen signal from the activated receptor to the nucleus (Rossomando *et al.*, 1989; Stokoe *et al.*, 1992; Nishimoto and Nishida, 2006; Morrison, 2012).

There are three families of MAP kinases identified in mammals: extracellular regulated signal kinases (Erks; consisting of the classical Erk1/2, Erk5 as well as the atypical Erk3, Erk4 and Erk7), p38 (p38α-δ) and c-Jun N-terminal kinases (Jnk1-3) (Cobb *et al.*, 1991; Cobb, 1999). Although each MAP kinase family regulates particular cellular events, for example p38 and Jnks have been implicated in apoptosis and inflammation, several reports have suggested that there is extensive cross-talk among them (Pearson *et al.*, 2001; Sharma *et al.*, 2003; McClean *et al.*, 2007). To set the ground for the focus of Paper I in this thesis, Erk5 signaling pathway will be discussed in more detail.

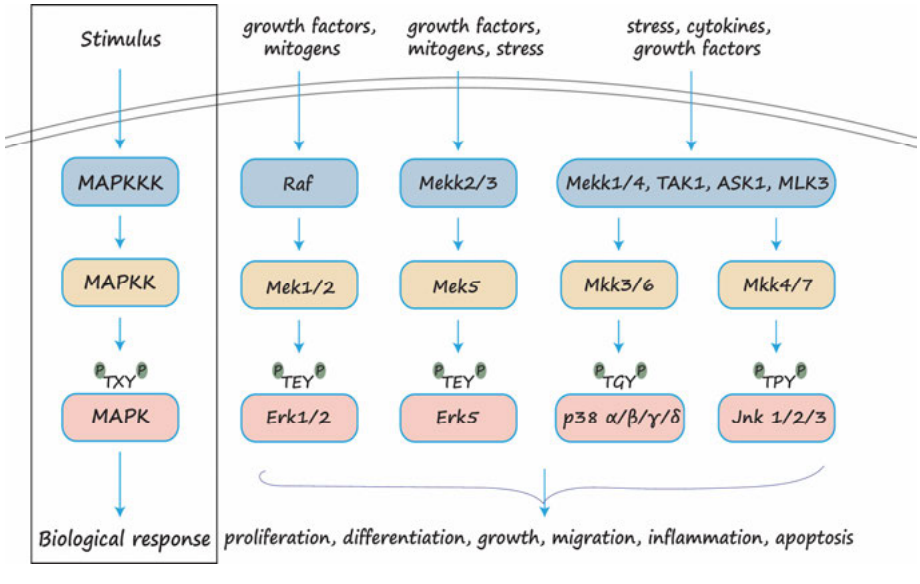

Figure 4. Simplified demonstration of the most studied MAP kinase pathways. The classical MAP kinase module consists of a three-tiered kinase cascade that can be activated by various extracellular stimuli and lead to a wide range of biological responses.

Structure and role in signaling of Erk5 pathway

One of the less understood MAP kinase modules is the extracellular signal regulated kinase 5 (Erk5) signaling cascade. Erk5, also known as big MAP kinase 1 (Bmk1), consists of 816 amino acid residues in humans and although its N-terminal and kinase domain share at least 50% identity with Erk2, it differs structurally from the other MAP kinases due to its unique C-terminal tail (Lee *et al.*, 1995; Zhou *et al.*, 1995; Nishimoto and Nishida, 2006). This large C-terminal tail consists of a nuclear localization signal (NLS), two proline-rich regions as well as a transcriptional activation domain, suggesting that Erk5 can act as both a kinase and a transcription factor (Lochhead *et al.*, 2012; Nithianandarajah-Jones *et al.*, 2012). Although Erk5 contains an NLS, many studies have shown that in non-stimulated conditions Erk5 is cytoplasmic (Kondoh *et al.*, 2006; Gomez *et al.*, 2016; Le *et al.*, 2018). This can be explained by an interaction between the N- and C-terminal halves of Erk5 that hides the NLS in unstimulated cells. Upon stimulation, Erk5 becomes phosphorylated leading to disruption of this interaction, resulting in translocation of activated Erk5 into the nucleus where it can activate transcription factors such as c-Fos, c-Myc, MEF2C and Fra-1 (Terasawa *et al.*, 2003; Nishimoto and Nishida, 2006; Yao *et al.*, 2010; Drew *et al.*, 2012).

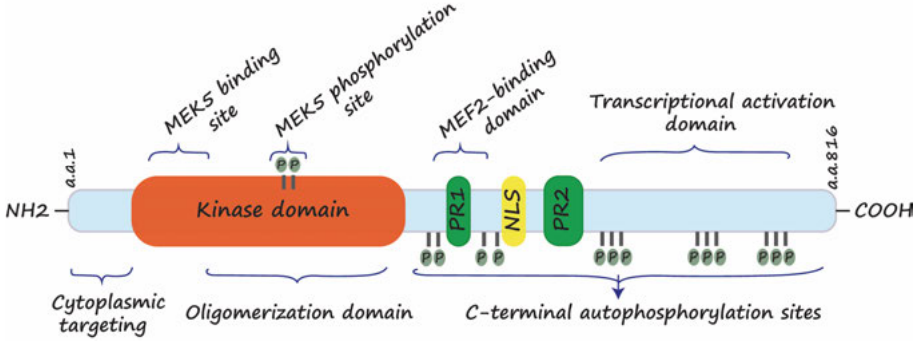

Figure 5. Schematic illustration of the functional domains in Erk5. The human Erk5 gene encodes a protein of 816 amino acid residues, which consists of an N-terminal kinase domain, similar to the one of Erk2, two proline-rich regions, a nuclear localization signal as well as a unique C-terminal tail of 400 amino acid residues that allows Erk5 to act as a transcriptional factor. Erk5 becomes activated by initial dual phosphorylation on Thr218 and Tyr220 by Mek5, its upstream activator, inducing autophosphorylation of several residues in its C-terminal tail, and thus, resulting in full activation of the protein. Additionally, various proteins, i.e. CDK1 and PKCζ, have been shown to phosphorylate Erk5, regulating its activity and leading to different physiological outcomes.

In contrast to the well characterized Erk1/2 pathway in which Erk1/2 (MAPK) is activated by Mek1/2 (MAPKK) that in turn is activated by Raf and Mos (MAPKKK), the signaling pathway leading to Erk5 activation still remain to be fully elucidated (Morrison, 2012). Due to the similarity between Erk5 and other MAP kinases, it is expected that Erk5 activation has the architecture of a three-layered kinase module. While it has been proposed that the upstream kinase of Erk5 is Mek5 (MAPKK) which in turn is activated by Mekk2/3 (MAPKKK), the exact mechanisms underlying Erk5 activation have not been clarified yet and may be cell type and stimulus specific (Sun *et al.*, 2001; Nakamura and Johnson, 2003; Díaz-Rodríguez and Pandiella, 2010; Yao *et al.*, 2010; Drew *et al.*, 2012; Le *et al.*, 2018). In this work (Paper I), we show that upon PDGF-BB stimulation, Erk5 becomes activated but remains cytosolic and apart from the proposed three-tiered cascade, various other signaling molecules are involved in its activation downstream of PDGFR. This highlights that the activation of Erk5 is complex and that it is involved in extensive crosstalk with other signaling pathways.

The Erk5 pathway plays an important role in cell growth, differentiation and proliferation, as well as in the regulation of vascular integrity and cardiovascular development (Regan *et al.*, 2002; Yan *et al.*, 2003; Hayashi and Lee, 2004; Hayashi *et al.*, 2004; Nithianandarajah-Jones *et al.*, 2014). Moreover, deregulation of Mek5/Erk5 pathway has been associated with various diseases, most importantly with tumor development and tumor cell invasion and migration (Lochhead *et al.*, 2012; Arias-González *et al.*, 2013; Finegan *et al.*, 2015; Simões *et al.*, 2016; Hoang *et al.*, 2017). Many reports have shown that overactive Erk5 drives proliferation and is correlated with highly aggressive forms of breast and prostate cancer among others (Esparís-ogando *et al.*, 2002; McCracken *et al.*, 2008; Montero *et al.*, 2009; Ramsay *et al.*, 2011; Kim *et al.*, 2012; Rovida *et al.*, 2014). These observations suggest that the Erk5 pathway is an attractive target for therapeutic intervention; therefore, it is important to define the components involved in this pathway.

PI3K pathway

The PI3K family is an important regulator of cellular growth and function. PI3Ks are activated by RTKs, such as PDGFR, but also by other cell surface receptors and are crucial for a wide range of cellular events, including proliferation, survival, intracellular trafficking, migration and metabolic processes (Vanhaesebroeck *et al.*, 1997). PI3Ks are divided into four classes based on their structure and regulation. An important substrate is phosphatidylinositol-4,5-bisphate (PIP_2) that becomes phosphorylated to phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 act as a docking site for various proteins containing protein-lipid binding domains that are recruited to the cell membrane, become activated and mediate signaling events (Vanhaesebroeck *et al.*, 1997;

Lien *et al.*, 2017). Known downstream effectors of the PI3K pathway are specific members of the protein kinase C (PKC) family, small GTPase family members, as well as serine/threonine kinases such as RAC-alpha serine/threonine-protein kinase (Akt) and its activator 3-phosphoinositide-dependent protein kinase-1 (PDK1), which have been studied extensively. Akt is recruited to the membrane, where it interacts with PIP_3 via its pleckstrin homology (PH) domains and undergoes conformational changes. These changes then result in phosphorylation of Akt at Thr308 by PDK1, followed by further phosphorylation at Ser473 by mTORC2, leading to full activation of Akt (Franke *et al.*, 1997; Manning and Cantley, 2007).

RTKs activate members of the PI3K class IA family. These members consist of a regulatory subunit (SH2 containing domain p85) and a catalytic subunit (p110) (Jean and Kiger, 2014). Upon PDGF stimulation, the p85 subunit of PI3K associates with specific phosphorylated tyrosine residues in the intracellular part of PDGFRα and PDGFRβ, becomes phosphorylated and subsequently promotes activation of the catalytic subunit p110. PI3K activation downstream of PDGFR is mainly essential for cell survival but is also implicated in actin reorganization, cell proliferation and migration (Heuchel *et al.*, 1999; Razmara *et al.*, 2012, 2013).

PLCγ/PKC pathway

PLC-γ associates with PDGFR via its two SH2 domains. Particularly, upon ligand stimulation, PLC-γ binds to two phosphorylated tyrosine residues in the C-terminal tail of the receptor, which leads to PLC-γ phosphorylation and partial activation (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989). For full activation of PLC-γ , PI3K plays an important role, as PIP_3 formed by PI3K may anchor PLC-γ in the plasma membrane by binding to its PH domains (Rameh *et al.*, 1998). PLC-γ and PI3K share the same substrate, i.e. PIP_2. However, PLC-γ cleaves PIP_2 into inositol-1,4,5-triphosphate (IP_3) and diacyglycerol (DAG), which act as second messengers promoting mobilization of Ca^{2+} ions as well as activation of PKC family members. Although PDGF-induced PLC-γ activation is associated with cell migration and growth *in vitro*, the importance of the pathway *in vivo* has not been elucidated yet (Wang *et al.*, 1998; Heldin *et al.*, 1999; Demoulin and Essaghir, 2014).

PKC family is a group of serine/threonine kinases that are classified into three subfamilies: the classical/conventional PKCs (α, βi, βii and γ isoforms) whose activation requires Ca^{2+}, DAG and phospholipids such as phosphatidylserine, the novel PKCs (δ, ε, η and θ isoforms) that depend on DAG but not Ca^{2+} for their activation, and the atypical PKCs (ζ and ι/ λ isoforms) that require phosphatidylserine but neither DAG or Ca^{2+} for their activation (Isakov, 2018;

Kikkawa, 2018). PKCs, by having numerous substrates, are important cell signaling regulators affecting various cell processes and their complex regulation will be discussed further later on in this thesis. It is interesting and noteworthy to mention that different isoforms can act on the same target but in an opposite fashion. This is demonstrated in Paper I of this thesis in which classical PKCs promote PDGF-induced Erk5 activation, whereas atypical PKCs are negative regulators of the pathway.

Crosstalk among signaling pathways

Cell signaling is a highly intricate system of communication among cells that involves numerous receptors, effector proteins and downstream players that regulate signaling pathways and lead to various biological responses. Although signaling pathways are usually described as a linear chain of molecules and events, an increasing number of studies demonstrates an extensive crosstalk among signaling pathways and formation of signaling networks, with various positive- and negative-feedback loops that ensure a fine-tuned response. Deciphering signaling networks does not only require understanding of individual signaling pathways and regulation and function of each component involved, but also understanding of the enormous variation that exists among cell types and also among species. The expression of receptors on the cell surface, scaffold proteins and second messengers downstream as well as transcriptional enhancers or repressors can vary in different cell types leading to different protein localization and function and thus, different cellular responses even to the same stimulus.

As many RTKs activate the same downstream effectors and initiate similar signaling cascades, it is not surprising that crosstalk between various RTK signaling pathways, i.e. epidermal growth factor receptor (EGFR), PDGFR, vascular endothelial growth factor receptor 2 (VEGFR2), insulin growth factor receptor (IGF-1R) and c-MET, in both normal and pathological conditions, has been presented (Lammers *et al.*, 1990; Rosenzweig, 2011; Castano *et al.*, 2013; Lan *et al.*, 2013). In addition to the interplay among RTK-activating pathways, reports have highlighted the existence of an extensive crosstalk between RTKs and other receptor families (Porsch *et al.*, 2014; Shi and Chen, 2017). Focusing on PDGFR, which is the key player of this thesis, a link with ECM, and specifically, a synergy between integrin αvβ3 and PDGFRβ during cell migration and chemotaxis has been demonstrated. This crosstalk is mediated by focal adhesion kinase (FAK), which is linked to PDGFRβ via the adaptor proteins NHERF that bind to the C-terminal tail of the receptor and act as intermediary bridging proteins between integrin/FAK and PDGFRβ (DeMali *et al.*, 1999; James *et al.*, 2004; Motegi *et al.*, 2011; Veevers-Lowe *et al.*, 2011). This is in agreement with reports indicating crosstalk also between

other RTKs (EGFR and IGF-1R) and integrins, providing a connection between these receptors and ECM (Cabodi *et al.*, 2004; Takada *et al.*, 2017).

Additionally, in glioblastoma multiforme (GBM), CXC-chemokine receptor 4 (CXCR4) has been shown to cooperate with PDGFRβ and modulate the migratory behavior of GBM cells, while other reports have shown interactions of CXCR4 and other G-protein-coupled receptors with EGFR and IGF-1R, indicating a crosstalk between the two receptor families (Akekawatchai *et al.*, 2005; Guo *et al.*, 2007; Sciaccaluga *et al.*, 2013). Furthermore, based on a recent study, neuropilin-1 binds PDGF-D and acts as a co-receptor for PDGFRβ (Muhl *et al.*, 2017). This report highlights a novel role for PDGF-D; however, it is not the first study proposing an interplay between neuropilin-1 and PDGFR (Ball *et al.*, 2010; Pellet-Many *et al.*, 2011).

It is now known that transforming growth factor (TGFβ) and RTK signaling pathways interact with each other and this interaction plays an important role especially during malignancy (Shi and Chen, 2017). There are various studies connecting the two receptor families; and RTKs have been proposed to stimulate the expression of TGFβ ligands, regulate Smad activity and modulate TGFβ-induced epithelial-to-mesenchymal transition (EMT). As TGFβ/Bone morphogenetic protein (BMP) signaling will be discussed in Paper I, to put it in context, a brief introduction to the pathway will be given below.

TGFβ/BMP signaling

The human TGFβ family consists of 33 members of extracellular ligands and is divided into the TGFβ subfamily, including TGFβ1, 2 and 3, activins, Nodal, growth and differentiation factors (GDFs), and the BMPs (Derynck *et al.*, 1985; Miyazono *et al.*, 2018). Ligand binding promotes the hetero-tetramerization of type I and type II serine/threonine kinase receptors and phosphorylation of cytoplasmic small mothers against decapentaplegic (Smad) transcription factors (R-Smads); Smad2 and Smad3 are activated by TGFβ ligands, whereas Smad1, Smad5 and Smad8 are activated by BMP ligand (Mathews and Vale, 1991; Heldin *et al.*, 1997). The activated R-Smads then associate with the common-mediator Smad, Smad4, and the complexes translocate to the nucleus where they regulate gene expression.

In addition to Smad signaling, TGFβ/BMP receptors activate alternative non-Smad signaling cascades, such as Erk, p38, JNK MAPK kinases and PI3K/Akt pathway (Aubin *et al.*, 2004; Heldin and Moustakas, 2016; Morikawa *et al.*, 2016), indicating that there is an overlap between signaling pathways activated by Ser/Thr kinase receptors and RTKs. This is in concurrence with studies showing that activation of Smads can occur upon stimulation with other growth factors, as EGF and PDGF-BB were found to induce Smad5 and

Smad2 phosphorylation and activation, respectively (Jin *et al.*, 2011; Porsch *et al.*, 2014). Paper I of this thesis also illustrates such an interplay between RTK and TGFβ signaling; PDGF-BB affects BMP signaling in an Erk5-dependent manner, expanding the repertoire of known targets downstream of PDGFRβ (Tsioumpekou *et al.*, 2016).

PDGFR downregulation

As PDGF is an important regulator of cellular functions, such as survival and proliferation, negative feedback mechanisms are essential to limit the signaling outcome of the receptor upon activation to ensure a proper biological response. Several protein tyrosine phosphatases have been suggested to dephosphorylate directly PDGFR, including TC-PTP, PTP1B, PTP-2C or SHP2 and DEP1, whereas other phosphatases have been proposed to act on effectors activated downstream of the receptor (Heldin *et al.*, 2018). Such examples include the phosphatase PTEN that is recruited to PDGFR via the adaptor proteins NHERF and controls PI3K activation, as well as DUSP6, which has been shown to be induced upon PDGF stimulation and dephosphorylate Erk1/2. Additional negative feedback loops have been proposed to modulate PDGF signaling. Activation of Ras after Grb2/Sos1 binding to activated PDGFR is negatively regulated by Ras-GAP; Ras-GAP binds to phosphorylated Tyr771 of PDGFRβ and suppresses Ras activation. This negative feedback mechanism is PDGFRβ-specific, as Ras-GAP does not bind to PDGFRα, allowing a stronger activation of Ras downstream of PDGFRα (Kaplan *et al.*, 1990; Kashishian *et al.*, 1992; Ekman *et al.*, 1999).

Finally, a major deactivation mechanism for RTKs, including PDGFR, involves the ligand-receptor complex internalization and degradation in lysosomes (Goh and Sorkin, 2013). A critical step for this internalization to occur is the receptor ubiquitination, which will be the focus of Paper II and III in this thesis. As in these two papers, we investigated the key enzymes involved in PDGFR ubiquitination and deubiquitination, a background to the internalization and sorting process of the receptor will be given, which will be followed by a section on ubiquitination and deubiquitination.

PDGFR endocytosis and sorting

PDGF binding to the receptor triggers receptor homo- or heterodimeration and autophosphorylation. Specific tyrosine residues in the intracellular part of the receptor act then as docking sites for members of the Casitas B-lineage lymphoma (Cbl) ubiquitin E3 ligase family, that bind to the receptor directly or via adaptor proteins and mediate the receptor ubiquitination (Levkowitz *et al.*, 1999). As illustrated in *figure 6*, the ligand-receptor complex is then internalized by endocytosis in a clathrin- and dynamin-dependent manner into the early endosomes where signaling continues. Pathways, such as MAPK and PI3K, have been proposed to be activated in endosomal compartments, and the signaling is terminated when pH decreases to such a low value that the ligand dissociates from the receptor and the receptors are monomerized and dephosphorylated. The ligands and receptor monomers are then sorted to the

inner membranes of multivesicular bodies (MVBs) which are fused with lysosomes, resulting in degradation by lysosomal hydrolases. A recycling pathway, in which PDGFR returns to the plasma membrane from the early endosomes and signals again, has been also proposed to take place in PKC- or PI3K-dependent manners (Wang *et al.*, 2004; Goh and Sorkin, 2013; Heldin, 2013; Villaseñor *et al.*, 2016). It should be noted that the knowledge regarding RTK internalization and sorting is mainly based on studies performed on EGFR and it is common to use EGFR in most reviews as an example for RTK downregulation. However, as failure of RTKs to be successfully deactivated contributes to tumor initiation (Bache *et al.*, 2004), an increasing number of studies investigating downregulation of other RTKs has provided additional information about this process. We, and others, have also demonstrated the importance of Cbl members in PDGFR internalization and signaling (Miyake *et al.*, 1999; Reddi *et al.*, 2007; Rorsman *et al.*, 2016), but as shown in paper II, surprisingly, degradation of PDGFRβ seems to occur mainly in proteasomes. This finding is in contrast to the proposed mechanism of RTK downregulation and lysosomal degradation, and to earlier reports where ^{125}I-labeled PDGF was shown to be degraded in lysosomes (Nilsson *et al.*, 1983), suggesting the existence of additional complex mechanisms regulating RTK downregulation and the need for further studies.

Apart from the clathrin-mediated endocytosis mentioned above, which is well documented, endocytosis can occur in a clathrin-independent manner and these mechanisms involve macropinocytosis, an actin-driven and PI3K-mediated process, as well as caveolae, which are small invaginations in the plasma membrane, enriched in caveolin (Pelkmans *et al.*, 2004; Orth and McNiven, 2006; Schmees *et al.*, 2012; Goh and Sorkin, 2013). Based on the localization of the receptor in different structures at the plasma membrane and its association with clathrin-dependent or -independent endocytosis, the signaling activity of the receptor can vary, affecting the overall biological output. As sorting of the receptor in endosomal compartments is dependent on its ubiquitination, various efforts have been made to identify the enzymes involved in this process.

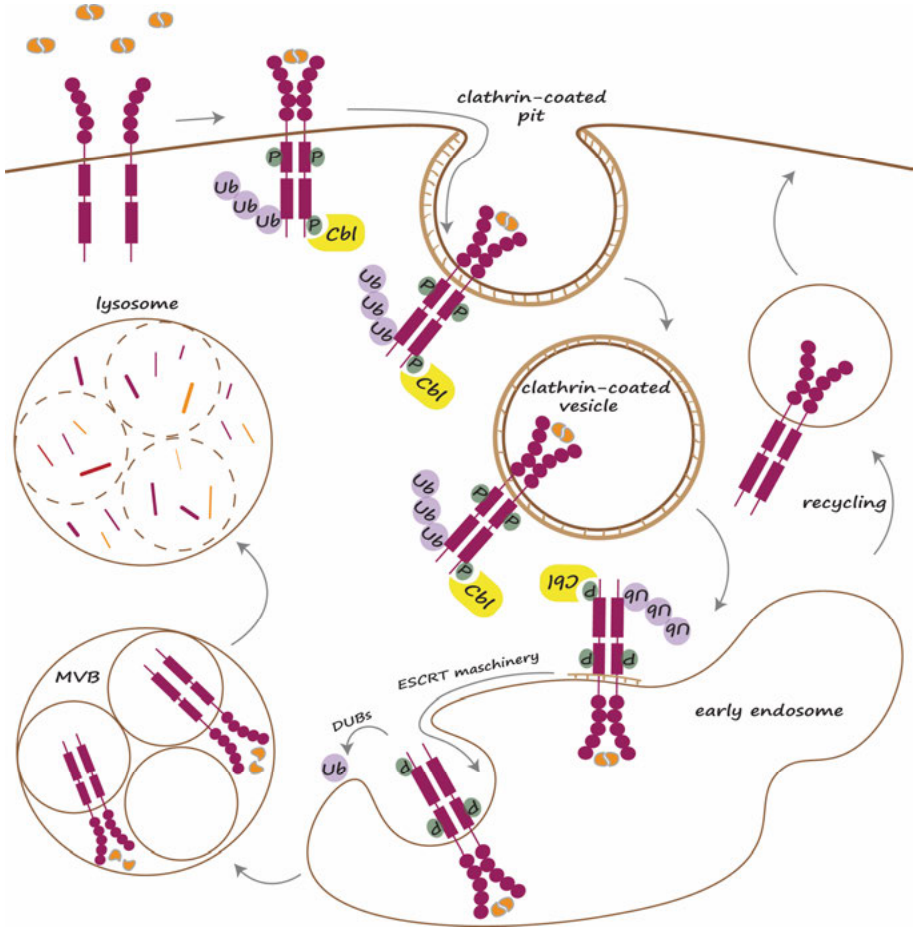

Figure 6. Simplified illustration of proposed RTK clathrin-mediated endocytosis. Upon ligand binding and subsequent RTK dimerization and autophosphorylation, Cbl binds to the receptor and leads to its ubiquitination. The ubiquitinated receptor then is internalized and sorted through the endosomal compartment to finally be degraded in the lysosomes. Alternatively, RTKs can be recycled back to the plasma membrane, where they can signal again.

Ubiquitination

Ubiquitin (Ub) is a highly conserved small protein that consists of 76 amino acid residues, encoded by four genes in humans, and through a three step process can be covalently linked to a target protein substrate regulating its fate inside the cell. This linkage of ubiquitin to lysine residues in a protein substrate is an enzymatic process carried out by Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes (Petroski, 2008; Komander and Rape, 2012). The first step includes the activation of ubiquitin by the E1 enzyme, which then transfers the ubiquitin onto the E2 conjugating enzyme. Finally, the E3 ligases mediate the transfer of the activated ubiquitin moiety from the E2 enzyme to a lysine residue on the target protein (Akutsu *et al.*, 2016).

There are three families of E3 ligases: really interesting new gene (RING), homologous to the E6AP carboxyl terminus (HECT) and RING between RING (RBR); RING family is the largest, with at least 600 members (Komander and Rape, 2012). Among these members, Cbl proteins are well characterized. Three Cbl isoforms exist in mammals: c-Cbl, Cbl-b and Cbl-c/Cbl-3, with c-Cbl and Cbl-b being structurally similar and most studied. Apart from their RING domain, Cbls consist of a phosphotyrosine-binding domain, proline-rich regions, several tyrosine residues that can be phosphorylated and a leucine zipper motif that is required for Cbl homodimerization and heterodimerization (Sanjay *et al.*, 2001). Studies on c-Cbl and Cbl-b knock-outs showed that mice with double knockout of the two isoforms were embryonic lethal at E10, whereas *c-Cbl*-null and *Cbl-b*-null mice survived and were fertile although they displayed immune system hyperactivation. These findings, taken together with various *in vitro* studies, where one or both isoforms are silenced, suggest that c-Cbl and Cbl-b have partially overlapping roles and act on the same or similar substrates (Mohapatra *et al.*, 2013). The Cbl family was early documented to be involved in RTK downregulation with the first reports relating c-cbl with EGFR in the late 1990s (Galisteo *et al.*, 1995). Since then, Cbl members have been shown to act on various RTKs, including PDGFR, regulating their internalization and trafficking and thus, influencing their biological response (Mori *et al.*, 1993; Miyake *et al.*, 1999; Yokouchi *et al.*, 1999; Reddi *et al.*, 2007; Rorsman *et al.*, 2016).

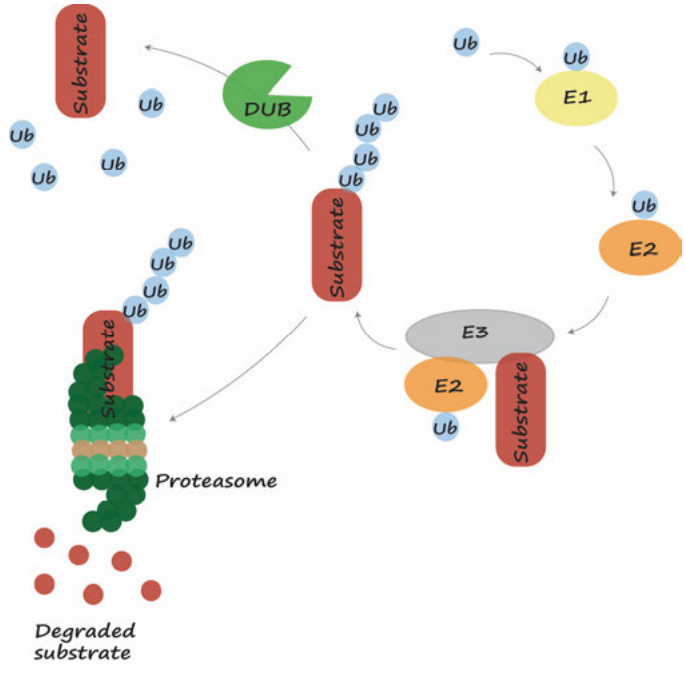

Figure 7. Ubiquitination-proteasome pathway. The attachment of ubiquitin to lysine residues of substrates is an ATP-dependent process, mediated by three types of enzymes, E1, E2 and E3, which based on the ubiquitination type can cause different outcomes. K48- linked polyubiquitination, for instance, is known to target proteins for proteasomal degradation. Ubiquitinated substrates either are deubiquitinated by deubiquitinating enzymes (DUBs) and escape degradation or are recognized by the 26S proteasome and are processed to short peptides.

Ubiquitin forms three types of ubiquitination: monoubiquitination, where one Ub moiety is attached to a single lysine residue of the target protein; multiubiquitination, i.e. single Ub moieties are attached to more than one lysine residues of the target protein; and polyubiquitination, where an ubiquitin chain is attached to the target protein and polymerized via any of the seven lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48 and K63) (Akutsu *et al.*, 2016; Swatek and Komander, 2016). Although initially ubiquitination was described as the "kiss of death" for proteins as it was targeting them for degradation, we now know that ubiquitination can cause three different outcomes: target proteins for degradation, alter their enzymatic activity and/or target them to specific locations inside the cell. Mono- and multi-ubiquitination are known to regulate processes, such as protein endocytosis (one single Ub has been shown to target EGFR and PDGFR for endocytosis (Hicke, 2001; Haglund *et al.*, 2003)), DNA damage response as well as protein signaling and localization. The outcome of polyubiquitination is still being explored and depends on the nature of polyubiquitin chains that are formed. K48 polyubiquitin

chains are associated with proteasomal degradation of proteins whereas K63-linked chains with signaling, DNA repair and endocytosis. K6, K27 and K33 polyubiquitin chains have been linked with DNA damage response, K29 with regulation of Wnt/β-catenin signaling whereas K11-linked ubiquitin chains appear to play an important role in cell division. Apart from the seven lysine residues of ubiquitin, the amino-terminal methionine (M1) of ubiquitin can act as an acceptor, leading to formation of M1-linear ubiquitin chains, whose role has been reported in tumor necrosis factor receptor function and innate immune signaling (Rieser *et al.*, 2013; Popovic *et al.*, 2014; Akutsu *et al.*, 2016; Critchley *et al.*, 2018).

Deubiquitination

Protein ubiquitination is a dynamic process that governs almost every cell function and its deregulation has been correlated with the development of various diseases, including cancer, indicating the need for a mechanism that keeps the balance inside the cell (Popovic *et al.*, 2014). Therefore, the human genome encodes approximately 100 deubiquitinating enzymes (DUBs), proteases that can remove the ubiquitin moiety from target proteins and reverse the effect of E3 ligases. Apart from reversing ubiquitination and rescuing proteins from degradation, these enzymes have additional activities, i.e. ubiquitin recycling, ubiquitin precursor processing where free ubiquitin moieties are generated from the ubiquitin precursor, as well as ubiquitin chain editing and thereby, alteration of signal (Clague *et al.*, 2013; Heideker and Wertz, 2015).

DUBs can be classified into six families: ubiquitin-specific processing proteases (USPs), which is the largest family; ovarian tumor proteases (OTU), ubiquitin C-terminal hydrolases (UCH), Machado-Josephin disease proteases (MJD), motif interacting with novel ubiquitin-containing novel deubiquitinase family (MINDY) and Jab1/Pab1/MPN domain-associated metalloisopeptidases (JAMM) (Komander *et al.*, 2009). Of the six families mentioned, USP, OTU, UCH, MJD and MINDY, are cysteine proteases whereas the JAMMs are zink metallopeptidases. DUBs have emerged as important regulators of various cellular processes, such as endocytosis and proteasomal degradation, signaling, RNA processing, cell cycle control and DNA damage repair and therefore, they have received attention as possible therapeutic targets (Komander *et al.*, 2009; Abdul Rehman *et al.*, 2016; Chen, 2016).

DUB specificity can be either target-oriented or substrate (ubiquitin moiety)-oriented; DUBs, such as USP4 and USP17, recognize specific target proteins and remove both mono- and polyubiquitination, whereas others are selective for specific ubiquitin chain linkages with prime examples MINDY-1/FAM63A, a highly specific K48-chain hydrolase, and BRCC36, a JAMM

member selective for K63-linkages (Nijman *et al.*, 2005; Clague *et al.*, 2013; Abdul Rehman *et al.*, 2016).

Deregulation of DUBs have been linked to pathogenesis and mutations on several DUBs are associated with genetic or sporadic disorders. In cancer, it has been proposed that DUBs contribute via few mechanisms; by displaying intrinsic oncogenic or tumor suppressor activity, by acting on oncogenes and/or tumor suppressors (with USP7 and ATXN3 as examples that act on p53) or by controlling epigenetic changes that promote tumor development, in which USP22 has been presented to play a role. Several low molecular weight inhibitors have been developed against various DUBs and tested with encouraging results, such as the USP9X inhibitors in chronic myeloid leukemia (CML) and GBM models; however, there is a need for identifying and developing more potent and selective inhibitors for clinical use (Sacco *et al.*, 2010; Sun *et al.*, 2011; D'Arcy *et al.*, 2015; Harrigan *et al.*, 2017).

As ubiquitination is a critical step for RTK sorting and trafficking, it is expected that several DUBs are involved in RTK regulation. Particularly, USP18, USP9X, USP8, Cezanne-1/OTUD7B and AMSH have been involved in EGFR regulation, either directly by acting on the receptor itself or indirectly by acting on proteins important for EGFR trafficking and downregulation (Pareja *et al.*, 2012; Critchley *et al.*, 2018). Although PDGFR ubiquitination has been extensively studied by us and others, there is no evidence regarding the mechanisms or key role enzymes underlying PDGFR deubiquitination. In Paper III, we identified two DUBs, USP4 and USP17, acting on PDGFRβ; a background of USP4 is given below.

USP4

USP4, otherwise known as UNP, is a cysteine protease that belongs to the USP family of DUBs. Human *USP4* is located on chromosome band 3p21.3, an area involved in lung neoplasms, encodes a 963 amino acid residue protein. Two USP4 isoforms have been identified in mammals, acting on a wide range of targets, important in various signaling pathways (Frederick *et al.*, 1998). Some of its targets include TGFβ type I receptor, p53, β-catenin as well as members of the NF-κB pathway (Yun *et al.*, 2015; Z. Li *et al.*, 2016; Mehić *et al.*, 2017). USP4 can remove or edit both K48- and K63-linked ubiquitin chains and shuttles between cytoplasm and nucleus. In breast cancer, Akt-mediated phosphorylation of USP4 has been proposed to affect its subcellular localization; upon phosphorylation, USP4 translocates to the membrane where it deubiquitinates TβRI, and thus results in an increase of pro-tumorigenic TGFβ signaling (Zhang *et al.*, 2012).

Although USP4 has been implicated in rheumatoid arthritis by affecting T_H17-activated T cells and is considered as a possible therapeutic target (Harrigan *et al.*, 2017), its role in cancer remains unclear. USP4 has been shown to inhibit breast cancer cell growth and lung cancer cell migration whereas in other tumors it is overexpressed and promotes metastasis (Y. Li *et al.*, 2016; Guo *et al.*, 2018; Li *et al.*, 2018; Zhong *et al.*, 2018). These controversial findings can be possibly explained by the growing list of USP4 targets and also illustrate the complex mechanism of action of DUBs in general, which is one of the issues that hinders their use as therapeutic targets in clinics.

PDGF signaling in disease

Deregulation of PDGF signaling has been associated with various pathological conditions, including fibrosis, vascular diseases, such as atherosclerosis, neurological disorders and cancer. Particularly, overexpression of PDGF ligands has been observed in lung, kidney and cardiac fibrosis, and several studies have tried to identify the mechanisms underlying these fibrotic reactions. PDGF overactivity in vascular smooth muscle cells has also been documented in vascular diseases, i.e. atherosclerosis, blood-brain barrier dysfunction and pulmonary artery hypertension (Ostman and Heldin, 2001; Heldin *et al.*, 2018). In contrast, *PDGFRB* and *PDGFB* loss-of-function mutations have been reported and characterized in idiopathic basal ganglia calcification/Fahr disease, a neurological disorder characterized by calcifications in the basal ganglia and other areas of the brain (Keller *et al.*, 2013; Arts *et al.*, 2015; Vanlandewijck *et al.*, 2015). Additionally, lack of PDGF-AA and PDGFRα activation has been associated with demyelinating lesions that occur in multiple sclerosis, strengthening the role of PDGF signaling in neurological diseases (Papadopoulos and Lennartsson, 2017).

Great effort has been made during the last two decades to unveil the contribution of the PDGF pathway to malignancies. Several mechanisms have been found to alter the expression and activity of both ligands and receptors, including amplification or deletion, fusion events due to chromosomal translocations and point mutations (Heldin *et al.*, 2018).

PDGFRA point mutations have been described in gastrointestinal stromal tumors (GIST), GBM, melanoma and acute myeloid leukemia (AML) and although most of these mutations are loss-of-function or passenger mutations, the transmembrane gain-of-function mutation V536E was found to constitutively activate PDGFRα in GBM (Demoulin and Essaghir, 2014; Velghe *et al.*, 2014; Heldin *et al.*, 2018). Similarly, *PDGFRB* mutations have also been linked to disease; prime examples are the heterozygous germ-line gain-of-function mutations causing infantile myofibromatosis, Kosaki overgrowth syndrome and Penttinen premature aging syndrome (Johnston *et al.*, 2015; Arts *et al.*, 2017; Pond *et al.*, 2018). In addition, amplification of *PDGFRA* is reported in GBM, arterial intimal sarcomas and esophageal squamous cell carcinoma (Demoulin and Essaghir, 2014; Jouenne *et al.*, 2017; Papadopoulos and Lennartsson, 2017), while translocations of *PDGFRA* and *PDGFRB* genes are associated with hematopoietic malignancies. More than 30 fusion partners have been discovered for *PDGFRA* and *PDGFRB*; fusions between *ETV6* and *PDGFRB* and between *FIP1L1* and *PDGFRA* in myeloid malignancies associated with hypereosinophilia are the most well- characterized (Toffalini and Demoulin, 2008, 2010; Toffalini *et al.*, 2010; Demoulin and

Montano-Almendras, 2012; Reiter and Gotlib, 2017). Apart from translocation events that involve the receptor genes, fusion between *PDGFB* and *collagen 1A1* genes has been shown in the rare skin tumor dermatofibrosarcoma protuberans (DFSP) (Kikuchi *et al.*, 1993; Greco *et al.*, 1998; Shimizu *et al.*, 1999).

Although PDGF receptors are not expressed on epithelial cells under physiological conditions, deregulation of the PDGF pathway in solid tumors has been observed. Overexpression of PDGFRs has been reported in advanced hepatocellular carcinoma, prostate, breast, colorectal and gastric cancer, among others, and correlates with poor survival prognosis (Pietras *et al.*, 2003; Östman and Heldin, 2007; Heldin *et al.*, 2018). Surprisingly and in contrast to the tumor types mentioned above, overexpression of PDGFRβ and PDGF-BB in mesothelioma and renal cell carcinoma and PDGFRα and PDGF-AA in Wilm's tumor, a rare kidney tumor in children correlated with good prognosis, illustrating the complex role of PDGF in cell signaling and cancer (Heldin *et al.*, 2018). Despite lack of expression of PDGFRs on epithelial cells (although induction of PDGFR expression in tumor cells is being investigated), PDGF signaling plays an important role in the stromal compartment of solid tumors, promoting angiogenesis and development of cancer-associated fibroblasts (CAFs) (Andrae *et al.*, 2008; Östman, 2017).

CAFs are a highly heterogeneous and secretory population of cells that play an important role in tumor progression as they can promote angiogenesis, cell proliferation and invasion, modulate ECM and lead to an increase of interstitial fluid pressure (IFP) that acts as a barrier for drug uptake in solid tumors (Santi *et al.*, 2018; Barbazán and Matic Vignjevic, 2019). CAFs, first mentioned in 1979 by Tremblay in the stroma of breast tumors (Tremblay, 1979), can originate from various cell types. A subset arises from activation of resident fibroblasts, which is triggered by a range of growth factors and cytokines and environmental stimuli, whereas others have been proposed to originate from various cell types, i.e. bone marrow-derived mesenchymal cells, epithelial cells that undergo EMT, endothelial cells, pericytes and smooth muscle cells. This may explain the heterogeneity of CAFs, as shown by single-cell analysis studies to different subpopulations in the same tumor microenvironment (Bartoschek *et al.*, 2018). Identification of CAFs is based on the expression of PDGFR, FSP, FAP or αSMA, but CAF-specific markers are still lacking (Alkasalias *et al.*, 2018; Belli *et al.*, 2018). Their role has been observed to be mostly tumorigenic and their expression correlates with poor prognosis, with only few studies showing a tumor suppressive role (Barbazán and Matic Vignjevic, 2019). By acquiring more knowledge about CAF heterogeneity as well as their interplay with other cells in the tumor microenvironment, targeting CAFs could be beneficial in future cancer therapy.

Besides CAFs, PDGFRβ is expressed in pericytes and smooth muscle cells, cell types present in the vasculature under physiological and pathological conditions (Hellstrom *et al.*, 1999). To put the work of Paper IV into context, an introduction to the tumor vasculature and its main components as well as the role of pericytes and PDGFRβ in tumor growth will be given below.

The role of PDGFRβ in tumor vasculature

Initial evidence of vascularization in tumors appeared in 1939, when Ide et al., proposed that tumors release specific factors, which stimulate growth of blood vessels. Various studies followed up on this finding, but it was not until 1971 that Judah Folkman, who is considered the pioneer of angiogenesis, noticed the importance of tumor vascularization. Based on his observations that tumors do not grow above two mm^3 without the existence of vessels, he hypothesized that tumor growth is dependent on tumor angiogenesis and that anti-angiogenic treatments could have a therapeutic potential (Folkman, 1974; Cao and Langer, 2008; Ribatti, 2008).

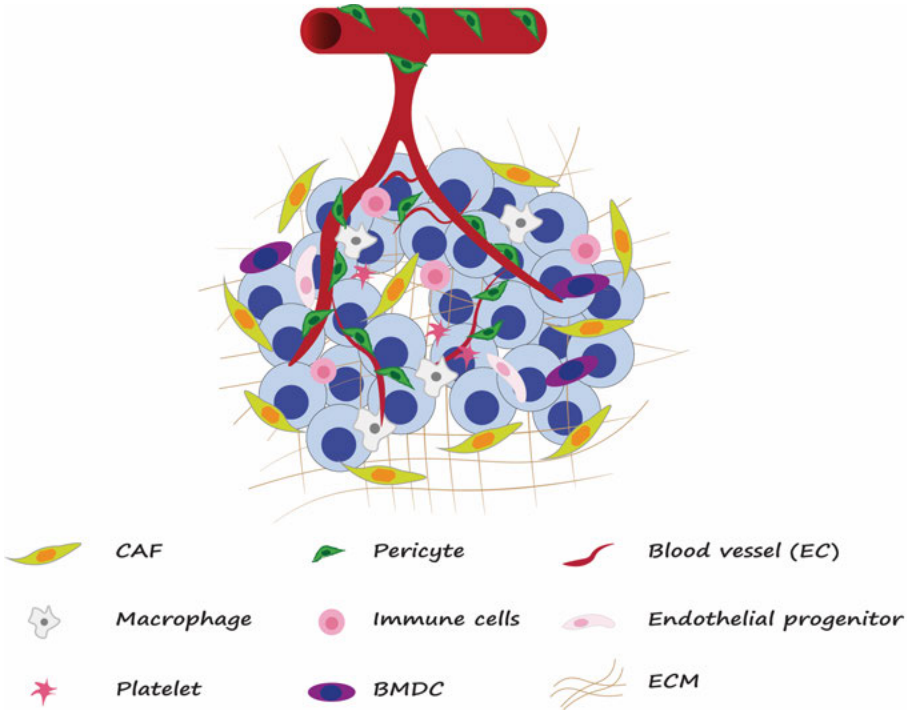

Figure 8. The complexity of tumor microenvironment, which consist not only of tumor cells (shown in blue), but also ECM, blood vessels (endothelial cells and pericytes), macrophages and other immune cells, platelets and bone marrow-derived cells, among others.

Angiogenesis is defined as the formation of new vessels from pre-existing ones and occurs in adulthood, under physiological conditions, only during pregnancy, female reproductive cell cycle and wound healing. However, during tumor development, angiogenesis is a critical step. Tumor vessels can originate via three different mechanisms: *de novo* formation from endothelial cell (EC) precursors inside the tumor (vasculogenesis), growth of pre-existing vessels that infiltrate the tumor (angiogenesis) as well as vessel co-option, where tumor cells hijack the existing vasculature. Vascular mimicry has also been observed and is an additional mechanism, where highly aggressive tumor cells form tubular structures themselves. Blood vessels in tumors serve two important roles; fuel the cancer cells with nutrients and oxygen so they can continue proliferating and provide a "path" for dissemination to other organs and formation of metastasis (Eichhorn *et al.*, 2007; Kubota, 2012; Donnem *et al.*, 2013).

Blood vessels consist of two interacting cell types; endothelial cells that form the inner lining of the vessel wall and mural cells which wrap around the vascular tube and promote integrity and function of the vessels. Mural cells can be further classified into vascular smooth muscle cells (VSMCs) and pericytes based on their location, morphology and expression of specific markers (Hirschi and D'Amore, 1996; He *et al.*, 2016).

Pericytes, also called as Rouget cells, are polymorphic, elongated, multi-branched peri-endothelial cells that wrap around the vessels of smaller diameter and are covered by the same basement membrane as endothelial cells. Pericyte-endothelial interaction occurs both by direct physical contact in a "peg-and-socket" fashion and also by paracrine signaling pathways (Armulik *et al.*, 2011). The origin of pericytes is still not fully understood; they can develop from various cells depending on their location, while it has been shown that they also have the ability to differentiate into several types of mesenchymal cells such as fibroblasts and VSMCs. There are various molecular markers used to identify pericytes, including among others intracellular proteins such as desmin, α-smooth muscle actin (αSMA) and regulator of G-protein signaling 5, and cell-surface proteins, such as neuron-glial antigen 2 (NG2), 3G5 ganglioside and PDGFRβ (Franco *et al.*, 2011; Cortez *et al.*, 2014; Geevarghese and Herman, 2014; Munde *et al.*, 2014). Although several markers have been identified, no pan-pericyte marker, absolutely specific for pericytes exists. In addition, the expression of these markers is dynamic and varies depending on species, tissue type and developmental stage, highlighting the difficulty to identify and study pericytes (Hirschi and D'Amore, 1996; Armulik *et al.*, 2011; Franco *et al.*, 2011; Geevarghese and Herman, 2014; Munde *et al.*, 2014; He *et al.*, 2016).

Pericytes are usually abundant in tumor vessels, but appear abnormal in shape and loosely attached to the endothelial cells, resulting in increased vessel leakiness and risk of hemorrhage. Although the role of pericytes in tumor angiogenesis is not fully understood, it has been shown that high amount of pericytes in tumor vessels associates with increased tumor growth and resistance to therapy, whereas low amount is associated with metastatic spread (Andrae *et al.*, 2008; Matsuo *et al.*, 2010; Raza *et al.*, 2010; Ribeiro and Okamoto, 2015). These findings highlight the various facets of pericyte effects on tumor angiogenesis and the need for careful selection of patients for therapy aimed at inhibiting tumor pericyte recruitment.

Although the initial focus of most anti-angiogenic treatments was targeting endothelial cells with various inhibitors or antibodies against VEGF and its receptors, the clinical results have been very modest, leading to efforts to understand the contribution of pericyte coverage. Several studies have demonstrated the involvement of pericytes in tumor development including one performed by Cheng *et al.*, 2013, where glioblastoma stem cells are recruited to endothelial cells and are induced to generate vascular pericytes to support vessel function and tumor growth; hence, combination of anti-endothelial and anti-pericyte agents might appear beneficial in anti-angiogenic therapy in specific tumor types (Bergers *et al.*, 2003; Bergers and Hanahan, 2008; Caspani *et al.*, 2014; Harrell *et al.*, 2018).

Pericyte function and homeostasis are regulated to a large extent by PDGF/PDGFRβ signaling as indicated by studies in which *pdgfb* and *pdgfrβ* were knocked out in mice (discussed in page 15). It has also been shown that bone marrow-derived $PDGFR^{+}$ cells have the ability to differentiate into NG2 or αSMA pericytes *in vivo*, illustrating the critical role of PDGF signaling in pericyte development (Song *et al.*, 2005; Lu *et al.*, 2008). Therefore, inhibition of PDGFRβ may represent an appealing approach to target pericytes.

Targeting PDGF signaling in cancer therapy

As overexpression/mutations of PDGF ligands and receptors are common in various cancers and PDGF signaling has proven to be important in the tumor microenvironment, it is desirable to develop PDGF/PDGFR antagonists and investigate their effect in cancer therapy. Therefore, antibodies, aptamers, kinase inhibitors and soluble extracellular parts of PDGFR used as traps for PDGF ligands, have been developed over the years with variable results. All approaches so far have shown advantages and drawbacks; i.e. antibodies are highly selective but expensive, whereas inhibitors are cost-effective but lack specificity (Papadopoulos and Lennartsson, 2017; Heldin *et al.*, 2018).
An approach to target PDGF/PDGFR signaling is by using PDGF- or PDGFR-blocking antibodies. Antibodies against PDGF-BB, PDGF-CC, PDGF-DD

and PDGF-AB have been developed and tested with encouraging results *in vitro* and *in vivo*. Similarly, PDGFRβ-blocking antibody was found to cause vessel regression in a model of corneal and choroidal neovascularization when administered together with VEGF aptamer, indicating therapeutic potential for ocular therapeutic disease (Jo *et al.*, 2006). However, the greatest success in respect to PDGFR antibodies is related to olaratumab, a human anti-PDGFRα monoclonal antibody. This antibody does not cross-react with PDGFRβ, and it has been successful in phase II clinical trials of advanced soft tissue sarcoma in combination therapy with doxorubicin. In 2016, olaratumab was granted approval for treatment of soft tissue sarcoma in USA (and conditional approval in EU) while now it undergoes phase III clinical trials, whose results will be announced in 2020. Although olaratumab improved survival of patients with soft tissue sarcoma, it showed no effect in other solid tumors, such as ovarian cancer, prostate cancer, glioma, metastatic GIST and non-small cell lung cancer (NSCLC), indicating the need for new therapeutic strategies in these tumors (Papadopoulos and Lennartsson, 2017; Antoniou *et al.*, 2018; Lowery *et al.*, 2018).

Aptamers have also been developed against PDGF ligands and receptors and are considered as alternatives to antibodies. They are single stranded DNA or RNA molecules that bind the target with high specificity while they are known for their thermal stability and low cost (Morita *et al.*, 2018). Anti-PDGF-B aptamer was reported to successfully downregulate PDGF signaling and thereby decrease IFP and increase drug uptake in rat and mouse tumor models. Moreover, a nuclease-resistant RNA aptamer targeting PDGFRβ showed promising results in both GMB and triple negative breast cancer xenografts (Pietras *et al.*, 2001; Lu *et al.*, 2010; Camorani *et al.*, 2014, 2017; Romanelli *et al.*, 2018). Taken altogether, anti- PDGF/PDGFR aptamers could possibly be of therapeutic value in cancer therapy, and it remains to be seen if they will make a successful transition into clinical trials.

A successful approach to target PDGFR signaling is to block its kinase activity with low molecular weight inhibitors. These inhibitors can penetrate the cell membrane and block the enzymatic activity of tyrosine kinases. A long panel of such inhibitors has been developed over the last decades, with imatinib, nilotinib, dasatinib, sorafenib, sunitinib, ponatinib as a few examples. It is notable that none of these inhibitors is specific for PDGFR, instead due to conservation of the ATP-binding pocket, they inhibit several targets including PDGFR (Velghe *et al.*, 2014; Heldin *et al.*, 2018).

Imatinib, the prototype of tyrosine kinase inhibitors (TKIs), targets in addition to PDGFRs, BCR-Abl and c-Kit, and was approved for clinical use in 2001 for CML; later on, it became the standard treatment also for GIST (which was resistance to chemo- and radiotherapy), DFSP, hypereosinophilia and other

myeloid neoplasms (Buchdunger *et al.*, 1996; Carroll *et al.*, 1997; Demoulin and Montano-Almendras, 2012; Iqbal and Iqbal, 2014; Pond *et al.*, 2018). Despite its beneficial effects in those tumor types, unfortunately imatinib was shown ineffective in trials for GBM, NSCLC, diffuse and systemic sclerodema, pulmonary arterial hypertension and prostate cancer among others; the reason still remains unclear (Papadopoulos and Lennartsson, 2017; Heldin *et al.*, 2018).

Furthermore, second- and third-generation TKIs were developed due to resistance to imatinib and are used in clinics. Prime examples are nilotinib (targets PDGFR, BCR-Abl, TEL-PDGFRβ, FIP1L1-PDGFRα and c-kit) for imatinib-resistant CML patients, ponatinib (targets PDGFR, BCR-Abl, FGFR, FLT3, TIE2 and VEGFR) for leukemia patients with T3151I mutation of BCR-Abl and sunitinib for GIST and RCC. In order to target the tumor stroma and specifically, the tumor angiogenesis, apart from VEGF/VEGFR monotherapies, inhibitors that target both VEGF and PDGF signaling pathways were developed such as sunitinib, sorafenib and axitinib, with beneficial effects in some cancers, but not all (Papadopoulos and Lennartsson, 2017; Heldin *et al.*, 2018).

Although targeting multiple kinases can be beneficial, the lack of selectivity of TKIs makes it difficult to evaluate the contribution of PDGFR inhibition in the beneficial effects observed in different tumor models. Therefore, development of inhibitors targeting PDGFR specifically is urgently required. Deciphering the role of PDGF signaling in tumor microenvironment with more selective approaches, and subsequently identifying biomarkers to select cohorts of patients for anti-PDGF therapy (in combination with other treatments) are critical steps for improving treatment efficacy and avoiding resistance.

Present investigations

As discussed in the previous chapters, PDGF signaling plays an important role in various cellular processes, i.e. proliferation and migration, and its perturbation has been implicated in several diseases including tumorigenesis. Deregulation of PDGF signaling, at either the receptor level or its downstream effectors, is a frequent hallmark of certain cancer types, such as glioblastoma and gastrointestinal tumors; therefore, it is important to understand in detail the signaling and regulation of PDGFR. Furthermore, paracrine PDGF signaling seems to play a critical role in the recruitment of stromal cell types in tumors, indicating a multifaceted role of PDGF in tumor development. We propose that increasing our understanding of PDGF signaling and identifying biomarkers within this pathway for either early diagnosis or as targets for therapeutic intervention will be beneficial in our fight against cancer.

The goal of this thesis has been to clarify the molecular mechanisms underlying PDGFRβ signaling regulation. We pursued and elucidated different aspects of PDGFRβ signaling, namely:

- Activation of Erk5, one of PDGFR downstream effectors, which is often associated with more aggressive and invasive behavior of cancer cells (Paper I)

- Regulation of the internalization and stability of PDGFRβ by studying the E3 ligases and DUBs implicated in these processes (Papers II and III)

- Role of PDGFRβ in tumor growth and angiogenesis by targeting specifically PDGFRβ and performing *in vivo* experiments using transgenic mice and different tumor models (Paper IV)

Paper I: Platelet-derived growth factor (PDGF)-induced activation of Erk5 MAP kinase is dependent on Mekk2, Mek1/2, PKC and PI3-K, and affects BMP signaling

Upon PDGF binding and subsequent PDGFR dimerization and autophosphorylation, a plethora of signaling molecules bind to the receptor, become activated and initiate signaling cascades downstream. Erk5, one of the less studied members of the MAP kinase family, is one of the downstream effectors of PDGFRβ; however, the mechanisms and key players involved in Erk5 activation upon PDGF stimulation remain unclear. Erk5 activation and expression is increased in various cancer types and has been associated with invasiveness and chemo-resistance of cancer cells, it therefore provides an attractive target for therapeutic intervention; hence, it is important to delineate the pathway from activated PDGFR to Erk5. By using siRNA downregulation, as well as a panel of inhibitors against signaling molecules activated downstream of PDGFRβ, we show that Erk5 is activated upon PDGF-BB stimulation in MOVAS cells, in a manner dependent on Mekk2, Mek5, Mek1/2, PI3K and classical PKCs. This indicates that apart from the proposed three-tiered kinase cascade, that characterizes MAPK activation (Mekk2-Mek5-Erk5 in this study), Erk5 activation is affected by PI3K, Erk1/2 and PKC signaling pathways. Furthermore, a complex formation between Erk5 and PKCζ was observed, consistent with other reports, and this may exert a negative feedback effect on Erk5 activation.

Erk5 contains an NLS, a transactivation domain and a serine/threonine kinase domain, suggesting that Erk5 has a function in the nucleus, either acting as a transcription factor or phosphorylating other transcription factors. Interestingly, we were unable to detect activated Erk5 in the nucleus in MOVAS cells; however, cytoplasmic Erk5 was shown to influence gene expression, as it was essential for PDGF-BB-mediated Smad1/5/8 signaling. Our findings demonstrate a complex mechanism underlying PDGF-BB-induced Erk5 activation with various stimulatory and inhibitory signals, and more surprisingly, a cross-talk between tyrosine kinase receptors- and serine/threonine receptor signaling, which expands the repertoire of signaling pathways activated in response to PDGF-BB.

Paper II: The ubiquitin ligases c-Cbl and Cbl-b negatively regulate PDGF-BB induced chemotaxis by affecting PDGFRβ internalization and signaling

Protein ubiquitination is a critical regulator of its fate inside the cell, by influencing protein stability, subcellular localization and enzymatic activity. Ubiquitination of RTKs is associated with their downregulation and signal termination, indicating the importance of this modification in signaling and subsequent biological response. As RTKs are often deregulated in various diseases, understanding the role of the key components involved in their downregulation is essential and may lead to novel therapeutic approaches. Two ubiquitin E3-ligases, Cbl-b and c-Cbl, have been shown to bind to RTKs, including PDGFRβ; however, their effect on PDGFRβ has not been fully elucidated. In this study, we show that siRNA-mediated downregulation of both Cbl-b and c-Cbl led to a decrease in the cell surface clearance of the receptor, which is in accordance to its observed reduced endocytosis. No effect on PDGFRβ degradation was observed, which was found to be mainly performed by proteasomes, in contrast with the central dogma regarding RTK degradation in the lysosomes.

Additionally, Cbl-b was shown to interact with both PDGFRβ and c-Cbl upon PDGF-BB stimulation. Tyrosine residues 1021 and 1009 in PDGFRβ were found to be the main binding sites for Cbl-b, whereas no complex formation was detected between c-Cbl and PDGFRβ. The reduced rate of PDGFRβ cell surface clearance was associated with prolonged Src, PLCγ and Stat3 signaling, which led to increased migration towards PDGF-BB. Taken together, our results provided further insights into the important role of ubiquitination in PDGFRβ internalization and signaling.

Paper III: The ubiquitin-specific protease 4 deubiquitinates and stabilizes PDGF receptor β

This part is a continuation of paper II where we studied PDGFRβ ubiquitination. As already addressed, ubiquitination is an important post-translational modification, which regulates various cellular processes, and can be reversed by deubiquitinases (DUBs). RTK deubiquitination is not fully understood, particularly, no information exists on the DUBs acting on PDGFRβ. Association of DUBs with malignant diseases has been proposed and it is known that deregulation of PDGFR signaling can lead to tumorigenesis; thereby, studying DUBs acting on PDGFR may provide a possibility for future drug development and combination therapies.

In order to identify DUBs acting on PDGFRβ, we performed a screening using a cDNA library of sixty-four DUBs and identified two hits that showed a strong effect on PDGFRβ ubiquitination. The study presented in this paper focuses on one of the positive hits, USP4 and its role in PDGFRβ regulation. Interestingly, we found that USP4 interacts with PDGFRβ, removes both K48- and K63-linked polyubiquitin chains and stabilizes its expression. Furthermore, USP4 depletion reduced the ability of PDGF to promote anchorage-independent growth in soft agar, suggesting a role for USP4 in tumorigenesis. Our findings shed light, for the first time, on the mechanism of PDGFRβ deubiquitination and the key components that are involved in its regulation.

Paper IV: Specific targeting of PDGFRβ inhibits growth and angiogenesis of tumors with high PDGF-BB expression

Blood vessels are essential for tumor development as they supply nutrients and oxygen, which are prerequisites for tumor growth and progression; hence, targeting tumor angiogenesis is an attractive strategy in cancer therapy. As PDGF/PDGFRβ signaling is crucial for pericyte recruitment to the vasculature, tumor therapy targeting PDGFRβ has attracted attention; however, as all available PDGFR inhibitors, even those that are clinically used, inhibit a range of other targets, the details underlying the importance of PDGFRβ in tumorigenesis remain unknown.

By specifically targeting PDGFRβ *in vitro* and *in vivo*, we identified a role of PDGFRβ as a tumor modulator with significant effects on tumor vasculature. This study used a transgenic knock-in mouse strain carrying a silent mutation in the PDGFRβ ATP binding site (Analogue-Sensitive Kinase Allele technology or ASKA mice) that allows very specific inhibition of PDGFRβ using the compound 1-NaPP1, which interferes uniquely with the ASKA mutant and not with any other wild-type kinases. This model is very informative and allowed us to study the effect of PDGFRβ inhibition in stromal cells on tumor growth, using several tumor models devoid of PDGFRβ expression (B16 melanoma, B16/PDGF-BB, Lewis lung carcinoma (LLC) and EO771 breast carcinoma) and in comparison with the multi-target kinase inhibitor imatinib as a reference compound. Unexpectedly, 1-NaPP1 suppressed growth of tumors with high PDGF-BB expression more efficiently than the clinically used imatinib; however, neither compound showed an effect on low PDGF-BB-expressing tumors. Although both treatments affected tumor vascularization and pericyte coverage, specific inhibition of PDGFRβ by 1-NaPP1 led to a more profound effect on tumor angiogenesis with increased vessel apoptosis compared to imatinib treatment. Overall, our study suggests for the first time

that specific inhibition of PDGFRβ signaling, targeting the tumor stroma, could be beneficial for cancer patients with high levels of tumor PDGF-BB compared to multi-kinase PDGFR inhibitors currently available, and highlights the need for identifying biomarkers and stratifying patients for anti-PDGF cancer therapy.

Future perspectives

The aim of this thesis has been to shed light on the mechanisms underlying PDGFRβ regulation and our findings have given insights into different aspects of PDGFRβ signaling. This has "opened up the floor" for various scientifically exciting questions that require further experiments in order to be addressed.

Paper I

How does PDGF induce BMP signaling in an Erk5-dependent manner?

In paper I, we have investigated the activation of Erk5, one of PDGFR downstream effectors, and have attempted to delineate a pathway from the activated receptor to Erk5. Interestingly, we found that upon PDGF-BB stimulation, apart from the classical MAPK cascade that had been proposed (Mekk2-Mek5-Erk5), various other signaling molecules, such as Mek1/2, PI3K and classical PKCs, were involved in PDGF-BB-induced Erk5 activation, indicating an extensive interplay among pathways activated downstream of the receptor.

However, the most surprising finding was the Erk5-dependent effect of PDGF-BB on Smad 1/5/8 signaling, raising questions regarding the mechanisms underlying this crosstalk. Specifically, upon PDGF-BB stimulation in MOVAS cells, there was a rapid and Erk5-dependent increase in activation of transcription factors Smad1/5/8 and subsequent increase of BMP-induced gene responses. These effects, but not Erk5 activation, were inhibited by the BMP antagonist Noggin and BMP receptor kinase inhibitor DMH1, suggesting an indirect mechanism by which PDGF-BB-induced Erk5 activation might promote BMP receptor signaling. The exact mechanism remains to be unraveled. In 2015, Budi *et al.* demonstrated that insulin, by activating Akt, promotes delivery of TGFβ receptors from intracellular compartments to the cell surface, and thus, enhances TGFβ signaling (Budi *et al.*, 2015). It would be interesting to explore if Erk5 could function in a similar manner. Specifically, our hypothesis is that PDGF-induced Erk5 could be required to induce, release or process BMP ligands that will act on BMP receptors and activate Smad1/5/8 pathway. To address this, analysis of expression of BMP ligands and receptors in these cells, in presence or absence of Erk5, would be the first

step, followed by experiments focusing on the trafficking of BMP ligands or receptors.

Papers II and III

How does USP4 affect PDGFRβ stability and signaling response in normal and cancer cells?

In papers II and III, we have investigated the E3 ligases and DUBs, which regulate PDGFRβ ubiquitination and downregulation. We have identified USP4 as one of the deubiquitinases that act on PDGFRβ; however, our understanding of the importance of USP4 and deubiquitination overall in PDGFRβ signaling is still in its infancy. Our experiments have been mainly performed in overexpressed conditions, and therefore, it is necessary to reproduce our findings in cells expressing endogenous USP4 and PDGFRβ. By using USP4 wild-type or knockout mouse embryonic fibroblasts we can explore the role of USP4 in PDGFRβ ubiquitination, internalization, trafficking, stability and activation of downstream signaling pathways. As we have observed an interaction between the two proteins by performing co-immunoprecipitation experiments, it would be great to complement this finding using immunofluorescence or proximity ligation assay and identify the localization of these complexes. Based on our finding that USP4 is mainly localized in endosomal compartments, the experiments above would verify our hypothesis that the two proteins interact while PDGFRβ is sorted in the endosomes and that USP4 may play a role in PDGFRβ trafficking. Furthermore, as we discovered that depletion of Cbl-b and c-Cbl led to increased PDGF-induced chemotactic responses, deubiquitination could also affect PDGF signaling responses and further experiments should be performed in both normal and cancer cell lines to investigate the effect of USP4 and deubiquitination on processes such as migration, proliferation and invasion.

What are the mechanisms underlying PDGFRβ degradation?

Upon ligand binding and receptor dimerization, subsequent autophosphorylation and ubiquitination, RTKs are internalized, sorted in endosomes and multivesicular bodies and finally degraded in lysosomes. In paper II, where we aimed to unveil the importance of ubiquitination and Cbl proteins in PDGFRβ signaling, it was surprising to observe that the receptor degradation is performed mainly by proteasomes and that depletion of both Cbl proteins did not affect its degradation. This finding is in contrast to both the central dogma that most RTKs are degraded in lysosomes and previous studies showing that ^{125}I-labeled PDGF was transported to lysosomes for proteolysis (Nilsson *et al.*,

1983). This discrepancy needs to be further explored. As reviewed by Merilanthi & Elenius, some RTKs can be cleaved by gamma-secretase and their intracellular domain can translocate to the proteasome for degradation (Merilahti and Elenius, 2019); however, the mechanisms leading to such events are still poorly understood. Such events could dramatically affect PDGFRβ signaling and downregulation; therefore, further experiments will focus on exploring this hypothesis and unravel possible unknown molecular events.

Papers IV

Why does a specific PDGFRβ inhibitor reduce tumor growth while the multi-targeting drug imatinib does not?

Based on our findings we conclude that 1-NaPP1, which only inhibits PDGFRβ in the tumor stroma of our transgenic mouse model, prevents tumor growth in tumors expressing high levels of PDGF-BB, whereas the clinically used multi-targeted inhibitor imatinib (inhibiting PDGFRs, Abl, c-Kit, and more) does not. Furthermore, neither 1-NaPP1 nor imatinib have any significant effect on growth of low PDGF-BB-expressing tumors. This is an important observation that suggests that for tumors with high levels of PDGF-BB, development of a highly specific PDGFR inhibitor may be of clinical value. We would like to investigate why a selective inhibitor is more potent than a multi-targeted inhibitor. It is possible that imatinib's secondary target inhibition could lead to tumor growth, and this would therefore neutralize the tumor growth suppression of PDGFRβ inhibition. For this reason, we plan to isolate cells from tumors (using GFP-tagged cancer cell lines and markers for pericytes and endothelial cells), and perform protein and gene expression profiling after treatment with vehicle, imatinib or 1-NaPP1 in these different cell populations. Using this approach, we aim to explain the differences on the effect of the selective PDGFRβ targeting and the multi-kinase inhibitor imatinib on different cell types.

How does selective PDGFRβ inhibition affect the metastatic process?

Studies have shown that targeting pericytes in cancer therapy should be assessed carefully as there could be a risk for increased metastatic potential (Keskin *et al.*, 2015). Therefore, it would be of importance to investigate possible metastasis to the lungs upon PDGFRβ treatment alone (selective or multi-targeted) or combined with VEGF inhibitors. mRNA (and if possible protein expression) analysis of the isolated metastases, compared to the primary tumor, could inform us about gene expression changes that could have taken place during the metastatic process and how they are influenced by the respective treatment.

Furthermore, to more closely mimic the clinical situation, it would be interesting to investigate the effect of 1-NaPP1 on the growth and metastatic potential of already established vascularized tumors. In this case, experiments where drug treatment would be initiated when the primary tumors have already reached 400 mm^3 would provide important information for the potential use of selective PDGFRβ treatment of solid tumors in the clinics.

Acknowledgements

I will start by saying that it is interesting to know that these pages will actually be the most read ones in this book… I would like to thank so many people that have been part of my life all these years in Sweden and I honestly would not like anyone to feel neglected. I hope I will mention most of you and I apologize if I forget someone, but I owe to each one of you a big "thank you", as a few years ago I would have never imagined my life as it turned out to be. I will try to be brief…well… who am I kidding? It is me, the "talkative Maria", so it will be tricky…Anyway, let's start!

As I mentioned, many people have made essential contribution in order for me to be able to defend this thesis and two of the most important ones are my two supervisors that gave me the opportunity to join the lab, **Carl-Henrik Heldin** and **Johan Lennartsson**.

Calle…Well…it is definitely not a coincidence that in every thesis acknowledgments, most PhD students, who were part of Ludwig Institute for Cancer Research, have written almost the same words about you; and I will not be the exception. It is unbelievable that someone with your time schedule, responsibilities, meetings/conferences, and Nobel dinners with the royals, finds the time for each one of us to discuss our results or overall concerns, provide feedback and solutions, and even read our thesis. Your door has always been open for everyone and you really pay attention to every person that has worked in our corridor even if it was just for a month. You are the example that important people can still be normal humans (although I am still amazed with the insane amount of papers you read in your free time and I cannot see how it is possible!), and I really need to thank you, not only for giving me the chance to become a PhD student while Ludwig was closing down, but also for all the help and fruitful discussions over the years.

Johan…Words are not enough to describe my gratitude or even explain to someone how these years have been with you as a supervisor. You are the reason I entered the institute for my master thesis, the reason I am doing my PhD in cancer research and one of the reasons I am scared about my next steps, as I know that it will be super hard to find a supervisor like you. I came to Sweden for my master thinking of continuing my bachelor thesis topic, meeting the professor that discovered the enzyme I was working on, and suddenly, change of plans… I still remember being in the lecture hall, listening to you talking about cell-cell communication and signaling pathways in cancer, and that was the moment I realized what I wanted to work with later on. Thank

you for giving me the opportunity to join the group for my master thesis, for trying to find a solution so I could register as a PhD student (although you were clear during my initial interview that you would not be able to keep me for PhD studies) and for handling my directness and honesty. It has been so much fun to discuss not only science but also life in general every day. Even though it has been a tough time during my last year with moving to FarmBio and starting from scratch, thank you for being there for me but at the same time allowing me to be independent and put my ideas into practice.

Talking about supervisors, I would also like to thank **Nikos Balatsos**, my Greek supervisor from my bachelor studies, as he has always supported me, believed in me and motivated me to continue in science.

My dear **ST group**, with its current and past members: **Natalia**, thank you very much for all the moments we have shared during the last years, from lab lunches, cookies and laughs, to discussions and advice (scientific or not) and our project collaborations. **Niki**, "my scientific baby", you have been an excellent student and I am so proud of you for becoming a PhD student in our group. You are one of the most cheerful people I have ever met and please, never lose your enthusiasm and honesty. **Kehuan**, our newest member, I hope you will love your PhD time, keep smiling and try to enjoy every moment that is coming! **Glenda**, thank you for being there when I joined the group, working side by side, and for all the dinners at your place. All the best to all four of you! **Sara** and **Haisha**, you may have left the lab, but this was not the end, you definitely could not get rid of me! Thank you so much for teaching me everything about the animal project and for all your help inside and outside the animal house, even now, after so many years. I really do not know how to thank you! **Haisha**, thanks also for trying to teach me how to make dumplings, although unfortunately I never mastered the technique! **Sara** (and **Naz** and **Sophia**); we have shared happiness and sadness, and we have always been there for each other, and this means a lot! Sara, it is crazy to think that because of you my personal life changed radically☺. Thank you for everything! **Runting**, **Marisse**, **Yanli**, **Mikaela**, **Shady**, I hope you are all doing great and you have fun memories from our time together in the lab! I left someone last for a reason, as she is not just a previous member of the group; she is much more than that. She is my Swedish family. **Lotti**, you belong to the few people that I am not sure what to write for. You are my Swedish mum (even my mum is calling you like that) and it does not matter where I will be next, this will never change. I cannot explain how this happened, but it did. Not only have you shared with me all your lab skill secrets about great western blots, you have made me part of your life, and I have missed you so much since you moved across the street! I cannot remember how many times I had entered our lab saying "we have to talk" and we would try to solve the mystery or drama over a quick cup of coffee. You and Fredrik, along with my Greek family in Sweden, are the main reasons I see Sweden as my second home and my heart is breaking whenever I think of maybe leaving. Thank you ♥

I just mentioned my Greek family in Sweden, and this is a chapter of my life that cannot be described with just words. Friends are your chosen family, people say, and they are right. It all started with **Ria**, and **George,** and an invitation for Christmas Eve so I would not spend my Christmas here alone…and that was it, life was never the same again… You mean so much to me that I will not be able to explain it to anyone. I trust you in everything, I thank you for everything, and you appeared in my life the moment I needed someone to trust the most. You have offered me one of the greatest gifts, to become **Elli**'s godmother. I would never believe in my life that I could love someone with all my heart without even meeting him/her, and it is insane when it happens; and of course, when the moment came and this sweetheart came into this world, I knew that love at first sight is real. I will never forget the night you called me to come and babysit Elli so you would deliver the second sunshine, **Areti**, or the moment you entered with her in the house. Thank you! As most people know…Greek families are quite big! Ria and George did not enter in my life alone, they brought **Kallia** and **Rafaello**! You, and of course little **Dafni**, have also brought so much happiness to my life that it is indescribable, so many moments that they cannot be counted, laughs, tears, moving(s), dinners and so on. I will never forget you, Kallia, telling me that you were pregnant at the BMC parking lot, our coffee before you gave birth or my first visit to see Dafnaki… Raf, we baptized Elli together, and I do not think there would be a better person to share that moment. I really wish that you both fulfill all your dreams in the future. All four of you and all the little ones, became my family here, the ones I trust with all my fears, all my secrets, all my happy and sad moments; σας αγαπώ και σας ευχαριστώ!

If I think of Kallia, it is hard not to think of a crazy Italian that swears a lot. My dear **Claudia**, you entered our family with your unique personality and sense of humor. You are a strong independent woman, that tries hard to hide and protect her soft, kind, sweet heart and I really miss our moments as neighbors and our laughs by the staircase. Miss you lots! **Manolya**, best of luck in your new adventure and thanks for everything! **Panos** and **Costas**, I do not think I have met many people so hardworking, dedicated to what they are doing, helpful and fun at the same time. Pano, good luck in France with **Cristina** and may everything turn out as you wish; Costa, good luck with the rest of the PhD and I have no doubt that it is going to be amazing. Thank you both for all the help, inside and outside BMC and the fun moments, and as both of you are meant to stay in academia, I wish you good reviewers always!

The Greek community of our corridor has had quite a few members: **George Divolis** (you are just amazing!), **Panagiotis B.**, **Michalis** etc., I wish you all the best. **Chrysa**, good luck with your PhD, try to enjoy every moment and good luck with the teaching! The same applies to you, **Caroline**!

Ludwig has been like home for me, with people that they are not just coworkers, but people I adore. **Anita**, you have always been there caring for me, giving me advice, teaching me Swedish and I really thank you for that, I mean

it, and **Aino**, the same applies to you. You are such a fun person to socialize with; I really thank you both. **Lasse**, well...how many times should I say thank you for all the help you have given me? You have helped me with equipment, bike, furniture etc. and cared for me throughout these years. Tack! I miss my daily Swedish lessons/morning coffee breaks, with the three of you along with **Lotti**, **Ulla**, **Eva Hallin** and **Uffe**! You all contributed to the nice Ludwig atmosphere! **Anders**, I think you could easily be considered as one of my unofficial co-supervisors, if we would count how many times I have entered your office asking for advice, and you were helping me every single time. You are brilliant, hardworking and I truly admire you so much! **Laia**, thank you for everything, from scientific advice to helping me with my moving and worrying about my stress levels. Moltes grácies! **Mariya** and **Ihor**, дуже тобі дякую ☺, and **Oleks**, it has been so nice to share my old office space with you, and дякую also for trying to teach me Ukrainian! **Chun**, good luck with the rest of your PhD and your career, you are one of the kindest people I have met! **Yutaro**, thanks for all our cell lab discussions and good luck with everything as well! To **Aris Moustakas**, I owe a big thank you for all the help, reagents and crucial advice and feedback during these years. To **Paraskevi Heldin** as well, many thanks for all the interesting discussions and advice over the years. To **Ingvar Ferby**, thanks for being the chairperson for my defense, as well as for your feedback, especially regarding the animal project. To **all members of B11:3 corridor,** current or past, thank you for making it such as a nice environment to work. Special thanks to **Anna-Karin Olsson's group**; thank you all for your help with reagents, protocols, techniques, feedback and our collaboration in my last project. **Anahita**, many thanks for all our non-scientific discussions we had over these years! Thanks also to **Staffan Johansson**, for all your interesting suggestions after my presentations, as well as **Kristofer Rubin** and **Vahid Reyhani** for our collaboration that resulted in a published paper. **Merima**, **Fred**, **Simon**, **Oskar**, **Linda**, **Eva**, **Jon**, **Noopur**, **Gad**, **Varun**, **Prathyusha, Anna** and **Yanshuang**, thank you all for all the fun moments we had together! In addition, I would like also to thank all the **IMBIM administration** for their kind help!

During my PhD, I also encountered a big "Mediterranean" community! **Giulia**, my super-fast-walking Italian, thanks for all the advice, discussions and dinners before Cat's corner, bringing me to Lindy hop and always motivating me to come out of my comfort zone and try new things. You are amazing! **Ana-Rosa**, I will never forget our laughs while driving the van for my moving, the first (ever) nation party you brought me to, or sharing the craziness of preparing our theses simultaneously and supporting each other. Thanks also to: **Alejandro**, we were meant to be friends when you saw me bleeding on the street after my bike accident and walked me to the hospital; **Guilherme** for trying to teach me so many latin dances; **Mahsa**, **Carmen**, **Dani**, **Andrea**, **Davide**, **Ana Migot** (and so many others), for our fun moments and parties; and **José**, for your insane directness and your great sense of humor that not

many understand. **Saga/Solstickan**, **Joakim**, **Benjaminas**, **Joran**, **Ilektra**, **Anna**, **Victoria** and **João**, thanks for all the amazing dances and Lindy hop moments, from Herräng camp to weekly Cat's! Friends from my master time: **Gergana**, **Maria** and **Tonia**, we may not meet often but every time we do, it is as if no day has passed. Thanks girls! My sweet **Juliane**, the same applies to you! Good luck with your PhD, it is almost over!

As you all know, I was introduced to a new department last year and I would like to thank **everyone at Farmbio** and specifically, all the members of the **three groups** (**Ola Södeberg**, **Sara Mangsbo** and **Greta Hultqvist**) for all the nice moments, laughs and help. Special thanks to **Axel** that introduced me to the world of CellProfiler, and **Ola** and **Johan H.** for their help with my last project.

To people across the street, many thanks to the groups of **Elisabetta Dejana**, **Peetra Magnusson**, **Anna Dimberg** and **Christer Betsholtz**, for all their help with equipment, reagents and advice.

Apart from my parents, my Swedish family and my Greek family in Sweden, there is also a Dutch one! **Sandra** and **Willem**, **Jerke** and **Silvia** and **Lizzy**, you have welcomed me in your family and made me feel that I belong there from the first moment. I am honestly thankful for all your care and love and I am so lucky to have you all in my life. Hartelijk bedankt!

Greece will always be my home and I would like to thank several people there for supporting me all over the years. However, the next few sentences will be addressed to the two people I owe everything in my life as they have sacrificed a lot to see me succeed and they have loved me unconditionally. The book is dedicated to them. **Μαμά** και **μπαμπά**, αυτό το βιβλίο είναι για εσάς. Δεν θα ήμουν αυτή που είμαι χωρίς την αμέριστη αγάπη και στοργή σας. Έχετε θυσιάσει τόσα πολλά για να με μεγαλώσετε, για να σπουδάσω, για να βρίσκομαι εδώ κυνηγώντας τα όνειρα μου που δεν νομίζω να υπάρχει τρόπος να σας το ανταποδώσω ποτέ. Είστε το λιμάνι μου, κι αν και ξέρω πόσο δύσκολο ήταν να αφήσετε το μοναχοπαίδι σας που αποκτήσατε με τόσο κόπο να φύγει μακριά και πόσο έχετε κλάψει, δεν παραπονεθήκατε ποτέ κι απλά λέγατε ότι τα παιδιά πρέπει να ανοίγουν τα φτερά τους και να είναι ευτυχισμένα. Σας αγαπώ τόσο όσο οι λέξεις δεν μπορούν να περιγράψουν και σας ευχαριστώ για όλα ♥

Last but definitely not least, I would like to thank someone that appeared in my life suddenly and changed it so much that I never would have imagined it beforehand. **Joppe**, καρδιά μου, dank je wel! Thank you for your help while I was writing this thesis, but mainly thank you for being there for me every single day during the last years, going through my "craziness" and nightmares, helping me deal with my stress and fears, and bringing constant happiness. You are my support, my strength and the reason why my heart smiles every day. We have been together through happy and stressful times, sickness and health, and I feel so blessed to have you in my life that I cannot imagine it without you. Ik hou van jou!

References

Abdul Rehman, S. A. *et al.* (2016) MINDY-1 is a member of an evolutionarily conserved and structurally distinct new family of deubiquitinating enzymes, *Molecular Cell*, 63: 146–155.

Akekawatchai, C., Holland, J. D., Kochetkova, M., Wallace, J. C. and McColl, S. R. (2005) Transactivation of CXCR4 by the insulin-like growth factor-1 receptor (IGF-1R) in human MDA-MB-231 breast cancer epithelial cells, *The Journal of Biological Chemistry*, 280: 39701–8.

Akutsu, M., Dikic, I. and Bremm, A. (2016) Ubiquitin chain diversity at a glance, *Journal of Cell Science*, 129: 875–80.

Alkasalias, T., Moyano-Galceran, L., Arsenian-Henriksson, M. and Lehti, K. (2018) Fibroblasts in the tumor microenvironment: shield or spear?, *International Journal of Molecular Sciences*, 19: E1532.

Andrae, J., Gallini, R. and Betsholtz, C. (2008) Role of platelet-derived growth factors in physiology and medicine, *Genes & Development*, 22: 1276–1312.

Antoniades, H. N. (1981) Human platelet-derived growth factor (PDGF): purification of PDGF-I and PDGF-II and separation of their reduced subunits, *Proceedings of the National Academy of Sciences of the United States of America*, 78: 7314–7317.

Antoniou, G., Lee, A. T. J., Huang, P. H. and Jones, R. L. (2018) Olaratumab in soft tissue sarcoma – Current status and future perspectives, *European Journal of Cancer*, 92: 33–39.

Arias-González, L. *et al.* (2013) ERK5/BMK1 is a novel target of the tumor suppressor VHL: implication in clear cell renal carcinoma, *Neoplasia (New York, N.Y.)*, 15: 649–59.

Armulik, A., Genové, G. and Betsholtz, C. (2011) Pericytes: developmental, physiological, and pathological perspectives, problems, and promises, *Developmental Cell*, 193–215.

Arts, F. A. *et al.* (2017) PDGFRB gain-of-function mutations in sporadic infantile myofibromatosis, *Human Molecular Genetics*, 26: 1801–1810.

Arts, F. A., Velghe, A. I., Stevens, M., Renauld, J.-C., Essaghir, A. and Demoulin, J.-B. (2015) Idiopathic basal ganglia calcification-associated PDGFRB mutations impair the receptor signalling, *Journal of Cellular and Molecular Medicine*, 19: 239–248.

Aubin, J., Davy, A. and Soriano, P. (2004) In vivo convergence of BMP and MAPK signaling pathways: Impact of differential Smad1 phosphorylation on development and homeostasis, *Genes & Development*, 18: 1482–1494.

Bache, K. G., Slagsvold, T. and Stenmark, H. (2004) Defective downregulation of receptor tyrosine kinases in cancer, *The EMBO journal*, 23: 2707–12.

Ball, S. G., Bayley, C., Shuttleworth, C. A. and Kielty, C. M. (2010) Neuropilin-1 regulates platelet-derived growth factor receptor signalling in mesenchymal stem cells, *The Biochemical Journal*, 427: 29–40.

Barbazán, J. and Matic Vignjevic, D. (2019) Cancer associated fibroblasts: is the force the path to the dark side?, *Current Opinion in Cell Biology*, 56: 71–79.
Bartoschek, M. *et al.* (2018) Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing, *Nature Communications*, 9: 5150.
Basciani, S., Mariani, S., Spera, G. and Gnessi, L. (2010) Role of platelet-derived growth factors in the testis, *Endocrine Reviews*, 31: 916–939.
Baxter, R. M., Secrist, J. P., Vaillancourt, R. R. and Kazlauskas, A. (1998) Full activation of the platelet-derived growth factor beta-receptor kinase involves multiple events, *The Journal of Biological Chemistry*, 273: 17050–5.
Belli, C. *et al.* (2018) Targeting the microenvironment in solid tumors, *Cancer Treatment Reviews*, 65: 22–32.
Bergers, G. and Hanahan, D. (2008) Modes of resistance to anti-angiogenic therapy, *Nature Reviews Cancer*, 8: 592–603.
Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E. and Hanahan, D. (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors, *The Journal of Clinical Investigation*, 111: 1287–95.
Bergsten, E. *et al.* (2001) PDGF-D is a specific, protease-activated ligand for the PDGF β-receptor, *Nature Cell Biology*, 3: 512.
Betsholtz, C. (1995) Role of platelet-derived growth factors in mouse development, *International Journal of Developmental Biology*, 39: 817–825.
Betsholtz, C. (2003) Biology of Platelet-Derived Growth Factors in Development, *Birth Defects Research Part C - Embryo Today: Reviews*, 272–285.
Boström, H. *et al.* (1996) PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis, *Cell*, 85: 863–873.
Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Druker, B. and Lydon, N. (1996) Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative, *Cancer Research*, 56: 100–104.
Budi, E. H., Muthusamy, B.-P. and Derynck, R. (2015) The insulin response integrates increased TGF-β signaling through Akt-induced enhancement of cell surface delivery of TGF-β receptors, *Science Signaling*, 8: ra96.
Cabodi, S. *et al.* (2004) Integrin regulation of epidermal growth factor (EGF) receptor and of EGF-dependent responses, *Biochemical Society Transactions*, 32: 438–442.
Camorani, S. *et al.* (2014) Inhibition of receptor signaling and of glioblastoma-derived tumor growth by a novel PDGFRβ aptamer, *Molecular therapy : the journal of the American Society of Gene Therapy*, 22: 828–41.
Camorani, S. *et al.* (2017) Inhibition of bone marrow-derived mesenchymal stem cells homing towards triple-negative breast cancer microenvironment using an anti-PDGFRβ aptamer, *Theranostics*, 7: 3595–3607.
Cao, Y. and Langer, R. (2008) A review of Judah Folkman's remarkable achievements in biomedicine, *Proceedings of the National Academy of Sciences*, 105: 13203–5.
Carroll, M. *et al.* (1997) CGP 57148, a Tyrosine Kinase Inhibitor, Inhibits the Growth of Cells Expressing BCR-ABL, TEL-ABL, and TEL-PDGFR Fusion Proteins, *Blood*, 90: 4947–52.
Caspani, E. M., Crossley, P. H., Redondo-Garcia, C. and Martinez, S. (2014) Glioblastoma: A pathogenic crosstalk between tumor cells and pericytes, *PLOS One*, 9: e101402.
Castano, Z., Marsh, T., Tadipatri, R., Kuznetsov, H. S., Al-Shahrour, F. and Paktinat, M. (2013) Stromal EGF and IGF-I together modulate plasticity of disseminated triple-negative breast tumors, *Cancer Discovery*, 3: 922–935.

Chen, I. (2016) New DUBs on the block, *Nature Structural & Molecular Biology*, 23: 623–623.
Chen, P.-H., Chen, X. and He, X. (2013) Platelet-derived growth factors and their receptors: structural and functional perspectives, *Biochimica et Biophysica Acta (BBA)-Proteins*, 1834: 2176–86.
Chiara, F., Bishayee, S., Heldin, C.-H. and Demoulin, J.-B. (2004) Autoinhibition of the platelet-derived growth factor β-receptor tyrosine kinase by its C-terminal tail, *The Journal of Biological Chemistry*, 279: 19732–19738.
Claesson-Welsh, L. *et al.* (1988) cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules, *Molecular and Cellular Biology*, 8: 3476–86.
Claesson-Welsh, L., Eriksson, A., Westermark, B. and Heldin, C. H. (1989) cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor, *Proceedings of the National Academy of Sciences of the United States of America*, 86: 4917–21.
Clague, M. J., Barsukov, I., Coulson, J. M., Liu, H., Rigden, D. J. and Urbé, S. (2013) Deubiquitylases From Genes to Organism, *Physiological Reviews*, 93: 1289–1315.
Cobb, M. H. (1999) MAP kinase pathways, *Progress in Biophysics and Molecular Biology*, 71: 479–500.
Cobb, M. H., Robbins, D. J. and Boulton, T. G. (1991) ERKs, extracellular signal-regulated MAP-2 kinases, *Current Opinion in Cell Biology*, 3: 1025–1032.
Cooper, D. M., Yu, E. Z., Hennessey, P., Ko, F. and Robson, M. C. (1994) Determination of endogenous cytokines in chronic wounds, *Annals of surgery*, 219: 688–692.
Cortez, E., Roswall, P. and Pietras, K. (2014) Functional subsets of mesenchymal cell types in the tumor microenvironment, *Seminars in Cancer Biology*, 25: 3–9.
Critchley, W. *et al.* (2018) Receptor tyrosine kinase ubiquitination and de-ubiquitination in signal transduction and receptor trafficking, *Cells*, 7: 22.
D'Arcy, P., Wang, X. and Linder, S. (2015) Deubiquitinase inhibition as a cancer therapeutic strategy, *Pharmacology & Therapeutics*, 147: 32–54.
DeMali, K. A., Balciunaite, E. and Kazlauskas, A. (1999) Integrins enhance platelet-derived growth factor (PDGF)-dependent responses by altering the signal relay enzymes that are recruited to the PDGF β receptor, *The Journal of Biological Chemistry*, 274: 19551–19558.
Demoulin, J.-B. and Essaghir, A. (2014) PDGF receptor signaling networks in normal and cancer cells, *Cytokine & Growth Factor Reviews*, 25: 273–283.
Demoulin, J.-B. and Montano-Almendras, C. P. (2012) Platelet-derived growth factors and their receptors in normal and malignant hematopoiesis, *American Journal of Blood Research*, 2: 44–56.
Demoulin, J.-B., Seo, J. K., Ekman, S., Grapengiesser, E., Hellman, U., Rönnstrand, L. and Heldin, C.-H. (2003) Ligand-induced recruitment of Na+/H+-exchanger regulatory factor to the PDGF (platelet-derived growth factor) receptor regulates actin cytoskeleton reorganization by PDGF, *The Biochemical Journal*, 376: 505–10.
Derynck, R. *et al.* (1985) Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells, *Nature*, 316: 701–705.
Díaz-Rodríguez, E. and Pandiella, A. (2010) Multisite phosphorylation of Erk5 in mitosis, *Journal of Cell Science*, 123: 3146–3156.
Ding, H. *et al.* (2004) A specific requirement for PDGF-C in palate formation and PDGFR-α signaling, *Nature Genetics*, 36: 1111–1116.

Donnem, T. *et al.* (2013) Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment?, *Cancer medicine*, 2: 427–36.
Drew, B. A., Burow, M. E. and Beckman, B. S. (2012) MEK5/ERK5 pathway: The first fifteen years, *Biochimica et Biophysica Acta - Reviews on Cancer*, 1825: 37–48.
Eichhorn, M. E., Kleespies, A., Angele, M. K., Jauch, K.-W. and Bruns, C. J. (2007) Angiogenesis in cancer: molecular mechanisms, clinical impact, *Langenbeck's Archives of Surgery*, 392: 371–379.
Ekman, S., Thuresson, E. R., Heldin, C.-H. and Rönnstrand, L. (1999) Increased mitogenicity of an αβ heterodimeric PDGF receptor complex correlates with lack of RasGAP binding, *Oncogene*, 18: 2481–2488.
Esparís-ogando, A., Díaz-rodríguez, E., Montero, C., Yuste, L., Crespo, P. and Montero, J. C. (2002) Erk5 participates in neuregulin signal transduction and is constitutively active in breast cancer cells overexpressing ErbB2, *Molecular and Cellular Biology*, 22: 270–285.
Finegan, K. G., Perez-Madrigal, D., Hitchin, J. R., Davies, C. C., Jordan, A. M. and Tournier, C. (2015) ERK5 is a critical mediator of inflammation-driven cancer, *Cancer Research*, 75: 742–753.
Folestad, E., Kunath, A. and Wågsäter, D. (2018) PDGF-C and PDGF-D signaling in vascular diseases and animal models, *Molecular Aspects of Medicine*, 62: 1–11.
Folkman, J. (1974) Proceedings: Tumor angiogenesis factor, *Cancer Research*, 34: 2109–2113.
Franco, M. *et al.* (2011) Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression., *Blood*, 118: 2906–17.
Franke, T. F., Kaplan, D. R. and Cantley, L. C. (1997) PI3K: Downstream AKTion Blocks Apoptosis, *Cell*, 88: 435–437.
Frederick, A., Rolfe, M. and Chiu, I. (1998) The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines, *Oncogene*, 16: 153–165.
Fredriksson, L. *et al.* (2012) Platelet-derived growth factor C deficiency in C57BL/6 mice leads to abnormal cerebral vascularization, loss of neuroependymal integrity, and ventricular abnormalities, *The American Journal of Pathology*, 180: 1136–44.
Fredriksson, L., Li, H. and Eriksson, U. (2004) The PDGF family: four gene products form five dimeric isoforms, *Cytokine & Growth Factor Reviews*, 15: 197–204.
Galisteo, M. L., Dikic, I., Batzer, A. G., Langdon, W. Y. and Schlessinger, J. (1995) Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation, *The Journal of Biological Chemistry*, 270: 20242–5.
Geevarghese, A. and Herman, I. M. (2014) Pericyte-endothelial crosstalk: Implications and opportunities for advanced cellular therapies, *Translational Research*, 163: 296–306.
Gladh, H. *et al.* (2016) Mice lacking platelet-derived growth factor D display a mild vascular phenotype, *PLOS One*, 11: e0152276.
Goh, L. K. and Sorkin, A. (2013) Endocytosis of receptor tyrosine kinases, *Cold Spring Harbor Perspectives in Biology*, 5: 1–17.
Gomez, N., Erazo, T. and Lizcano, J. M. (2016) ERK5 and Cell Proliferation: Nuclear Localization Is What Matters, *Frontiers in Cell and Developmental Biology*, 4: 105.

Greco, A., Fusetti, L., Villa, R., Sozzi, G., Minoletti, F., Mauri, P. and Pierotti, M. A. (1998) Transforming activity of the chimeric sequence formed by the fusion of collagen gene COL1A1 and the platelet derived growth factor b-chain gene in dermatofibrosarcoma protuberans, *Oncogene*, 17: 1313–1319.
Guo, W. *et al.* (2018) Up-regulated deubiquitinase USP4 plays an oncogenic role in melanoma, *Journal of Cellular and Molecular Medicine*, 22: 2944–2954.
Guo, Z. *et al.* (2007) The synergistic effects of CXCR4 and EGFR on promoting EGF-mediated metastasis in ovarian cancer cells, *Colloids and Surfaces B: Biointerfaces*, 60: 1–6.
Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P. and Dikic, I. (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation, *Nature Cell Biology*, 5: 461–466.
Hanahan, D. and Coussens, L. M. (2012) Accessories to the crime: Functions of cells recruited to the tumor microenvironment, *Cancer Cell*, 21: 309–322.
Hanahan, D. and Weinberg, R. A. (2000) The hallmarks of cancer, *Cell*, 100: 57–70.
Hanahan, D. and Weinberg, R. A. (2011) Hallmarks of cancer: The next generation, *Cell*, 144: 646–674.
Harrell, C. R., Simovic Markovic, B., Fellabaum, C., Arsenijevic, A., Djonov, V. and Volarevic, V. (2018) Molecular mechanisms underlying therapeutic potential of pericytes, *Journal of Biomedical Science*, 25: 21.
Harrigan, J. A., Jacq, X., Martin, N. M. and Jackson, S. P. (2017) Deubiquitylating enzymes and drug discovery: emerging opportunities, *Nature Reviews Drug Discovery*, 17: 57–78.
Hayashi, M. *et al.* (2004) Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure, *Journal of Clinical Investigation*, 113: 1138–1148.
Hayashi, M. and Lee, J. D. (2004) Role of the BMK1/ERK5 signaling pathway: Lessons from knockout mice, *Journal of Molecular Medicine*, 82: 800–808.
He, L. *et al.* (2016) Analysis of the brain mural cell transcriptome, *Scientific Reports*, 6: 35108.
Heideker, J. and Wertz, I. E. (2015) DUBs, the regulation of cell identity and disease, *The Biochemical Journal*, 465: 1–26.
Heldin, C.-H. (2013) Targeting the PDGF signaling pathway in tumor treatment, *Cell Communication and Signaling*, 11: 97.
Heldin, C.-H. and Lennartsson, J. (2013) Structural and functional properties of platelet-derived growth factor and stem cell factor receptors, *Cold Spring Harbor Perspectives in Biology*, 5: a009100.
Heldin, C.-H. and Moustakas, A. (2016) Signaling receptors for TGF-β family members, *Cold Spring Harbor Perspectives in Biology*, 8: a022053.
Heldin, C. H. *et al.* (1988) Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types, *The EMBO journal*, 7: 1387–93.
Heldin, C. H. *et al.* (1999) Mechanism of action and in vivo role of platelet-derived growth factor, *Physiological reviews*, 79: 1283–316.
Heldin, C. H., Ernlund, A., Rorsman, C. and Ronnstrand, L. (1989) Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation, *The Journal of Biological Chemistry*, 264: 8905–8912.
Heldin, C. H., Hellberg, C. and Östman, A. (2010) PDGF and vessel maturation, *Recent Results in Cancer Research*, 103–114.

Heldin, C. H., Lennartsson, J. and Westermark, B. (2018) Involvement of platelet-derived growth factor ligands and receptors in tumorigenesis, *Journal of Internal Medicine*, 283: 16–44.
Heldin, C. H., Miyazono, K. and ten Dijke, P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins, *Nature*, 390: 465–71.
Heldin, C. H., Westermark, B. and Wasteson, A. (1979) Platelet-derived growth factor: purification and partial characterization, *Proceedings of the National Academy of Sciences of the United States of America*, 76: 3722–3726.
Heldin, C. H., Westermark, B. and Wasteson, A. (1981) Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia, *Proceedings of the National Academy of Sciences of the United States of America*, 78: 3664–3668.
Hellstrom, M., Kal n, M., Lindahl, P., Abramsson, A. and Betsholtz, C. (1999) Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse, *Development*, 126: 3047 LP-3055.
Heuchel, R. *et al.* (1999) Platelet-derived growth factor beta receptor regulates interstitial fluid homeostasis through phosphatidylinositol-3' kinase signaling, *Proceedings of the National Academy of Sciences of the United States of America*, 96: 11410–11415.
Hicke, L. (2001) Protein regulation by monoubiquitin, *Nature Reviews Molecular Cell Biology*, 2: 195.
Hirschi, K. K. and D'Amore, P. A. (1996) Pericytes in the microvasculature, *Cardiovascular Research*, 687–698.
Hoang, V. T., Yan, T. J., Cavanaugh, J. E., Flaherty, P. T., Beckman, B. S. and Burow, M. E. (2017) Oncogenic signaling of MEK5-ERK5, *Cancer Letters*, 392: 51–59.
Hoch, R. V and Soriano, P. (2003) Roles of PDGF in animal development, *Development*, 130: 4769 LP-4784.
Iqbal, N. and Iqbal, N. (2014) Imatinib: a breakthrough of targeted therapy in cancer, *Chemotherapy, Research and Practice*, 2014: 357027.
Isakov, N. (2018) Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression, *Seminars in Cancer Biology*, 48: 36–52.
James, M. F., Beauchamp, R. L., Manchanda, N., Kazlauskas, A. and Ramesh, V. (2004) A NHERF binding site links the betaPDGFR to the cytoskeleton and regulates cell spreading and migration, *Journal of cell science*, 117: 2951–61.
Jean, S. and Kiger, A. A. (2014) Classes of phosphoinositide 3-kinases at a glance, *Journal of Cell Science*, 127: 923–8.
Jin, X. *et al.* (2011) EGFR-AKT-Smad signaling promotes formation of glioma stem-like cells and tumor angiogenesis by ID3-driven cytokine induction, *Cancer Research*, 71: 7125–34.
Jo, N. *et al.* (2006) Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization, *The American Journal of Pathology*, 168: 2036–53.
Johnston, J. J. *et al.* (2015) A Point Mutation in PDGFRB Causes Autosomal-Dominant Penttinen Syndrome, *The American Journal of Human Genetics*, 97: 465–474.
Jouenne, F. *et al.* (2017) Germline CDKN2A/P16INK4A mutations contribute to genetic determinism of sarcoma, *Journal of Medical Genetics*, 54: 607–612.
Kaplan, D. R., Morrison, D. K., Wong, G., McCormick, F. and Williams, L. T. (1990) PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex, *Cell*, 61: 125–133.

Kashishian, A., Kazlauskas, A. and Cooper, J. A. (1992) Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo, *The EMBO journal*, 11: 1373–82.
Kazlauskas, A. and Cooper, J. A. (1989) Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins, *Cell*, 58: 1121–1133.
Kazlauskas, A., Durden, D. L. and Cooper, J. A. (1991) Functions of the major tyrosine phosphorylation site of the PDGF receptor beta subunit, *Cell Regulation*, 2: 413–25.
Keller, A. *et al.* (2013) Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice, *Nature Genetics*, 45: 1077–1082.
Keskin, D. *et al.* (2015) Targeting Vascular Pericytes in Hypoxic Tumors Increases Lung Metastasis via Angiopoietin-2, *Cell Reports*, 10: 1066–1081.
Kikkawa, U. (2018) The story of PKC: A discovery marked by unexpected twists and turns, *IUBMB Life*.
Kikuchi, K. *et al.* (1993) Dermatofibrosarcoma protuberans: Increased growth response to platelet-derived growth BB in cell culture, *Biochemical and Biophysical Research Communications*, 196: 409–415.
Kim, S. M., Lee, H., Park, Y. S., Lee, Y. and Seo, S. W. (2012) ERK5 regulates invasiveness of osteosarcoma by inducing MMP-9, *Journal of Orthopaedic Research*, 30: 1040–1044.
Klinghoffer, R. A., Mueting-Nelsen, P. F., Faerman, A., Shani, M. and Soriano, P. (2001) The two PDGF receptors maintain conserved signaling in vivo despite divergent embryological functions, *Molecular Cell*, 7: 343–354.
Komander, D., Clague, M. J. and Urbé, S. (2009) Breaking the chains: structure and function of the deubiquitinases, *Nature Reviews Molecular Cell Biology*, 10: 550.
Komander, D. and Rape, M. (2012) The ubiquitin code, *Annual Review of Biochemistry*, 81: 203–229.
Kondoh, K., Terasawa, K., Morimoto, H. and Nishida, E. (2006) Regulation of nuclear translocation of extracellular signal-regulated kinase 5 by active nuclear import and export mechanisms, *Molecular and Cellular Biology*, 26: 1679–1690.
Kubota, Y. (2012) Tumor angiogenesis and anti-angiogenic therapy, *Keio Journal of Medicine*, 61: 47–56.
Lammers, R., Obberghen, E. Van, Ballotti, R., Schlessinger, J. and Ullrich, A. (1990) Transphosphorylation as a Possible Mechanism for Insulin and Epidermal Growth Factor Receptor Activation, *The Journal of Biological Chemistry*, 265: 16886–16890.
Lan, S., Wu, S., Raghavaraju, G., Chow, N. and Liu, H. (2013) The crosstalk of c-MET with related receptor tyrosine kinases in urothelial bladder cancer, in Persad, R. and Ranasinghe, W. (eds) *Intech open*, 21–39.
Le, N.-T., Hoang, N. M., Fujiwara, K. and Abe, J. (2018) MEK5/ERK5, in *Encyclopedia of Signaling Molecules*, 3052–3074.
Lee, J. D., Ulevitch, R. J. and Han, J. (1995) Primary structure of BMK1: a new mammalian map kinase., *Biochemical and Biophysial Research Communications*, 715–24.
Lemmon, M. A. and Schlessinger, J. (2010) Cell signaling by receptor tyrosine kinases, *Cell*, 141: 1117–34.
Lennartsson, J., Wardega, P., Engström, U., Hellman, U. and Heldin, C. H. (2006) Alix facilitates the interaction between c-Cbl and platelet-derived growth factor β-receptor and thereby modulates receptor down-regulation, *The Journal of Biological Chemistry*, 281: 39152–39158.

Levéen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E. and Betsholtz, C. (1994) Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities, *Genes & Development*, 8: 1875–1887.

Levkowitz, G. *et al.* (1999) Ubiquitin Ligase Activity and Tyrosine Phosphorylation Underlie Suppression of Growth Factor Signaling by c-Cbl/Sli-1, *Molecular Cell*, 4: 1029–1040.

Li, T. *et al.* (2018) Ubiquitin-specific protease 4 promotes hepatocellular carcinoma progression via cyclophilin A stabilization and deubiquitination, *Cell Death & Disease*, 9: 148.

Li, X. *et al.* (2000) PDGF-C is a new protease-activated ligand for the PDGF α-receptor, *Nature Cell Biology*, 2: 302.

Li, Y., Jiang, D., Zhang, Q., Liu, X. and Cai, Z. (2016) Ubiquitin-specific protease 4 inhibits breast cancer cell growth through the upregulation of PDCD4, *International Journal of Molecular Medicine*, 38: 803–11.

Li, Z. *et al.* (2016) USP4 inhibits p53 and NF-κB through deubiquitinating and stabilizing HDAC2, *Oncogene*, 35: 2902–2912.

Lidén, A., Berg, A., Nedrebø, T., Reed, R. K. and Rubin, K. (2006) Platelet-Derived Growth Factor BB–Mediated Normalization of Dermal Interstitial Fluid Pressure After Mast Cell Degranulation Depends on β3 but Not β1 Integrins, *Circulation Research*, 98: 635–641.

Lien, E. C., Dibble, C. C. and Toker, A. (2017) PI3K signaling in cancer: beyond AKT, *Current Opinion in Cell Biology*, 45: 62–71.

Lindahl, P. (1997) Pericyte Loss and Microaneurysm Formation in PDGF-B-Deficient Mice, *Science (New York, N.Y.)*, 277: 242–245.

Lindahl, P. and Betsholtz, C. (1998) Not all myofibroblasts are alike: revisiting the role of PDGF-A and PDGF-B using PDGF-targeted mice, *Current Opinion in Nephrology and Hypertension*, 21–6.

Lindblom, P. *et al.* (2003) Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall, *Genes & Development*, 17: 1835–1840.

Lochhead, P. A., Gilley, R. and Cook, S. J. (2012) ERK5 and its role in tumour development, *Biochemical Society Transactions*, 40: 251–256.

Lowery, C. D. *et al.* (2018) Olaratumab exerts antitumor activity in preclinical models of pediatric bone and soft tissue tumors through inhibition of platelet-derived growth factor receptor α, *Clinical Cancer Research*, 24: 847–857.

Lu, C. *et al.* (2010) Targeting pericytes with a PDGF-B aptamer in human ovarian carcinoma models, *Cancer Biol Ther*, 9: 176–182.

Lu, K., Lamagna, C. and Bergers, G. (2008) Chapter 3 Bone marrow–derived vascular progenitors and proangiogenic monocytes in tumors, *Methods in Enzymology*, 445: 53–82.

Manning, B. D. and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream, *Cell*, 129: 1261–1274.

Mathews, L. S. and Vale, W. W. (1991) Expression cloning of an activin receptor, a predicted transmembrane serine kinase, *Cell*, 65: 973–982.

Matsuo, K., Lu, C., Shazad, M. K., Coleman, R. and Sood, A. (2010) Role of pericytes in resistance to antiangiogenic therapy, in *The Tumor Microenvironment*, 311–323.

McClean, M. N., Mody, A., Broach, J. R. and Ramanathan, S. (2007) Cross-talk and decision making in MAP kinase pathways., *Nature genetics*, 39: 409–414.

McCracken, S. R. C. *et al.* (2008) Aberrant expression of extracellular signal-regulated kinase 5 in human prostate cancer, *Oncogene*, 27: 2978–88.

Mehić, M., de Sa, V. K., Hebestreit, S., Heldin, C.-H. and Heldin, P. (2017) The deubiquitinating enzymes USP4 and USP17 target hyaluronan synthase 2 and differentially affect its function, *Oncogenesis*, 6: e348.

Meisenhelder, J., Suh, P.-G., Rhee, S. G. and Hunter, T. (1989) Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro, *Cell*, 57: 1109–1122.

Merilahti, J. A. M. and Elenius, K. (2019) Gamma-secretase-dependent signaling of receptor tyrosine kinases, *Oncogene*, 38: 151–163.

Miyake, S., Mullane-Robinson, K. P., Lill, N. L., Douillard, P. and Band, H. (1999) Cbl-mediated negative regulation of platelet-derived gowth factor receptor-dependent cell proliferation: A critical role for Cbl tyrosine kianse-binding domain, *The Journal of Biological Chemistry*, 274: 16619–16628.

Miyazawa, K. *et al.* (1998) Role of immunoglobulin-like domains 2-4 of the platelet-derived growth factor alpha-receptor in ligand-receptor complex assembly, *The Journal of Biological Chemistry*, 273: 25495–502.

Miyazono, K., Katsuno, Y., Koinuma, D., Ehata, S. and Morikawa, M. (2018) Intracellular and extracellular TGF-β signaling in cancer: some recent topics, *Frontiers of Medicine*, 387–411.

Mohapatra, B. *et al.* (2013) Protein tyrosine kinase regulation by ubiquitination: Critical roles of Cbl-family ubiquitin ligases, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1833: 122–139.

Montero, J. C., Ocaña, A., Abad, M., Ortiz-Ruiz, M. J., Pandiella, A. and Esparís-Ogando, A. (2009) Expression of Erk5 in early stage breast cancer and association with disease free survival identifies this kinase as a potential therapeutic target, *PLOS One*, 4: e5565.

Mori, S., Heldin, C. H. and Claesson-Welsh, L. (1993) Ligand-induced ubiquitination of the platelet-derived growth factor beta-receptor plays a negative regulatory role in its mitogenic signaling, *The Journal of Biological Chemistry*, 268: 577–83.

Morikawa, M., Derynck, R. and Miyazono, K. (2016) TGF-β and the TGF-β family: Context-dependent roles in cell and tissue physiology, *Cold Spring Harbor Perspectives in Biology*, 8: a021873.

Morita, Y., Leslie, M., Kameyama, H., Volk, D. E. and Tanaka, T. (2018) Aptamer therapeutics in cancer: Current and future., *Cancers*, 10: E80.

Morrison, D. K. (2012) MAP kinase pathways, *Cold Spring Harbor Perspectives in Biology*, 4: a011254–a011254.

Motegi, S. I., Garfield, S., Feng, X., Sárdy, M. and Udey, M. C. (2011) Potentiation of platelet-derived growth factor receptor-β signaling mediated by integrin-associated MFG-E8, *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31: 2653–2664.

Muhl, L., Folestad, E. B., Gladh, H., Wang, Y., Moessinger, C., Jakobsson, L. and Eriksson, U. (2017) Neuropilin 1 binds PDGF-D and is a co-receptor in PDGF-D–PDGFRβ signaling, *Journal of Cell Science*, 130: 1365–1378.

Munde, P. B., Khandekar, S. P., Dive, A. M. and Upadhyaya, N. R. (2014) Pericytes in health and disease, *International Journal of Oral & Maxillofacial Pathology*, 5: 02–07.

Nakamura, K. and Johnson, G. L. (2003) PB1 domains of MEKK2 and MEKK3 interact with the MEK5 PB1 domain for activation of the ERK5 pathway, *The Journal of Biological Chemistry*, 278: 36989–36992.

Nijman, S. M. B., Luna-Vargas, M. P. A., Velds, A., Brummelkamp, T. R., Dirac, A. M. G., Sixma, T. K. and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes, *Cell*, 123: 773–786.

Nilsson, J., Thyberg, J., Heldin, C. H., Westermark, B. and Wasteson, A. (1983) Surface binding and internalization of platelet-derived growth factor in human fibroblasts, *Proceedings of the National Academy of Sciences of the United States of America*, 80: 5592–5596.

Nishimoto, S. and Nishida, E. (2006) MAPK signalling: ERK5 versus ERK1/2, *EMBO reports*, 7: 782–6.

Nithianandarajah-Jones, G. N., Wilm, B., Goldring, C. E. P., Müller, J. and Cross, M. J. (2012) ERK5: Structure, regulation and function, *Cellular Signalling*, 24: 2187–2196.

Nithianandarajah-Jones, G. N., Wilm, B., Goldring, C. E. P., Müller, J. and Cross, M. J. (2014) The role of ERK5 in endothelial cell function, *Biochemical Society Transactions*, 42: 1584–9.

Omura, T., Heldin, C. H. and Ostman, A. (1997) Immunoglobulin-like domain 4-mediated receptor-receptor interactions contribute to platelet-derived growth factor-induced receptor dimerization, *The Journal of Biological Chemistry*, 272: 12676–82.

Orth, J. D. and McNiven, M. A. (2006) Get off my back! Rapid receptor internalization through circular dorsal ruffles, *Cancer Research*, 66: 11094–6.

Östman, A. (2017) PDGF receptors in tumor stroma: Biological effects and associations with prognosis and response to treatment, *Advanced Drug Delivery Reviews*, 121: 117–123.

Ostman, A. and Heldin, C. H. (2001) Involvement of platelet-derived growth factor in disease: development of specific antagonists, *Advances in cancer research*, 80: 1–38.

Östman, A. and Heldin, C. H. (2007) PDGF receptors as targets in tumor treatment, *Advances in Cancer Research*, 97: 247–274.

Paget, S. (1889) The distribution of secondary growths in cancer of the breast, *Lancet*, 133: 571–573.

Papadopoulos, N. and Lennartsson, J. (2017) The PDGF/PDGFR pathway as a drug target, *Molecular Aspects of Medicine*, 62: 75–88.

Pareja, F. *et al.* (2012) Deubiquitination of EGFR by Cezanne-1 contributes to cancer progression, *Oncogene*, 31: 4599–608.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K. and Cobb, M. H. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions, *Endocrine Reviews*, 22: 153–183.

Pelkmans, L., Bürli, T., Zerial, M. and Helenius, A. (2004) Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membranet traffic, *Cell*, 118: 767–780.

Pellet-Many, C., Frankel, P., Evans, I. M., Herzog, B., Jünemann-Ramírez, M. and Zachary, I. C. (2011) Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130Cas, *The Biochemical Journal*, 435: 609–18.

Petroski, M. D. (2008) The ubiquitin system, disease, and drug discovery, *BMC Biochemistry*, 9: S7.

Pietras, K., Ostman, A., Sjoquist, M., Buchdunger, E., Reed, R., Heldin, C. and Rubin, K. (2001) Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors, *Cancer Research*, 61: 2929–2934.

Pietras, K., Sjöblom, T., Rubin, K., Heldin, C. H. and Östman, A. (2003) PDGF receptors as cancer drug targets, *Cancer Cell*, 3: 439–443.

Polyak, K., Haviv, I. and Campbell, I. G. (2009) Co-evolution of tumor cells and their microenvironment, *Trends in Genetics*, 25: 30–38.

Pond, D., Arts, F. A., Mendelsohn, N. J., Demoulin, J.-B., Scharer, G. and Messinger, Y. (2018) A patient with germ-line gain-of-function PDGFRB p.N666H mutation and marked clinical response to imatinib, *Genetics in Medicine*, 20: 142–150.

Popovic, D., Vucic, D. and Dikic, I. (2014) Ubiquitination in disease pathogenesis and treatment, *Nature Medicine*, 20: 1242.

Porsch, H., Mehic, M., Olofsson, B., Heldin, P. and Heldin, C.-H. (2014) PDGF β-receptor, TGFβ type I receptor and CD44 modulate each others signaling and stability, *The Journal of Biological Chemistry*, 289: 19747–57.

Rameh, L. E., Rhee, S. G., Spokes, K., Kazlauskas, A., Cantley, L. C. and Cantley, L. G. (1998) Phosphoinositide 3-kinase regulates phospholipase Cgamma-mediated calcium signaling, *The Journal of Biological Chemistry*, 273: 23750–7.

Ramsay, a K. *et al.* (2011) ERK5 signalling in prostate cancer promotes an invasive phenotype, *British Journal of Cancer*, 104: 664–72.

Raza, A., Franklin, M. J. and Dudek, A. Z. (2010) Pericytes and vessel maturation during tumor angiogenesis and metastasis, *American Journal of Hematology*, 85: 593–598.

Razmara, M., Eger, G., Rorsman, C., Heldin, C. H. and Lennartsson, J. (2012) MKP3 negatively modulates PDGF-induced Akt and Erk5 phosphorylation as well as chemotaxis, *Cellular Signalling*, 24: 635–640.

Razmara, M., Heldin, C.-H. and Lennartsson, J. (2013) Platelet-derived growth factor-induced Akt phosphorylation requires mTOR/Rictor and phospholipase C-γ1, whereas S6 phosphorylation depends on mTOR/Raptor and phospholipase D, *Cell Communication and Signaling : CCS*, 3.

Reddi, A. L. *et al.* (2007) Binding of Cbl to a phospholipase Cgamma1-docking site on platelet-derived growth factor receptor beta provides a dual mechanism of negative regulation, *The Journal of Biological Chemistry*, 282: 29336–47.

Regan, C. P., Li, W., Boucher, D. M., Spatz, S., Su, M. S. and Kuida, K. (2002) Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects, *Proceedings of the National Academy of Sciences of the United States of America*, 99: 9248–53.

Reigstad, L. J., Varhaug, J. E. and Lillehaug, J. R. (2005) Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family, *FEBS Journal*, 272: 5723–5741.

Reiter, A. and Gotlib, J. (2017) Myeloid neoplasms with eosinophilia, *Blood*, 129: 704–714.

Ribatti, D. (2008) Judah Folkman, a pioneer in the study of angiogenesis, *Angiogenesis*, 11: 3–10.

Ribatti, D., Mangialardi, G. and Vacca A (2006) Stephen Paget and the seed and soil theory of metastatic dissemination, *Clinical Experimental Medicine*, 6: 145–149.

Ribeiro, A. L. and Okamoto, O. K. (2015) Combined effects of pericytes in the tumor microenvironment, *Stem Cells International*, 2015: 868475.

Rieser, E., Cordier, S. M. and Walczak, H. (2013) Linear ubiquitination: a newly discovered regulator of cell signalling, *Trends in Biochemical Sciences*, 38: 94–102.

Robinson, D. R., Wu, Y. M. and Lin, S. F. (2000) The protein tyrosine kinase family of the human genome, *Oncogene*, 19: 5548–5557.

Rodt, S. a, Ahlén, K., Berg, A., Rubin, K. and Reed, R. K. (1996) A novel physiological function for platelet-derived growth factor-BB in rat dermis, *The Journal of Physiology*, 495 (Pt 1: 193–200.

Romanelli, A. *et al.* (2018) An anti-PDGFRβ aptamer for selective delivery of small therapeutic peptide to cardiac cells, *PLOS One*, 13: e0193392.

Rorsman, C., Tsioumpekou, M., Heldin, C. H. and Lennartsson, J. (2016) The ubiquitin ligases c-Cbl and Cbl-b negatively regulate platelet-derived growth factor (PDGF) BB-induced chemotaxis by affecting PDGF receptor β (PDGFRβ) internalization and signaling, *The Journal of Biological Chemistry*, 291: 11608–11618.

Rorsman, F., Bywater, M., Knott, T. J., Scotth2, J. and Betsholtzl, C. (1988) Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: Alternative exon usage predicts two different precursor proteins, *Molecular and Cellular Biology*, 8: 571–577.

Rosenzweig, S. A. (2011) Receptor Cross-Talk, in Schwab, M. (ed.) *Encyclopedia of Cancer*, 3194–3198.

Ross, R., Glomset, J., Kariya, B. and Harker, L. (1974) A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro, *Proceedings of the National Academy of Sciences of the United States of America*, 71: 1207–10.

Rossomando, A. J., Payne, D. M., Weber, M. J. and Sturgill, T. W. (1989) Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase, *Proceedings of the National Academy of Sciences of the United States of America*, 86: 6940–3.

Rovida, E. *et al.* (2014) The mitogen-activated protein kinase ERK5 regulates the development and growth of hepatocellular carcinoma, *Gut*, 64: 1–12.

Sacco, J. J., Coulson, J. M., Clague, M. J. and Urbé, S. (2010) Emerging roles of deubiquitinases in cancer-associated pathways, *IUBMB Life*, 62: 140–157.

Sanjay, A., Horne, W. C. and Baron, R. (2001) The Cbl family: ubiquitin ligases regulating signaling by tyrosine kinases, *Science's STKE : signal transduction knowledge environment*, 2001: pe40.

Santi, A., Kugeratski, F. G. and Zanivan, S. (2018) Cancer Associated Fibroblasts: The Architects of Stroma Remodeling, *Proteomics*, 18: e1700167.

Schmees, C., Villaseñor, R., Zheng, W., Ma, H., Zerial, M., Heldin, C.-H. and Hellberg, C. (2012) Macropinocytosis of the PDGF β-receptor promotes fibroblast transformation by H-RasG12V, *Molecular biology of the cell*, 23: 2571–82.

Sciaccaluga, M. *et al.* (2013) Functional cross-talk between CXCR4 and PDGFR on glioblastoma cells is essential for migration, *PLOS One*, 8: e73426.

Sharma, G. D., He, J. and Bazan, H. E. P. (2003) p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing. Evidence of cross-talk activation between map kinase cascades, *The Journal of Biological Chemistry*, 278: 21989–21997.

Shi, Q. and Chen, Y.-G. (2017) Interplay between TGF-β signaling and receptor tyrosine kinases in tumor development, *Science China Life Sciences*, 60: 1133–1141.

Shimizu, A. *et al.* (1999) The dermatofibrosarcoma protuberans-associated collagen type I1/platelet-derived growth factor (PDGF) B-chain fusion gene generates a transforming protein that is processed to functional PDGF-BB, *Cancer Research*, 59: 3719–3723.

Simões, A. E. S., Rodrigues, C. M. P. and Borralho, P. M. (2016) The MEK5/ERK5 signalling pathway in cancer: a promising novel therapeutic target, *Drug Discovery Today*, 21: 1654–1663.

Song, S., Ewald, A. J., Stallcup, W., Werb, Z. and Bergers, G. (2005) PDGFRβ+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival, *Nature Cell Biology*, 7: 870–879.

Soriano, P. (1994) Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice, *Genes & Development*, 8: 1888–1896.
Soriano, P. (1997) The PDGFα receptor is required for neural crest cell development and for normal patterning of the somites, *Development*, 124: 2691–2700.
Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C. and Cohen, P. (1992) MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase, *The EMBO journal*, 11: 3985–94.
Sun, H. *et al.* (2011) Bcr-Abl ubiquitination and Usp9x inhibition block kinase signaling and promote CML cell apoptosis., *Blood*, 117: 3151–62.
Sun, W. *et al.* (2001) MEKK2 associates with the adapter protein Lad/RIBP and regulates the MEK5-BMK1/ERK5 pathway, *The Journal of Biological Chemistry*, 276: 5093–5100.
Swatek, K. N. and Komander, D. (2016) Ubiquitin modifications, *Cell Research*, 26: 399–422.
Takada, Y., Takada, Y. K. and Fujita, M. (2017) Crosstalk between insulin-like growth factor (IGF) receptor and integrins through direct integrin binding to IGF1, *Cytokine & Growth Factor Reviews*, 34: 67–72.
Tallquist, M. and Kazlauskas, A. (2004) PDGF signaling in cells and mice, *Cytokine and Growth Factor Reviews*, 15: 205–213.
Terasawa, K., Okazaki, K. and Nishida, E. (2003) Regulation of c-Fos and Fra-1 by the MEK5-ERK5 pathway, *Genes to Cells*, 8: 263–273.
Toffalini, F. and Demoulin, J.-B. (2008) The transmembrane domain of PDGFR-β plays an important role in ETV6-PDGFR-β activation, *Blood*, 112:.
Toffalini, F. and Demoulin, J.-B. (2010) New insights into the mechanisms of hematopoietic cell transformation by activated receptor tyrosine kinases, *Blood*, 116: 2429–37.
Toffalini, F., Hellberg, C. and Demoulin, J.-B. (2010) Critical role of the platelet-derived growth factor receptor (PDGFR) beta transmembrane domain in the TEL-PDGFRbeta cytosolic oncoprotein, *The Journal of Biological Chemistry*, 285: 12268–78.
Tremblay, G. (1979) Stromal aspects of breast carcinoma, *Experimental and Molecular Pathology*, 31: 248–260.
Tsioumpekou, M., Papadopoulos, N., Burovic, F., Heldin, C. H. and Lennartsson, J. (2016) Platelet-derived growth factor (PDGF)-induced activation of Erk5 MAP-kinase is dependent on Mekk2, Mek1/2, PKC and PI3-kinase, and affects BMP signaling, *Cellular Signalling*, 28: 1422–1431.
Vanhaesebroeck, B., Leevers, S. J., Panayotou, G. and Waterfield, M. D. (1997) Phosphoinositide 3-kinases: A conserved family of signal transducers, *Trends in Biochemical Sciences*, 22: 267–272.
Vanlandewijck, M. *et al.* (2015) Functional characterization of germline mutations in PDGFB and PDGFRB in primary familial brain calcification, *PLOS One*, 10: e0143407.
Veevers-Lowe, J., Ball, S. G., Shuttleworth, A. and Kielty, C. M. (2011) Mesenchymal stem cell migration is regulated by fibronectin through α5β1-integrin-mediated activation of PDGFR-β and potentiation of growth factor signals, *Journal of Cell Science*, 124: 1288–1300.
Velghe, A. I. *et al.* (2014) PDGFRA alterations in cancer: characterization of a gain-of-function V536E transmembrane mutant as well as loss-of-function and passenger mutations, *Oncogene*, 33: 2568–2576.
Villaseñor, R., Kalaidzidis, Y. and Zerial, M. (2016) Signal processing by the endosomal system, *Current Opinion in Cell Biology*, 39: 53–60.

Wahl, M. I., Olashaw, N. E., Nishibe, S., Rhee, S. G., Pledger, W. J. and Carpenter, G. (1989) Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-gamma in quiescent BALB/c 3T3 cells, *Molecular and Cellular Biology*, 9: 2934–43.

Wang, Y., Pennock, S. D., Chen, X., Kazlauskas, A. and Wang, Z. (2004) Platelet-derived growth factor receptor-mediated signal transduction from endosomes, *The Journal of Biological Chemistry*, 279: 8038–46.

Wang, Z., Glück, S., Zhang, L. and Moran, M. F. (1998) Requirement for phospholipase C-gamma1 enzymatic activity in growth factor-induced mitogenesis, *Molecular and Cellular Biology*, 18: 590–7.

Westermark, B. and Wasteson, Å. (1976) A platelet factor stimulating human normal glial cells, *Experimental Cell Research*, 98: 170–174.

Yan, L., Carr, J., Ashby, P. R., Murry-Tait, V., Thompson, C. and Arthur, J. S. C. (2003) Knockout of ERK5 causes multiple defects in placental and embryonic development, *BMC developmental biology*, 3: 11.

Yao, Z., Yoon, S., Kalie, E., Raviv, Z. and Seger, R. (2010) Calcium regulation of EGF-induced ERK5 activation: Role of Lad1-MEKK2 interaction, *PLOS One*, 5: 1–10.

Yokouchi, M. *et al.* (1999) APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis, *Oncogene*, 18: 759–767.

Yun, S.-I. *et al.* (2015) Ubiquitin specific protease 4 positively regulates the WNT/β-catenin signaling in colorectal cancer, *Molecular Oncology*, 9: 1834–51.

Zhang, L. *et al.* (2012) USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-β type I receptor, *Nature Cell Biology*, 14: 717–726.

Zhong, M., Jiang, Q. and Jin, R. (2018) *USP4* expression independently predicts favorable survival in lung adenocarcinoma, *IUBMB Life*, 70: 670–677.

Zhou, G., Bao, Z. Q. and Dixon, J. E. (1995) Components of a new human protein kinase signal transduction pathway, *The Journal of Biological Chemistry*, 270: 12665–9.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1538

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-376248

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2019