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Mechanisms of modulation of PDGFRβ signaling

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Abstract

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Platelet-derived growth factors (PDGF) constitute a family of five functional dimers that bind to two structurally related tyrosine kinase receptors i.e. PDGF receptor α and β (PDGFRα and PDGFRβ, respectively), controlling cell growth, proliferation, and migration in cells of mesenchymal origin. However, the aberrant activation of PDGF-induced intracellular signalling pathways is a frequent event in cancer. Therefore, the aim of this thesis has been to discover novel molecular mechanisms of modulation of PDGFRβ signalling.

Since mitogen-activated protein (MAP) kinases are activated in PDGF signalling and their spatiotemporal activity is defined by a balance in phosphorylation and dephosphorylation events, in paper I we focused on dual-specificity MAPK phosphatases (MPKs or DUSPs). We found MKP2/DUSP4 to be induced in response to PDGF-BB stimulation. We then demonstrated that the expression of MKP2/DUSP4 was dependent on ERK1/2 activation and on the STAT3/p53 signalling.

Endocytosis of RTKs is another mechanism that serves for signal attenuation and termination and this process can be regulated by ubiquitination or deubiquitination of cell-surface receptors. In paper II, we have identified that ubiquitin specific proteases USP4 and USP17 act as deubiquitinases (DUBs) for PDGFRβ. Both deubiquitinases impacted the timing of PDGFRβ trafficking and prolonged STAT3 activation. Consequently, high transcriptional activity of STAT3 led to the increased expression of STAT3-inducible genes *c-MYC*, *CSF1*, *JUNB* and *CDKN1A*. USP4 deletion attenuated cell proliferation in response to PDGF-BB stimulation.

The family of Cbl E3 ligases is essential for ubiquitination of PDGFRβ upon ligand stimulation, followed by the receptor internalization from the cell surface and downregulation of signalling. In paper III, we have identified a new E3 ligase, i.e. tripartite motif-containing protein TRIM21, that deubiquitinates PDGFRβ and regulates its basal levels and its availability on the cell surface in a PDGF-BB independent manner.

In paper IV, we described a regulatory role of the endoribonuclease Ras GTPase-activating protein-binding protein 1 (G3BP1) in PDGF signalling. G3BP1 was identified as a PDGFRβ interacting protein which also interacts with BAF155, a core component of SWI/SNF chromatin remodelling complex. G3BP1 depletion upregulated *c-FOS*, *c-MYC* and *c-JUN* mRNA and negatively affected *STAT3* and *ERK1/2* mRNA and protein levels, stalling cell proliferation.

Collectively, we present new mechanisms that regulate PDGF signalling by controlling either PDGFRβ protein levels, availability on the cell surface, subcellular trafficking or activation of downstream signalling affecting regulation of cell proliferation.

Keywords: PDGF signalling, phosphatase, MKP2, DUSP4, deubiquitinase, USP4, USP17, E3 ligase, Cbl, TRIM21, endoribonuclease, G3BP1

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Αφιερωμένο στην οικογένειά μου
To my family

List of Papers

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

I. Yin R, Eger G, **Sarri N**, Rorsman C, Heldin CH, Lennartsson J. Dual specificity phosphatase (DUSP)-4 is induced by platelet-derived growth factor -BB in an ERK1/2-, STAT3- and p53-dependent manner. *Biochem Biophys Res Commun*. 2019 Nov 12;519(3):469-474.

II. **Sarri N**, Wang K, Tsioumpekou M, Castillejo-López C, Lennartsson J, Heldin CH, Papadopoulos N. Deubiquitinating enzymes USP4 and USP17 finetune the trafficking of PDGFRβ and affect PDGF-BB-induced STAT3 signalling. *Cell Mol Life Sci.* 2022 Jan 21;79(2):85.

III. **Sarri N**, Papadopoulos N, Lennartsson J, Heldin CH. The E3 ubiquitin ligase TRIM21 modulates the basal levels of PDGFRβ (2022). *Manuscript*

IV. **Sarri N**, Lennartsson J, Witek B, Heldin CH, Papadopoulos N. G3BP1 is a SWI/SNF-bound regulator of PDGFR signalling that controls cell proliferation via ERK1/2, c-MYC and STAT3 (2022). *Manuscript*

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Other publications not included in the thesis:

I. Zarei O, **Sarri N**, Dastmalchi S, Zokai F, Papadopoulos N, Lennartsson J, Heldin CH, Hamzeh-Mivehroud M. Structure-based discovery of novel small molecule inhibitors of platelet-derived growth factor-B. *Bioorg Chem.* 2020 Jan;94:103374.

II. Olsson F, **Sarri N**, Papadopoulos N, Lennartsson J, Norlin M. Effects of 1α,25-dihydroxyvitamin D3 and tacalcitol on cell signaling and anchorage-independent growth in T98G and U251 glioblastoma cells. *Biochem Biophys Rep.* 2022 Jul 31;31:101313.

Contents

Abbreviations

ARF-BP1	ADP ribosylation factor - binding protein 1
ATP	Adenosine triphosphate
BAP1	BRCA1 associated protein-1
BLAST	Basic local alignment search tool
Cbl	Casitas B-lineage lymphoma
cDNA	complementary deoxyribonucleic acid
CYLD	Cylindromatosis
DAG	Diacylglycerol
DBCL	Diffuse B cell lymphoma
DUBs	Deubiquitinases
DUSP	Dual-specificity MAP kinase phosphatases
Dvl	Dishevelled
ERAD	Endoplasmic reticulum associated degradation
ERK	Extracellular signal-regulated kinases
ESCRT	Endosomal sorting complexes required for transport
EST	Expressed Sequence Tag
Grb2	Growth factor receptor-bound protein 2
HDAC	Histone deacetylase
HECT	Homologous to the E6-AP carboxyl terminus
IgG	Immunoglobulin
IKK	IκB kinase
ILVs	Intraluminal vesicles
IP3	Inositol trisphosphate
IRF	Interferon-regulatory factor
JNK	c-Jun N-terminal kinase
KIM	Kinase-interaction motif
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MEF	Mouse embryonic fibroblast
MINDY	Motif Interacting with Ub-containing novel DUB family
MJD	Machado-Joseph deubiquitinase
MKP	Mitogen-activated protein kinase phosphatase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MT-Sp1	Matriptase 1

MVEs	Multivesicular endosomes
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
OTU	Ovarian tumour proteases
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent protein kinase-1
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (Zo-1)
PEST	Proline, glutamic acid, serine, and threonine rich domain
PH	Pleckstrin-homology
PI3K	Phosphoinositide 3´-kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PTB	Phosphotyrosine-binding domain
PTEN	Phosphatase and tensin homolog
RBR	RING between rING
RING	Really interesting new gene
SH2	Src Homology 2
SH3	Src Homology 3
SHP-2	Src homology region 2 domain-containing phosphatase-2
Sos1	Son of Sevenless 1
STAT	Signal transducer and activator of transcription
TAK1	Transforming growth factor-β-activated kinase 1
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
tPA	Tissue-type plasminogen activator
TRAF	TNF receptor-associated factor
TRIM	Tripartite motif-containing protein
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like domain
UCH	Ubiquitin C-terminal hydrolases
UNP	Ubiquitous nuclear protein
uPA	Urokinase-type plasminogen activator
USP	Ubiquitin specific protease
Wnt	Wingless-related Integration Site
ZnF	Zinc finger
ZUP1	Zinc finger-containing ubiquitin peptidase 1

Introduction

Cells are the fundamental units of a living organism. They need to sense their dynamic surroundings and respond to diverse environmental cues and internal stimuli in order to survive. In other words, cells need to communicate with their environment. Cell communication is mediated through cell surface receptors that transduce the message within the cell by detecting signal molecules, unable to diffuse passively across the plasma membrane. These stimuli, that originate at the plasma membrane, control vital cellular processes such as growth, proliferation, differentiation, migration and apoptosis. At the heart of these cell-cell and cell-environment communication systems lies a highly complex but concerted network of signal transduction pathways that ensures finely-tuned appropriate responses.

Receptor tyrosine kinases (RTKs) comprise a subclass of receptors endowed with intrinsic tyrosine kinase activity. Fifty-eight RTKs have been identified in the human genome that are organized into twenty subfamilies based on their structure homology (Robinson et al., 2000). All RTKs share common structural features. They have an extracellular ligand binding domain, a hydrophobic helix that spans the plasma membrane, and an intracellular region which encompasses a juxtamembrane region, a tyrosine kinase domain (TKD) and a carboxyl-terminal (C-terminal) tail. Ligand binding to the extracellular domain of RTKs triggers the receptor dimerization and autophosphorylation of the receptors, leading to conformational changes that activate the kinase and enable RTKs to auto-phosphorylate and recruit several secondary messengers to its intracellular domain. These interactions lead to activation of different signalling pathways that ultimately culminate in the binding of transcription factors in the nucleus to specific gene promoters affecting the expression of specific genes (Du and Lovly, 2018). Finally, signalling pathways have to be attenuated and terminated, which is achieved by various mechanisms that decrease the cell sensitivity to external stimuli. These mechanisms rely on ligand elimination, proteolytic cleavage, endocytosis and subsequent degradation of receptors, as well as inhibition of receptors by activation of proteins that act as negative regulators, including specific kinases and tyrosine phosphatases (Margiotta, 2021).

Receptor tyrosine kinases (RTKs) play fundamental roles in embryonic development and cellular homeostasis and the tight regulation of their enzymatic activity is required to prevent abnormal cell behaviour that drives pathogenetic

diseases, e.g. cancer. Dysregulated temporospatial activity of RTKs has been observed in human cancers and is attributed to gain-of-function mutations, chromosome rearrangements, gene amplification and autocrine signalling (Du and Lovly, 2018). Although ligand-mediated activation of RTKs has been extensively studied and numerous components involved in the downstream signal transduction pathways of distinct RTKs have been characterized, less is known about the downregulation of RTK signalling (Margiotta, 2021). In the present thesis, we focused on the transduction pathways of platelet-derived growth factor-BB (PDGF-BB)/PDGF receptor β (PDGFRβ) with the aim to elucidate novel molecular mechanisms that negatively impact on the activation of downstream signalling. Thus, we have described processes that fine-tune the availability of PDGFRβ on plasma membrane both in resting conditions (via the E3 ligase TRIM21) and in response to ligand stimulation (via DUBs), which consequently affect the cell sensitivity to PDGF-BB. In addition, we discovered two proteins, MKP2/DUSP4 and G3BP1, that act as negative regulators of downstream signalling pathways induced by PDGFRβ.

PDGF ligands and receptors

PDGF was first discovered as a significant component of alpha granules of platelet exerting mitogenic activity for cells of mesenchymal and oligodendrocyte origin (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976). Purification of PDGF revealed that it is a dimeric protein of two disulphide-bonded A- and B-polypeptide chains, forming PDGF–AA, -AB and –BB isoforms . Based on these findings, the PDGF family was suggested to produce three biologically active dimers. However, in the early 2000s *PDGF-C* and *PDGF-D* genes were discovered by cDNA sequencing, showing homology to *PDGFA* and *PDGFB* (Bergsten et al., 2001; Li et al., 2000). The PDGF-C and -D polypeptides have only been shown to form homodimers, i.e., PDGF-CC and -DD.

The genes for PDGF ligands are located on different chromosomes; *PDGFA* and *PDGFB* are located on chromosomes 7 and 22, respectively, (Betsholtz et al., 1986; Dalla-Favera et al., 1982; Swan et al., 1982) whereas *PDGFC* and *PDGFD* are mapped to chromosomes 4 and 11, respectively (Dijkmans et al., 2002; Uutela et al., 2001). All PDGF isoforms are synthesized as latent precursors which share the highly conserved growth factor domain (Figure 1A). The novel PDGF-CC and -DD isoforms are structurally different from PDGF -AA and -BB as they contain an amino-terminal CUB (C1r/C1s, Uegf, Bmp1) domain. PDGF activation requires proteolytic cleavage; PDGF-AA and BB are cleaved intracellularly by furin proteases of ER before secretion, while PDGF-CC and -DD are most likely cleaved extracellularly by tissue-type plasminogen activator (tPA), plasmin, and urokinase-type PA (uPA), or matriptase (MT-Sp1) (Chen et al., 2013; Fredriksson et al., 2004; Reigstad et al., 2005).

A variety of cell types including activated platelets, endothelial, epithelial, glial or inflammatory cells secrete PDGF isoforms that act locally in a paracrine manner. PDGF isoforms then bind to two structurally related receptors, PDGFRα and PDGFRβ, respectively, that are expressed in oligodendrocyte progenitors and in cells of mesenchymal origin, such as fibroblasts, pericytes and vascular smooth muscle cells. The expression of PDGF ligands and receptors is dynamic and sensitive to various external stimuli, such as hypoxia, cytokines and growth factors (Andrae et al., 2008; Heldin et al., 2018). PDGFRα and PDGFRβ together with the receptors c-KIT, c-FMS and FLT3 compose the class III receptor tyrosine kinase (RTK) family (Heldin et al., 2018; Heldin and Lennartsson, 2013; Heldin and Westermark, 1999). PDGF receptors contain five extracellular immunoglobulin-like (IgG) domains, a single transmembrane helix and an intracellular part consisting of a juxtamembrane

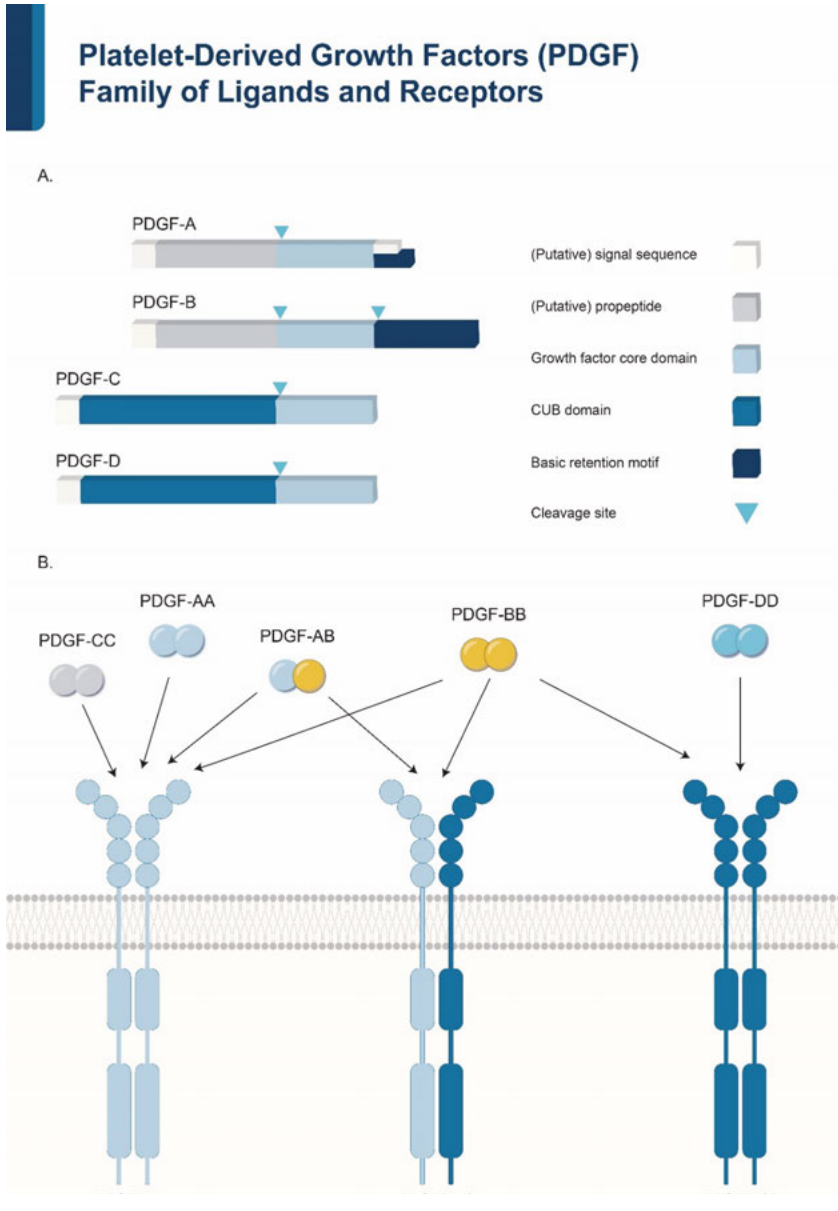

Figure 1. The family of PDGF ligands and their receptors. A. All PDGF precursor isoforms contain a highly conserved growth factor core domain, whereas in the N- and C-terminus there are different sequences; the classic PDGF-A and PDGF-B chains with basic retention motifs, the novel PDGF-C and PDGF-D with CUB domains. PDGF isoforms require proteolytic cleavage at the indicated sites in order to be activated. B. PDGF ligands bind to the receptors with distinct specificities. Specifically, PDGF -AA, -AB, -BB, and -CC bind to PDGF αα receptor dimers, PDGF-BB and -DD to PDGF ββ receptor dimers, and PDGF-AB, -BB, -CC and -DD to PDGF αβ receptor dimers.

segment, a split tyrosine kinase domain and a carboxyl-terminal tail. The binding site of PDGF ligands lies at the interface between the IgG domains 2 and 3. The A-, B-, C- and D-polypeptide chains of PDGF ligands create different types of receptor dimers (i.e. αα, αβ and ββ receptor dimers) by binding to them with distinct affinities (Figure 1B); the dimers are further stabilized by interactions between Ig-like domains 4 and 5 (Heldin et al., 2018; Heldin and Lennartsson, 2013).

PDGFR activation and biological function

In the resting state the kinase domain of PDGFRs is inactive due to the folding of the activation loop, the juxtamembrane segment, and the carboxyl-terminal tail over the catalytic cleft. The binding of the ligand triggers dimerization and trans autophosphorylation of receptor kinase domains, thus lifting all the aforementioned inhibitory interactions (Heldin, 2013; Heldin and Lennartsson, 2013). In particular, phosphorylation of a conserved tyrosine residue (Y849 in the α-receptor and Y857 in the β-receptor) in the activation loop enhances the catalytic activity of tyrosine kinase domains in PDGFRs. Apart from the conformational changes of the receptor's intracellular part that leads to the kinase activation, the phosphorylated tyrosine residues in the receptors also serve as docking sites for downstream signalling molecules. PDGFRα and PDGFRβ have ten and eleven known phosphorylated tyrosine residues, respectively. These phosphorylation sites recruit signalling molecules with intrinsic enzymatic activity (Src, Fer, PI3K, PLCγ), adaptor molecules that lack enzymatic activity (Grb2, Nck, Shc, Crk) mediating the binding of other signalling molecules, as well as STAT proteins that translocate to the nucleus and act as transcription factors (Heldin et al., 2018; Heldin and Westermark, 1999) (Figure 2). Importantly, the different dimeric PDGFR complexes initiate overlapping but not identical cellular processes. Activation of ββ homodimers and the αβ heterodimers induces chemotaxis of smooth muscle cells and fibroblasts, whereas activation of αα homodimers has the opposite effect (Siegbahn et al., 1990; Yokote et al., 1996). In addition, although all dimeric PDGFRs are able to rearrange actin filaments, only ββ and αβ receptor dimers mediate the formation of cortical actin at the dorsal surface of the cell (Eriksson et al., 1992).

Several knockout and transgenic mouse models have revealed the functional significance of PDGF signalling in both homeostasis and pathogenesis. Similar phenotypes were observed in mice with *PDGF-B* or *PDGFRβ* knockouts that demonstrated a series of organ and vascular defects, including impaired kidney mesangial cell development, cardiac and placental failure, decrease in pericyte number, poor smooth muscle cell development and weakening in vessel wall structure that ultimately culminated to haemorrhage and

death (Levéen et al., 1994; Lindahl et al., 1997; Ohlsson et al., 1999; Soriano, 1994; Van den Akker et al., 2008). PDGF-DD inactivation resulted only in a mild phenotype, suggesting that PDGF-BB compensates in binding to PDGFRβ. Several studies have also reported that loss-of-function mutations in *PDGF-B* or *PDGFRβ* human genes are correlated with brain calcification (Arts et al., 2015; Keller et al., 2013; Vanlandewijck et al., 2015; Wang et al., 2017). PDGFRβ signalling is important for embryonic development and wound healing, proper intestinal fluid pressure and blood vessel maintenance in adults. In contrast, mice with *PDGF-A* or *PDGFRα* knockouts developed more severe phenotypes; prenatal death was common along with abnormal development of gastrointestinal (GI) tract (Karlsson et al., 2000), defects in oligodendrocytes (Calver et al., 1998; Woodruff et al., 2004), impaired development of the facial skeleton, hair follicles, spermatogenesis (Ding et al., 2004; Soriano, 1997), and retina (Gerhardt et al., 2003). In addition, mice with *PDGF-A* knockout died in the perinatal period due to poor development of lung alveoli and subsequent lung emphysema (Boström et al., 1996). Mice with PDGF-C knockout also did not survive past the perinatal period due to feeding and respiratory difficulties (Ding et al., 2004). Collectively, PDGFRα signalling is essential for embryonal development.

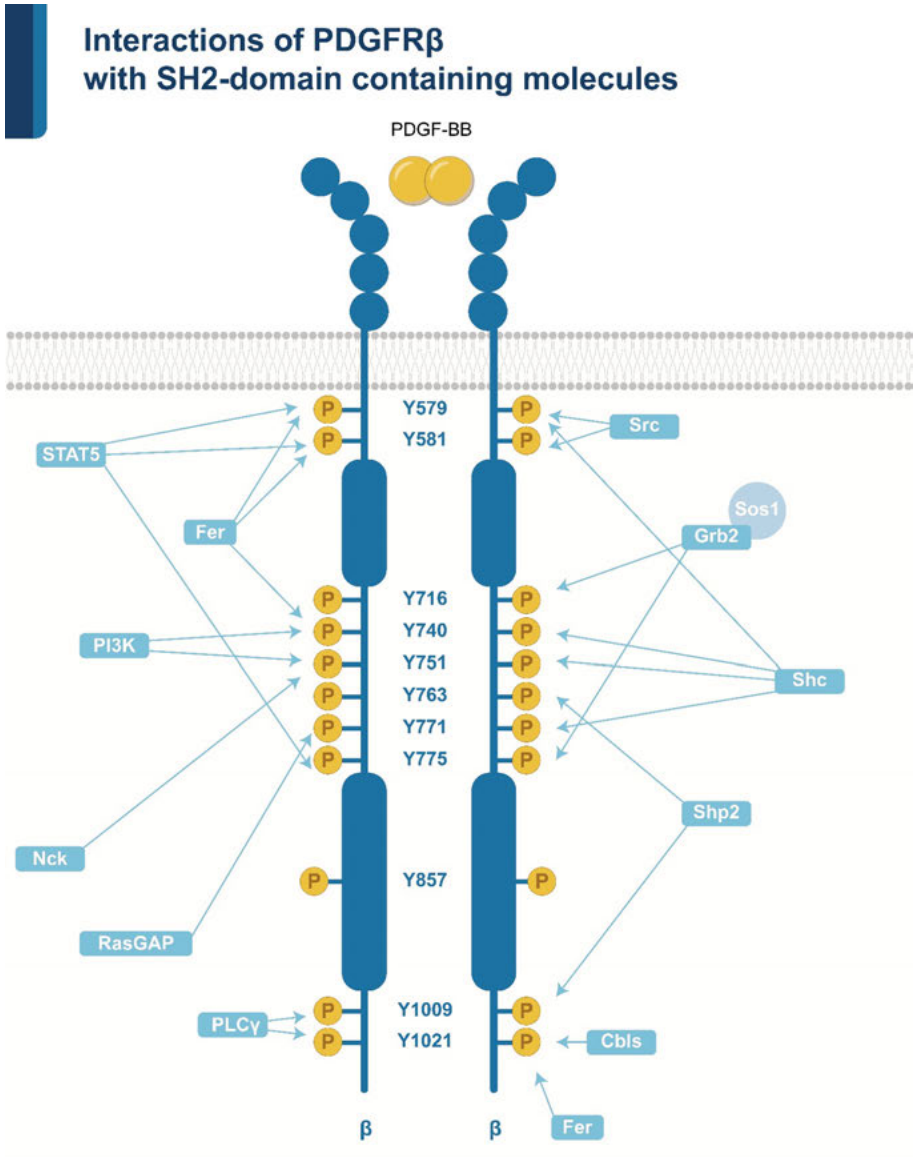

Figure 2. Activated PDGFRβ recruits various downstream signalling molecules which contain the SH2 domain. The homo-dimeric complex of PDGFRβ is depicted with the focus on the intracellular region across which phosphorylated tyrosines are presented as docking sites for downstream SH2-containing proteins.

PDGF signalling pathways

Upon activation PDGFRs stimulate downstream signalling pathways such as MAPK/ERK, PI3K/AKT and PLCγ/PKC (Figure 3).

A variety of specific domains in the downstream molecules are essential for their recruitment to PDGFRs. The most common is the Src homology 2 (SH2)-domain, which interacts with phosphorylated tyrosine residues in the intracellularl part of PDGFRs. Other domains involved in signal transduction are the Src homology 3 (SH3), the pleckstrin homology (PH), and post synaptic density protein (PDZ) domains. These domains recognize proline-rich areas, membrane phospholipids, and a C-terminal valine residue, respectively. Known cellular responses of the PDGF signal transduction pathways are cell proliferation and survival, actin reorganization and migration (Heldin et al., 1998a).

MAPK/ERK1/2 pathway

The mitogen-activated protein (MAP) kinases are a family of highly evolutionary conserved serine/threonine kinases. MAPKs are activated by a three-tiered kinase (MAPK kinase kinase, MAPK kinase, and MAPK itself) cascade in response to diverse stimuli in the cell environment, including mitogenic signals, oxidative stress, and inflammatory cytokines. The activation of MAPKs requires a dual phosphorylation on both threonine and tyrosine residues within the activation loop with the signature motif T–X–Y. The classical MAPK signalling kinases are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the family of p38 (p38α, p38β, p38γ, and p38δ), c-Jun N-terminal kinases (JNKs) 1, 2 and 3, and ERK5. The duration and magnitude of these signalling pathways determine the biological outcomes, such as cell proliferation, differentiation, cell survival, and apoptosis (Chen et al., 2019; Kondoh and Nishida, 2007; Plotnikov et al., 2011).

Upon ligand binding PDGFRβ induces the transduction of the classical MAP kinase cascades out of which the MEK/ERK1/2 pathway is the most extensively studied. In resting cells, the adaptor protein Grb2 resides in the cytoplasm in a preformed complex with a guanine nucleotide exchange factor, i.e. Son of Sevenless 1 (Sos1), which in response to mitogenic stimuli migrates to the plasma membrane. There the SH2 domain of Grb2 binds to phosphorylated sites of PDGFRβ, such as Tyr716 and Tyr775 which are located in phospho-Tyr-X-Asn consensus motif (Arvidsson et al., 1994; Heldin et al., 1998b; Ruusala et al., 1998). Grb2 can also indirectly bind to PDGFRβ via the Shc adaptor which associates with PDGFRβ phospho-Tyr579, Tyr740, Tyr751, and Tyr771 (Yokote et al., 1994). Sos1 then activates Ras, a small GTPase protein, by catalysing GDP exchange to GTP. Active GTP-bound Ras initiates signalling that involves sequential phosphorylation of the Serine/Threonine kinase Raf-1, dual-specificity MAP-ERK kinase (MEK)1/2,

and extracellular-regulated kinase (ERK)1/2. To this end, kinase suppressor of Ras (KSR) is released from a retention complex with 14-3-3 and the Impedes Mitogenic signal Propagation (IMP) E3 ligase upon growth-factor treatment (Matheny et al., 2004; Müller et al., 2001). Following the binding to MEK1/2, KSR then migrates to caveolin-rich microdomains in the plasma membrane (Kortum et al., 2014; Pudewell et al., 2021), where its serves as a molecular scaffold facilitating interactions between Raf and MEK1/2 proteins (McKay et al., 2009). The MAPK pathway culminates in the dimerization of phosphorylated ERK1/2 and its rapid migration into the nucleus where ERK1/2 activates the transcription factors c-Fos, Elk1, Ets1 and SP-1 (Lenormand et al., 1993). Although phosphorylation of ERK1/2 is proven to be essential for nuclear translocation (Fukuda et al., 1997; Khokhlatchev et al., 1998; Lenormand et al., 1998; Lidke et al., 2010), interestingly, the role of its dimerization in nucleocytoplasmic shuttling has been contentious. *Adachi et al.* reported that ERK monomer passively diffuses into the nucleus while Ras-related Nuclear protein (Ran) mediates the nuclear transport of active ERK dimers after its phosphorylation and concomitant dissociation from upstream MEK1/2 proteins which are known to anchor ERK1/2 in the cytoplasm (Adachi et al., 1999; Burack and Shaw, 2005). Moreover, *Zehorai et al.* showed that importin7 (imp7) associates with phosphorylated ERK1/2 escorting it into the nucleus (Zehorai et al., 2010). In contrast, independent studies provided a carrier- and energy-independent import mechanism in which ERK2 directly interacts with nuclear pore complex proteins such as nucleoporins (Matsubayashi et al., 2001; Whitehurst et al., 2002).

The subcellular localization of ERK1/2 determines the biological outcome of the MAPK signalling pathway. In resting cells inactive ERK1/2 assembles cytoplasmic complexes with anchoring and scaffolding proteins such as MEK1/2 (Adachi et al., 1999; Burack and Shaw, 2005), KSR (Yu et al., 1998), PEA-15 (Formstecher et al., 2001), β-arrestin (Tohgo et al., 2002), MP1 (Schaeffer et al., 1998), and IQGAP1 (Roy et al., 2004). Upon phosphorylation and activation ERK1/2 dissociates from these complexes and rapidly migrates into the nucleus where it promotes cell growth, cell migration, and differentiation (Brunet et al., 1999; Pouysségur and Lenormand, 2003). However, a pool of activated ERK1/2 remains in the cytoplasm where it phosphorylates cytoplasmic targets and participates in negative feedback loops. Importantly, MEK/ERK1/2 pathway can signal from different subcellular compartments such as endosomes, Golgi, mitochondria, cytoskeleton and caveolae (Yao and Seger, 2009).

PI3K/AKT pathway

Phosphatidylinositol 3'-kinases comprise a conserved family of lipid kinases that phosphorylate phosphatidylinositols at their 3' position. PI3Ks are classified into three main groups according to their structure and function (Engelman et al., 2006; Vanhaesebroeck et al., 1997). PDGFR activates class I_A PI3Ks consisting of a p85 regulatory subunit and a p110 catalytic subunit (Demoulin and Essaghir, 2014; Heldin et al., 1998a). The two SH2 domains of p85 mediate interactions with PDGFR by recognizing the phosphorylated Tyr740 and Tyr751 within the sequence motif pYxxM on the activated receptor (Kashishian et al., 1992; Zhou et al., 1993). PDGFR, then, phosphorylates p85 on Tyr508 leading to conformational changes, which is critical for PI3K enzymatic activity (Kavanaugh et al., 1994; Panayotou et al., 1992). The basal inhibition on the p110 catalytic subunit is then relieved, and PI3K phosphorylates its preferred substrate phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Heldin et al., 1998a; Whiteford et al., 1996).

PIP_3 is a lipid second messenger that modulates a plethora of downstream effector molecules through interaction with their PH domains, including 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Levina et al., 2022), certain members of the protein kinase C (PKC) family (Akimoto et al., 1996; Moriya et al., 1996; Nakanishi et al., 1993; Toker et al., 1994), the p70 S6 kinase (Cheatham et al., 1994; Chung et al., 1994), c-Jun N-terminal kinase (JNK) (Lopez-Ilasaca et al., 1997), and small GTPases of the Rho family (Hawkins et al., 1995). Moreover, an important binder of PIP_3 is the serine/threonine kinase AKT/PKB, which is recruited to the cell membrane upon PIP_3 binding (Burgering and Coffer, 1995; Franke et al., 1995; Klippel et al., 1997). There the mammalian target of rapamycin (mTOR) rictor kinase complex and PDK1 respectively phosphorylate the Ser473 and Thr308 of AKT leading into its full activation (Alessi et al., 1997; Sarbassov et al., 2005). In turn, activated AKT phosphorylates a range of critical proteins involved in cellular functions as diverse as cell metabolism, cell motility, cell survival, and proliferation (Demoulin and Essaghir, 2014).

PLCγ/PKC pathway

PLCγ is one of the target enzymes of PDGFRs (Meisenhelder et al., 1989). The phosphorylated Tyr1021 and Tyr1009 in the carboxyl-terminal tail of PDGFRs bind cytosolic PLCγ via its two SH2 domains recruiting it to the plasma membrane (Kashishian et al., 1992; Rönnstrand et al., 1992; Valius et al., 1993). Upon interaction with the receptors, PLCγ becomes phosphorylated, amplifying its catalytic activity (Kumjian et al., 1991; Morrison et al., 1990; Valius et al., 1993). PLCγ acquires full catalytic activity by interacting

via its PH domain with PIP_3 which anchors PLCγ to the cell membrane (Falasca et al., 1998). There PLCγ acts on the same substrate as PI3K, i.e. PIP_2. The cleavage of PIP_2 gives rise to two second messengers, inositol 1,4,5-trisphosphate (IP_3) that diffuses away in the cytoplasm and 1,2-diacylglycerol (DAG) that remains at the plasma membrane. IP_3 binds to calcium- channel receptors on the endoplasmic reticulum membrane leading to a release of Ca^{2+} into the cytoplasm that together with DAG can activate calcium-dependent proteins such as distinct protein kinase C (PKC) isoforms (Berridge, 1993). In addition, PLCγ-mediated activation of PKC and Ca^{2+} release are required for Na^+/H^+ exchange (Ma et al., 1994). In certain cell types, PLCγ activity elicits cell growth responses and cell motility (Heldin et al., 2018). However, the importance of this PDGF-mediated signalling pathway for the migration and proliferation of cells in culture remains elusive, since the independent roles of PLCγ and PI3K could not be distinguished (Demoulin and Essaghir, 2014; Tallquist, 2000).

STAT

STATs are a family of cytoplasmic transcription factors that comprise seven members - STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Yu and Jove, 2004). Cytokine and growth-factor receptors phosphorylate inactive STAT monomers triggering their dimerization and activation. Upon ligand binding PDGFRβ recruits and activates STAT1 (Choudhury et al., 1996; Yamamoto et al., 1996), STAT3 (Vignais et al., 1996; Wang et al., 2000), STAT5 (Valgeirsdóttir et al., 1998, p. 5), and STAT6 (Patel et al., 1996). In particular, STAT5 binds to the phosphorylated Tyr579, Tyr581 and Tyr775 of PDGFRβ (Valgeirsdóttir et al., 1998). Whether PDGFRβ directly activates STATs or do so by mobilizing another protein tyrosine kinases remains elusive. Since STATs interact with PDGFRβ, one possibility is that STATs are directly phosphorylated by the receptor at the plasma membrane. However, different mechanisms for STAT activation have been described. Vignais et al. suggested that PDGF-induced STAT phosphorylation is dependent on the activation and redundant function of JAKs (Vignais et al., 1996; Vignais and Gilman, 1999) while the non-receptor tyrosine kinase Src was reported to mediate STAT phosphorylation upon PDGF stimulation (Cirri et al., 1997; Wang et al., 2000). In contrast, *Sachsenmaier et al.* observed that the phosphorylated Tyr579 and Tyr581 in the juxtamembrane region of PDGFRβ are required for STAT activation and not Src (Sachsenmaier et al., 1999). Moreover, Fer kinase that also strongly binds to PDGFRβ phospho-Tyr579 and Tyr581 was found to serve as an adaptor protein mediating phosphorylation of STAT3 (Tyr705) (Lennartsson et al., 2013). Activation of STAT3 has been reported to emanate from different subcellular compartments besides

plasma membrane, such as perinuclear endosomes in c-Met signalling (Kermorgant and Parker, 2008), Golgi in endomyocardiocytes (Chen et al., 2006), endoplasmic reticulum (Avalle et al., 2019) and mitochondria for cellular respiration (Wegrzyn et al., 2009). Importantly, full activation of STAT3 in PDGF signalling has been shown to rely on the internalization of PDGFRβ (Jastrzębski et al., 2017).

Following tyrosine phosphorylation and activation STATs form stable homo- or hetero-dimers and translocate to the nucleus. Distinct from other STATs, STAT1, STAT3 and STAT5 continuously shuttle between the cytoplasm and the nucleus independent of their phosphorylation state (Liu et al., 2005; Marg et al., 2004; Pranada et al., 2004). Unphosphorylated latent STAT1 is reported to migrate into the nucleus by interacting with nucleoporins at the nuclear envelope (Marg et al., 2004) while a role of various importins and Ran in the nuclear translocation of STAT3 has been suggested although there is no consensus in the exact STAT3 import mechanism (Cimica et al., 2011; Ernst and Müller-Newen, 2019; Ushijima et al., 2005). Interestingly, a pool of latent STAT1 and STAT3 proteins has been found in pre-associated homodimers indicating that dimerization might be independent of cytokine and growth factor stimulation (Braunstein et al., 2003; Haan et al., 2000). In the nucleus the phosphorylation and dimerization status of STAT1 and STAT3 proteins can determine the formation of complexes with transcription factors that dictate the expression of certain target genes (Chatterjee-Kishore et al., 2000; Sehgal, 2008; Yang et al., 2005). In particular, active STAT3 dimer drives the expression of genes related to cell survival (Survivin, Bcl-xL, Mcl-1, Bcl-2), proliferation (c-Fos, c-Myc, Cyclin-D1), invasion (matrix metalloproteinases), and angiogenesis (VEGF-A, bFGF) (Carpenter and Lo, 2014). Apart from phosphorylation at Tyr705, maximal transcriptional activity of STAT3 is achieved by an additional phosphorylation event at Ser727 which has been implicated in promoting prostate tumorigenesis (Qin et al., 2008; Wen et al., 1995). Moreover, STAT3 is involved in fibroblast transformation by autocrine PDGF signalling (Demoulin and Essaghir, 2014; Lennartsson et al., 2013). STAT molecules, especially STAT3 and STAT5, are constitutively activated in various human cancers (Yu and Jove, 2004).

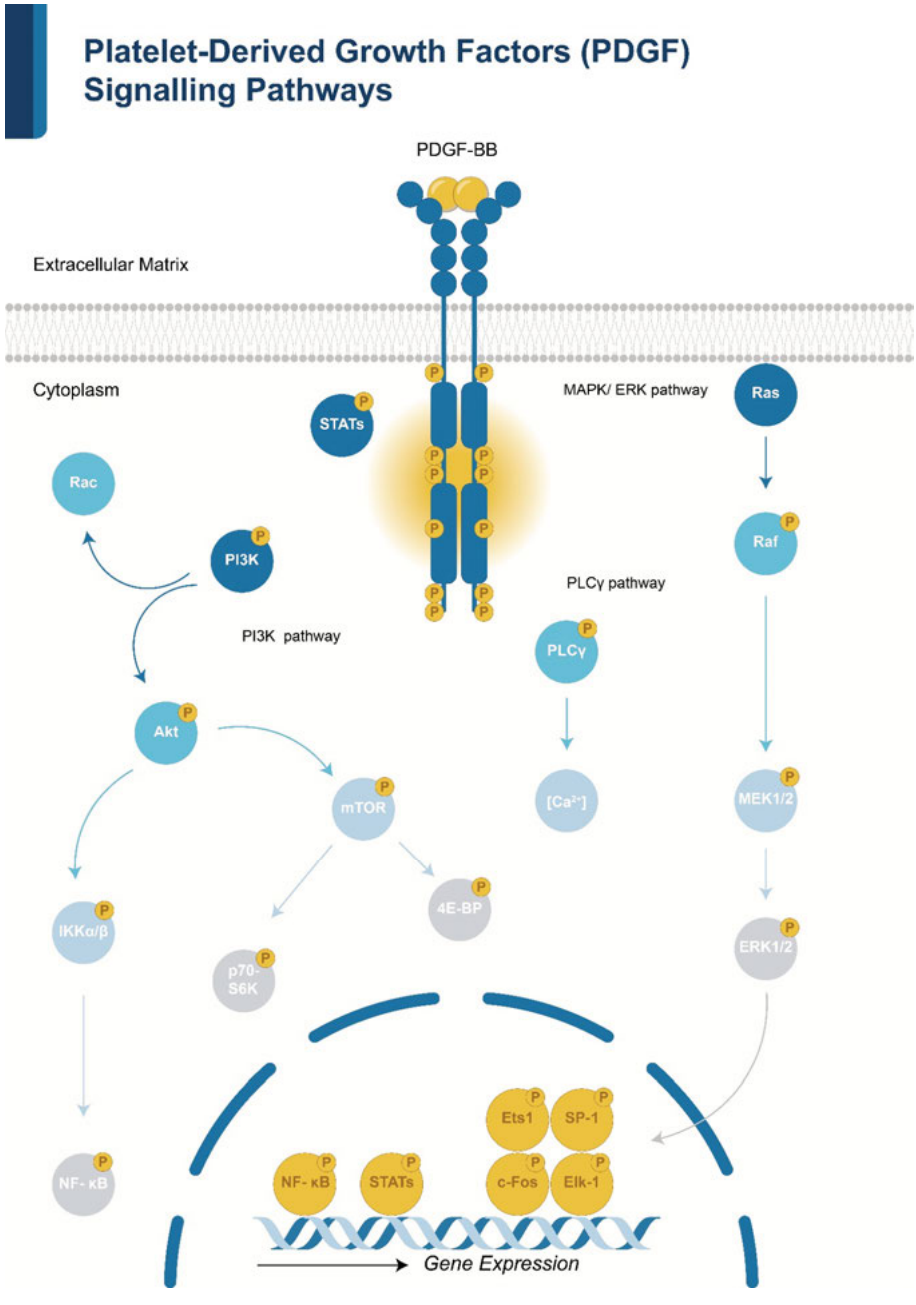

Figure 3. Schematic illustration of PDGFR-β signalling pathways. Upon activation, PDGFR-β recruits downstream signalling molecules initiating various signalling pathways that control cell proliferation, migration, survival, cell differentiation and cell metabolism.

Downregulation of PDGF signalling

Feedback control mechanisms regulate PDGF signal transduction. Stimulatory and inhibitory cues arise simultaneously, and their overall effect determines the ultimate response, which is cell- and context-dependent. For example, RasGAP binds to PDGF receptors and downregulates Ras activity (Ekman et al., 1999), whereas the Grb2/Sos1 complex activates Ras. Moreover, several protein tyrosine phosphatases have been reported to directly act on the PDGF receptors, including PTP1B, TC-PTP, PTPRJ/DEP-1, and SHP-2 (Heldin and Lennartsson, 2013). SHP-2 plays a dual signalling role. It dephosphorylates PDGFRβ and its substrates, providing an example of a negative feedback mechanism. However, SHP-2 also dephosphorylates the inhibitory carboxyl-terminal phosphorylation site of Src, thus activating it. Apart from its dephosphorylating activity, SHP-2 serves as an adaptor for the binding of the Grb2/Sos1 complex (Heldin, 2013; Heldin and Lennartsson, 2013).

Moreover, there are other phosphatases that act on downstream effectors of the activated pathways. For instance, PTEN controls the activation of PI3-kinase by the PDGF receptors. MKP3 (DUSP6) dephosphorylates ERK1/2, suppressing MAPK signalling pathway after an extended PDGF stimulation (Heldin and Lennartsson, 2013).

Dual-specificity MAP kinase phosphatases (DUSPs/MKPs)

Dual-specificity MAPK phosphatases (DUSPs or MPKs) attenuate MAPK signalling providing a negative feedback regulatory mechanism which shapes the spatiotemporal activity of MAPKs. Several DUSPs also participate in the crosstalk between the different MAPK pathways and other intracellular signalling molecules. So far, it is known that the human genome encodes twenty-five DUSP genes (Huang and Tan, 2012). DUSPs are classified into the atypical DUSPs lacking the kinase-interaction motif (KIM) domain and the classical DUSPs or MAP kinase phosphatases (MKPs) that possess this domain. The former ones are not well characterized, and their effect on MAPKs has not been clarified yet.

Ten catalytically active enzymes represent the MKPs, which share a similar structure. In the amino-terminus, there is a kinase-interaction motif (KIM) conferring the MAPK substrate selectivity, flanked by the non-catalytic domain, while in the carboxyl-terminus there is the catalytic domain. Besides, some MKPs contain nuclear localization (NLS) or export (NES) signals, and a C-terminal sequence, rich in proline, glutamic acid, serine, and threonine (PEST domain). Based on structural similarity, subcellular localization, and substrate specificity, the ten MKPs can be further subclassified into three

groups; 1) the growth-factor and stressed induced DUSP1 (MKP1), DUSP2, DUSP4 (MKP2), and DUSP5 which reside in the nucleus; 2) the cytoplasmic DUSP6 (MKP3), DUSP7 (MKP-X) and DUSP9 (MKP4), and 3) the JNK/p38- specific DUSP8, DUSP10 (MKP5) and DUSP16 (MKP7), which are found both in the nucleus and in the cytoplasm. Regarding the substrate specificity, DUSP1 mainly dephosphorylates JNKs and p38s, DUSP2 targets ERK1/2 and JNKs whereas DUSP6, DUSP7, DUSP9, DUSP4 and DUSP5 are more specific for ERK1/2. In contrary, DUSP8, DUSP10, and DUSP16 dephosphorylate JNKs and p38s MAPKs.

DUSP4/MKP2

DUSP4/MKP2 is a nuclear phosphatase that selectively acts on ERK1/2, JNK, and p38; albeit with a higher affinity for ERK1/2 and p38 (Seternes et al., 2019).

Despite much effort, the role of DUSP4 in cell homeostasis and tumour progression has not been fully elucidated. Numerous studies report that the expression levels of DUSP4 are up- or downregulated in a wide array of human cancer cell lines and primary tumours. Elevated mRNA levels of *DUSP4* are found in pancreatic, liver, thyroid, breast, ovarian, and colon cancer (Low and Zhang, 2016). Moreover, DUSP4/MKP2 is overexpressed in colorectal cancer, associated with high microsatellite instability. It is still unknown whether the increased induction of DUSP4 in malignant cells acts as a compensatory mechanism of the cell to reach homeostasis or if it is linked to the progression of malignancies (e.g., liver cancer, acute myeloid leukaemia). In contrast, DUSP4 has been found to be epigenetically silenced in glioma and in the overwhelming majority of diffuse B cell lymphoma (DBCL), suggesting a role as a tumour suppressor, lack of which is an adverse prognostic factor for patients (Seternes et al., 2019). Underpinning the idea that *DUSP4* is a candidate tumour suppressor gene, evidence shows that its gene maps to chromosome arm 8p, a region that frequently loses its heterozygosity in breast and lung cancer (Armes et al., 2004).

DUSP4 is further associated with chemotherapy resistance both to doxorubicin in gastric cancer and to the anti-Her2 antibody, Trastuzumab, in breast cancer (Seternes et al., 2019). Interestingly, in MCF-7 breast cancer cells, treatment with a non-steroid anti-estrogen tamoxifen induces the phosphatase expression indicating its negative regulatory role in ERK1/2-mediated proliferation and tamoxifen resistance (Haagenson et al., 2014). DUSP4 has also been shown to play a role in cellular senescence and apoptosis (Torres et al., 2003). Despite these findings, the mechanisms underlying the regulatory roles of DUSP4 are still poorly understood.

In paper I, we have identified MKP2/DUSP4 to be induced in response to PDGF-BB stimulation. We have provided further evidence that the expression of MKP2/DUSP4 was dependent on ERK1/2, STAT3 and p53 signalling.

Ubiquitination

Ubiquitin is a small globular protein of 76 amino acid residues highly conserved across bacteria, archaea, and eukarya (Foot et al., 2017). It was first identified during the isolation of bovine thymus in 1974 and was misperceived to be a thymus hormone (Goldstein, 1974). The regulatory function of ubiquitin was, however, discovered in the early 1980s as a post-translational modification, in which ubiquitin covalently modified lysine residues of protein substrates and targeted them for degradation in proteasomes, in what had been described as a molecular kiss of death.

Ubiquitination is a diverse and dynamic post-translational modification. Protein substrates can bear only a single ubiquitin, on a single lysine residue (mono-ubiquitination) or numerous ubiquitin molecules on multiple lysine residues (multi-, mono-, ubiquitination). Mono-ubiquitination accounts for the majority of ubiquitin modification that regulates the biological processes of DNA repair, gene expression, endocytosis, and viral budding. Furthermore, ubiquitin has seven internal lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63), and the N-terminus methionine residue (Met1) which can serve as acceptor sites for further ubiquitin moieties, giving rise to the formation of homotypic, heterotypic or branched poly-ubiquitin chains of variable length. This adds another layer of complexity vastly increasing the versatility of the ubiquitin code which has not been fully deciphered yet (Komander, 2009). The most extensively studied poly-ubiquitinations have been the homotypic Lys48- and Lys63-linked poly-ubiquitin chains. Lys48-linked chains have been characterized to target protein substrates to proteasomal degradation whereas Lys63-linked chains are involved in autophagy, endocytic trafficking, inflammation, and DNA damage repair (Komander, 2009; Komander and Rape, 2012).

Ubiquitin has in the C-terminus a glycine residue, that attaches to the ε-amino group of a substrate lysine residue forming an isopeptide bond. This enzymatic reaction occurs in three sequential steps (Figure 4), catalysed by an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). In an ATP-dependent first step, E1 loads and activates ubiquitin. So, the ubiquitin C-terminal glycine links to the E1 active site cysteine via a thioester bond. E1, then charges E2 with the activated ubiquitin via a trans-thioesterification reaction on E2 active cysteine.

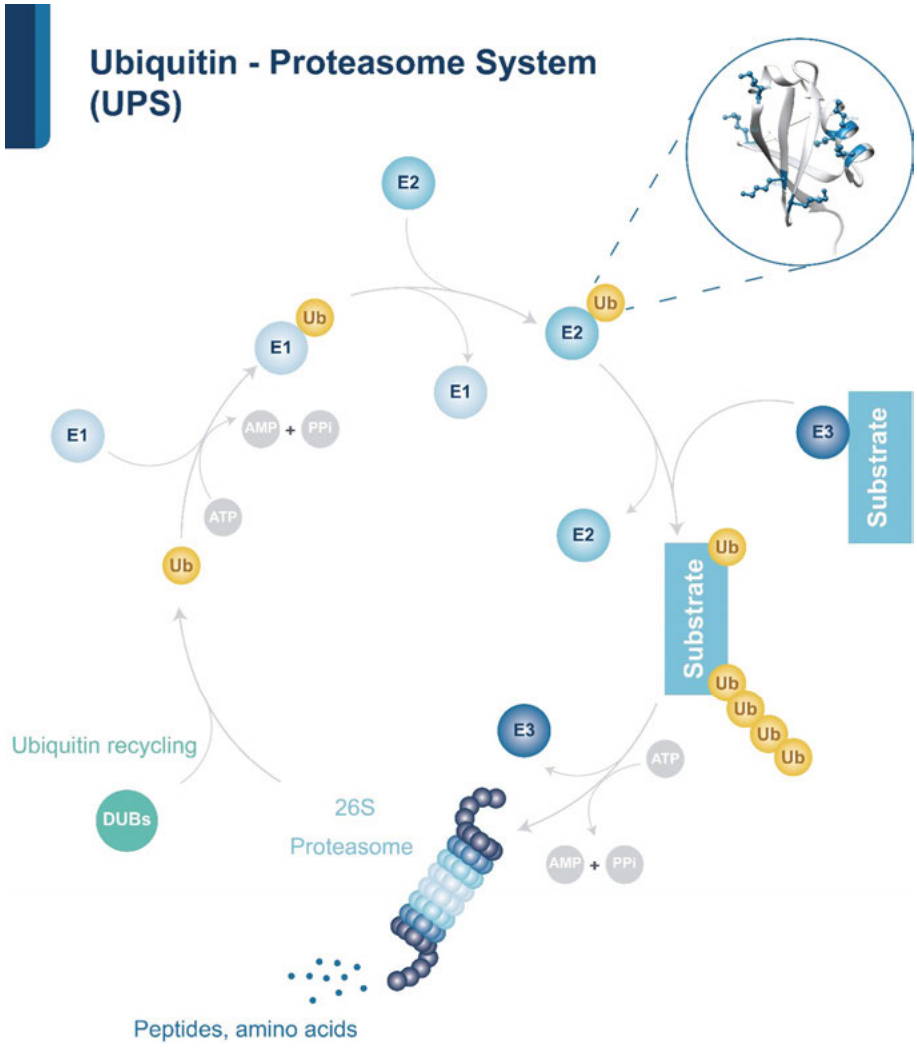

Figure 4. Schematic illustration of the ubiquitination - proteasome system. The E1, E2 and E3 enzymes mediate the covalent attachment of ubiquitin to the lysine residues of substrates, which requires ATP hydrolysis. Depending on the number of ubiquitin moieties and the type of linkages, ubiquitinated substrates can be targeted to 26S proteasomal degradation. Ubiquitination can be removed by deubiquitinases (DUBs); thus, rescuing protein substrates and maintaining the total pool of ubiquitin.

In the last step, an E3 ligase mediates the transfer of ubiquitin from the charged E2 enzyme to a lysine residue in the substrate facilitating the formation of an isopeptide bond. The human genome encodes two activating E1, forty conjugating E2 enzymes, and more than 600 E3 ligases, which confer high substrate specificity in the final step (Foot et al., 2017; Liu and Walters, 2010).

E3 ligases can be organized in three groups based on the mechanism of ubiquitin transfer. The really interesting new gene (RING) type E3 ligases

constitute the largest subfamily, that act as a molecular scaffold between the E2 conjugating enzyme and the target protein substrate catalysing a direct ubiquitin transfer. The second subfamily that contains 30 proteins is defined by the presence of the homologous to E6-AP carboxyl terminus (HECT) domain. Distinct from the RING E3 ligases, HECT E3 ligases exhibit intrinsic catalytic activity and perform substrate ubiquitination in two steps. First, they load activated ubiquitin at the active site cysteine residue within the HECT domain, thus forming an E3-Ub thioester intermediate. In the second step, protein ubiquitination occurs. Last, the RING-between-RING (RBR) E3s with 12 representatives share features with both RING- and HECT-type E3 ligases and employ a RING/HECT hybrid mechanism to conjugate ubiquitin to the substrate (Kannt and Đikić, 2021).

Cbls E3 ligase

Casitas B-lineage lymphoma (Cbl) family of enzymes (c-Cbl, Cbl-b and Cbl-3) is the most well characterized E3 ubiquitin ligase of the RTK family members (Tang et al., 2022).

The *c-Cbl* proto-oncogene was first identified as *v-Cbl* in the genome of murine Cas NS-1 retrovirus in 1989, able to transform fibroblasts and induce pre-B-cell lymphomas and myelogenous leukaemia in mice (Langdon et al., 1989). Later studies showed that v-Cbl was a truncated counterpart of a larger protein known as c-Cbl and that overexpression of full-length c-Cbl could not promote tumorigenesis (Blake et al., 1991). Consequently, three mammalian homologues (c-Cbl, Cbl-b and Cbl-3) have been discovered. All Cbl proteins share a highly conserved amino-terminal region that comprises a tyrosine-kinase-binding (TKB) domain, an alpha helix linker and a catalytic RING finger domain. In contrast, their carboxyl-terminal region is more variable and encompasses proline-rich motifs, tyrosine phosphorylation sites and an ubiquitin-associated/leucine zipper domain all of which mediate a wide range of interactions with signalling proteins (Schmidt and Dikic, 2005; Thien and Langdon, 2001). Cbls can negatively modulate the signalling of activated RTKs by targeting them for degradation. Apart from the negative regulation of RTKs, Cbls can act as a multidomain adaptor protein promoting positive signalling events in bone resorption, glucose uptake in response to insulin, and cell spreading in response to integrin activation (Thien and Langdon, 2001).

Cbl E3 ligases are ubiquitously expressed and are found to localize in the cytoplasm. Upon growth factor stimulation Cbl proteins translocate to the plasma membrane where their TKB domain binds to phosphorylated tyrosine residues of RTKs. Concurrently, the RING domain of Cbl proteins recruits E2 enzymes loaded with ubiquitin; thus facilitating the transfer of ubiquitin moieties from the E2 ubiquitin-conjugating enzyme to the targeted RTKs. The Cbl E3 ligases negatively regulate various RTKs (Tang et al., 2022). Levkowitz et

al. reported that c-Cbl binds to phosphorylated Tyr1045 in the cytoplasmic tail of EGFR promoting the ubiquitination and lysosomal degradation of the receptor. Moreover, overexpression of Cbl-b or Cbl-3 accelerated the cell-surface clearance of epidermal growth factor receptor (EGFR), indicating that Cbl-mediated ubiquitination of EGFR is critical for endocytosis (Levkowitz et al., 1999). In addition, c-Cbl and Cbl-b contribute to the ubiquitination of nerve growth factor (NGF) receptor TrkA which is then committed to degradation limiting signalling (Chen et al., 2022; Emdal et al., 2015; Takahashi et al., 2011). Similarly, the activity of MET is also downregulated by c-Cbl, which together with the endophilin–CIN85 complex affect the internalization and signalling of MET (Petrelli et al., 2002; Taher et al., 2002). Furthermore, independent studies have shown the regulatory role of c-Cbl in colony-stimulating factor-1 (CSF-1) signalling. c-Cbl binds to the phosphorylated Tyr973 of CSF-1R in a ligand-dependent manner, polyubiquitinates the receptor initiating its endocytosis and degradation which stalls macrophage proliferation (Lee et al., 1999; Wilhelmsen et al., 2002).

Previous studies reported that Cbls ubiquitinate PDGFRα and PDGFRβ receptors, enhancing their internalization and degradation rate (Miyake et al., 1999; Reddi et al., 2007). In a similar mechanism, high Cbl levels in recessive dystrophic epidermolysis bullosa attenuated MAPK and AKT downstream signalling pathways (Martínez-Martínez et al., 2021). Our group has extensively studied the Cbl-mediated ubiquitination of PDGFRβ providing a better understanding of PDGFRβ ubiquitination, downregulation and signalling (Rorsman et al., 2016). It was found that Cbl-b and not c-Cbl binds to the phosphorylated Tyr1021 in the carboxyl tail of PDGFRβ *in vitro*. Interestingly, Cbl-b and c-Cbl form a complex in response to PDGF-BB stimulation. Based on these data, Cbl-b binds directly to the receptor while c-Cbl binds indirectly via Cbl-b. Regarding the chain preferences that E3 ligases might exhibit, it was shown that Cbl-b mediates the formation of both Lys48- and Lys63-polyubiquitin chains whereas c-Cbl conveys only Lys63-polyubiquitination. Surprisingly, the Cbl-induced ubiquitination of PDGFRβ triggered its internalization without affecting its degradation rate. Several downstream signalling pathways of PDGFRβ, such as Src, STAT3, and PLCγ, are fine-tuned by a change in receptor's subcellular localization, which consequently impacts the chemotactic response of cells (Rorsman et al., 2016).

TRIM21 E3 ligase

The only E3 ligases for PDGFRβ that have been discovered so far are members of the Cbl protein family and their function and regulatory roles have been extensively studied. However, in paper III we found another E3 ligase that acts on PDGFRβ, i.e. TRIM21.

TRIM21, also known as Ro52 or SSA1, is a member of the tripartite motif (TRIM) family of RING-finger E3 ligases widely expressed in most tissues and cells. More than eighty TRIM proteins are known in humans, which are implicated in the modulation of various cellular events like cell cycle, protein quality control, autophagy, and innate immunity (Hatakeyama, 2017; Jones et al., 2021). Although most members of the TRIM family possess a RING-finger domain and are therefore characterized as E3 ubiquitin ligases, eight TRIM molecules lack this domain (Hatakeyama, 2017). TRIMs are further classified into eleven different subgroups based on their domain composition (Hatakeyama, 2017). Moreover, TRIMs exist as monomers or can undergo homo-dimerization, which is required for the catalytic activity of the RING domain (Fiorentini et al., 2020; Hatakeyama, 2017; Jones et al., 2021). As a member of the tripartite motif family, TRIM21 contains a RING-finger domain at the amino-terminus, followed by a zinc-finger type II B-box, a central coiled-coil region and a PRY/SPRY domain in the carboxyl-terminal region (Jones et al., 2021). The RING domain confers the E3 ligase activity of TRIMs through interactions with E2 enzymes, thus, catalysing the transfer of ubiquitin to substrates (Metzger et al., 2014). However, TRIM21 is not constitutively active, since its E3 ligase activity is auto-inhibited by its B-Box domain, which antagonizes the binding site of the RING domain for E2 enzymes (Dickson et al., 2018). Phosphorylation of Ser80 relieves the B-Box mediated inhibition restoring the activation of TRIM21 (Dickson et al., 2018). Zeng et al. has proposed that TRIM21 exists as a catalytically inactive dimer (Zeng et al., 2021). The coiled-coil domain participates in TRIM21 dimerization enhancing the catalytic activity of its RING domain (Zeng et al., 2021). The PRY/SPRY domain is critical for protein interactions and subcellular localization (James et al., 2007; Reymond et al., 2001), and binds to the Fc region of IgG antibody with high affinity (Keeble et al., 2008; Takahata et al., 2008).

TRIM21 has been shown to ubiquitinate several protein substrates, including itself, and to build both Lys48- and Lys63-polyubiquitin chains, suggesting that it can alter the enzymatic activity of substrates, affect protein interactions and target proteins for proteasomal degradation (Espinosa et al., 2011). Generally, the role of TRIMs in the clearance of misfolded proteins via different mechanisms has been well characterized (Zhang et al., 2020). Those mechanisms are the ubiquitin-proteasome pathway, autophagy, and ER-associated degradation (ERAD) (Zhang et al., 2020). TRIM21, particularly, has been reported to interact with the molecular chaperone p97/VCP contributing to the quality control of IgG1 through the ERAD system (Takahata et al., 2008).

Furthermore, TRIM21 neutralizes seeds of misfolded tau aggregates, a cytoplasmic protein that accumulates in patients with Alzheimer's disease, by delivering them to proteasomes (McEwan et al., 2017; Zhang et al., 2020). Importantly, TRIM21 is also involved in autophagy. TRIM21 ubiquitinates and sequesters SQSTM1/p62, a receptor of autophagic cargo, thus suppressing the cellular antioxidant response (Pan et al., 2016). In addition, TRIM21 interacts with the autophagy regulators ULK1 and Beclin1 and with GABARAP autophagy effectors (Kimura et al., 2015). Interestingly, TRIM21 can act as a direct cargo receptor for active IRF3 and assemble autophagic machinery, hence, initiating selective autophagy degradation (Kimura et al., 2017, 2015). In osteosarcoma cells TRIM21 has been shown to promote autophagy via the TRIM21/ANXA2/TFEB axis (H.-T. Zhang et al., 2021).

Apart from being an E3 ligase, TRIM21 has been described as an autoantigen, targeted by anti-TRIM21 autoantibodies, which are present in patients with systemic autoimmune diseases, specifically lupus erythematosus and Sjögren's syndrome. The autoantibodies bind the RING domain of TRIM21 at the binding interface site of the E2 conjugating enzyme UBE2E1. This sterically blocks the E2/ E3 critical interaction, inhibiting the E3 ligase activity of TRIM21 (Espinosa et al., 2011).

TRIM21 is a ubiquitously expressed protein that predominantly localizes in the cell cytoplasm with small amounts detected in the nucleus (Ohlsson et al., 2002). However, TRIM21 is mainly expressed in hematopoietic cells in which interferons (IFNs) indirectly induce the E3 ligase in response to viral infections and in systemic autoimmune diseases (Sjöstrand et al., 2013). The induction of TRIM21 by IFNs serves as a negative feedback loop mechanism to prevent overactivation of the innate immune system. In support of this idea, embryonic fibroblasts and immune cells of a TRIM21 knockout mouse model are characterized by increased levels of pro-inflammatory cytokines (Espinosa et al., 2009). Therefore, the hypersensitivity of TRIM21 knockout mice to innate immune response consolidates the important role of TRIM21 as a regulator in immunity in vivo.

Although the role of TRIM21 in intracellular immunity is established, in tumorigenesis the significance of this E3 ligase is still emerging. TRIM21 is reported to display dual functions, both tumour suppressive and tumour promoting, which are cell type- and context-dependent. As a tumour suppressor, TRIM21 can increase the ubiquitination and degradation of octamer-binding transcription factor 1 (Oct-1), hence, restricting the self-renewal capacity and maintenance of cancer stem cells (Du et al., 2016). Moreover, TRIM21 attenuates the NF-κB signalling, which is often implicated in tumour development and progression, by mono-ubiquitinating and sequestering the IκB kinase β (IKKβ) to autophagosomes for degradation (Niida et al., 2010). Interestingly, TRIM21 promotes apoptosis either by downregulating anti-apoptotic factors, including c-FLIP(L) (Zhang et al., 2012), B-cell lymphoma 2 (BCL2) (Jauharoh et al., 2012), lifeguard protein (Müller et al., 2015), caspase-3, caspase-

7, and PARP (Sun et al., 2022) or by positively regulating the TRAIL-induced apoptosis (Simoes Eugénio et al., 2021). In triple-negative breast cancer, TRIM21 has been reported to act as a suppressor via different mechanisms. TRIM21 impairs metastasis by targeting the type II receptor for TGFβ for proteasomal degradation (Liu et al., 2022), while downstream of TGFβ receptors TRIM21 ubiquitinates the Snail transcription factor, thus, suppressing cell migration and invasion (Jin et al., 2020).

Despite its tumour suppressive functions, TRIM21 also has a pro-oncogenic role. In breast cancer TRIM21 enhances cell proliferation and colony formation (Guha et al., 2020), and negatively regulates p53 (Guha et al., 2019; Reddy et al., 2014). In glioma cells, high expression levels of TRIM21 have been reported to inhibit the p53-p21 senescence pathway regulating, hence, the cell cycle, apart from cell proliferation and migration (Zhao et al., 2020). TRIM21-mediated ubiquitination and subsequent degradation of the cyclin-dependent kinase (CDK) inhibitor p27 drive G1/S-phase progression (Sabile et al., 2006).

Due to the presence of TRIM21 autoantibodies in patients with autoimmune diseases, especially the systemic lupus erythematosus (SLE), and Sjögren's syndrome (SS), TRIM21 has been used as a diagnostic biomarker for decades (Dugar et al., 2010). In addition, since the dual role of TRIM21 in tumorigenesis is emerging, TRIM21 can also serve as a prognostic factor with different outcomes depending on the type of cancer. The downregulation of TRIM21 in hepatocellular carcinoma and diffuse large B cell lymphoma is correlated with poorer survival (Brauner et al., 2015; Ding et al., 2015), whereas in glioma (Wang et al., 2021), pancreatic cancer (Nguyen and Irby, 2017; Zhou et al., 2020), soft tissue sarcoma (Lin et al., 2021) and oesophageal squamous cell carcinoma (Kuboshima et al., 2006), it is correlated with long-term survival outcome.

Endocytosis

Dephosphorylation of the receptors is not the only mechanism to dampen signalling. Endocytosis also negatively regulates the duration of receptor signalling cascades that initiate at the cell surface affecting the ability of cells to constitutively respond to external stimuli. During endocytosis a cell engulfs the extracellular domains of cell surface receptors with its plasma membrane (Haglund and Dikic, 2012). Ubiquitination is believed to be a key post-translational modification that drives endocytosis of RTKs. Although there are several endocytic pathways, the clathrin-mediated mechanism is the most extensively studied (Doherty and McMahon, 2009).

Activated signalling receptors are covalently marked with ubiquitin moieties at the cytoplasmic tails while they are anchored at the cell surface membrane. The attachment of the ubiquitin tag attracts the clathrin coat adaptor AP2 and accessory adaptor proteins which interact with clathrin heavy and light chains assembling clathrin triskelions in small areas of the plasma membrane. The signalling receptors accumulate in clathrin-coated pits which invaginate inwards until they pinch off forming clathrin-coated vesicles (CCVs) which are routed to early endosomes. The early endosomes are organelles characterized by a highly dynamic network of tubules and vesicles and are located near the plasma membrane, in the periphery of the cell (Goh and Sorkin, 2013). Importantly, Rab GTPases coordinate vesicular trafficking. Rab5 localizes in early endosomes where it facilitates the fusion of clathrin-coated vesicles with endosomes which serves as an initial sorting nexus determining the fate of receptors and ligands along the endocytic pathway. The lumen of early endosomes is mildly acidic because of the activity of vacuolar ATPase (V-ATPase), a multi-subunit transmembrane complex that pumps protons from the cytoplasm. This slight endosomal acidification allows the dissociation of receptors from their bound ligands. Depending on the type of receptors and their ubiquitination status, they are then concentrated into tubular elements of early endosomes from where vesicles pinch off transporting the receptors back to the cell surface for repeated rounds of receptor activation (Sorkin and von Zastrow, 2009). In this process Rab4 is involved in fast endocytic recycling directly from early endosomes while Rab11 and Rab35 are responsible for slow recycling through special recycling compartments (Stenmark, 2009).

As early endosomes progressively mature, they travel to inner cytoplasm, and are finally accumulated in a perinuclear area as multivesicular bodies (MVBs) known as late endosomes where Rab7 localizes. This transition from an early to a late endosome involves biochemical and structural changes that involve a decrease in pH, an exchange of Rab proteins and inward invaginations of the limiting membrane for the formation of intraluminal vesicles that are also accompanied by the fission of tubular extensions (Huotari and Helenius, 2011; Scott et al., 2014). Signalling receptors that do not recycle back to the plasma membrane, are further sequestered in the intraluminal vesicles (ILVs) of late endosomes, that act as a second sorting station. Interestingly, ILVs can release exosomes that migrate and fuse with the plasma membrane. Alternatively, ILVs eventually traffic to the lysosomes for cargo degradation (Huotari and Helenius, 2011; Scott et al., 2014; Sorkin and von Zastrow, 2009). Importantly, signalling is sustained during intracellular trafficking of RTKs which continue to signal off the endosomes, even off the lysosomes (Deng et al., 2019; Sorkin and von Zastrow, 2009).

The trafficking and sorting of cargo along the endocytic pathway is orchestrated by the endosomal sorting complex required for transport (ESCRT) protein machinery. Four distinct ESCRT complexes are recruited to endosomes

via a vast protein-protein and protein-lipid interaction network, that act in sequence. Initially, ESCRT-0 composed of the proteins Hrs (hepatocyte growth factor regulated tyrosine-kinase substrate), and Stam1/2 (signal transducing adaptor molecule 1 and 2) selects the ubiquitinated receptors at the endosomal membrane. Next, Hrs interacts via its FYVE domain with the 3'-phosphoinositides lipids in the endosomal membrane, and via its UIM/ UBDs with ubiquitinated receptors. Hrs then binds to TSG101, which together with Vps28 and Vps37 forms the ESCRT-I complex. The post endocytic sorting progresses with the downstream recruitment of ESCRT-II, which captures both the ubiquitinated substrates and the membrane lipids. Finally, ESCRT-III recognizes the super complex of ESCRT-I and ESCRT-II. Lacking UBD protein modules, the role of ESCRT-III is to incorporate the cargo into intraluminal vesicles (ILVs) of late endosomes. Therefore, the ESCRT-III complex recruits a deubiquitinase (DUB) which removes an ubiquitin tag from the substrate before its inclusion into the lumen of the MVB. ESCRT-0, -I and -II facilitate the invagination of the endosomal membrane while ESCRT-III, characterized as the molecular scissors of the endocytic machinery, drives the scission of ILVs (Acconcia et al., 2009; Haglund and Dikic, 2012).

PDGFRβ endocytosis

Upon binding of PDGF-BB ligand to PDGFRβ, the receptor is mono-ubiquitinated on multiple sites which promotes its internalization (Haglund et al., 2003). PDGFRβ is also ubiquitinated by addition of Lys48- and Lys63-linked polyubiquitin chains, with different kinetics, involving the E3 ubiquitin ligases Cbl-b and c-Cbl (Rorsman et al., 2016). Activated PDGFRβ is predominantly internalized via the clathrin-mediated route, but it has also been found to internalize through clathrin-independent pathways (Boucrot et al., 2015; De Donatis et al., 2008; Jastrzębski et al., 2017). In both pathways, the receptors reach the early endosomes where the first sorting events occur. Some receptors can be recycled back to the plasma membrane for another round of activation (Haglund and Dikic, 2012). In comparison with other receptors, PDGFRβ does not normally recycle. However, a transient recycling can occur in response to activation of protein kinase C α (PKCα) and Rab4α (Hellberg et al., 2009) or activation of PI3K (Schmees et al., 2012). For the attenuation of the signalling pathways receptors are sorted further into ILVs of multivesicular endosomes and degraded in lysosomes upon endosomal fusion (Haglund and Dikic, 2012) .

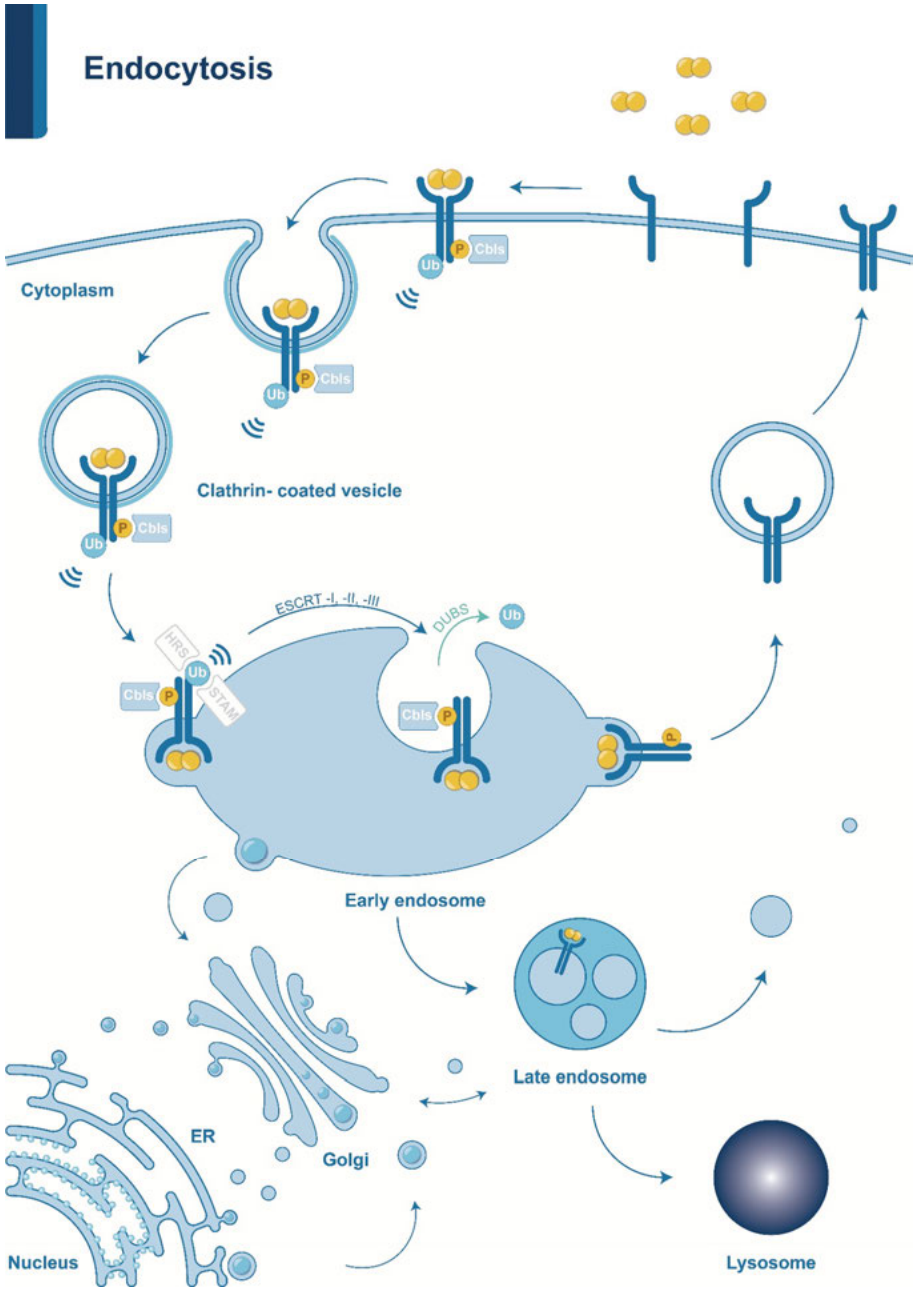
Endocytosis
Cytoplasm
Ub
P
Cbls
Clathrin- coated vesicle
ESCRT -I, -II, -III
DUBs
HRS
STAM
Early endosome
Late endosome
ER
Golgi
Nucleus
Lysosome

Figure 5. The role of ubiquitination in receptor tyrosine kinase (RTK) endocytosis and endosomal trafficking. Following RTKs' dimerization and activation, Cbls E3 ligases are recruited and confer ubiquitination on receptors. This ubiquitination signal then initiates the internalization of receptors which reach the early endosomes through clathrin-dependent or -independent mechanisms. At the early endosomes, the ubiquitination of receptors is recognized by the following components of the endocytic machinery; the ESCRT-0 molecules eps15b, HRS and STAM, the ESCRT-I molecules TSG101, MVB12, VPS37, and VPS28, the ESCRT-II molecules VPS36, VPS25, VPS22, and VPS25, the ESCRT-III molecules CHMP6, CHMP4A, CHMP3, and the CHMP2. The receptors are, then, sorted further into the mature multivesicular endosomes and ultimately, degraded in lysosomes. Alternatively, deubiquitinases (DUBs) can cleave the ubiquitin tag rescuing receptors from degradation allowing their recycling back to the cell surface membrane.

Deubiquitinases (DUBs)

To regulate the stability, subcellular localization, and enzymatic activity of target substrates, DUBs remove the ubiquitin tag, and modulate the signalling outcome counteracting the activity of E3 ligases. The initially accepted function of the DUBs was to save proteins that were targeted for degradation. Modulation of the signalling outcome became evident later. The human genome encodes nearly 100 DUBs, which display different specificity towards both substrates and particular ubiquitin chain linkages (Clague et al., 2019).

Based on sequence conservation and domain architecture, DUBs are organized in seven structurally distinct families. These are the cysteine proteases ubiquitin C-terminal hydrolases (UCHs; 4 members), ubiquitin-specific proteases (USPs; 55 members), ovarian tumour proteases (OTUs; 17 members), Josephins (MJDs; 4 members), the motif interacting with ubiquitin (MIU)-containing novel DUB family (MINDYs; 5 members), Zinc finger with UFM1-specific peptidase domain protein (ZUFSP/ZUP1; 1 member), and the metalloproteases JAB1/MPN/MOV34 (JAMMs; 14 members) (Clague et al., 2019). Of the currently known DUBs 11 are considered as pseudo-enzymes lacking enzymatic activity.

DUBs demonstrate various layers of specificity in order to distinguish between different substrates and ubiquitin chain topologies. Numerous proteins are mono-ubiquitinated on one or multiple sites, and DUBs might exist that specifically recognize them to remove mono-ubiquitin. Moreover, many DUBs such as the proteasome-associated USP14, UCHL5, and PSMD14 that can directly bind to a target protein, may clip off an entire ubiquitin chain, leaving the substrate mono-ubiquitinated, or completely unmodified. Alternatively, unlike DUBs that are promiscuous and do not discriminate between chain linkages, there are several enzymes such as OTUB1 (Lys48), Cezanne (Lys11) and OTULIN (Met1) that recognize specific ubiquitin chain assemblies. Importantly, this layer of specificity is not limited to a particular DUB family, since such linkage-specific proteases have been found in the USPs, the

OTUs and the JAMM metalloenzymes. DUBs can also cleave from the distal end, gradually disassembling the chain (exo-deubiquitinase activity) or within chains (endo-deubiquitinase activity). In addition, chain length is another variable for the binding selectivity of DUBs. Last, DUBs might be specifically directed against branch points, Ub-Ubl bonds, or posttranslationally modified ubiquitin (Mevissen and Komander, 2017).

Like ubiquitination, deubiquitination fine-tunes fundamental cellular events, such as signal transduction, cell-cycle progression, DNA repair, and gene expression, and many deubiquitinases form multimolecular complexes to exert their physiological roles. In mammals, ubiquitin is encoded either as a linear polyubiquitin chain of multiple moieties or as an amino-terminal fusion to two ribosomal proteins (Komander et al., 2009). A key function of UCHL3, USP9X, USP7, USP5, and Otulin deubiquitinases is to process newly synthesized ubiquitin precursors and generate free ubiquitin monomers (Grou et al., 2015). Apart from the precursor processing, DUBs maintain the pool of free ubiquitin by rescuing it from substrates that are targeted for proteasomal degradation. Proteasome-associated DUBs USP14, UCHL5, and PSMD14, cleave ubiquitin from proteins prior to being unfolded by ATPases, and further processed into the 20S proteasome core for degradation (Deol et al., 2020; Hu et al., 2005). As such, free ubiquitin molecules are recycled being available for another target substrate to be modified (Snyder and Silva, 2021).

Importantly, DUBs participate in the internalization, endosomal sorting and lysosomal degradation of cell surface receptors, opposing the function of E3 ligases and modulating the outcome of signal transduction. DUBs can not only be recruited to the plasma membrane influencing the initial steps of endocytosis but also localize in various organelles affecting the trafficking of receptors (McCann et al., 2016). Two known DUBs found on endosomes are USP8 and AMSH that associate with components of ESCRT-0, and -III complexes (Wright et al., 2011). Moreover, a rage of DUBs at different stages determines the fate of EGFR, which is the most intensively studied receptor in terms of internalization and trafficking. Particularly, USP25 (Niño et al., 2020), USP22 (Zhang et al., 2018), USP17 (McCann et al., 2018), USP9X (Savio et al., 2016), USP8 (Berlin et al., 2010), USP2a (Liu et al., 2013), Cezanne-1 and -2 (Pareja et al., 2012), UCHL1 (Jin et al., 2015) and AMSH (McCullough et al., 2004) have been implicated in the regulation of EGFR, either directly deubiquitinating the receptor itself or indirectly exerting their effect on proteins essential for EGFR intracellular sorting and downregulation.

The ability of DUBs to reverse ubiquitination allows them to govern a wide range of cellular processes. Due to the multifaceted role of DUBs as key enzymes in all ubiquitin-dependent processes, different mechanisms based on allosteric regulation and PTMs, have evolved to tightly control the expression levels, subcellular localization, and catalytic activity of deubiquitinases.

Dysregulation of DUBs is associated with several human diseases, including cancer, neurodegenerative disorders, and inflammatory conditions, thus highlighting the significance of their function. Therefore, DUBs have emerged as novel therapeutic agents (Harrigan et al., 2018).

Although the ubiquitination of PDGFRβ has been studied before and the role of Cbl family of E3 ligases in the internalization, stability and PDGF signalling has been elucidated, the role of DUBs in this pathway remained elusive. In paper II, we identified USP4 and USP17 as DUBs acting on PDGFRβ, affecting trafficking of the receptor and sustaining STAT3 activation.

USP4

USP4, also known as ubiquitous nuclear protein (UNP), belongs to the ubiquitin specific proteases (USPs) (Hu et al., 2021). Discovered almost three decades ago USP4 was originally characterized as a nuclear oncoprotein due to the location of human *UNP* gene on chromosome 3p21.3, a locus often involved in malignancies, and due to its high expression levels in human lung adenocarcinomas (Gray et al., 1995; Gupta et al., 1994, 1993). Later studies revealed the deubiquitinating activity of USP4 and its sequence similarity with other USPs (Frederick et al., 1998; Gilchrist et al., 1997; Gilchrist and T. Baker, 2000). The tumour suppressor retinoblastoma pRb protein and the p107 and p130 pocket proteins were the first identified interacting partners of USP4, indicating a potential USP4-mediated cell transformation mechanism (Blanchette et al., 2001; DeSalle et al., 2001).

USP4 features at the amino-terminus a tandem DUSP-UBL domain, which is separated from the catalytic core by a connecting linker, and at the carboxyl-terminus a USP catalytic domain bifurcated by a second UBL domain and an insert (Clerici et al., 2014). USP4 as a cysteine protease employs a papain-like mechanism to remove the ubiquitination from target proteins (Cstorer and Ménard, 1994; Mevissen and Komander, 2017). USP4 contains in the USP domain the catalytic triad composed of three highly conserved residues i.e. Cys89, His334 and Asp350 which are responsible for its deubiquitinating activity (Ye et al., 2009). After substrate hydrolysis ubiquitin is strongly retained compromising the catalytic turnover of USP4. The DUSP-Ubl domain restores the full enzymatic activity of USP4 *in vitro* by facilitating ubiquitin dissociation (Clerici et al., 2014). Although the catalytic core at the carboxyl-terminus is required for the recognition and recruitment of the majority of substrates, few interactions are mediated through the tandem DUSP-UBL domain (Hu et al., 2021). Such examples are the interactions of the amino-terminal DUSP and UBL domains of USP4 with SART3, an element for spliceosome assembly, and the S9/Rpn6 subunit of the 19S proteasome, respectively (Song

et al., 2010; Zhao et al., 2012). USP4 shares high sequence similarity and domain organization with two structurally related paralogues, USP15 (56.9% identity) and USP11 (44.6% identity) (Vlasschaert et al., 2015).

USP4 possesses both nuclear export signal (NES) and nuclear localization signal (NLS) motifs rendering it capable of shuttling between nucleus and cytoplasm (Soboleva et al., 2005). In the cytoplasm, USP4 deubiquitinates protein substrates of several signalling pathways, such as NF-κB (Xiao et al., 2012), TGF-β (Zhang et al., 2012), Wnt/β-catenin (Yun et al., 2015), p53 (Zhang et al., 2011), and adenosine A2A receptor (Milojević et al., 2006), whereas in the nucleus USP4 targets spliceosome proteins determining spliceosome dynamics (Park et al., 2016; Song et al., 2010) .The proportions of nuclear and cytoplasmic USP4 localization are different for distinct cell types, cell cycle phases, or expression levels (Soboleva et al., 2005). Importantly, phosphorylation of DUBs in response to growth factors determines their subcellular localization and therefore substrate availability (Das et al., 2020). In particular, USP4 undergoes AKT-mediated phosphorylation at its Ser445 following TGF-β stimulation, which results in the translocation of USP4 from nucleus to cytoplasm, mostly to the plasma membrane in breast cancer cells (Zhang et al., 2012). Similarly, EGF treatment triggers AKT-dependent phosphorylation of USP4 at Ser445 (Deng et al., 2019). In addition, cyclin dependent kinases (CDKs) are suggested to play a role in USP4 phosphorylation at Thr149 and Thr219 in Hela cells in which dephosphorylated USP4 accumulates in the nucleus, in support of the previous findings (Das et al., 2019).

Ubiquitination is another post-translational modification that controls the stability and abundance of deubiquitinases (Das et al., 2020). The ubiquitination sites of USP4 have been mapped and studies showed that USP4 can autodeubiquitinate itself without affecting its stability (Liu et al., 2015). Unusually, unlike ubiquitination that occurs on lysine residues of target proteins, USP4 can be ubiquitinated on cysteine residues, i.e. Cys461, Cys464, Cys799, and Cys802 that form a flexible zinc-binding area stabilizing the catalytic core of the deubiquitinase (Wijnhoven et al., 2015). Interestingly, protein complexes between DUBs and E3 ligases are commonly observed. USP4 is known to interact with and deubiquitinate the E3 ligase TRIM21 (Ro52) (Di Donato et al., 2001; Hayes et al., 2012a; Wada and Kamitani, 2006). The activity of USP4 may prevent auto-ubiquitination of E3 ligases, a common feature of these ligating enzymes, and prevent their degradation by prolonging the half-life of E3 enzymes. Alternatively, E3 ligases might down-regulate DUBs. These observations suggest that there is an intricate interplay between ubiquitination and deubiquitination in the regulation of the expression levels of DUBs (Komander, 2010).

USP4 participates in the regulation of various signalling pathways, as mentioned above. In the TGFβ signalling pathway, USP4 can stabilize target substrates, enhancing the pro-tumorigenic features of the pathway. USP4 directly

deubiquitinates the type I receptor for TGFβ (TβRI) stabilizing its levels on the cell surface membrane (Zhang et al., 2012). Moreover, USP4 counteracts the ligase activity of SMURF2 by removing the inhibitory mono-ubiquitination of SMAD4, hence enhancing SMAD complex formation and sustaining downstream activation in the TGFβ/activin/ bone morphogenetic protein signalling pathways (Dupont et al., 2009; Zhou et al., 2017).

USP4 has an established role as a negative regulator of the NF-κB signalling pathway, which is critical for immune response, cell proliferation, and apoptosis. In contrast with the TGF-β signalling pathway, USP4 does not affect the stability of NF-κB, rather the recruitment of downstream signalling molecules such as TAK1 and IKK after deubiquitinating both E3 ligases TRAF2 and TRAF6, and the scaffold protein RIP1, respectively (Hou et al., 2013; Xiao et al., 2012; Zhou et al., 2012). In addition, USP4 has been reported to directly deubiquitinate TAK1 and abrogate the TAK1/IKK complex, thus, suppressing the signalling pathway downstream of TAK1 (Fan et al., 2011; Wang et al., 2020).

Moreover, USP4 enhances the suppressive activity of histone deacetylases (HDACs) on p53 by deubiquitinating and stabilizing HDAC2. The USP4-mediated stabilization of HDAC2 contributes to the downregulation of NF-κB activation upon tumour necrosis factor-α (TNFα) stimulation (Li et al., 2016). Apart from deacetylation, ubiquitination by the E3 ligase ARF-binding protein 1 (ARF-BP1) downregulates p53 levels (Chen et al., 2005). ARF-BP1 interacts directly with the p53 protein and induces p53 ubiquitination, targeting it for degradation. USP4 binds directly with and stabilizes ARF-BP1 via deubiquitination, promoting ARF-BP1-dependent ubiquitination and degradation of p53. Furthermore, knockout of USP4 in Usp4 $^{-/-}$ mouse embryonic fibroblasts (MEFs) leads to the activation of p53, upregulation of apoptosis, premature cell senescence, and reduced oncogene-associated transformation (Zhang et al., 2011).

Wnt/β-catenin signalling is another pathway that is fine-tuned by USP4 at different levels. USP4 has been reported to bind to Dishevelled (Dvl) scaffold removing its Lys63-polyubiquitination chain, eventually hindering β-catenin accumulation and its transcriptional activity in response to Wnt (Zhou et al., 2016). In addition, following binding to Nlk, a negative regulator of Wnt signalling, USP4 translocates into the nucleus where USP4 deubiquitinates the transcription factor T-cell factor (TCF) inhibiting the transcription of Wnt target genes (Zhao et al., 2009). Distinct from the role of USP4 as a suppressor of the Wnt pathway, USP4 has been found to positively regulate Wnt signalling by deubiquitinating and directly stabilizing β-catenin which facilitates β-catenin accumulation and further transcriptional activity in colorectal cancer and brain metastatic lung adenocarcinoma (Hwang et al., 2016; Yun et al., 2015).

The expression levels of USP4 are elevated in the majority of human cancers including breast, colorectal, urinary bladder, prostate and lung cancer,

brain metastatic lung adenocarcinoma, glioblastoma (GBM), melanoma, hepatocellular (HCC), and adrenocortical carcinoma (Hu et al., 2021; Wang et al., 2020; Zhang et al., 2011). High expression of USP4 is correlated with tumour progression and therefore poor survival rate in cancer patients, although it can be associated with favourable prognosis in some cases, e.g. in lung adenocarcinoma exhibiting tumour-suppressive functions (Hu et al., 2021; Wang et al., 2020; Zhong et al., 2018). Due to the multifaceted roles of USP4 in tumorigenesis via regulation of distinct signalling pathways, USP4 has been proposed as a potential therapeutic agent and a small compound called vialinin A has been identified as a non-selective inhibitor of USP4 (Okada et al., 2013; Wang et al., 2020).

USP17

USP17, also known as DUB3, comprises a subfamily of thirteen members (USP17A-N), which are encoded with unique expression patterns by the tandemly repeated megasatellite sequence RS447 in the human genome and are characterized by high copy numbers (Alkan et al., 2009; Ducker and Shaw, 2021; Saitoh et al., 2000). Members of the USP17 subfamily were originally identified as immediate early genes whose expression was rapidly and transiently induced in response to stimulation with IL-2 and IL-3 cytokines in hematopoietic murine cells (Baek et al., 2004, 2001; Zhu et al., 1997, 1996). Sequence homology analysis later revealed the human orthologue of murine USP17 and the location of *USP17* genes was mapped mainly on chromosome 4 (4p16.1) and partly on chromosome 8 (8p23.1) (Burrows et al., 2010, 2005, 2004; Okada et al., 2002). Due to their high sequence identity and genetic complexity, different USP17 deubiquitinating enzymes and their corresponding functions have not been fully distinguished yet and thus are collectively referred as USP17 (Ducker and Shaw, 2021).

The full-length human USP17 contains 530 amino acid residues, which comprise two functionally distinct structural domains, an amino-terminal catalytic ubiquitin specific protease (USP) domain and two carboxyl-terminal hyaluronan (and RNA) binding motifs (HABMs) at amino acid residues 401–409 and 445–453 (Ducker and Shaw, 2021; Shin et al., 2006; Yang et al., 2021). HABMs have been shown to be essential for the interactions between USP17 and hyaluronan, hence hindering cell proliferation and anchorage-independent tumour growth (Ramakrishna et al., 2012). Out of the thirteen deubiquitinases of this subfamily, only USP17N, which is a truncated variant of USP17L, lacks HABMs (Lim et al., 2013; Shin et al., 2006). USP17 unlike other ubiquitin specific proteases lacks ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains (Komander et al., 2009). Instead, two interaction motifs with TRAF2 and TRAF3 proteins have been identified at the carboxyl-

terminus of USP17 that allow the deubiquitinase to disrupt the TRAF2/3 complex and thus enhance the inflammation-related gene expression (Lu et al., 2018). In general, interaction partners bind to different regions of USP17. For example, TRAF2, SETD8 and SDS3 bind to the carboxyl-terminus of USP17, whereas ELK-1, BRD4 and MCL-1 interact with the amino-terminus where the catalytic USP domain resides (Ducker et al., 2019; Fukuura et al., 2019; Jin et al., 2018; Lu et al., 2018; Ramakrishna et al., 2011; Wu et al., 2019).

The expression of USP17 and its activity is regulated by mitogenic stimuli, cell cycle and posttranslational modifications (Ducker and Shaw, 2021). In response to cytokine (IL-4, IL-6, IL-8), chemokine (SDF-1) and growth factor (EGF) stimuli, both the mRNA and protein expression levels of USP17 increase (Burrows et al., 2004, p. 3; de la Vega et al., 2011; Jaworski et al., 2014). In addition, USP17, controlled in a cell cycle-dependent manner, is expressed during late G1 phase and plays a fundamental role in the successful transition from G1 phase of the cell cycle to S phase, which is critical for cell proliferation (McFarlane et al., 2010). Therefore, USP17 depletion can block proliferation by accumulating cells in the G1 phase and thus impairing cell cycle progression (McFarlane et al., 2010). Interestingly, analysis of USP17 promoter region disclosed the existence of binding sites for various transcription factors, including HIF-1, STAT3, STAT6, and NF-κB, which implies that they may regulate USP17 expression (Lu et al., 2018). Furthermore, CDK4/6-mediated phosphorylation of USP17 on Ser41 has been reported to be critical for the activation of the deubiquitinating activity of USP17 (Liu et al., 2017).

Following its expression and activation, USP17 influences cell cycle progression and cell proliferation by exerting its deubiquitinating activity towards various substrates at different levels (Ducker and Shaw, 2021). Among the pro-proliferative substrates of USP17 are cyclin A, a regulatory subunit for the activity of Cyclin-dependent kinase complexes (CDKs), and Cdc25A, a dual specificity tyrosine/threonine phosphatase, which governs the phosphorylation status of Cyclin-CDKs. USP17 is reported to deubiquitinate and stabilize both cyclin A and Cdc25A, promoting transition in cell cycle phases and proliferation (Hu et al., 2019; Pereg et al., 2010). In the MAPK/ERK signalling pathway USP17 acts on mono-ubiquitinated ELK-1, a transcription factor downstream of ERK1/2. Therefore, USP17 enhances the transcription of ELK-1 target genes and promotes cell proliferation (Ducker et al., 2019). Similarly, USP17 stabilizes c-MYC which activates several pro-proliferative genes augmenting cell proliferation (Nagasaka et al., 2022). In addition, in the same signalling pathway the Ras-converting enzyme 1 (RCE1) is known to activate the upstream protein Ras by cleaving its carboxyl-terminal motif, which allows Ras to translocate from the ER to the cell membrane and transduce downstream signalling. USP17 negatively regulates the MAPK/ ERK signalling pathway by removing the Lys63-polyubiquitin chains from RCE1 and consequently blocking RCE1 activity. Distinct from the previous inde-

pendent studies, USP17-mediated deubiquitination of RCE1 resulted in impaired cell proliferation (Burrows et al., 2009). The contradictory functions of USP17 in enhancing or attenuating pro-proliferative signalling might reflect dependency on the cell type and the context under which those effects are observed.

Although the subcellular localization of USP17 varied depending on the different sequences used in the overexpression system and on the diverse cell lines employed in biochemical assays (Ducker and Shaw, 2021), endogenous USP17 is reported to be widely distributed both in the endoplasmic reticulum (ER) and the nucleus of HeLa cells (Burrows et al., 2009; Ramakrishna et al., 2011). Furthermore, the mRNA and protein expression levels of USP17 are elevated in numerous cancer types in which the role of USP17 in tumour development and progression is emerging (McFarlane et al., 2010). For example, in osteosarcoma cells USP17 is shown to contribute to epithelial to mesenchymal transition (EMT) and subsequent migration and invasion by stabilizing SMAD4, a transcription factor involved in the canonical TGFβ pathway (Song et al., 2017). Although USP17 has been suggested as a promising therapeutic target, up to date the partly selective compound WP1130 has only been developed while specific inhibitors for USP17 have not been discovered yet (Ducker and Shaw, 2021; Kapuria et al., 2010). Despite a growing list of studies displaying oncogenic roles of USP17, it has also been characterized as a tumour suppressor by deubiquitinating and stabilizing components of the Hippo pathway (Ducker and Shaw, 2021; Yang et al., 2021).

RNA-binding proteins

Growth factor signal transduction culminates in the activation of transcription factors which promote gene expression in the nucleus. Shutoff of transcription and short half-lives of mRNAs control which genes will be turned off. RNA-binding proteins (RBP) have emerged as regulators of gene expression participating in many aspects of mRNA metabolism such as mRNA trafficking to subcellular sites, mRNA translation, splicing, stability and degradation. During the assembly of the basic translational machinery RBPs have been reported to exert control over initial translational stages by preventing the recruitment of 43S preinitiation complex (Muckenthaler et al., 1998) or over late translational stages by inhibiting the elongation of specific mRNAs (Stebbins-Boaz et al., 1999); thus stalling translation. Moreover, RBPs provide a quality control mechanism by degrading RNAs via nonsense-mediated mRNA decay in which 3' UTR helicases or chaperones contribute to decay efficiency (Lykke-Andersen and Jensen, 2015; Stalder and Mühlemann, 2008). In contrast, RBPs such as AUF1, TTP, and Hu proteins can stabilize mRNAs by binding to A/U-rich elements (AREs) within 3'UTR region (Barreau et al.,

2005). An increasing body of evidence reveals the implication of RBPs in tumour progression (Iino et al., 2020; Qin et al., 2020). Therefore, unravelling the roles of RBPs in cell homeostasis and pathogenesis might lead to the discovery of novel targets for cancer treatment.

G3BP1

Ras-GTPase activating protein SH3-domain-binding (G3BP1) protein 1 is a small evolutionary conserved family of RNA-binding proteins that modulates gene expression in response to environmental changes by controlling mRNA degradation and translation. In addition, G3BPs appear as important components of stress granules even though not all stress granules contain them. G3BP was first identified in a screen for interactors of the SH3 domain of Ras GTPase Activating Protein (RasGAP) (Parker et al., 1996). There are two homologous proteins with their isoforms; G3BP1, G3BP2a and the alternatively spliced isoform G3BP2b, which are encoded by two distinct genes, mapped on human chromosomes 5 and 4, respectively (Kennedy et al., 2002).

All G3BPs share five distinct structural motifs. These are the N- terminal nuclear transport factor 2 (NTF2 domain), the central acid-rich region, and proline-rich (PxxP) motif, and the C-terminal RNA recognition motif (RRM), and RGG domain (arginine-, glycine- rich boxes). Due to the high arginine content of RGG, this domain is also readily methylated, which increases the binding affinity of G3BPs to RNA, and facilitates protein interaction, overall stabilizing the G3BP-RNA complex. Furthermore, the RRM domain has two short consensus sequences, RNP1 and RNP2, which contain conserved hydrophobic amino acid residues distributed throughout the motif that are essential for RNA binding. Regarding the central region, the main difference between the three G3BP variants is the varying numbers of PxxP motifs, which are able to interact with the SH3-domain of Ras-GAP. Unlike G3BP1 that has one PxxP motif, G3BP2a and G3BP2b encompass four and five PxxP motifs, respectively (Kennedy et al., 2002). G3BP2b also lacks 33 amino acid residues compared to G3BP2a in the central region. Last, the NTF2 domain, the most conserved region in G3BP, is implicated in nuclear transport and subcellular localization.

G3BPs are ubiquitously expressed in all normal cells and primarily localize in the cytoplasm. However, G3BP1 has been found to localize in the nuclei of quiescent cells following its phosphorylation at Ser149 (Gallouzi et al., 1998; Tourrière et al., 2003) whereas G3BP2 translocate into the nucleus upon serum stimulation (French et al., 2002).

The presence of G3BPs in the nucleus indicate their role as endoribonucleases regulating the stability and degradation of target mRNAs and thus, controlling gene expression. In Wnt/ β-catenin signalling, for example, G3BP1 normally associates with the Dishevelled3 (Dvl3) scaffold and docks to

CTNNB1, the mRNA encoding β-catenin subjecting it to degradation (Bikkavilli and Malbon, 2011). In response to Wnt3a stimulation, however, G3BP1 is robustly methylated in the RGG region triggering its release from the Dishevelled (Dvl3) based complex, thus allowing, the translation of *CTNNB1* into β-catenin and the activation of Wnt signalling pathway (Bikkavilli and Malbon, 2011). Interestingly, the regulation of the endoribonuclease activity has been reported to be dependent on the phosphorylation of G3BP Ser149, which initiates translocation of the protein to the nucleus (Gallouzi et al., 1998; Tourrière et al., 2001). There, G3BP cleaves the 3'-untranslated region (3'-UTR) of the mRNA encoding c-Myc mediating rapid mRNA decay (Gallouzi et al., 1998; Tourrière et al., 2001). Absence of Ser149 phosphorylation correlates with a delay in *c-MYC* mRNA decay, thus validating the essential role that this modification holds in the regulation of G3BPs as endoribonucleases (Tourrière et al., 2001). Besides serine phosphorylation, tyrosine phosphorylation of G3BP1 has been also reported (Rahmouni et al., 2005). In addition, G3BP interacts with Ras GTPase-activating protein p120 (RasGAP) in proliferating, but not quiescent cells, which contain hyperphosphorylated G3BPs (Parker et al., 1996; Tourrière et al., 2001).

The expression of G3BPs is upregulated in a variety of human cancers where G3BPs are associated with aberrant cell proliferation, abnormal cell growth, invasion and metastasis via distinct mechanisms in such cancers as (Kang et al., 2021). G3BPs particularly participate in various signalling pathways such as the Ras, the NF-κB, and the MAPK/ERK signalling pathways. High levels of G3BP1 in oesophageal cancer cells promote cell proliferation, migration, and invasion via upregulation of Wnt/β□catenin and PI3K/AKT signalling pathways (Zhang et al., 2019). Similarly, G3BP1 is also involved in the Ras/ MEK/ERK and PI3K/AKT signalling pathways by sustaining the activation of ERK1/2 and AKT in several other types of cancer including gastric cancer, breast cancer, and lung adenocarcinoma (Alam and Kennedy, 2019; Min et al., 2015; Zhang et al., 2013; Zheng et al., 2019). Interestingly, G3BP1 has been reported to play contradictory roles in the Wnt/β-catenin signalling pathway. Although elevated expression levels of G3BP1 stabilize β-catenin by inactivating GSK-3β suppressor in breast cancer cells (Zhang et al., 2021), G3BP1 has been characterized as a negative regulator of Wnt/β-catenin signalling because of its ability to degrade β-catenin mRNA (*CTNNB1*) in totipotent mouse embryonic F9 cells (Bikkavilli and Malbon, 2011). This discrepancy suggests that the function of G3BP1 is context and cell dependent. In line with the G3BP1-mediated stabilization of β-catenin in breast cancer cells, Li et. al demonstrated that the upregulation of G3BP1 is linked to increased levels of β-catenin and c-MYC, which consequently promote the progression of colon cancer (Li et al., 2020). Furthermore, the downregulation of G3BP1 impairs the NF-κB and JAK/STAT pathways in lung cancer (Omer et al., 2020). In contrast, G3BP1 increases the phosphorylation levels of STAT3

in response to the pro-inflammatory cytokine IL-6 in primary renal cell carcinomas (Wang et al., 2018). Last, multiple studies have reported that G3BP1 acts as a positive regulator of metastasis via the SMAD-pathway, which suppresses the expression of the epithelial marker E-cadherin and activates the expression of mesenchymal markers Snail1 (Snail), Snail2 (Slug), and ZEB1 (Dou et al., 2016; Wang et al., 2018; Zhang et al., 2015). Given that high levels of G3BP1 expression are correlated with low survival rates of patients, G3BP1 has been proposed as a prognostic biomarker in some types of cancers (Hu et al., 2021).

Targeting PDGF signalling in cancer

Aberrant activation of PDGF signalling is associated with cancerogenesis and the progress of other diseases that are characterized by excessive cell proliferation, such as atherosclerosis and various fibrotic conditions (Östman and Heldin, 2007). Constitutive activation of PDGF signalling pathways can result from gain-of-function mutations, chromosomal rearrangements as well as epigenetic changes that increase the expression of PDGF ligands and their receptors. In the rare skin tumour dermatofibrosarcoma protuberance (DFSP), the *PDGF-B* gene is fused to the *collagen 1A1* gene (O'Brien et al., 1998; Simon et al., 1997) while in certain types of leukaemia the genes of PDGFRs are found to be translocated (Toffalini and Demoulin, 2010). Point mutations in the *PDGFRα* gene are seen in 5% of gastrointestinal stromal tumours (GIST) (Heinrich et al., 2003). In fact, cancer genome sequencing revealed that mutational events related to PDGF family of ligands and receptors account for 20% of melanomas, glioblastomas and other types of cancers (lung, bladder, prostate, colorectal and ovarian) (Farooqi and Siddik, 2015; Heldin et al., 2018). Interestingly, in the stromal compartment of tumours, cells can secrete PDGF ligands themselves that in turn act in a paracrine manner on normal neighbouring cells. In a dynamic tumour microenvironment the paracrine signalling by cancer cells recruits to vessels various cell types, like stromal fibroblasts, pericytes, vascular smooth muscle cells and protumorigenic inflammatory cells which modulate the components of extracellular matrix promoting abnormal growth, angiogenesis and invasion (Heldin et al., 2018; Song et al., 2005). In addition, PDGF signalling enhances the increased interstitial fluid pressure, a common feature of solid tumours, which forms a barrier to transcapillary transport rendering tumour treatment with chemotherapeutical agents inefficient (Heldin et al., 2004).

The overactivity of PDGF signalling that is often observed in malignancies has prompted attempts to develop PDGF/PDGFR antagonists like antibodies, DNA aptamers and kinase inhibitors. These antagonists disrupt the PDGF/PDGFR pathway targeting either the ligand, the extracellular domain

of the receptors or the intracellular kinase activity of PDGFRs (Papadopoulos and Lennartsson, 2018). Different types of antagonists now undergo preclinical and clinical evaluation with variable results. Antibodies are highly specific but are also expensive and difficult to administer. Olaratumab, a monoclonal antibody against PDGFRα, received first global approval for the treatment of adult patients with soft tissue sarcoma in combination with doxorubicin (Papadopoulos and Lennartsson, 2018; Shirley, 2017). An effective approach to inhibit PDGFR signalling is to hinder its enzymatic activity by employing low molecular weight compounds. These compounds penetrate cells and compete with ATP in occupying the binding pocket of kinases. Although the high conservation of the ATP binding pocket across RTKs has imposed challenges in the design of specific tyrosine kinase inhibitors (TKI), clinical experience has shown that inhibition of more than one RTK can be advantageous (Heldin et al., 2018; Papadopoulos and Lennartsson, 2018). Imatinib (sold under the trade name Gleevec) was the first tyrosine kinase inhibitor to be approved for the treatment of chronic myelogenous leukaemia (CML) in 2001 (Iqbal and Iqbal, 2014). Despite the remarkable effectiveness of imatinib clinical resistance has been increasingly reported leading to the development of second- and third-generation tyrosine kinase inhibitors (dasatinib, nilotinib, bosutinib, and ponatinib). Due to the poor oral bioavailability of tyrosine kinase inhibitors the focus has shifted on designing high permeable drugs that are delivered to tumour sites without adversely affecting the neighbouring cells. Such examples are the development of nanoparticles, thermosensitive liposomes and cell-penetrating peptides. Although animal models have been extensively used for evaluating drug efficacy, culturing human organoids in 3D conditions hold great promise for successful drug development by providing more accurate and reliable drug screening (Papadopoulos and Lennartsson, 2018).

Present Investigations

"Say not, "I have found the truth," but rather, "I have found a truth.""
Khalil Gibran

As addressed in the previous chapters, the primary function of PDGF is to stimulate cell growth, proliferation and migration, which is essential for embryonic development, wound healing and tissue homeostasis in adults. However, constitutive activation of PDGFRs has been observed in diseases characterized by aberrant cell proliferation, such as cancer. Gain-of-function mutations, high expression levels of ligands and receptors, and activating translocations of their relative genes account for the sustained PDGF signalling, which obviates negative regulatory feedback mechanisms in different types of cancer. Due to the over-activity of PDGF signalling often seen in malignancies, efforts have been made to treat patients with PDGF/PDGFR antagonists. Therefore, dissecting PDGF signalling pathways is critical in having a deeper understanding in how these pathways are activated and regulated and can prompt attempts to develop effective inhibitors, which can be directed against the ligand, the receptor itself or its downstream effectors.

The goal of this thesis has been to investigate novel molecular mechanisms underpinning modulation of PDGFRβ signalling. We explored different approaches of PDGFRβ signalling regulation, namely:

- Activation of MKP2/DUSP4, a specific phosphatase of ERK1/2, in response to PDGF-BB stimulation (Paper I)

- USP4- and USP17-mediated deubiquitination of PDGFRβ affecting its internalization, intracellular trafficking and STAT3 signaling pathway (Paper II)

- Regulation of the basal levels of PDGFRβ and its availability at the plasma membrane by TRIM21, a new E3 ligase of PDGFRβ (Paper III)

- The role of G3BP1, a novel interactor of PDGFRβ, in the regulation of PDGF-BB-mediated ERK1/2 and STAT3 signaling pathways (Paper IV)

Paper I

Yin R, Eger G, **Sarri N**, Rorsman C, Heldin CH, Lennartsson J. Dual specificity phosphatase (DUSP)-4 is induced by platelet-derived growth factor -BB in an Erk1/2-, STAT3- and p53-dependent manner. Biochem Biophys Res Commun. 2019 Nov 12;519(3):469-474.

Growth factor-mediated MAPK signalling pathways culminate in the activation of MAP kinases that translocate into the nucleus and phosphorylate transcription factors involved in cell proliferation, survival and cell death. Although their aberrant activity has been correlated with tumorigenesis, the study of their negative regulators, DUSPs, has been poorly explored.

In paper I, we investigated the PDGF-BB-induced regulation of MKP2/DUSP4 expression.

We report that PDGF-BB induced the DUSP4 expression along with the expected activation of STAT3, Src, AKT, PLCγ, and ERK1/2 and p38 MAP kinases in primary human fibroblasts (AG1523). In order to delineate the predominant signalling pathway underlying DUSP4 expression, we used the following inhibitors: CI-1040 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), XMD8-92 (ERK5 inhibitor), SP600125 (JNK inhibitor) and Stattic (STAT3 inhibitor). In agreement with previous studies, we observed that inhibition of ERK1/2 decreased DUSP4/MKP2 mRNA levels, suggesting that ERK1/2 activation was necessary for DUSP4/MKP2 transcription.

Moreover, we found that the STAT3-p53 pathway, independent of ERK1/2 pathway, could regulate DUSP4 expression. We observed that Stattic, an inhibitor of STAT3, as well as p53 silencing had a downregulating effect on PDGF-BB-induced DUSP4 expression. Interestingly, in the absence of STAT3, the levels of p53 and DUSP4 were depleted as well. However, upon treatment with a p53 stabilizer nutlin-3, DUSP4 expression was rescued, suggesting that p53 is an upstream regulator of DUSP4. In conclusion, we provide evidence that prolonged PDGF-BB stimulation induces the expression of DUSP4/MKP2 and this regulation requires the involvement of ERK1/2, STAT3 and p53.

Paper II

Sarri N, Wang K, Tsioumpekou M, Castillejo-López C, Lennartsson J, Heldin CH, Papadopoulos N. Deubiquitinating enzymes USP4 and USP17 finetune the trafficking of PDGFRβ and affect PDGF-BB-induced STAT3 signalling. Cell Mol Life Sci. 2022 Jan 21;79(2):85.

Our group has previously characterized the Cbl-mediated ubiquitination of PDGFRβ. In the present study, we were interested in identifying deubiquitinating enzymes that may be regulating receptor's stability, endocytosis, trafficking and downstream signalling. To that end, we screened a cDNA library of sixty-four DUBs and identified USP4 and USP17 acting on PDGFRβ. By performing co-immunoprecipitation under denaturing conditions, we validated that USP4 and USP17 act as deubiquitining enzymes directly on PDGFRβ. Due to the ubiquitin linkage-specificity that some deubiquitinases exhibit, we further utilized mutant ubiquitin molecules that are refractory to Lys48- or Lys63-ubiquitination and demonstrated that USP4 is able to remove both Lys48- and Lys63-linked ubiquitin chains from PDGFRβ, which is in agreement with the literature (Zhao et al., 2009).

To examine whether the decreased ubiquitination of ligand-stimulated PDGFRβ prolonged the half-life of the receptor, we pre-treated cells with a protein translation inhibitor cycloheximide and found that USP4- and USP17-mediated deubiquitination of PDGFRβ did not impact the stability of PDGFRβ. To further investigate the possibility that deubiquitination of PDGFRβ is linked to the regulation of endocytosis, we biotinylated the remaining receptors after ligand stimulation in the presence and absence of DUBs. Interestingly, USP4 did not affect the internalization rate of PDGFRβ from the cell surface, whereas USP17 delayed its endocytosis. These findings suggest that USP17 is recruited to the plasma membrane to regulate the internalization of the receptor, while USP4 might act on the sorting of PDGFRβ further in the intracellular trafficking pathways. Analysis of downstream signalling events revealed that both deubiquitinases affected the timing of STAT3 activation. USP4 slightly shifted the STAT3 phosphorylation peak within the first ten minutes of PDGF-BB stimulation while USP17 sustained its activation from fifteen to forty-five minutes. To exclude the possibility that the deubiquitinases act directly on STAT3 mediating the changes in the activation frame of STAT3, we immunoprecipitated STAT3 and did not detect any ubiquitination of STAT3 or changes in its ubiquitination status upon induction of USP4 and USP17. Our results confirm that it is the deubiquitinated PDGFRβ that conveys the changes in STAT3 activation. We then evaluated the transcriptional activity of STAT3 and observed that the expression of *c-MYC* and *CDKN1A* genes in particular was upregulated upon induction of DUBs. Finally, to examine whether altered gene expression affected the proliferative response of

cells to PDGF, we performed the CYQUANT proliferation assay. Depletion of USP4 inhibited cell proliferation in response to PDGF-BB. Based on our findings we have provided insight in the key molecular components of PDGFRβ deubiquitination that affect its internalization and downstream signalling pathways that culminate in altered gene expression and dysregulated cell proliferation.

Paper III

Sarri N, Papadopoulos N, Lennartsson J, Heldin CH. The E3 ubiquitin ligase TRIM21 modulates the basal levels of PDGFRβ (2022). *Manuscript*

In this study we have identified a new E3 ligase of PDGFRβ, i.e. TRIM21, in a mass spectrometry screen and we have characterized its mode of action on PDGFRβ. Our group has previously discovered the family of Cbl E3 ligases to be critical for the ubiquitination, internalization from the cell surface and downstream signalling of PDGFRβ (Rorsman et al., 2016). Despite the robust decrease in the ubiquitination status of the receptor upon dual depletion of c-Cbl and Cbl-b, residual ubiquitination was observed, suggesting that another E3 ligase might function. To explore the possibility that TRIM21 ubiquitinates PDGFRβ, we transiently knocked down TRIM21 in U2OS osteosarcoma cells and AG1523 fibroblasts and noticed a reduction in the ubiquitination of PDGFRβ, which indicated that TRIM21 is a novel E3 ligase of PDGFRβ. Then we sought to investigate whether the two families of E3 ligases function complementarily. To this end, we performed siRNA-mediated individual and/or triple knockdown of TRIM21, c-Cbl and Cbl-b in U2OS and AG1523 cells. While downregulation of TRIM21 impacted the ubiquitination of PDGFRβ in both cell lines, depletion of Cbl molecules in U2OS was sufficient to completely deubiquitinate the receptor, suggesting that TRIM1 and Cbl E3 ligases do not possess redundant functions, but rather specialized ones which might depend on the context and cell type.

Ubiquitination of RTKs in response to growth factors initiates internalization from cell surface, intracellular trafficking, and degradation which culminates in signal termination. E3 ligases are known to prolong or shorten the half-life of target proteins, to affect their stability and to determine their abundance by mediating their ubiquitination. To evaluate the potential stabilizing effects of TRIM21 on PDGFRβ, we pre-treated U2OS cells with cycloheximide and did not observe any long-lasting stabilization effects on PDGFRβ in the course of stimulation with PDGF-BB upon depletion or induction of TRIM21. The internalization rate of cell-surface receptor did not change in

the presence or absence of TRIM21. Interestingly, induction of TRIM21 protein expression consistently decreased basal levels of PDGFRβ while siRNA depletion of TRIM21 led to increased presence of the receptor on the cell surface. In addition, the decreased basal levels of receptor upon induction of TRIM21 correlated with reduced activation of the PLCγ downstream signalling pathway.

Furthermore, we found that blockage of the autophagy pathway with bafilomycin A1 upon induction of TRIM21 in tet-inducible U2OS cells leads to the accumulation of PDGFRβ thus suggesting that ubiquitination of PDGFRβ by TRIM21 directs a portion of receptor for degradation by the autophagy pathway. Based on our findings we propose a ligand-independent regulatory pathway in which TRIM21 controls the total pool of PDGFRβ on cell surface under basal conditions.

Paper IV

Sarri N, Lennartsson J, Witek B, Heldin CH, Papadopoulos N. G3BP1 is a SWI/SNF-bound regulator of PDGFR signalling that controls cell proliferation via ERK1/2, c-MYC and STAT3 (2022). *Manuscript*

We have previously performed a mass spectrometry (MS) screen to identify novel interaction partners of PDGFRβ. To that end, PDGFRβ was immunoprecipitated from the nuclear fraction of BJ-hTERT fibroblasts, and eluted proteins were subjected to MS. Apart from TRIM21, MS analysis suggested that G3BP1 is a nuclear interactor of PDGFRβ in resting and PDGF-BB stimulated cells. Other studies have shown G3BP1 to have a phosphorylation-dependent endoribonuclease activity that cleaves the 3'-untranslated region of *c-MYC* mRNA triggering its rapid mRNA decay. High expression levels of G3BP1 have been observed in a variety of human cancers, where G3BP1 participates in different pro-proliferative signalling pathways.

In this study we sought to explore the role of G3BP1 in the regulation of PDGFRβ signalling. In order to validate the MS results we performed co-immunoprecipitation of endogenous PDGFRβ, BAF155 and BAF170 in AG1523 fibroblasts. BAF155 and BAF170 are core components of the SWI/SNF chromatin remodelling complex that also appeared as positive hits of MS screen. Surprisingly, immunoblotting for G3BP1 revealed the formation of an interaction complex with BAF155 and not with the receptor, although Papadopoulos *et al.* (2018) have shown that the translocation of the full-length PDGFRβ is important for the reassembly of the SWI/SNF complex in the nucleus.

To determine whether G3BP1 affects PDGFRβ signalling, we depleted G3BP1 in AG1523 fibroblasts, stimulated serum-starved cells with PDGF-BB and observed that transient knockdown of G3BP1 significantly decreased the phosphorylated levels of STAT3 between five and thirty minutes of stimulation. G3BP1-mediated regulation of STAT3 phosphorylation status has been also reported in lung cancer and primary renal adenocarcinoma (Omer et al., 2020; Wang et al., 2018). While the majority of studies have described impaired activation of ERK1/2 in the absence of G3BP1, we noticed that downregulation of G3BP1 robustly decreased total ERK1/2 protein, interestingly without affecting the levels of phosphorylated ERK1/2 in the cell.

An important feature of G3BP1 is its ability as endoribonuclease to degrade (e.g. *c-MYC*, *BART, CTNNB1, ATP5B, PMP22, IGF-II, GAS5*) or stabilize (e.g. *TAU*) mRNAs. To investigate the role of G3BPs in the regulation of genes of interest we performed siRNA-mediated knockdown of G3BP1 in non-stimulated and stimulated AG1523 fibroblasts. We found that depletion of G3BP1 upregulated PDGF-BB-induced gene expression of *c-FOS2, c-MYC* and *c-JUN*, whereas PDGF-BB-induced gene expression levels of *STAT3* and *ERK1/2* were slightly decreased. G3BP1 is known to bind to target mRNAs, mostly through 3′-UTRs. In order to assess whether the observed changes on the mRNA levels of the genes were linked to growth factor-mediated mRNA decay, a post-transcriptional regulatory mechanism of gene expression, we treated control and G3BP1-depleted AG1523 cells with the transcriptional inhibitor actinomycin D. Interestingly, no stabilization effects on the mRNA half-lives upon depletion of G3BP1 were observed. To relate these findings to a functional role of G3BP1, we then performed a WST-1 based colorimetric assay to assess cell proliferation upon G3BP1 downregulation and found that G3BP1-depleted cells grew and proliferated at a slower rate as compared to control cells.

Future Perspectives

Paper I

What does MKP2/DUSP4 do in the nucleus in the PDGF-BB signalling pathway?

In paper I we have reported that MKP2/DUSP4 is induced in the nucleus in response to PDGF-BB stimulation. MKP2/DUSP4 is known to dephosphorylate ERK1/2, JNK and p38 MAP kinases in different contexts (Seternes et al., 2019). However, the biological role of MKP2/DUSP4 induction in PDGF-BB signal transduction is not well understood. To address this, we consider to assess the phosphorylation levels of ERK1/2, JNK and p38 upon overexpression and downregulation of MKP2/DUSP4 in the nuclear fractions of resting and PDGF-BB-stimulated AG1523 fibroblasts. Then we would like to explore which transcription factors downstream of ERK1/2, JNK and p38 depend for their activation on MKP2/ DUSP4. Due to the fact that Elk-1 is a transcription factor activated by all the aforementioned MAP kinases, it would be worth evaluating whether Elk-1 phosphorylation levels are also affected by MKP2/DUSP4. To further determine to which promoters putative MKP2/DUSP4-dependent transcription factors bind, we could perform chromatin immunoprecipitation (ChIP) with Elk1 transcription factor as a bait as well as use luciferase reporter assays to test the binding to the consensus element. Additionally, we could analyse the expression levels of genes of interest in the presence and absence of MKP2/DUSP4 by q-PCR. Our group has previously found cytoplasmic MKP3/ DUSP6 to be also induced upon PDGF-BB stimulation (unpublished data). Interestingly, MKP2/DUSP4 and MKP3/DUSP6 display functionally redundant roles according to recent evidence (Ito et al., 2021). To explore the possibility that MKP2/DUSP4 and MKP3/DUSP6 function redundantly in the PDGF context, we could perform double knock- down of both phosphatases and determine changes in MAP kinases expression levels and phosphorylation status. Individual knock-down of one MKP/DUSP and overexpression of the other would further validate the results.

Which cellular processes is MKP2/DUSP4 essential for?

MKP2/DUSP4 as a specific phosphatase of MAP kinases provides a negative feedback mechanism that affects cell growth, proliferation, and cell survival

in different cancer cell lines. Therefore, it would be of interest to perform functional assays that would characterize the role of MKP2/DUSP4 in PDGFRβ signalling. To that end, cells depleted of the MKP2/DUSP4 phosphatase could be employed in the PDGF-induced migration which can be performed with both the chemotaxis and the cell culture wound healing assays. Proliferation can be evaluated by WST-1 based colorimetric assay. We could further study cell cycle and apoptosis via flow cytometry in cells overexpressing or depleted of MKP2/DUSP4. Last, a hallmark of cancer is the anchorage-independent growth of cells. Thus, we could perform the soft-agar colony assay and observe the changes in the formed number of colonies and their size in the presence or absence of MKP2/DUSP4.

Paper II

How do USP4 and USP17 interact with PDGFRβ?

In paper II we have identified for the first time the deubiquitinating enzymes of PDGFRβ, i.e. USP4 and USP17. DUBs are often reported to be self-associated or found in complexes with E3 ligases or other DUBs (Sowa et al., 2009). Previous studies have shown that USP4 interacts with the E3 ligases, TRIM21 and BRAP, a negative regulator of MAP kinases pathway (Di Donato et al., 2001; Hayes et al., 2012b; Wada and Kamitani, 2006). Currently, there are no known E3 ligases that are found bound to USP17. Therefore, it would be of interest to examine whether USP17 interacts with Cbl E3 ligases that are also recruited to the plasma membrane in response to PDGF-BB stimulation, whereas USP4 forms a complex with TRIM21, BRAP or its paralog enzymes USP15 and USP11.

How is the deubiquitinating activity of USP4 and USP17 regulated in PDGF signalling?

In the TGFβ signalling pathways, AKT phosphorylates USP4 at Ser445 upon ligand stimulation and translocates from the nucleus to the cytoplasm, where USP4 targets TβRI at the plasma membrane (Zhang et al., 2012). On the other hand, CDK4/6-mediated phosphorylation of USP17 in the nucleus is essential for the activation of the deubiquitinase in order to control SNAIL1 protein levels (Liu et al., 2017). To explore the phosphorylation-dependent regulation of USP4 and USP17 in response to PDGF-BB stimulation, we could immunoprecipitate the deubiquitinases and assess their phosphorylation status by generic phospho-serine/threonine antibodies. Utilizing phospho-specific antibodies for Ser445 and Ser41 would then validate the exact phosphorylation sites of USP4 and USP17, respectively. Moreover, immunoprecipitation of DUBs followed by mass spectrometry analysis would elucidate potentially new phosphorylation sites that are involved in PDGF signalling as well as

novel proteins that might interact with these DUBs. To explore whether the phosphorylation is critical for the localization of DUBs, we then could employ phospho-deficient and phospho-mimetic mutants of USP4 and USP17 in nuclear and cytoplasmic fractions. Furthermore, we have shown that induction of USP17 sustains the activation of STAT3 at later time points. Interestingly, the STAT3 transcription factor is reported to bind to the USP17 promoter region (Lu et al., 2018). Therefore, luciferase reporter assays would determine whether there is a possible positive feedback loop between USP17 induction and prolonged STAT3 activation in PDGF signalling.

Paper III

Does TRIM21 catalyse the formation of specific linkage chains? Which sites of PDGFRβ does TRIM21 ubiquitinate? Where inside the cell do TRIM21 and PDGFRβ meet?

In paper III we have identified a novel E3 ligase of PDGFRβ, i.e. TRIM21, and attempted to characterize its regulatory role in PDGFRβ signalling. By depletion of TRIM21 in both U2OS cells and AG1523 fibroblasts we observed that TRIM21 affects the levels of ubiquitination of PDGFRβ. It has been previously reported that addition of Lys48-linked ubiquitin chains targets proteins for degradation by the proteasome, while Lys63-linked chains are involved in such processes as signalling, protein interactions and endocytic trafficking. It would be of interest to determine what type of ubiquitin chains (Lys48- or Lys63-linked) Trim21 E3 ligase can add to the receptor and whether it works directly on PDGFRβ or indirectly on other proteins that affect PDGFRβ ubiquitination status. To determine the ubiquitin chain linkage specificity of TRIM21, we would like to employ Lys48- or Lys63-chain specific antibodies in TRIM21 depleted cells and observe which type of chains might be affected. In addition, TRIM21 is reported to predominantly localize in the cytoplasm. Immunofluorescence techniques and in particular, triple co-localization of TRIM21, PDGFRβ, and different subcellular organelles would allow us to understand where inside the cell the TRIM21-mediated ubiquitination of PDGFRβ occurs.

Is TRIM21 required for the clearance of misfolded PDGFRβ?

The family of TRIMs is reported to be involved in the misfolded protein clearance through different degradation pathways. To explore the possibility that TRIM21 eliminates misfolded PDGFRβ, we could use an inhibitor of N-linked glycosylation of proteins, i.e. tunicamycin, which causes endoplasmic reticulum (ER) stress and consequently evokes the unfolded protein response (UPR). Prolonged strong ER stress overloads proteasomes and accumulates polyubiquitinated proteins (Bence et al., 2001), on the other hand, long term

inhibition of proteasomal degradation has been reported to cause ER stress (Park et al., 2011). Therefore, we could determine the ubiquitination status and the total amounts of PDGFRβ in the presence and absence of TRIM21 under conditions of ER stress. The levels of E3 ligases that are involved in ER-associated degradation (ERAD), have been often found to be elevated. Therefore, evaluating the expression levels of TRIM21 upon ER stress would be also helpful in deciphering its role in ERAD. Moreover, to assess which TRIM21-mediated degradation pathway is undertaken for misfolded PDGFRβ, we would like to employ a combinatorial treatment of tunicamycin and proteasomal, lysosomal or autophagic inhibitors. Interestingly, a previous study has shown that TRIM21 interacts with the molecular chaperone p97/VCP and targets IgG heavy chain for proteasomal degradation (Takahata et al., 2008). Although our attempts to identify direct interactions between PDGFRβ and TRIM21 in vivo have not been successful, it is worth considering the possibility that nascent PDGFRβ forms an indirect interaction complex with TRIM21 through the molecular chaperone p97/VCP.

How does TRIM21 affect cell proliferation, migration and anchorage-independent soft agar colony formation in response to PDGF-BB stimulation?

Several independent studies have reported that TRIM21 impacts cell proliferation, migration and soft agar colony formation (Guha et al., 2020; Kunishita et al., 2020; Sun et al., 2022; Zhao et al., 2020). Therefore, we are interested in unravelling the functional role of TRIM21 by performing the following assays upon downregulation or induction of TRIM21; the WST-1 based colorimetric assay to assess cell proliferation, the scratch wound healing assay to study cell migration, and the soft agar colony formation assay to evaluate the anchorage-independent growth ability of cells. Furthermore, TRIM21 has been found to ubiquitinate p21, a cyclin-dependent kinase (CDK) inhibitor, promoting cell proliferation (Wang et al., 2021). Therefore, we consider assessing the levels of p21 in TRIM21-depleted or induced cells in response to PDGF-BB stimulation.

Paper IV

What is the functional significance of G3BP1-BAF155 interactions?

In paper IV, we have explored the role of G3BP1 in the regulation of PDGFRβ signalling cascades. Interestingly, we found that G3BP1 interacts with BAF155, a core component of the SWI/SNF chromatin remodelling complex, in a ligand independent manner in the nucleus. Other core components of the SWI/SNF complex are the ATPases Brahma related gene 1 (BRG1) and BRM

which are mainly related to initiation of transcription. Our group has previously shown that the SWI/SNF complex disassembles upon PDGF-BB stimulation, which culminates in altered expression of target genes (Papadopoulos et al., 2018). Therefore, to delineate whether G3BP1 contributes to the SWI/SNF-mediated regulation of transcription we are interested in identifying putative interactions between G3BP1, BRG1 and BRM by co-immunoprecipitation of the respective endogenous proteins in nuclear extracts of resting and stimulated cells. Deletion mutant analysis would determine which domain in the G3BP1 structure is required for BAF155 interactions. An important step would also be to transiently silence individual G3BP1 and/or these core elements in order to observe whether G3BP1 is essential for the formation of the SWI/SNF complex. In the same study our group has also reported that changes in the SWI/SNF complex assembly led to increased levels of p21, a cyclin-dependent kinase inhibitor. Hence, we would like to look for p21 protein and mRNA levels upon depletion of G3BP1.

Is G3BP1 involved in gene transcription, mRNA splicing, mRNA translocation or translation?

Although the functional significance of the G3BP1-BAF155 interaction is not fully clear, we believe it to be important for cell growth and division. Due to their ability to interact with numerous molecules, SWI/SNF complex elements can exhibit diverse functions one of which has been reported to regulate mRNA splicing by recruiting not only the essential splicing factors but also RNA binding proteins (RBPs) (Ito et al., 2008). Therefore, RNA-Seq analysis would allow us to have a more comprehensive look at the alternative gene spliced transcripts that might be affected by G3BP1 downregulation. In addition, we have demonstrated that G3BP1 depletion resulted in the mRNA upregulation of *c-FOS, c-MYC*, and *c-JUN*, and in the downregulation of *STAT3* and *ERK1/2* mRNA levels. Treatment with the transcriptional inhibitor actinomycin D (ActD) revealed that G3BP1 does not convey these effects on the aforementioned genes post-transcriptionally via mRNA decay. To examine whether G3BP1 controls translation, we would like to treat G3BP1-depleted cells with cycloheximide, an inhibitor of translation, and observe any changes in protein levels of PDGF-BB-inducible target genes. Importantly, G3BP1, belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) superfamily (Sidibé et al., 2021). RNPs are known to selectively shuttle mRNAs between nucleus and cytoplasm (Piñol-Roma and Dreyfuss, 1993; Sloan et al., 2016). Therefore, the possibility that G3BP1 participates in the transport of RNAs should be addressed as well.

How is G3BP1 regulated upon PDGF-BB stimulation?

The function of G3BP1 is determined by post-translational modifications. Phosphorylation at Ser149 is essential for its endoribonuclease activity (Tourrière et al., 2003), Lys63-conjugated polyubiquitin chains are required for the

dissociation of heat-shocked stress granules (Gwon et al., 2021), and arginine methylation in response to Wnt stimulation is critical for the expression of β-catenin (*CTNNB1*) mRNA enhancing Wnt/ β-catenin signalling (Bikkavilli and Malbon, 2011). Additionally, G3BP1 has been reported to be phosphorylated on tyrosine that could be performed by PDGFRβ itself or other tyrosine kinases that act downstream of PDGFRβ. Antibodies that specifically detect the methylation, phosphorylation, and ubiquitination status would be helpful in determining the function, localization and interaction partners of G3BP1 in response to PDGF-BB.

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"O, wonder! How many goodly creatures are there here! How beauteous mankind is! O brave new world, That has such people in't!"
W. Shakespeare

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