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Analysis of PDGF receptor internalization and signaling using proximity ligation assays

MARIE RUBIN SANDER



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Abstract

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Cell signaling is mediated by signaling proteins that relay the signal in an intricate network of interactions before the signal is translated into a biological response. Short linear motifs (SLiMs) in intrinsically disordered regions of proteins serve as docking sites for protein interaction in all aspects of cell regulation including signal transduction. SLiM-mediated interactions are transient and low affinity and can be hijacked by virus. Only a small fraction of SLiMs have been described, but many more exist. Platelet derived growth factor receptor β (PDGFR β) belongs to the family of receptor tyrosine kinases (RTKs) and controls cell growth, proliferation and migration. Dysregulation of PDGFR β mediated signaling pathways is seen in many cancer types. To discover and characterize protein interactions, large scale high through-put methods are needed in concert with low through-put methods, that can characterize the interaction in a cellular context. The aim of this thesis has been to study protein-protein interactions in internalization and signaling of PDGFR β and motif-mediated host-virus interactions through the use of in situ proximity ligation assay (PLA).

Signaling via PDGFR β is compartmentalized and depends on receptor internalization. In paper I we investigated the effects of dynamin inhibition for activation and signaling of PDGFR β , and found that dynamin inhibition leads to impaired dimerization of the PDGFR β . The results indicate that membrane localization of PDGFR β is affected by dynamin.

In paper II we developed a new method, Molboolean, for localized simultaneous detection of both free protein and protein in complex in cells. Molboolean is based on the principles from PLA with a fluorescent read out detectable with fluorescence microscopy.

In paper III we mapped SLiM based host-virus interactions and explored their mechanisms. Using proteomic peptide phage display, we identified 1712 potential virus-host interactions by screening a library covering intrinsically disordered regions of the proteome for 229 RNA viruses. Clathrin mediated endocytosis was found to be a common target for viral hijacking, and viral binding of clathrin impaired PDGFR β internalization.

Some RTKs are proteolytically cleaved following ligand activation. In paper IV we characterized the Ca²⁺-dependent proteolytic cleavage of PDGFR β . The cleavage resulted in two PDGFR β fragments and was dependent on receptor internalization. Inhibition of the proteasome with bortezomib prevented internalization and cleavage and resulted in increased activation of PLC γ and STAT3.

This thesis provides insight in the regulation of PDGFR β signaling and internalization, and highlights contributions of both large-scale screenings and low through-put methods for studying protein-protein interactions.

Keywords: PDGFR β , dynamin, proteolytic cleavage, signaling, internalization, protein-protein interaction, short linear motif, viral hijack, in situ PLA, Molboolean

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List of Papers

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

- I. Heldin, J., **Sander, M.R.**, Thomsson, S., Lennartsson J., Söderberg, O. (2019) Dynamins inhibitors impair platelet-derived growth factor β -receptor dimerization and signaling. *Experimental Cell Research*, 380(1):69–79
- II. Raykova, D., Kerpatsou, D., Malmqvist, T., Harrison, P.J., **Sander, M.R.**, Stiller, C., Heldin, J., Leino, M., Ricardo, S., Klemm, A., David, L., Spjuth, O., Vermuri, K., Dimberg, A., Sundqvist, A., Norlin, M., Klaesson, A., Kampf, C., Söderberg, O. (2022) A method for Boolean analysis of protein interactions at a molecular level. *Nature Communications*, 13(1):1–17
- III. Mihalič, F., Simonetti, L., Giudice, G., **Sander, M.R.**, Lindqvist, R., Peters, M.B.A, Benz, C., Kassa, E., Badgujar, D., Inturi, R., Ali, M., Krystkowiak, I., Sayadi, A., Andersson, E., Aronsson, H., Söderberg, O., Dobritzsch, D., Petsalaki, E., Överby, A.K., Jemth, P., Davey, N.E., Ivarsson, Y.(2022) Large-scale phage-based screening reveals extensive pan-viral mimicry of host short linear motifs. *Submitted manuscript*.
- IV. **Sander, M.R.**, Wang, K., Papadopoulos, N., Rorsman, C., Heldin, J., Tsiatsiou, K., Söderberg, O., Heldin, C-H., Lennartsson, J. (2023) PDGF induced internalization promotes proteolytic processing of PDGFR β which can be inhibited by borte-zomib. *Manuscript*.

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Other publications not included in this thesis.

- I. Leino, M., Heldin, J., **Sander, M.R.**, Kerpatsou, D., Raykova, D., Koos, B., Söderberg, O. (2019) Optimization of proximity-dependent initiation of hybridization chain reaction for improved performance. *Molecular Systems Design & Engineering*, 4(5):1058–1065

Contents

Introduction.....	9
Chapter 1: Protein interactions in cell signaling	11
Protein-protein interactions	11
Short linear motifs	12
PPIs in cell signaling are modular	12
Virus can hijack SLiM mediated interactions.....	13
Chapter 2: Methods for detection of protein-protein interactions.....	15
Methods for mapping of novel interactions	15
Yeast-two-hybrid.....	15
AP-MS	16
Proteomic phage display.....	18
Methods for studying known interactions in cells.....	20
FRET based assays	20
Proximity ligation assay	21
Molboolean.....	23
Chapter 3: Introduction to platelet derived growth factor receptor β	27
PDGF receptors and ligands.....	27
Structure and mechanism of PDGFR β activation	28
Signaling via PDGFR β	29
PI3K/Akt.....	30
PLC γ	31
STATs.....	32
MAPK-Erk1/2	32
PDGFR β downregulation.....	33
Endocytosis of PDGFR β	33
Sorting and degradation.....	35
Proteolytic cleavage.....	37
PDGFR β disease and treatment.....	37
Present investigations.....	39
Paper I	39
Paper II.....	39
Paper III.....	40
Paper IV	42

Discussion and future perspectives	45
Paper I	45
Dynamins role in PDGFR β dimerization	45
Paper II	46
Improved 3D analysis to prevent false positives	46
Paper III	46
Validation of host-virus interactions	46
Effects of viral hijack of clathrin	47
Paper IV	48
Identify proteolytic cleavage site and protease	48
Elucidate functional consequences of increased PLC γ and STAT3 signaling	49
Signaling from the fragment	49
Populärvetenskaplig sammanfattning	50
Acknowledgements	53
References	55

Abbreviations

AD	Activation domain
ADAM	A disintegrin and metalloproteinase
AP-MS	Affinity purification coupled with mass spectrometry
BD	Binding domain
BRET	Bioluminescence resonance energy transfer
cPLA2	Cytosolic phospholipase A ₂
CSF1R	Colony stimulating factor 1 receptor
EEEV	Eastern equine encephalitis virus
EGFR	Epidermal growth factor receptor
ELM	The Eukayotic Linear Motif database
Erk	Extracellular-signal-regulated kinases
ESCRT	Endosomal sorting complex required for transport
FLIM	Fluorescence lifetime imaging microscopy
Flt3	Fms-like tyrosine 3 receptor
FOXO	Forkhead box O
FRET	Fluorescence resonance energy transfer
GPCR	G-protein coupled receptors
HDAg-L	Large hepatitis antigen-L
JAK	Janus kinase
JNK	Jun amino-terminal kinases
MaMTH	Mammalian membrane two-hybrid
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MRV1	Mammalian Reovirus type 1
mTOR	Mechanistic target of rapamycin
MYTH	Membrane yeast two hybrid
NTD	N-terminal domain
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLA	Proximity ligation assay
PLC γ	Phospholipase C γ
PPI	Protein-protein interaction

ProP-PD	Proteomic peptide phage display
ProxHCR	Proximity-dependent initiation of hybridization chain reaction
PTB	phosphotyrosine binding
PTM	post translational modifications
PX	Phox homology
RCA	Rolling circle amplification
RiboVD	Riboviria Viral Disorderome
RTK	Receptor tyrosine kinase
SH2	Src homology-2
SH3	Src homology-3
SLiM	Short linear motif
STAT	Signal transducer and activator of transcription
UAS	Upstream activating sequence
Y2H	Yeast-two-hybrid

Introduction

For a multicellular organism to function, the cells need to communicate. This communication is mediated by extracellular signal molecules that bind to receptor proteins either on the cell surface or inside the cell. Receptor proteins transduce the signal and a number of intracellular signaling proteins are then involved in relaying the signal in an intricate network of interactions before activating effector proteins that initiate a response. Cells receive and respond to a combination of signals either promoting or inhibiting a certain cellular response, and cell signaling controls key biological processes such as cell survival, growth, division and differentiation [1].

Proteins make up the phenotype of a cell, and behind the function of a cell is a complex network of protein interactions. Some interactions may be more static than others, and for cell signaling in particular, transient and dynamic interactions are key to a rapid response to changes in signals. Many of these interactions are mediated by short linear motifs (SLiMs), linear stretches of 3-10 amino acids, that serve as binding or recognition site for proteins involved in signal transduction and other important cellular processes [2].

One example of a signaling network is the platelet derived growth factor receptor (PDGFR β), which belongs to the receptor tyrosine kinase (RTK) family of receptors and have important functions in regulation of cell proliferation, migration and survival. Dysregulation with increased activity or amount of PDGFR β is associated with diseases with excessive cell proliferation such as cancers [3]. Viral infection too can disturb cell signaling. Viruses hijack the cell's regulatory machinery and rewires the signaling network for its own benefit [4]. Hence, more knowledge is needed about the protein interactions in signaling and other regulatory events in the cell and how they are rewired in cancer and infectious disease, so we can develop efficient anti-cancer and antiviral treatment. One aspect of obtaining the knowledge needed, is to have methods available to study protein-protein interactions at endogenous levels in a native environment.

The aim of this thesis has been to study protein-protein interactions in internalization and signaling of PDGFR β and motif-mediated interactions between viral and host cell proteins through the use of antibody-based methods such as proximity ligation assay.

The work includes studies of the effect of dynamin inhibition on activation of PDGFR β (**Paper I**), and characterization of the proteolytic cleavage of

PDGFR β and the implications of proteasome inhibition on receptor cleavage and signaling (**Paper IV**). For studying protein-protein interactions, we have developed a new method of detection, Molboolean (**Paper II**), which visualize both protein interactions and free unbound protein. Finally, the work includes studies of SLiM mediated interactions between virus and host cell proteins (**Paper III**), where we identify and characterize interactions between proteins from RNA viruses and human proteins.

Chapter 1: Protein interactions in cell signaling

Protein-protein interactions

A DNA sequence is made up of four different bases, and has highly predictable structure and binding specificity. In comparison, the function of proteins are much harder to predict. Protein consists of sequences of amino acids, of which there are 20 different side chains, some are charged, some are hydrophobic, polar uncharged or special cases like proline and cysteine. How proteins bind to each other depends on which amino acids are exposed at the binding interface, which depends on the structure of the protein. And the structure of a protein is determined by interactions of the side chains, these include ionic interactions, hydrogen bonds, hydrophobic interactions and van der Waals forces. The secondary structure can, to some extent, be predicted from the amino acid sequence alone, but information on tertiary structure is dependent on data from x-ray crystallography, cryo-electron microscopy and prediction models such as AlphaFold [5]. For long the general notion was that protein structure dictates function, but structural disorder in proteins is increasingly recognized as also contributing to function [6]. Intrinsically disordered regions are regions of a protein with no stable well defined tertiary structure [7]. It is estimated that around 30% of the proteome is intrinsically disordered [8].

Protein-protein interactions (PPIs) can be roughly classified in three groups. 1) Interaction between two globular domains, either with preformed or induced interaction surface, 2) interaction between a peptide and a globular domain with either rigid or induced interaction surface, or 3) between two peptides. For all groups, the interaction surface may then be either continuous or discontinuous. [9]. PPIs can be permanent interactions that are strong and irreversible, usually with a dissociation constant K_D in the nanomolar range, or transient interactions that easily changes state. Transient interactions can further be classified as strong or weak, with a K_D in the micromolar range, where the strong transient interactions are induced from an unbound or weak transient interaction by a triggering event such as ligand binding [10]. Structural disorder in a region of a protein is associated with transient interactions [10] and regions of disorder are found to be enriched in proteins involved in transient interactions, as for example for proteins involved in signaling [11].

Short linear motifs

For protein interactions consisting of a globular domain interacting with a peptide, the interaction is determined by only a few residues constituting a binding motif. A binding motif is a recurring pattern of residues from different peptides that forms a consensus sequence for binding to a certain protein [2]. Short linear motifs (SLiMs) are sequences of 3 to 10 amino acids with no defined secondary structure and they are frequently occurring in intrinsically disordered regions of proteins [12]. Because of the small number of residues involved and thus limited surface area for binding, SLiM mediated interactions fall into the category of low affinity transient interactions [10]. This is an advantage in regulatory processes in the cell, such as signaling, where tight dynamic regulation is needed. SLiM-mediated interactions are found in nearly all aspects of cell regulation including signaling, trafficking, and degradation [2]. Because of the small size, only 3-4 residues are key determinants of binding, SLiMs can easily arise *de novo* by a single or few mutations [2], [12]. The small size also means that residues flanking the motif are important in the recognition process [12]

SLiMs can be overlapping by a few residues or they can be situated only a few residues apart and bind functionally distinct proteins. This pose a way of controlling interaction and formation of protein complexes based on the local concentrations of the interacting proteins [13]. SLiM interactions may also be controlled by post translational modifications (PTMs), either in the motif or near the motif, and the PTM then functions as a switch for binding. This can be either as an on/off switch for binding, or the PTM changes the specificity of the SLiM to favor binding of another interactor [14]. It is important to note that SLiM binding and PTM binding may not be the same thing. For SLiM binding, it is the peptide sequence that determines binding specificity, but it is common for binding via a PTM site to not depend on residues in the flanking regions, only the PTM residue is recognized.[2].

The Eukaryotic Linear Motif (ELM) database is a resource that contains annotated information on nearly 4000 annotated SLiM based interactions from different species with more than 2000 being human [15], but it is suggested that more than 100.000 SLiMs exist in the eukaryotic proteome [2], [13]. Hence, many motifs and interactions are to be explored.

PPIs in cell signaling are modular

Signal transduction can be viewed upon as consisting of modules made up of protein domains that, via interaction with other proteins or by having intrinsic enzymatic activity, can relay or modulate the signal [16], [17]. A protein domain is a part of a protein, a stretch of 50-400 amino acids that folds into a stable tertiary structure and has a function of its own, for example enzymatic activity such as phosphatase or kinase activity, or it can function as a docking

domain to direct enzymatic activity to the right location [18]. The docking domains bind to specific binding motifs in the upstream signaling protein. Some common docking domains in cell signaling are Src homology-2 (SH2) and phosphotyrosine binding (PTB) domains that recognize and bind to a phosphorylated tyrosine residue and the Src homology-3 (SH3) domain that recognizes proline-rich motifs [17], [18]. Docking domains may also bind lipids, as for instance Phox homology (PX) and pleckstrin homology (PH) domains that recognize membrane lipids [17], [18]. Phospholipase C γ (PLC γ) is an example of a signaling molecule that contains many of the mentioned docking domains. PLC γ contains a single full PH domain, a split PH domain, a split SH2 domain, an SH3 domain and C2 domain [19]. PLC γ associates with lipid membrane via the PH domain, and binds a phosphotyrosine residue in an activated receptor tyrosine kinase (RTK) via one part of the split SH2 domain. This induces conformational changes and assembles the split catalytic domain that will hydrolyse lipids in the membrane to create second messengers [19]. PLC γ signaling is further described in chapter 3.

Viruses can hijack SLiM mediated interactions

As previously mentioned, signal transduction is in large part mediated by transient low affinity protein-protein interactions in order to maintain and fine tune regulatory control of the signaling networks. Viruses consist of proteins and nucleic acids packed in a capsid of proteins. They are obligate parasites and therefore need the host cell for completion of their life cycle [20]. By hijacking endogenous interactions of the host cell, viruses can use and redirect the regulatory machinery of cell for their own advantage. One way of achieving this, is by mimicking host protein SLiMs [4], [21]. Because of the few key residues in a SLiM it can easily arise *de novo* [12], a virus may only need to acquire a single point mutation to mimic a host cell SLiM [4]. Considering the high mutation rate in viruses, mimicking SLiMs is thus a smart strategy for adaptation to a host cell [4], [21].

Examples of viral hijacking of host cell functionality include the transport system, signal transduction and transcriptional regulation [4]. Hijacking of the transport system is for example seen with the binding of the large hepatitis antigen-L (HDAg-L) from hepatitis delta virus to clathrin via LFXAD corresponding to a clathrin box motif used by several host cell clathrin adaptor proteins [22]. The interaction of HDAg-L with clathrin inhibits clathrin-mediated internalization of proteins and membrane, and facilitates maturation of virus particles in the trans-Golgi network [22]. In **paper III**, we show similar effects on clathrin mediated endocytosis are caused by viral hijack of clathrin by non-structural protein 3 (NSP3) from Eastern Equine Encephalitis virus (EEEV).

Chapter 2: Methods for detection of protein-protein interactions

The human interactome is estimated to consist of 650.000 protein interactions [23]. To map and explore this vast number of interactions, methods for large scale discoveries of both stable and high affinity, and transient and low affinity interactions are needed, as well as methods for localized detection of the protein interactions in their native environment in the cell, at endogenous expression levels. Large scale mapping of protein interactions is performed using high throughput methods to detect interactions, like yeast-two-hybrid, affinity purification coupled with mass spectrometry and proteomic peptide phage display (described in more detail below). The interaction data obtained may then be visualized with specific software for showing interaction networks, like the open source software Cytoscape [24]. Subsequent analysis of the interaction network may include cluster analysis and annotation of gene ontology terms [25], [26] to gain information on cellular processes and location of the proteins [27]. Other methods focus directly on one interaction at the time in the context of the cell. Methods for this include fluorescence (or Förster) resonance energy transfer (FRET) based assays and *in situ* proximity ligation assay (*in situ* PLA). These are low throughput, but can give information on the protein interaction and its location under various conditions, at single cells level, and for *in situ* PLA also for endogenous proteins.

There are to date, a vast repertoire of methods available for studying protein-protein interactions, which all comes with different advantages and disadvantages. Selected methods important to this thesis are described below.

Methods for mapping of novel interactions

Yeast-two-hybrid

Yeast-two-hybrid (Y2H) has been extensively used for detection of protein-protein interactions as it can both be used for checking a hypothesis of interaction between two proteins or it can be adapted to a high through put system for detection of new protein-protein interactions by screening of cDNA libraries [28]. Y2H was originally developed for use in yeast cells, taking advantage of the ability of Gal4 to bind an upstream activating sequence (UAS) DNA

sequence and thereby activate transcription of a downstream reporter gene [29]. By splitting the Gal4 in two fragments that does not function as transcription activators on their own, a binding domain (BD) and an activation domain (AD), and expressing them as fusion proteins coupled to proteins of interest. If the proteins interact, the two fragments of Gal4 comes in proximity and are able to bind UAS and activate the reporter gene (see **Figure 1**).

Y2H is a robust and easy-to-use method for screening of protein interactions and has the advantage of being able to detect both strong and weak interactions, but it suffers from a high rate of false positives due to non-specific interactions [28] or from overexpression of the fusion proteins. Additionally, false negatives may arise because of steric hindrance of the fusion constructs, preventing the proper interaction of the proteins of interest and transcription of the reporter gene, or because of different post translational modifications in the yeast system when analyzing proteins from other organisms [28].

The classical Y2H setup described above requires that the interacting proteins can translocate from the cytoplasm where they are expressed to the nucleus. This makes it unsuitable e.g. for membrane proteins. Therefore, several methods based on the Y2H idea has been developed, including adaptations for use with membrane proteins with the membrane yeast two hybrid (MYTH) [30], [31], and for use in mammalian cells with mammalian membrane two-hybrid (MaMTH) [32] which both are based on the split-ubiquitin two hybrid system [33]. MYTH and MaMTH has been used for mapping the interaction network of RTKs with protein phosphatases [34], and classical and adaptations of Y2H has been used for mapping the human proteome with near full coverage [35].

AP-MS

Like Y2H, affinity purification coupled with mass spectrometry (AP-MS) can be used for detection of new protein interactions in a high throughput setting, as well as to verify interactions detected with other methods. AP-MS has been used in large scale efforts to map the proteome of different organisms, from yeast [36] to human cells [37], [38].

The method links affinity-based purification of proteins with mass spectrometry, which is a powerful method for analyzing proteins. Mass spectrometry measures the mass to charge ratio of ionized molecules and can be used to obtain information on the mass of molecules, from which the molecule identity can be inferred. With mass spectrometry it is possible to examine multiple interactions of the bait protein at the same time [27].

With AP-MS, a protein of interest is captured on beads, either via tag recognition or antibodies, and used as bait to pull down interacting proteins from cell lysate (see **Figure 1**). The purification step can be performed either as a single step purification which better preserves weak interactions, with the disadvantage that it also pulls down some non-specific interactions, or using a

tandem affinity purification approach, which limits the numbers of non-specific interactions, at the cost of losing some weaker interactions [39], [40]. The purified protein complexes are then eluted from the beads, trypsinized and subject to mass spectrometry analysis. AP-MS may in some cases fail to co-purify interaction partners of the bait protein due to the engineering of the bait protein, which is expressed with a tag for purification. This may interfere with proper folding of the protein and thus its ability to bind interaction partners [40].

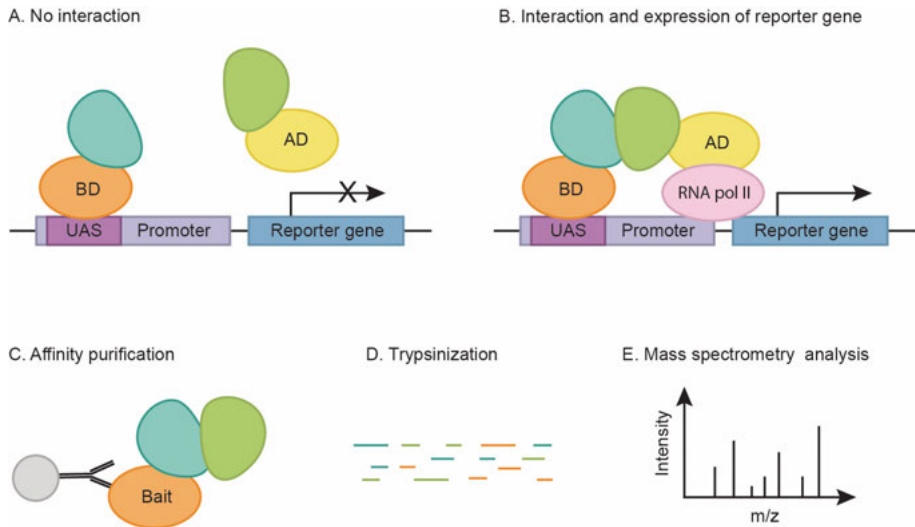


Figure 1: Schematic illustration of Y2H and AP-MS. **A-B)** Y2H. The proteins of interest are expressed in yeast as fusion proteins with binding domain (BD) and activation domain (AD). **A)** If no interaction occurs, the reporter gene is not expressed. **B)** If interaction occurs, the BD will bind to the UAS site in the promoter region upstream of the reporter gene, and AD will recruit RNA polymerase II for transcription of the reporter gene. **C-E)** AP-MS. **C)** Affinity purification of proteins. Bait proteins are immobilized on beads and used for purification of proteins from cell lysate **D-E)** Bound proteins are trypsinized and subject to mass spectrometry.

It can be challenging to preserve weak interactions and at the same time keep non-specific interactions at a reasonable level during the affinity purification, but advances in instrumentation combined with protocols for affinity enrichment rather than purification has made it possible to detect weaker interactions [41]. For investigation of transient low affinity interaction partners of a protein of interest, as is often the case for signaling proteins, proximity labeling followed by MS is becoming increasingly popular. This method utilizes engineered enzyme based on biotin ligase fused to the protein of interest. The

biotin ligase will biotinylate all proteins in the close vicinity. The biotinylated proteins can then be pulled down, using streptavidin coated beads, and analyzed with mass spectrometry [42].

Proteomic phage display

Due to the transient nature and weak affinity of SLiM mediated interactions, they may not be detected in Y2H and standard AP-MS [10]. Hence, alternative methods are required.

Phage display utilizes the biology of filamentous phages to screen for peptides or protein to which the phages bind. By engineering the phage DNA, peptides or proteins can be expressed on the surface of the phage via the phage coat proteins [43]. A great advantage with this method is that it provides a DNA template for the proteins expressed on the phages surface, allowing large libraries of alternative coat proteins to compete for binding to the bait protein. The binding phages can be replicated in bacteria and generate a new library with a more restricted repertoire of phages. After multiple rounds of selection, the library will contain a limited number of clones with the highest affinity. The identity of the coat proteins can be determined by sequencing the phages DNA. Since its invention [44], [45], phage display has become a powerful tool in the selection of peptide or protein fragments based on affinity and has led to the development of new antibodies [43], [46].

Proteomic peptide phage display (ProP-PD) is based on the phage display technique and has been developed as a tool to identify SLiM based interactions and binding motifs [47]. In ProP-PD, a peptide library is designed covering disordered regions of the proteome [48], [49] (see **Figure 2**). The peptides from the library are expressed as part of the coat proteins P8 or P3 on the surface of the M13 phage. The phages are then used in selections where pools of phages displaying the different peptides from the library are allowed to bind bait protein immobilized on a surface. Unbound phages are washed away, and the bound phages are eluted and amplified for repeated rounds of selection to allow enrichment of the binding phages. DNA from enriched phage pools are then amplified by PCR and sequenced with next generation sequencing, and the sequences bioinformatically analyzed against the library design in order to get information on the parent proteins and against databases of interactions and binding motifs, e.g. using the software tool Peptools [49].

ProP-PD has successfully been used for screening of SLiM mediated interactions of the human proteome [47]–[49] and corona viruses [50]. In **paper III** we used ProP-PD to screen for SLiM mediated interactions between human proteins and peptides from a library of disordered protein regions of 229 RNA viruses.

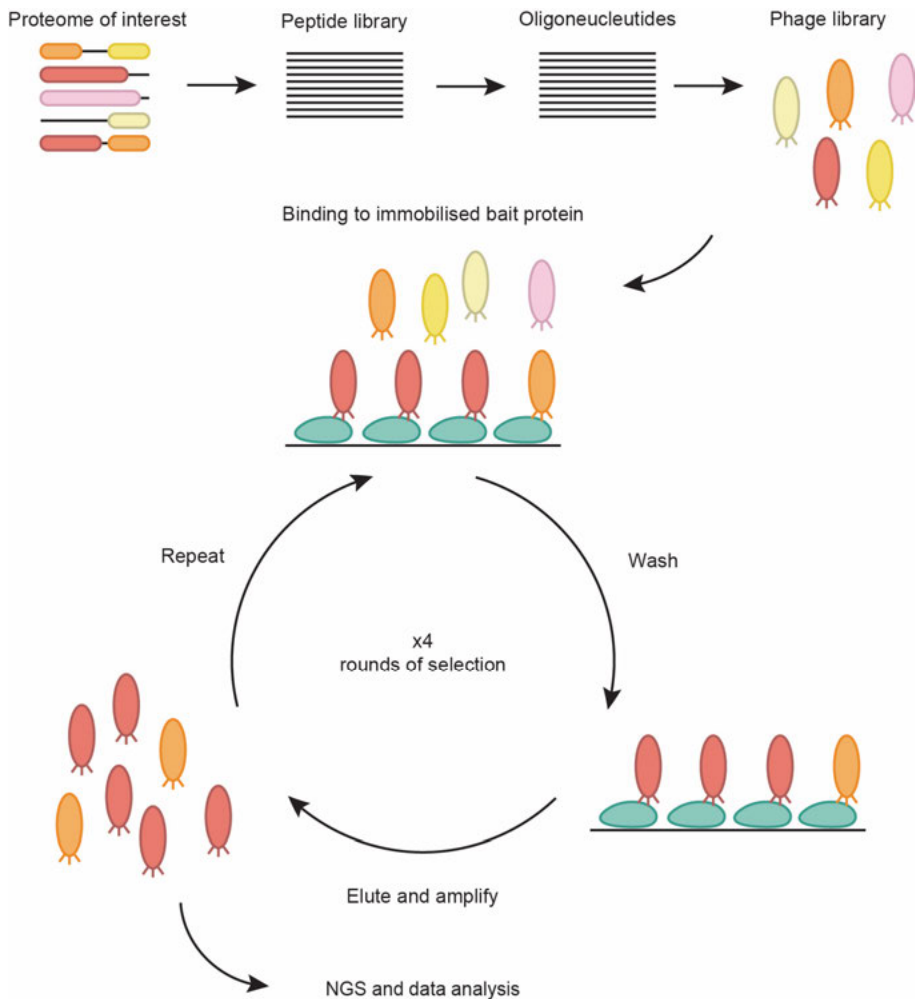


Figure 2: Schematic overview of ProP-PD. Disordered regions of the proteome of interest are identified, and the sequences broken down to peptides to create a computational peptide library. The peptide library is translated to nucleotide sequences and corresponding oligonucleotide library is synthesized, genetically fused to a gene for the phage protein, and electroporated into E.coli which then produce phages displaying the desired peptides on their surface. The phage library is screened for binding to immobilized bait protein, and through repeated rounds of washing, eluting, amplifying and binding, phages with a strong affinity for the bait protein are selected for. The DNA from the enriched pool of phages are then subject to next-generation sequencing and sequence analysis to determine the identity of the peptide.

For all the presented high throughput methods for detection of protein-protein interaction, Y2H, AP-MS and ProP-PD, there is the risk of detecting

interactions which are not biologically relevant. It is therefore important to validate the potential interaction partners identified using high throughput methods, and also determine where in a cell the interactions take place. For that purpose, a number of methods exist, such as FRET based assays or *in situ* PLA, which will be presented below. Common for these methods is that prior knowledge of the identity of both proteins involved in the interactions is required.

Methods for studying known interactions in cells

FRET based assays

Fluorescence resonance energy transfer (FRET) is the nonradiative energy transfer from one molecule in an excited state to another nearby molecule. The efficiency of the energy transfer depends on the distance between the two molecules [51] which enables FRET to be used for measuring distances between molecules [52]. When applying FRET for analysis of protein-protein interaction, popular fluorophores are variants of green fluorescent protein, and the proteins of interest are expressed as fusion proteins with the fluorescent proteins attached (see **Figure 3**). For FRET to occur there needs to be an overlap in donor emission and acceptor absorbance spectra for the fluorophores, the fluorophore dipoles should not be perpendicular to each other, and the donor and acceptor fluorophores should be within a 10 nm distance [53].

There are different ways to measure FRET, but a popular method is Fluorescence lifetime imaging microscopy (FLIM), where the lifetime of the donor fluorophore is measured and used for calculations of the FRET efficiency. The donor lifetime will decrease if FRET occurs [53].

FRET microscopy methods have the advantage of detecting donor-acceptor pairs that are within a 10 nm distance, can be used in live cells and offers real time localized detection of interaction events. In addition, because of the 10 nm resolution and the possibility to calculate the distance between the fluorophores from the measured FRET efficiency, FRET can also be useful for obtaining information on structural rearrangements of proteins (intramolecular FRET) [54], for example when investigating when the dynamics of transmembrane receptors [55]. A limitation to FRET is the need for expression of a fusion construct, which makes it unsuitable for investigating interactions of endogenous proteins. This also means that constructs used for FRET needs functional validation to assure that fluorophores do not hinder proper folding of the protein. This can be overcome by using fluorophore labelled antibodies [56], [57], but this is at the cost of the possibility to monitor FRET in live cells [58]. Technical limitations include risk of phototoxicity, autofluorescence, and false negative results due to perpendicular orientation of the donor and acceptor dipoles. Bioluminescence resonance energy transfer (BRET) does

not have the same issues with phototoxicity and autofluorescence, since a light source to excite the donor is not needed. Instead, luciferase acts as an energy donor and a fluorescent protein as energy acceptor. Upon addition of a substrate the donor luciferase is oxidized, energy is emitted, and the acceptor fluorophore is excited if spaced within 10 nm [59], [60].

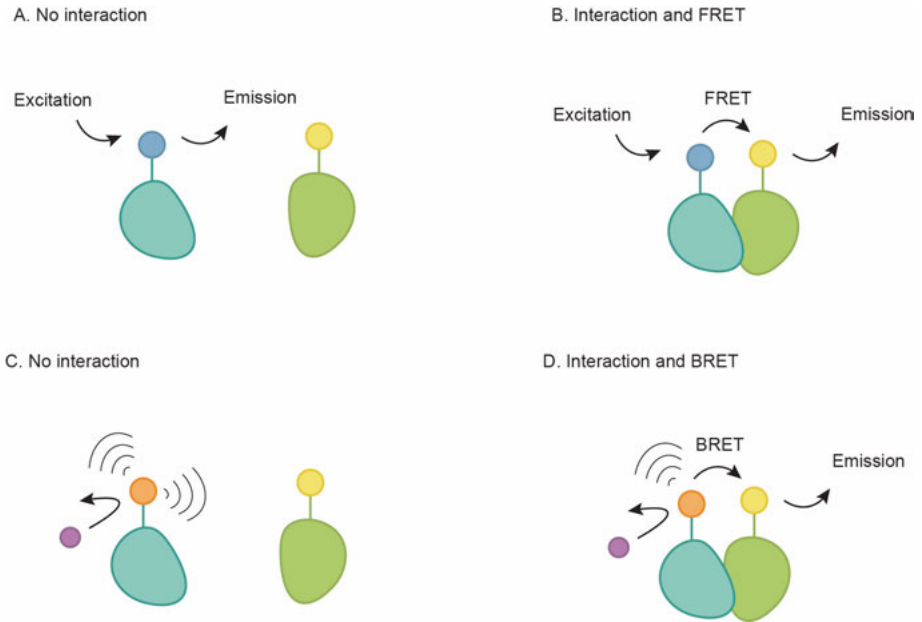


Figure 3: FRET and BRET for detection of protein-protein interaction. **A-B)** Proteins of interest are expressed in cells as fusion proteins tagged with donor (blue) and acceptor (yellow) fluorescent proteins. **A)** The donor fluorophore is excited and emits light. **B)** The donor fluorophore is excited and because the target protein interacts with an acceptor tagged protein, transfer of energy, FRET, to the acceptor fluorophore happens. This can be measured as a decrease fluorescence emitted from the donor (not shown). **C-D)** Proteins of interest are expressed as fusion proteins tagged with luciferase (orange) and fluorescent protein (yellow). **C)** Upon addition of a substrate luciferase is oxidized and energy is emitted. **D)** If the proteins of interest interact, bioluminescent energy will be transferred to the acceptor fluorophore and light will be emitted.

Proximity ligation assay

In situ PLA is a method for localized detection of protein-protein interactions or proteins in fixed cells or tissue sections based on the use of DNA as reporter for an interaction event and as a tool to generate a detectable signal [61]. Compared to other methods of detecting protein-protein interactions in cells, *in situ* PLA has the advantage of detecting endogenous protein-protein interactions at the site of interaction without the need for protein engineering. For studying

protein-protein interactions, *in situ* PLA can be used to obtain localized information of the interaction in question, or information of PTM status of proteins involved in cell signaling [62]. Since *in situ* PLA is performed in fixed and permeabilized cells, the method cannot be used to monitor dynamic events in real time, the sample present a snapshot of the proteins at a given time. However, fixation allows for detection of low affinity transient interactions [63]. We have used *in situ* PLA in **paper I, II and III** to study signaling and internalization of PDGFR β under various conditions.

In *in situ* PLA a set of antibodies are conjugated to oligonucleotides (arm 1 and 2) to make a proximity probe (see Figure 4). After the proximity probes bind their target, two circularization oligonucleotides are added. Only if the target proteins in questions are in close proximity can the circularization oligonucleotide hybridize to a pair of proximity probes that, upon ligation with T4 DNA ligase, will join the circularization oligonucleotides into a circular DNA molecule. This circular DNA molecule can now act as template for rolling circle amplification (RCA) by Phi 29 DNA polymerase, utilizing the oligonucleotides of the proximity probes as primers. The RCA products will hence be an elongation of the proximity probes, containing repetitive elements of reverse complementarity to the circular DNA template. The RCA products can then be visualized by hybridization of fluorophore-labelled oligonucleotides, reverse complementary to the RCA product. A one-hour RCA generates a product consisting of several hundred concatemeric repeats, generating bright objects easily detected also by standard epifluorescence microscopy.

Further development of methods for detection of protein proximity includes development of Unfold, an improvement of the *in situ* PLA method to increase efficiency of signal generation by having all DNA reagents incorporated in the proximity probes [64], and proximity-dependent initiation of hybridization chain reaction (ProxHCR), an enzyme free method for detection of protein-protein interactions [65], [66].

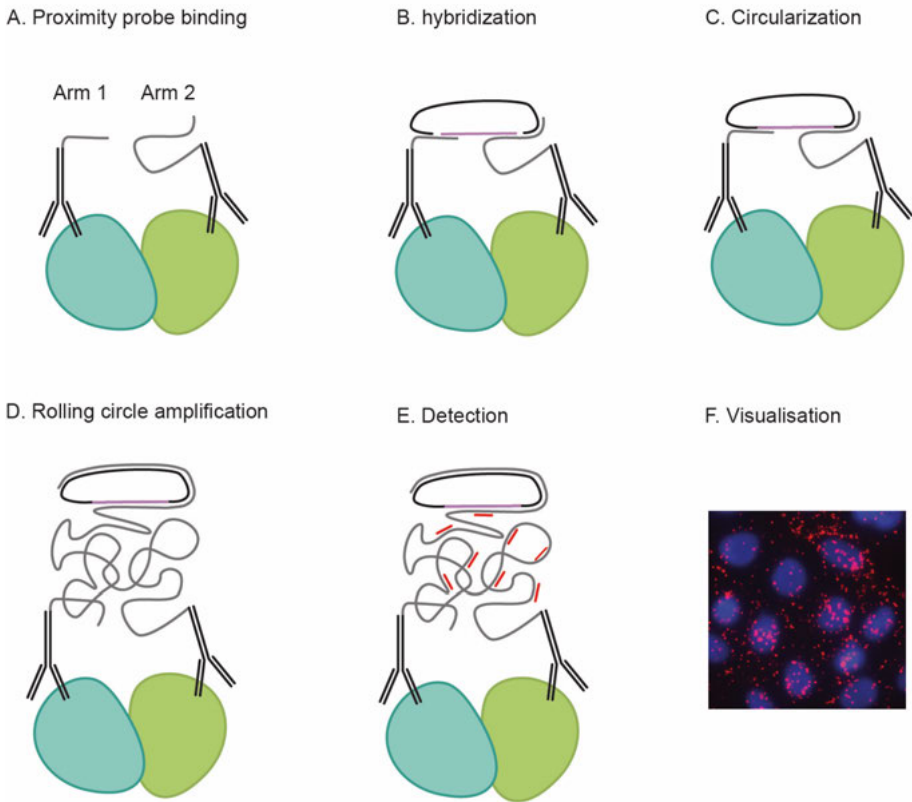


Figure 4: *In situ* proximity ligation assay step by step. A) Binding of proximity probes containing sequence specific arm 1 and arm 2 to proteins. B) Circularization oligonucleotides hybridize and bridge arm 1 and arm 2, only if the two proteins are in proximity. C) Circularization oligonucleotides are ligated to create a DNA circle. D) The DNA circle serves as template for rolling circle amplification (RCA) to create an RCA product. E) The RCA product is detected by hybridization of fluorescently labelled detection oligonucleotides. F) The RCA product can now be visualized by fluorescence microscopy. Each red dot in the microscopy image corresponds to an interaction event.

Molboolean

In situ PLA is a good method for detection of proteins in proximity, it has been optimized so that it is easy to use, and it is commercially available in kit format (Sigma, Navinci). One of the limitations of *in situ* PLA is that it only reports on the amount of interactions, an increase in amount of *in situ* PLA signals may reflect either an increased affinity of the interacting proteins or an increased concentration of the interacting proteins. For proteins expressed at high levels, the risk of false positive signal is increased simply because of

proximity by chance. In order to interpret *in situ* PLA data, it is important to have information about how much of the total pool of proteins actually is engaged in interaction. Expression levels of the individual proteins can be determined by parallel immunofluorescence, or immuno-RCA [67], or by introducing additional immunofluorescence/immuno-RCA steps downstream of the RCA step in the *in situ* PLA protocol. This will give information of the total pool of proteins, both interacting and non-interacting. With the development of a new method, Molboolean, which is described in **paper II**, we have created a method that is based on the same principles as *in situ* PLA, but it reports both protein-protein interactions and protein expression at the same time [68]. The advantage with Molboolean is that it uses the same approach to stain both free and complexed proteins, thereby circumventing problems with different read-out and/or efficiencies that will be a consequence of combining *in situ* PLA with immunofluorescence or immuno-RCA.

The Molboolean proximity probes are antibodies with oligonucleotide arms conjugated to them, similar as for *in situ* PLA (see **Figure 5**). The oligonucleotide arms contain specific tag sequences that are hidden by the structure of the oligonucleotide arm and a sequence motif for recognition by a nickase. The arms are reverse complementary to a preformed DNA circle, which functions as information receiver. When two target proteins A and B are in proximity, the DNA circle can hybridize to the arms of both proximity probes, creating stretches of double stranded DNA which include sites for nickase cleavage. The nickase will cleave one of the DNA strands, i.e., only in the DNA circle. Once the DNA circle is cut, tag sequences, reverse complementary to the hidden tag sequences in the arms can hybridize to the proximity probes, filling the gaps in the cut DNA circle. Following ligation of the circle, two tags will now be incorporated in the circle, marking it with a sequence specific for protein A and B. After rolling circle amplification, fluorophore-labelled detection oligonucleotides designed to hybridize to the tags, can hybridize to the RCA product and be used for visualization with fluorescence microscopy. The RCA product will then be detected in two colors corresponding to protein A and B. If protein A and B are not in proximity, the circle binds only one of the proximity probes and as a consequence only one tag, A or B, will be incorporated, corresponding to which proximity probe the DNA circle have bound. Free protein A or B, will hence be visible by only one of the colors, while the dual stained RCA products will represent proximity events.

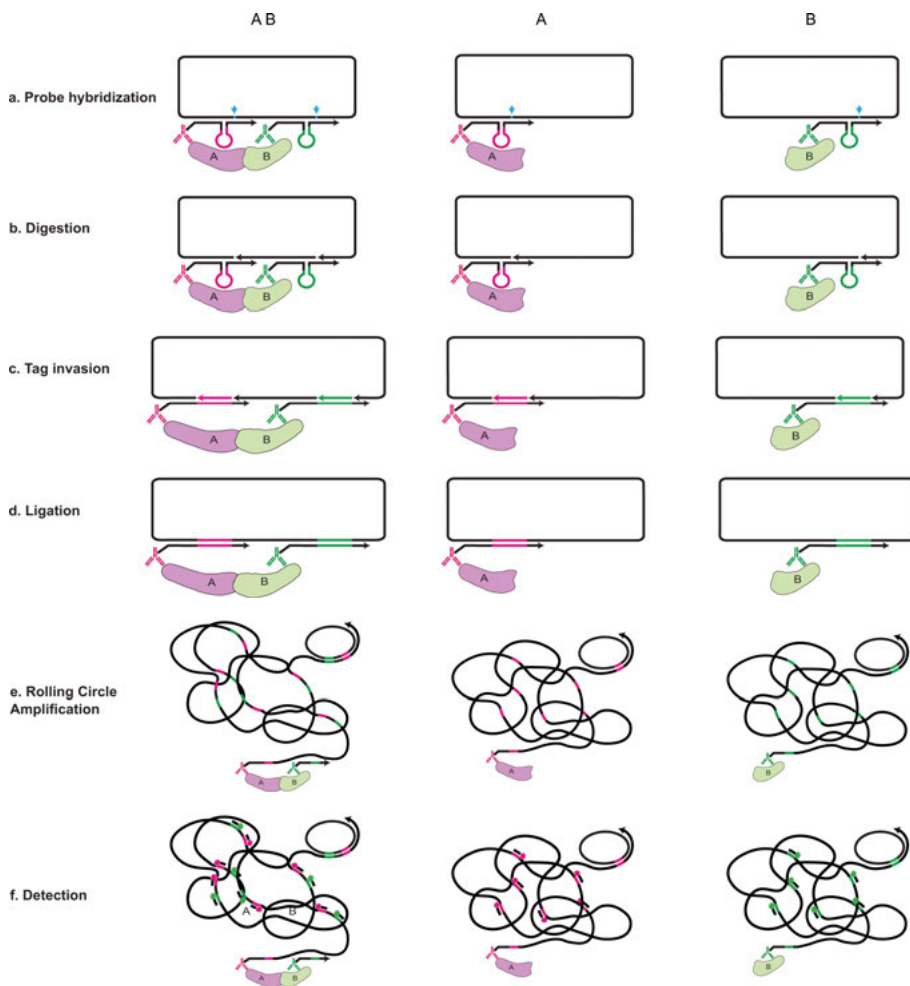


Figure 5: Molbooleen step by step for the protein complex AB and free protein A and B. **A)** After binding of proximity probes to protein A and B, the preformed DNA circle hybridizes to the proximity probes creating stretches of double stranded DNA. **B)** The DNA circle is nicked by the enzyme nickase. **C)** Tags can now invade the DNA circle and fill in the gaps. **D)** The DNA circle and tags are ligated to form a complete circle. **E)** The DNA circle now acts as template in rolling circle amplification (RCA) to create a rolling circle product. **F)** The RCA product can be detected by fluorophore labelled detection oligonucleotides that are specific for the incorporated tag sequence. Figure from Raykova et al. [68] reprinted in compliance with CC BY 4.0.

Chapter 3: Introduction to platelet derived growth factor receptor β

PDGF receptors and ligands

RTKs are a family of transmembrane receptors that share a conserved tyrosine kinase domain in their intracellular part. Upon ligand binding, the tyrosine kinase domain becomes auto-phosphorylated which creates binding sites for SH2 and PTB domain containing signaling and adaptor proteins to transduce the signal. [69]. PDGFR α and PDGFR β belong to the type III class of RTKs together with the structurally similar colony stimulating factor 1 receptor (CSF1R), c-Kit and Fms-like tyrosine 3 receptor (Flt3). They share a common overall domain structure consisting of an extracellular part for ligand binding made up of five immunoglobulin-like domains D1-5, a helical transmembrane part and an intracellular part consisting of a juxta membrane region, a split tyrosine kinase domain and a disordered C-terminal tail [69].

Platelet derived growth factor receptor (PDGFR) is expressed in cells of mesenchymal origin like fibroblasts, pericytes and vascular smooth muscle cells where they stimulate cell growth and migration. PDGFRs are involved in embryonic development and wound healing. Dysregulation of PDGFR is linked to cancer diseases, fibrotic conditions and vascular disorders [3]. PDGFRs exist in two isoforms, PDGFR α and PDGFR β . Upon ligand binding, receptors dimerize to form the homomers PDGFR $\alpha\alpha$ and PDGFR $\beta\beta$, or a PDGFR $\alpha\beta$ heterodimer [3].

The ligands for PDGFR are expressed in four distinct polypeptide chains, PDGF-A, PDGF-B, PDGF-C and PDGF-D [70]–[72]. These polypeptides form either homodimers PDGF-AA, -BB, -CC, -DD, or a heterodimer -AB, although expression of the homodimers seems to dominate [72], [73]. The PDGF dimers have different preference for binding to the PDGFR isoforms in vitro, with PDGF-AA, -AB, -BB and -CC being able to interact with PDGFR α , PDGF-BB and -DD to PDGFR β , and PDGF-AB, -BB, -CC, and -DD to PDGFR $\alpha\beta$ [73] (see **Figure 6**). This preference of PDGF isoforms to bind to PDGFRs determines whether homo- or heterodimers form.

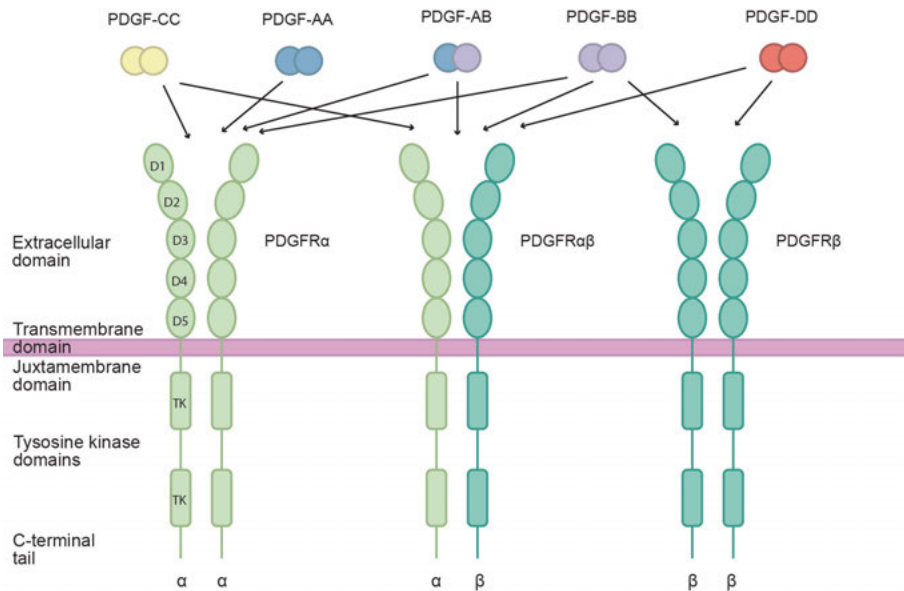


Figure 6: Schematic illustration of PDGFR α and - β isoforms, their domain structure and ligand specificity. PDGF ligand bind in the immunoglobulin D2-D3 domain. PDGF isoforms bind with varying affinity to the homomeric or heteromeric receptors. The arrows indicate experimentally validated and putative binding.

Structure and mechanism of PDGFR β activation

The proposed model for activation of PDGFR β are based on structures of similar RTKs and mutational analysis of key residues [74]. For PDGFR β , crystal structures exist for domains D1-2-3 in complex with ligand [75], for D4-D5 [76], and cryo-EM of the full length receptor [77]. Much information is also derived from the crystal and cryo-EM structures of extracellular and kinase domains and full length c-Kit [78], [79] and the crystal structure of the kinase domain of PDGFR α and c-Kit [80], [81].

PDGF binds in the D2-D3 domain of PDGFR β , and brings the receptor to dimerize [75], [77] and the dimerization is further stabilized by homotypic contacts of the D4-D5 domains [76], [77]. The transmembrane helices are in close proximity, and in the 2D view they cross each other near their N-terminal and are wider apart at the C-terminal. This is thought to allow for more space for movement and change of configuration for the downstream kinase domains [77]. The kinases of the intracellular domains adopt a tilted conformation, which disrupts the overall symmetry of the dimer [77].

The split tyrosine kinase domain consists of an N- and C-terminal part (N and C lobe) divided by a kinase insertion domain. In the inactive form, there are extensive contacts between the juxtamembrane domain and the N- and C-lobes of the kinase. A long flexible loop, called the activation loop which is

conserved throughout the type III RTKs, is in an outward conformation for PDGFR α [80]. This is likely to be the case for PDGFR β as well. For type III RTKs the juxtamembrane region covers the cleft between the N- and C-lobes of the kinase that contains the active site, thus preventing the activation loop from changing to its active conformation. This is likely to be the same for PDGFR α and PDGFR β , considering that the length of the juxtamembrane domain and structurally important residues are conserved for all RTK III [80], [82]

Activation of the kinase domains depends on phosphorylation of key residues Y579 and Y581 in the juxtamembrane region [83], [84], a phosphorylation which takes place in trans [85]. Based on structural evidence on the kinase domain from c-Kit and PDGFR α it is assumed that the in trans phosphorylation of Y579 and Y581 results in release of the juxtamembrane domain from its autoinhibitory conformation making it possible for the activity loop to change to its active conformation [79], [80], [82]. Y857 is situated in the activation loop, and its phosphorylation changes the conformation of the activation loop, which is necessary for the activation of kinase activity of the receptor [83].

Several conserved tyrosine residues throughout the intracellular domain are phosphorylated upon activation of PDGFR β [86] (see **Figure 7**). The specific order of phosphorylation of tyrosine residues in PDGFR β is not known, but has been described for the RTK fibroblast growth factor receptor 1 (FGFR1) [87]. One mechanism that has been suggested for the transphosphorylation of tyrosine residues in the intracellular domain is that one kinase first acts as an enzyme and the other kinase as substrate. The roles then switch in an alternating fashion, and via several asymmetric arrangements of sequential docking and phosphorylating, the tyrosine residues will be phosphorylated in an organized way [77], [79].

The c-terminal tail of PDGFR β contains a number of regulatory motifs and tyrosine residues that are phosphorylated upon activation, and is assumed to take a disordered conformation. Additionally, it has been suggested that the c-terminal tail takes part in the autoinhibition of the kinase domain when PDGFR β is in the inactive state [88].

Signaling via PDGFR β

The phosphorylation of tyrosine residues in the intracellular domain of PDGFR β creates docking sites for SH2 domain containing proteins [89], [90] (see **Figure 7**). These are either SH2-domain containing adaptor molecules such as Grb2, Shc, Nck and Crk that mediate interaction with signaling molecules via additional binding domains, or they are SH2 domain containing signaling molecules with enzymatic activity such as PI3-kinase and PLC γ that directly interact with the phosphotyrosine site on the receptor [86], [90]. The binding

of adaptors and signaling molecules to the activated receptor leads to activation of PLC γ , PI3K/Akt, STAT, MAPK-Erk1/2 signaling pathways and cell growth and migration [3]. The signaling pathways of importance to this thesis are introduced in the following sections.

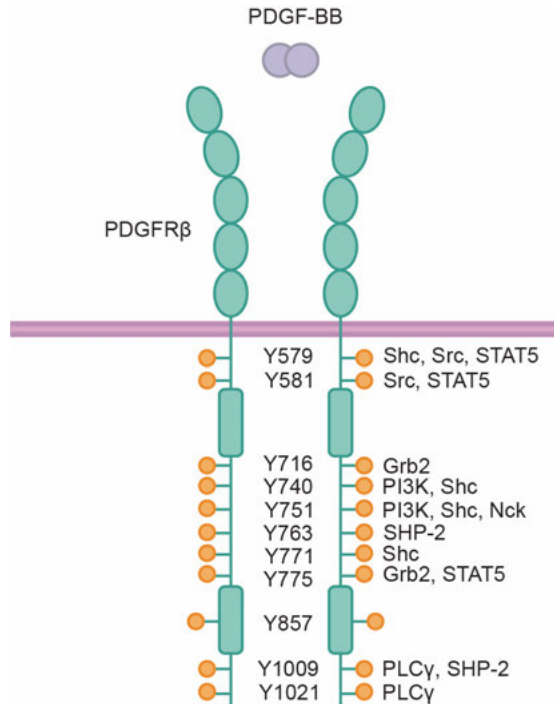


Figure 7: Phosphorylation and activation of signaling pathways. Upon ligand induced activation and dimerization, PDGFR β is phosphorylated at several tyrosine residues (orange) throughout the intracellular domain. This creates binding sites for SH2-domain containing adaptor and signaling molecules.

PI3K/Akt

The PI3K/Akt pathway is activated by RTKs and G-protein coupled receptors (GPCRs). PI3Ks are grouped in three classes I-III, with class I being activated by RTKs.

PDGF-induced activation of PI3K occurs via the phosphorylated residues Y740 and Y751 on PDGFR β that can bind the SH2-domain containing p85 subunit of PI3K [91], [92]. This activates the catalytic p110 subunit of PI3K resulting in phosphorylation of membrane lipids to create second the messengers phosphatidylinositol-3,4-bisphosphate (PI3,4P₂) and phosphatidylinositol-3,4,5-triphosphate (PIP₃) [93]. Cytosolic Akt can now bind to PI3,4P₂ at the plasma membrane via its PH domain [94]. Akt also exist at endosomal

membranes where it is suggested to bind PI3,4P₂ produced from PIP₃ by the SH2-domain-containing inositol 5'-phosphatase (SHIP) [95], [96]. Akt is activated by phosphorylation of threonine and serine residues by phosphoinositide-dependent protein kinase 1 (PDK1) that also localizes to PI3,4P₂ and PIP₃ and mechanistic target of rapamycin (mTOR) complex 2 [96]. In addition, PI3K can also get activated directly via binding of Ras to its catalytic p110 subunit [93]. Akt itself is a serine/threonine kinase and phosphorylates a large number of substrates including kinases, transcription factors, metabolic enzymes, E3 ubiquitin ligases, and cell cycle regulators [96].

PDGF induced signaling via PI3K has been shown to be important in mediating cell motility responses such as chemotaxis, actin reorganization, and membrane ruffling [97], [98]. PDGF induced signaling furthermore promotes cell survival via Akt, as Akt phosphorylates BAD, a member of the BCL-2 family and thereby inhibits apoptosis [99] and can phosphorylate the Forkhead box O (FOXO) transcription factors to prevent them from inducing cell growth arrest and apoptosis, thereby promoting survival [100]–[102].

PLC γ

Phospholipase C γ (PLC γ) belongs to a family of enzymes that are able to hydrolyze the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) that both functions as second messengers in signal transduction from the plasma membrane. DAG activates protein kinase C (PKC), while IP₃ is released from the plasma membrane and signal to influx of Ca²⁺ from the ER to the cytosol, which also activates PKC [19], [103].

PDGFR β can induce phosphorylation of both PLC γ 1 and PLC γ 2 [104], but PLC γ 1 is the isoform that is predominantly expressed in cells of mesenchymal origin [105]. PLC γ 1 is located in the cytosol, but activation by PDGFR β takes place at the plasma membrane rather than at endosomal membranes [106]. Upon ligand induced activation of PDGFR β , PLC γ binds to phosphorylated Y1009 and Y1021 in the extreme C-terminal of PDGFR β via its two SH2 domains [107]–[109] and PLC γ becomes activated by phosphorylation at several tyrosine residues [110], [111]. Signaling via PLC γ seems to be modulated by PIP₃ produced by PI3K. Binding of PIP₃ to PLC γ via the PH domain is shown to target PLC γ to the membrane [112] and binding to PIP₃ via the N-terminal SH2 domain has been shown to increase the activity of PLC γ and lead to dissociation of PLC γ from PDGFR β [113].

Activation of PLC γ via PDGFR β has been associated with cell migration [114], but the importance of PLC γ signaling induced by PDGFR β has proven difficult to elucidate [74].

STATs

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway can be activated via cytokine receptors, GPCRs and RTKs. The classical activation of the pathway is via ligand induced activation of a receptor causing the receptor to dimerize thereby bringing inactive JAK associated with the receptor in proximity for JAK to activate itself by phosphorylation *in trans*. The activated JAK subsequently phosphorylates tyrosine residues providing docking sites for SH2-domain containing molecules, like STAT. STAT is then phosphorylated by JAK, causing STAT to dimerize and translocate to the nucleus where it acts as transcription factors and induce expression of genes promoting responses related to cell survival and proliferation [115], [116].

For PDGFR β induced activation of STAT, the mechanism seems to differ from the classical JAK/STAT activation. As PDGFR β has intrinsic kinase activity it is not dependent on the JAKs in the same way as e.g. cytokine receptors. PDGF has been shown to induce phosphorylation of the JAK family members JAK1, JAK2, and Tyk2 [117], [118] and Stat family members Stat1, Stat3, Stat5 and Stat6 [117]–[121]. For Stat5, the activation of Stat5 happens directly via binding and phosphorylation by PDGF β and does not seem to depend on JAKs [119]. For Stat3, PDGFR β does not contain a consensus binding motif YXXQ for Stat3 [15], [74]. Instead, Src, JAK2 and Fer has been suggested to mediate the activation of Stat3 [74], [117], [122]–[124]. Src is a non-receptor tyrosine kinase that can associate with the plasma membrane via a lipid modification [125] and bind to phosphorylated Y579 and Y581 in PDGFR β [84]. Fer is an intracellular tyrosine kinase that possibly functions as an adaptor in the PDGF induced activation of STAT3 [123]. Furthermore the PDGF induced activation of STAT3 is dependent on internalization of PDGFR β , even if STAT3 could be activated at the plasma membrane [126], [127]. The bottom line is, the exact mechanism for activation of STAT3 by PDGFR is not fully known, but the cell level PDGF induced Stat3 activation has been shown to lead to activation of c-Myc and cell proliferation [124] and induction of cytosolic phospholipase A₂ (cPLA₂) expression and cell migration (Neeli et al., 2004).

MAPK-Erk1/2

The mitogen-activated protein (MAP) kinase pathways serve important roles in the transduction of signal from various receptor types at the plasma membrane. MAP kinase pathways can be initiated by activation of RTKs, G-protein coupled receptors (GPCRs), ion channels and more. Four groups of MAP kinases exist; Jun amino-terminal kinases (JNK), p38MAPK, extracellular-signal-regulated kinases (Erk) 5 and the prototypical Erk1/2 [130]. Erk1 and 2 (Erk1/2) are activated in the MAP kinase (MAPK) cascade, where the MAP

kinases Raf, MEK1/2 and Erk1/2 activate each other by phosphorylation in the order Raf-MEK1/2-Erk1/2. Activated Erk1/2 phosphorylate a large number of both cytosolic and nuclear substrates including, but not limited to, kinases, phosphatases, other signaling molecules and transcription factors [131]. This leads to cell specific responses including proliferation, migration, survival and growth [132].

For initiation of the MAPK-Erk1/2 pathway via PDGFR β , the SH2 and SH3 domain containing adaptor molecule Grb2 binds to activated PDGFR β via a pYNSA motif at Y716 [133], [134]. Grb2 can also bind indirectly to PDGFR β via the adaptor protein Shc that can associate with PDGFR β at phosphorylated Y759 and to a lesser extent Y740, Y751 and Y771 [135]. The tyrosine phosphatase SHP-2 that binds to phosphorylated Y763 and Y1009 can also associate with Grb2 [136], [137]. Grb2 in turn binds to and activate the nucleotide exchange factor Sos1, which activates the GTPase Ras [138]. Ras activates Raf, and Raf as the first MAP kinase in the MAP kinase cascade activates MEK1/2 which in turn activates Erk1/2.

PDGFR β downregulation

PDGFR β activation and subsequent signaling leads to a number of cellular processes that ultimately results in a biological response such as cell proliferation or migration. A variety of mechanisms seem to modulate and downregulate PDGFR β signaling at different levels of receptor signaling. First, dephosphorylation of phosphorylated tyrosine residues on the receptor by phosphatases that act directly on the receptor can deactivate the receptor. Tyrosine phosphatases acting directly on PDGFR β include PTP1B, TC-PTP and PTPRJ/DEP-1 [3]. Second, dephosphorylation of signaling proteins downstream of PDGFR β by phosphatases like PTEN dephosphorylating NHERF to control activation of PI3K, DUSP which dephosphorylates ERK1/2 and SHP-2 which dephosphorylates Grb2 [3]. Third, internalization of activated receptors via endocytosis to remove the receptor from the plasma membrane, induce signaling via signaling molecules activated at cellular locations other than the plasma membrane and sort the receptor ligand complex for degradation. Fourth, proteolytic cleavage of the receptor may pose both a regulation of the signaling and be part of a degradation pathway.

Endocytosis of PDGFR β

Endocytosis is a way to negatively regulate expression of proteins at the cell surface, but is also a way of trafficking cell surface receptors like PDGFR β to other cellular compartments for activation of the signaling molecules that are expressed there, e.g. at the early endosome. Endocytosis of EGFR as the standard example of an RTK has historically been more studied, and for PDGFR

some of the knowledge of especially internalization and sorting of the receptor in the cell is derived from studies of EGFR (Goh, Sorkin 2013). Some of the features of internalization, like dependence on dimerization is the same for PDGFR and EGFR [139].

Several motifs in the C-terminal tail of PDGFR β has been shown to be involved in mediating endocytosis, and some overlap with binding motifs for signaling proteins. PDGFR β has an YXXM motif at Y740 and Y751 which mediate internalization, but is also the binding site for PI3K, although not at the same time [140]. The same way of overlapping is seen with Y579 in the juxtamembrane region of PDGFR β , which is important for internalization [141], but is also part of the binding site for Src [84]. Residues 952-965 in PDGFR β is a hydrophobic signal also important for internalization [139], [142].

After ligand induced activation, PDGFR β is internalized via clathrin mediated endocytosis (see **Figure 8**) [143], [144]. The internalization is promoted by ubiquitination of PDGFR β by members of the Cbl family of E3 ubiquitin ligases, Cbl-b and c-Cbl [145], [146]. Cbl-b and c-Cbl monoubiquitinate PDGFR β at multiple positions [147], which creates binding sites for adaptor proteins containing a ubiquitin binding domain [148]. As monoubiquitin as a signal for clathrin mediated endocytosis is a field still being explored, it is not known exactly which adaptor proteins bind, but Epsin and Eps15 are considered good candidates as linkers between ubiquitin on cargo proteins and the clathrin coated pit [149], either directly binding to clathrin, AP-2 or other adaptors [150]. So with aid from accessory proteins and other adaptor proteins clathrin forms a lattice around the membrane, where PDGFR β and adaptor proteins have accumulated, to form a curvature which is then pinched off from the plasma membrane via scission by dynamin [151].

Dynamin is a GTPase consisting of an N-terminal G domain harboring the GTPase activity, a bundle signaling element, a “stalk” domain, a PH domain for association with membrane lipids, a GTPase effector domain that interacts with the G domain, and a proline rich domain in the C-terminal containing PXXP motifs for binding of SH3-domain-containing proteins [152], [153]. Three isoforms exist: dynamin I, II, and III. Dynamin I is expressed in neurons and dynamin III is mostly expressed in the brain, both of them are expressed at low levels in in other tissues. Dynamin II is ubiquitously expressed [153]. The exact mechanism of dynamins ability to mediate membrane fission is discussed, but it is known that in the absence of nucleotide, dynamin dimerizes via the stalk domain, and polymerizes into helices around the neck of a forming vesicle. When GTP is bound, the G-domains dimerize, and upon GTP hydrolysis the dynamin changes conformation so the helix constricts and the vesicle is pinched off the membrane [152], [153].

Clathrin mediated endocytosis of PDGFR β has been more studied and it is also suggested that this is the main internalization route for PDGFR β , but PDGFR β has also been demonstrated to internalize via clathrin independent

endocytosis [127], [154], [155]. The amount of receptor internalizing via this pathway and via clathrin mediated endocytosis is not known [150]. So to what extend the differences in internalization pathways affect or give rise to alternative signaling outcome, or whether it is merely a way of regulating internalization, is not known. For EGFR which internalize via both routes, it has been suggested to be a strategy to regulate internalization. With low doses of EGF, EGFRs will not be ubiquitinated and will internalize via clathrin mediated endocytosis, but with higher doses of EGF and thus larger amounts of activated receptor, receptors will be ubiquitinated and clathrin independent endocytosis will be the main internalization route and serve as a degradation route rather than a way of stratifying signaling [150], [156], [157]. For PDGFR β , as response to increased gradient of PDGF stimulation the cells change phenotype from migrating to proliferating, and it has been suggested that the choice of phenotype depends on the endocytic route which again depends on ligand concentration [158]. In fact, high doses of PDGF has been reported to result in receptor internalization via both clathrin mediated and independent routes, whereas with low doses of PDGF, PDGFR β will predominantly internalize via clathrin mediated endocytosis [127].

Sorting and degradation

Once internalized (see **Figure 8**), PDGFR β is sorted via the endosomal/lysosomal pathway [144], [159] while still actively signaling from endosomes [160], and signaling is terminated in late endosomes and lysosomes where the receptor is degraded [159]. There is, however, also evidence for a role of the proteasome in degradation of PDGFR β [146], although the exact mechanism remains to be elucidated.

PDGFR β can be detected at early endosomes within 5 minutes of ligand stimulation [161]. On the surface of early endosomes PDGFR β will encounter signaling molecules such as Akt [162] and Erk1/2 [163] which are activated here. In contrast to e.g. EGFR, PDGFR β does not normally recycle back to the plasma membrane after internalization, but recycling can be induced by loss of the tyrosine phosphatase TC-PTP [164], and has been suggested to be dependent on the activation of PKC [165] and possibly PI3K [155].

During the passage through the endosomal system, PDGFR β will also encounter proteins of the endosomal sorting complex required for transport (ESCRT) pathway. The ESCRT pathway consist of protein complexes ESCRT-0, -I, -II, and -III, which recognizes ubiquitinated cargo, prevents it from recycling and retrograde trafficking and catalyzes the process of forming intraluminal vesicles containing cargo for sorting [166]. The protein complexes of the ESCRT pathway will recognize and bind to the ubiquitinated residues in the receptor C-terminal, and destine PDGFR β for intraluminal vesicles, which are vesicles inside the early endosomes [150], [167]. Deubiquitinating enzymes (DUBs) associated with ESCRT-III deubiquitinate receptors

before the receptors enter intraluminal vesicles [157]. The early endosome will gradually mature into a late endosome/multivesicular body which will then fuse with lysosomes that has a low pH in the lumen and contains proteolytic enzymes to degrade the receptor [167].

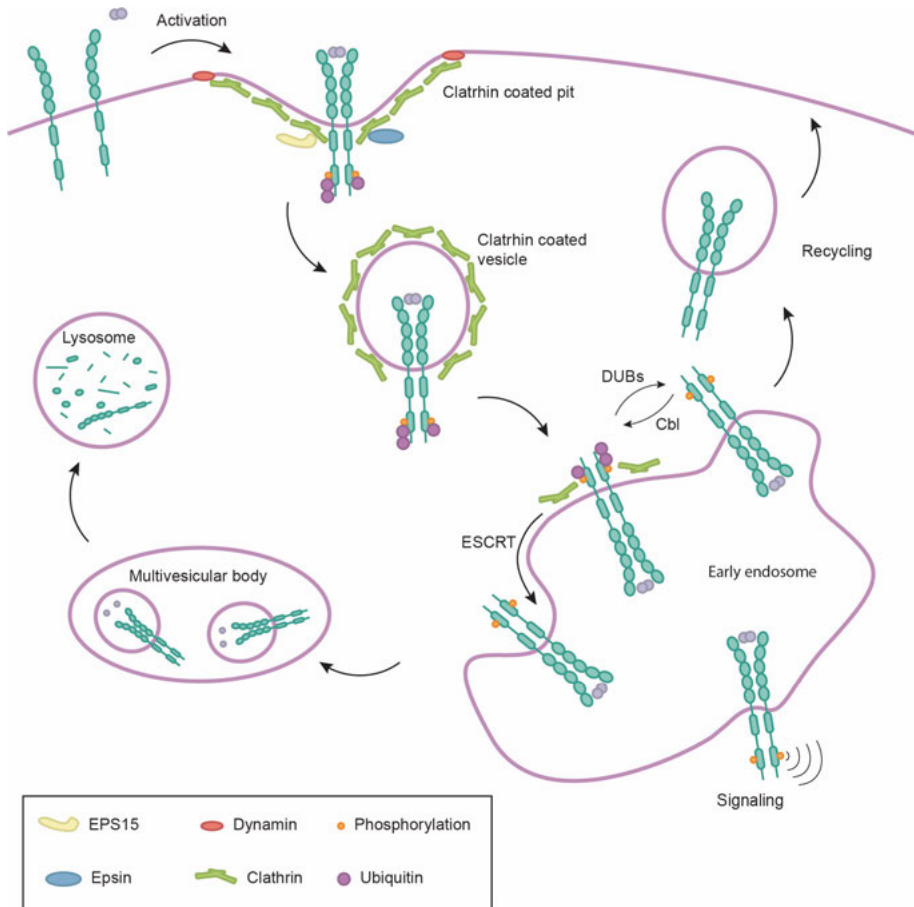


Figure 8: Simplified overview of clathrin mediated endocytosis of PDGFR β . Upon ligand stimulation, PDGFR β dimerizes and is activated by phosphorylation. PDGFR β is monoubiquitinated at several residues, which promotes internalization via clathrin coated pits. Association of PDGFR β and clathrin is mediated via adaptor proteins EPS15 and Epsin. Dynamin constrict the plasma membrane to release the clathrin coated vesicle. PDGFR β sorts to the early endosome where PDGFR β can associate with the ESCRT complex to enter intraluminal vesicles. The endosome then matures to a multivesicular body, which will mature into lysosome where the receptor is degraded. PDGFR β can be deubiquitinated by DUBs and re-ubiquitinated by Cbl at the early endosome, and the phosphorylated receptor can engage with signaling molecules residing here. In some cases, PDGFR β is recycled from early endosomes and back to the plasma membrane for reuse.

Proteolytic cleavage

Some RTKs go through proteolytic cleavage in response to ligand stimulation as a mechanism of signal regulation [168]. In some cases proteolytic cleavage of transmembrane receptors includes ectodomain shedding, where the extracellular domain is cleaved by proteases from the disintegrin and metalloprotease (ADAM) family, especially ADAM10 and ADAM17 (also known as TACE), or by matrix metalloproteases resulting in a release of the a receptor fragment to the extracellular space [168]. The released fragment may then serve as a soluble receptor in the extracellular space to bind and neutralize ligand, thereby modulating signal transduction as a negative feedback mechanism [169]. Ectodomain shedding has been observed in several RTKs [168], among these c-Kit and colony stimulating factor 1 receptor (CSF1R) which are cleaved by ADAM17 [170], [171], EGFR which is cleaved by the serine protease prostatin [172] and VEGFR2 which is cleaved by matrix metalloproteases [173]. Ectodomain shedding has been observed for PDGFR β in cerebral pericytes [174], and is suggested to be mediated by ADAM10 and ADAM17 [175], but the exact mechanism is unknown.

Regulated intramembrane proteolysis is a process in which the receptor ectodomain is first shed and then the remaining membrane bound receptor is cleaved by γ -secretase residing in the membrane to form a soluble intracellular fragment [176]. The intracellular fragment may then translocate to the nucleus. This process has been observed for a variety of proteins like Notch [177] and some RTKs like ErbB4 [178] and CSF1R [179], but γ -secretase does not seem to cleave PDGFR β [180].

RTKs may also undergo ligand induced proteolytic cleavage by other mechanisms. For instance, VEGFR2 has been reported to be cleaved in its intracellular domain [181], [182], however the protease has not been identified and the functional consequences of the cleavage are not fully understood. Proteolytic cleavage of PDGFR β by a Ca²⁺-dependent protease from a 185 kDa to a 135 kDa form has previously been reported [183] which we further characterize in **paper IV**.

PDGFR β disease and treatment

Dysregulation of PDGFR is associated with a number of diseases, including fibrosis, vascular disease and cancer [3].

In cancers, dysregulation includes overexpression of PDGFR β seen in stroma from prostate cancer patients [184]. Another mechanism is translocation of the PDGFRB gene which results in expression of fusion proteins of PDGFR β with another protein, and it is common for these fusion proteins to have a constitutively active kinase [3]. In chronic monomyelocytic leukemia, PDGFR β is fused with the transcription factor Tel [185].

Disease related mutations have also been described, especially in the juxtamembrane and kinase domains of PDGFR β . These are both gain- and loss of function mutations, and are associated with a range of diseases including myofibromas (constitutively active PDGFR β) and primary familial brain calcification (impaired PDGF-BB and PDGFR β signaling) [186].

The strategies to inhibit the PDGF/PDGFR pathway includes targeting the ligand and PDGF to neutralize it, target the receptor-ligand binding interface to prevent PDGF to bind PDGFR β , or targeting the kinase domain to inhibit its function [73], [187].

Currently no selective PDGFR β inhibitor exists, as all tyrosine kinase inhibitors have multiple targets among RTKs [3], [187]. As also mentioned above, the structure and sequence of PDGFR closely resembles the other RTKS class III members c-Kit, Flt3 and CSF1R (Verstraete & Savvides, 2012), and these similarities even extend to RTKS of other classes as for example VEGFR in class IV [187], [188].

Current approved small molecule inhibitors of the tyrosine kinase domain include among others Imatinib (Gleevec, Novartis), Nilotinib (Tasigna, Novartis) and Sunitinib (Sutent, Pfizer) [187]. Imatinib was the first tyrosine kinase inhibitor approved for clinical use. Imatinib was developed for the treatment of chronic myeloid leukemia, in which a translocation between chromosome 9 and 22 gives rise to the constitutively active fusion protein bcr-Abl [189], but is now indicated for use in a variety of cancer diseases [187]. Imatinib targets the binding site for ATP in the kinase domain of Abl [190], [191], and additionally inhibits PDGFR c-kit and FLt3 with different efficacy [187], [189].

Present investigations

Paper I

Dynamin has a key function in clathrin mediated endocytosis as it promotes the budding off of vesicles from the plasma membrane [192]. PDGFR β is internalized via clathrin mediated endocytosis [143]. In paper I we investigated the effects of dynamin inhibition on activation and signaling of PDGFR β . Using dynamin inhibitors Dynasore and Dyngo-4a that both inhibits dynamin I and II, we found that inhibition of dynamin prevents activation of PDGFR β because the ligand induced dimerization of PDGFR β monomers was impaired. This in turn lead to lack of phosphorylation of receptor and downstream signaling proteins Akt and Erk1/2. Inhibition of dynamin also affected cellular localization of PDGFR β , and resulted in PDGFR β forming large structures independent of stimulation with the ligand PDGF-BB. The results indicate a change in membrane localization of PDGFR β as a consequence of dynamin inhibition. Inhibition of dynamin affected PDGFR β internalization by impairing recruitment of PDGFR β to clathrin coated pits and PDGFR β internalization to EEA1-coated early endosomes in PLA experiments, whereas proximity between PDGFR β and dynamin II was unchanged upon dynamin inhibition. In contrast, the effects of dynamin inhibitors on dimerization, signaling and internalization were not observed for epidermal growth factor receptor (EGFR), and our findings thus suggests a link between PDGFR β activation and plasma membrane dynamics, that is not shared with EGFR. For this project I performed *in situ* PLA experiments to investigate effects of dynamin inhibition on ligand induced proximity of PDGFR β to marker proteins of early endosomes and proteins associated with clathrin mediated endocytosis. The results from these experiments helped demonstrate the importance of dynamin for internalization of PDGFR β . Based on our findings we suggest that dynamin activity is important for PDGFR β distribution to clathrin coated pits in the plasma membrane and subsequent sorting to early endosomes.

Paper II

Current available methods for analysis of protein-protein interaction does not control for expression levels of the proteins in question. Knowing the expression levels of the interacting proteins is important for the interpretation of the

obtained data as overexpression can lead to false positive interaction. In this project we developed a new method, Molboolean, for analysis of protein-protein interactions. In contrast to existing methods for analysis of protein-protein interactions such as *in situ* PLA, Molboolean not only detects the pool of interacting proteins, but also the pools of non-interacting protein. The Molboolean method is based on the same design principles as *in situ* PLA as described in more detail in chapter 2. Following validation of the Molboolean specificity in solution and test of ability to detect single proteins using two antibodies for the same protein, Molboolean was performance tested on a panel of well described interactions with different cellular location and all tested interactions were validated against *in situ* PLA and immunofluorescence and technical controls.

The interaction between E-cadherin and β -catenin which under normal conditions is observed at the plasma membrane [193], was tested under different conditions; wildtype conditions with technical controls, with a mutation (V832M) in E-cadherin for disruption of the interaction [194], and in cells treated with transforming growth factor β (TGF- β) which also disrupts the interactions [195]. The E-cadherin and β -catenin interaction was furthermore tested in FFPE tissue sections. Molboolean also detected interaction between Lamin B1 and Emerin [196] which takes place at the nuclear membrane and detected a fraction of free Emerin in the ER region and free Lamin B1 in the nucleoplasm as expected. In the nucleus, Molboolean detected the co-localization and free protein of FUS and HNRNPM. Dynamic changes in protein interaction was detected with Molboolean for the interaction between PDIA3 and Calreticulin [197] after treatment with siRNA to silence PDIA3 expression. That Molboolean is also capable of detecting the induction of complex formation was shown based on the ligand induced proximity between PDGFR β and clathrin [161]. And finally, we showed that Molboolean can also be used on FFPE tissue sections, and the method may therefore find use in the clinic.

In conclusion, we present a method can be used for detection of protein-protein interactions while at the same time providing information of the levels of non-interacting protein with a reduced the risk of false positives compared to *in situ* PLA.

Paper III

Viruses are obligate parasites that hijack and take advantage of the host cell machinery by mimicking short linear motifs (SLiMs) [4]. Most of SLiM-mediated virus-host protein interactions already reported are identified using low throughput methods [15]. Several virus-host interactions have already been reported through use of Y2H and AP-MS, but these methods are not optimized for capturing transient low affinity SLiM mediated interactions as also

discussed in chapter 2. The aim of paper III was therefore to map SLiM mediated host-virus interactions and explore their mechanisms.

To map SLiM-mediated virus-host interactions, we created a library, Riboviria Viral Disorderome (RiboVD), covering disordered regions of the proteome from 229 RNA viruses. Using phage display, the RiboVD library was screened against 139 human bait proteins that were selected based on previous reports of interaction with viral proteins, interaction with human SLiMs and proteins involved in cellular processes relevant for the viral life cycle. We identified 1712 potential SLiM based host-virus interactions through screening of the RiboVD library. These came from 1285 viral peptides and 97 human protein domains. Global analysis of the interaction data revealed that cell processes associated with protein transport and sorting are common targets for viral hijacking, and we could identify both common strategies of hijacking used by unrelated viruses, and distinct strategies used by closely related viruses. Selected host-virus interactions involved in ESCRT machinery, endocytosis and protein translation were further validated using biophysical, biochemical and structural methods and investigated in more depth.

We next turned to hijacking of clathrin binding motifs to which I contributed with *in situ* PLA experiments concerning clathrin and PDGFR β . From screening of the RiboVD library against the N-terminal domain (NTD) of the clathrin heavy chain, three interactions were obtained with high confidence; the μ -NS protein from the non-enveloped dsRNA virus Mammalian Reovirus type 1 (MRV1), NSP3 protein from Eastern equine encephalitis virus (EEEV) and RNA-directed RNA polymerase from Seneca Valley virus. The peptides bound by clathrin NTD all contained a classical clathrin box motif (L ϕ x ϕ [DE], where ϕ is a hydrophobic amino acid and x is any amino acid [15]. The clathrin box motif in the μ -NS protein from MRV1 is previously described [198], but for NSP3(EEEV) the found clathrin box motif was new. The binding was confirmed using FP, GST-pull down, and *in situ* PLA experiments. The peptides NSP3₁₇₆₅₋₁₇₈₀(EEEV) and μ -NS₇₀₅₋₇₂₀(MRV1) were also co-crystallized with clathrin for further characterization of the binding mode. We further characterized the effects of NSP3(EEEV) binding to clathrin in HEK293 cells using *in situ* PLA. Using PDGFR β as a model system and example of an RTK that mainly internalizes via clathrin mediated endocytosis (see chapter 3), we hypothesized that if NSP3 binds clathrin, the ligand induced phosphorylation of PDGFR β will not be reduced due to degradation after 60 minutes of ligand stimulation as usually seen [161]. Indeed, we found that NSP3(EEEV) prolonged the ligand induced phosphorylation of PDGFR β at Tyr751. Using a cell surface fluorescence assay, we confirmed that the interaction between NSP3(EEEV)s and clathrin prevents internalization of PDGFR β from the plasma membrane.

Another interesting host protein was polyadenylate binding protein 1 (PABP1), which binds to the poly(A) tail of mRNA and acts as a translation initiator [199]. Three viral peptides that interacted with the PABP1 PABPC

domain were identified. The interactions were validated with fluorescence polarization-based measurements, GST pull down, AP-MS and further structurally characterized by co-crystalizing the peptides in complex with the PABP1 PABPC domain. The results showed direct competition between endogenous and viral ligands of PABC. The biological effects of viral hijacking of PABP1 was further investigated by creating a lentiviral construct expressing repeats of the PV/V/C₁₈₃₋₁₉₈ Hendravirus peptide fused to EGFP and testing it against a panel of RNA viruses for the ability of the expressed peptide to inhibit infection. Overall, infection by most of the tested viruses was reduced. The EGFP-peptide was further tested for its effect on viral replication complexes in tick borne encephalitis virus infected cells, which resulted in a more diffuse subcellular distribution of the replication complexes. These results suggest that the sequence from the PV/V/C₁₈₃₋₁₉₈ Hendravirus peptide found in the screening, could serve as a starting point for development of a pan-viral inhibitor of viral replication.

To conclude, we present a large-scale dataset of SLiM mediated RNA virus-host protein interactions. We showed that SLiM mimicry is widely used strategy and that virus can use different SLiMs spaced closely together to achieve temporal control of interactions with host cell proteins. Hijacking of clathrin, clathrin adaptors and different transport processes is common. Finally, we showed that the PABP1 binding PV/V/C₁₈₃₋₁₉₈ Hendra virus peptide is able to inhibit replication of a many of the tested viruses.

Paper IV

It has been observed that some RTKs, including PDGFR β , are proteolytically cleaved following ligand activation (see chapter 3) [168], [183]. In paper IV we have characterized the proteolytic cleavage of PDGFR β using site- and phospho-specific antibodies. The proteolytic cleavage resulted in two fragments of PDGFR β , a 130 kDa extracellular fragment, and a 70 kDa intracellular fragment. More specifically, we could narrow down the region where cleavage takes place to be between tyrosine residues 581 and 857. In line with previous observations [183], the cleavage was Ca²⁺-dependent, since treating cells with the Ca²⁺-chelator BAPTA AM which chelates both outside and inside the cell prevented cleavage of PDGFR β , whereas EDTA which does not pass the cell membrane, did not prevent cleavage. By preventing receptor internalization by stimulation on ice, or by treating the cells with dynamin inhibitors Dynasore and Dyngo-4a to prevent clathrin-mediated endocytosis, we found that receptor internalization was required for proteolytic processing. This was also confirmed with cell surface biotinylation.

PDGFR β is sorted to lysosomes where it is degraded [144], [159]. There are, however, also indications of a role for the proteasome in the degradation of PDGFR β [146]. To investigate the role of the proteasome in proteolytic

processing of PDGFR β , we used the proteasome inhibitor bortezomib. Treatment with bortezomib prevented both the proteolytic cleavage and decreased internalization of PDGFR β . We also found that treatment with bortezomib resulted in increased phosphorylation of PDGFR β at Y751 and the downstream signaling proteins PLC γ and STAT3, while Akt was unaffected. Erk1/2 phosphorylation was initially increased compared to the DMSO treated control, but was mildly reduced after PDGF-BB stimulation. The increased ligand induced phosphorylation of PDGFR β upon treatment with bortezomib corresponds well with the prolonged presence of PDGFR β at the plasma membrane observed in the cell surface biotinylation experiment. Both PLC γ and STAT3 can be activated at the plasma membrane [84], [106], [122], [125]. Prolonged presence of PDGFR β at the plasma can therefore explain why phosphorylation of PLC γ and STAT3 was increased. It also explains why Akt and Erk1/2 was reduced, as they are activated at the endosome [162], [200].

In an attempt to identify the protease responsible for cleavage, we tested different protease inhibitors targeting cysteine proteases calpain and cathepsin for their ability to block the proteolytic cleavage of PDGFR β . The results showed no effect of the inhibitor on the ligand induced proteolytic cleavage of PDGFR β . The same was found for inhibitors of γ - and β -secretase.

Based on these results we suggest that proteolytic cleavage of PDGFR β is Ca²⁺-dependent and does not occur at the plasma membrane, but is dependent on receptor internalization.

Discussion and future perspectives

Paper I

Dynamins role in PDGFR β dimerization

In **paper I** we showed that the dynamin inhibitor Dynasore negatively affects the internalization of PDGFR β , because it prevents dimerization of PDGFR β , and we suggest that dynamin is important for distribution of PDGFR β to clathrin coated pits in the plasma membrane. We showed that inhibition of dynamin inhibited dimerization of PDGFR β , but we have not shown if dynamin is inducing dimerization and if so the mechanism behind it. PDGFR β can be co-immunoprecipitated with dynamin 2 [201], but a direct binary interaction has not been reported in the Uniprot database [202]. The effect of dynamin on PDGFR β dimerization may therefore be an indirect effect via clathrin and the adaptor and scaffold proteins that constitute clathrin coated pits. Off-target effects of Dynasore cannot be excluded either [203].

Dynamin directly interacts with the actin cytoskeleton and promote actin polymerization [204]. Dynamin is also involved in clathrin mediated endocytosis where it accumulates in the clathrin coated pit as it forms, and eventually performs the scission of the clathrin coated vesicle from the plasma membrane [153], and in the internalization of caveolae [205]. Dynamin furthermore localizes to the trans-Golgi network [206] and is involved in secretory transport from the trans-Golgi network to the plasma membrane [207]. Because of dynamins function in different compartments of the cell, answering whether dynamin plays a role in the dimerization of PDGFR β and elucidating the mechanism, will require the use of advanced microscopy methods with better resolution than standard fluorescence microscopy, and possibly in combination with fluorescence lifetime imaging to measure FRET changes in the receptor, dynamin, other adaptor proteins, and membrane components involved in organization of proteins in the plasma membrane.

Paper II

Improved 3D analysis to prevent false positives

CellProfiler software [208] was used for analysis of images from Molboolean experiments in **paper II**. Image analysis with CellProfiler is performed in 2D. Prior to the CellProfiler analysis, images were deconvolved as to minimize blurring of the image. The deconvolution also helped minimize out of focus fluorescence from RCA signal. Images were acquired for each fluorophore, and after segmentation of each RCA signal, the signal intensity for each fluorophore in the area assigned to the RCA signal was plotted against each other in a scatter plot, with fluorophore A on the x-axis and fluorophore B on the y-axis. This helps distinguish four groups of signal originating from protein AB in complex, free protein A and B and background. By thresholding the data, low intensity signal that was considered background from fluorophore A and B was removed from the analysis, and where intensities correlated, this was considered signal from protein A and B in complex. The non-correlated signal was assigned to free protein A and B.

When imaged, a fluorophore will emit light in all directions. Emitted light from an RCA product that have its center in a different plane on the Z-axis can therefore appear in the focal plane that is imaged. Image deconvolution may not always be enough to prevent this. Since Molboolean images were analyzed in 2D, it was therefore not possible to distinguish whether overlapping fluorescence intensities were a true positive for an interaction between protein A and B, or whether the overlap originated from RCA signal from individual free protein A and B that were just situated in different focal planes. Analyzing the images in 3D using a software suitable for 3D analysis would help on this.

Paper III

Validation of host-virus interactions

For **paper III** we build a ProP-DP library, RiboVD, covering disordered regions of the proteome of 229 RNA viruses, screened the library against 139 human protein domains and found 1712 SLiM-mediated host-virus interactions. Of the 1712 interactions, a subset of 25 interactions were biophysically validated, and of them a subset was thoroughly validated structurally and in cell-based assays. Further validations can be added by *in situ* PLA in the future. Validation of all the found interaction will be an enormous task, but the interaction data is now available and can be explored by the community. A

big task also lies ahead when it comes to choosing interactions for further validation and investigation of the biological consequences of these interactions.

Effects of viral hijack of clathrin

Among many things in **paper III**, we investigated the effect of the SLiM-mediated interaction between viral protein NSP3(EEEV) and clathrin on PDGFR β activation and internalization. We found that the ligand induced phosphorylation of PDGFR β which increase after 10 minutes of stimulation and then gradually decreases, was prolonged after 60 minutes in cells transfected with full length NSP3(EEEV). Similarly, PDGFR β expression at the plasma membrane did not decrease after 60 minutes, as it normally would, due to ligand induced clathrin mediated endocytosis of the receptor. In other words, PDGFR β was stuck at the plasma membrane when clathrin was not available. The reduced availability of clathrin is probably not specific to PDGFR β , and may affect other transmembrane receptors that rely on clathrin for their internalization. Some receptor may be able switch pathway to clathrin independent internalization. Other members of the RTK family such as EGFR internalize via both clathrin mediated and independent pathways and may therefore be able to self-regulate the lack of clathrin by internalizing via clathrin independent mechanisms instead [156]. The effect of viral hijack of clathrin on other cell surface receptors remains to be tested.

Clathrin is involved not only in endocytosis, but also in vesicular transport from the *trans*-Golgi network and in the cargo sorting via ESCRT at the endosomal membrane [151], [209], [210]. Clathrin is needed for viral assembly and release [211], [212]. For μ -NS (MRV1), which also interact with clathrin via a clathrin box motif, it has been described how clathrin is important for the formation of viral factories in cells [198]. A similar mechanism could exist for NSP3(EEEV).

Finally, the prolonged phosphorylation of PDGFR β observed when PDGFR β is retained at the plasma membrane likely alters it's signaling pattern. As also described in chapter 3, signaling via PDGFR β is compartmentalized [213], meaning that different signaling molecules are present at certain sub-cellular locations, e.g. Akt at early endosomes [162] and internalization of the activated receptor is therefore necessary for activation of some of the signaling proteins. In papers I and IV, we observed a change in signaling pattern when as a result of dynamin and proteasome inhibitors, endocytosis of PDGFR β was prevented. The presence of NSP3(EEEV) in cells may have a similar effect and it would be interesting to explore if this is the case and if so how it would affect cells in functional assays.

Paper IV

Identify proteolytic cleavage site and protease

It remains to be answered from **paper IV** what protease cleaves PDGFR β , and at which residue. In paper IV we found the region of cleavage to be between Y581 and Y857. Taking the 130 kDa size of the extracellular fragment into consideration, this would lead towards a cleavage site close to Y581 in order for the molecular weights to add up to a 180 kDa full length receptor.

The prime suspects in this case would be calpains since the cleavage was Ca²⁺-dependent, and so are calpains. Calpains are a family of calcium dependent cysteine proteases that do not have a consensus cleavage site, but prefers cleaving after a large a hydrophobic residue [214], [215]. We speculated and tested whether inhibitors of different calpains could block the cleavage, but none of the tested calpain inhibitors did.

Caspases (cysteine-aspartic proteases) are another option. Caspases are proteolytic enzymes involved in apoptosis, but can also mediate cell death without apoptosis, and can play a role in cell proliferation, tissue regeneration and tumorigenesis [216]. There is also no consensus motif for cleavage by caspases as they are very promiscuous, but they do have a tendency to cleave after aspartic acid residues [217]. A starting point for investigating this, could be to locate aspartic acid residues in the juxtamembrane part of the cleaved region and mutate them. Proteolytic cleavage by caspases in the intracellular domain is common for dependence receptors, a group of receptors including some RTKs that in the absence of ligand stimulation signals to apoptosis. The caspases cleavage is essential to the apoptotic function of these receptors, but PDGFR does not belong to this group of receptors [218].

To identify the protease responsible for cleavage of PDGFR β , proximity labelling followed by MS as introduced in chapter 2 is currently being pursued.

To map the cleavage site, sequential deletion constructs of PDGFR β with deletions in the cleaved region Y581-Y758 could be tested for ligand induced cleavage, followed by an alanine or glycine scan to identify the exact cleavage site. Mapping the cleavage site is also possible by performing AP-MS of the cleaved receptor fragments. Once the cleavage site is known we hope to make a mutated PDGFR β construct that cannot be cleaved, but is kinase active, for use in investigating consequences of receptor cleavage in regard to PDGF-induced functions such as proliferation and migration.

During the work with **Paper IV** we also noticed that the ligand induced cleavage of PDGFR β seem to be cell type specific for fibroblasts. To gain information that can help identify the protease in question, it could be beneficial to perform a comparison of expression profiles between fibroblast cell

lines Bj-hTERT and AG01523 and e.g. the osteosarcoma cell line U2OS which also endogenously express PDGFR β , but PDGFR β is not cleaved.

Elucidate functional consequences of increased PLC γ and STAT3 signaling

PLC γ and STAT3 phosphorylation was found to be increased in cells treated with the proteasome inhibitor bortezomib. PDGFR β phosphorylation was also increased, likely due to a prolonged presence at the plasma membrane. The functional consequences of increased PLC γ and STAT3 would be interesting to elucidate. Activation of PLC γ is associated with cell migration [114] and VEGFR2 is cleaved in a very similar manner as PDGFR β , and it was shown that preventing VEGFR2 cleavage with a proteasome inhibitor promoted cell migration [181]. An increase in chemotactic response towards PDGF-BB for cells treated with bortezomib may potentially be a due to increased duration of active PDGFR β at the plasma membrane, but it should nevertheless be explored.

Signaling from the fragment

It is an interesting idea that the PDGFR β intracellular fragment should sort and signal in parallel with full length PDGFR β . For some RTKs undergoing regulated intramembrane proteolysis, it has been shown that the intracellular fragment can translocate to the nucleus [168], as is the case with another RTK ErbB4 [178]. PDGFR β has previously been reported to also locate to the nucleus and regulate proliferation [219]. This was however using an antibody recognizing the intracellular domain of PDGFR β , so whether it is full length or intracellular fragment that translocate to the nucleus is not clear. It would therefore be interesting to explore the possibility that the intracellular fragment is translocated to the nucleus and affects transcription of specific genes.

Populärvetenskaplig sammanfattning

För att en flercellig organism som en människa ska kunna fungera, behöver cellerna kunna kommunicera med varandra. Det gör de genom signalmolekyler som binder till receptor-proteiner på cellytan eller inne i cellen. Receptor-proteinerna sitter i cellmembranet, och har en del som pekar utåt, och en del som pekar inåt i cellen, och deras främsta funktion är som signalgivare. När en signalmolekyl binder till receptorproteinet, ändras strukturen i receptorproteinet från inaktiv till aktiv. Detta gör att signaleringsproteiner inne i cellen kan binda till den del av receptorproteinet som finns på cellens insida. Signalen kommer då att skickas vidare genom ett antal av interaktioner i ett nätverk av signaleringsproteiner och sedan översätts till en biologisk respons, till exempel cellväxt. PDGFR β (eng. platelet-derived growth factor receptor β) är ett receptorprotein som finns främst i fibroblaster (bindvävsceller) i celler runt blodkärl, så kallade pericyter och glatta muskelceller. PDGFR β signalerar genom en rad signaleringsvägar till cellväxt och migration. I olika typer av cancer är genom PDGFR β -signaleringen ur balans.

Det är vanligt för proteiner involverade i cellsignalering att binda till varandra genom bindningsställen, så kallade korta linjära motiv eller SLiMs (eng. short linear motifs). Dessa är streckkodslänkande bindningsställen bestående av 3–10 aminosyror som binder till en proteindomän på ett annat protein. Bindningar genom SLiMs är svaga och bryts snabbt igen, vilket är en fördel i cellsignalering. Det är estimerat att det finns runt 100 000 SLiMs bland alla människans proteiner, men bara 4 000 har beskrivits hittills. Virus består av genetiskt material omslutet av protein, och kan inte formera sig utan en värdcell. Virus kan imitera SLiMs som finns i värdcellens proteiner och på så sätt binda till värdcellens proteiner, och omdirigera deras funktion till egen fördel.

För utveckling av framtida behandlingar mot cancer och virusinfektion behövs kunskap om proteinbindningarna i cellsignalering och om hur virala proteiner binder till värdcellens proteiner. Det finns en rad metoder för att ta reda på hur proteiner binder till varandra som var och en har sina fördelar och nackdelar. Det finns metoder som kan detektera nya proteininteraktioner i stor skala, bland annat fagdisplay där man utnyttjar bakteriofager, som är virus som infekterar bakterier, till att uttrycka de proteiner eller peptider man är intresserad av att testa. Andra metoder är bra att använda för att undersöka proteinbindningen i celler, och kan visualisera proteinerna så att man med hjälp av ett mikroskop kan se var i cellen proteinbindningarna man är

intresserad av pågår. I vårt labb jobbar vi med att ta fram sådana metoder. Ett av dessa är *in situ* Proximity ligation assay (PLA). Med *in situ* PLA kan man undersöka om de två proteiner man är intresserad av binder till varandra och se i mikroskop var i cellen det sker.

I arbetet med denna avhandling har jag använt *in situ* PLA för att undersöka hur PDGFR β -signalering påverkas under olika förhållanden. Jag har också varit med om att ta fram en ny metod som, till skillnad från PLA, kan påvisa att två proteiner binder till varandra och samtidigt visar nivån av obundet protein.

I paper 1 har vi undersökt hur proteinet dynamin påverkar PDGFR β . Dynamin hjälper till med transporten av PDGFR β från cellytan in i cellen efter PDGFR β har aktiverats av signaleringsmolekylen PDGF. När dynamins funktion hämmas, såg vi att PDGFR β blev kvar på cellytan när vi behandlade cellerna med PDGF. Vi såg också PDGFR β inte aktiveras som vanligt och att två signalmolekyler Akt och Erk1/2 som PDGFR β i sin tur vanligtvis aktiverar förblev inaktiva. Vi misstänker därför att dynamin förutom att vara viktig för transport in i cellen, också ser till att PDGFR β och andra proteiner som finns i cellmembranet fördelas i cellmembranet på rätt sätt.

I paper 2 tog vi fram en ny metod, Molboolean, som detekterar mängden proteininteraktion samtidigt som den ger information om mängden obundet protein i cellerna. Detta minskar risken för falskt positiva resultat jämfört med PLA. Metoden är baserad på igenkänning av antikroppar hos målproteiner. Med DNA som reporter-molekyl kan proteinerna detekteras och visualiseras i ett fluorescensmikroskop. Vi testade Molboolean på en rad välkända proteininteraktioner under olika förhållanden båda i celler och på vävnadssnitt och jämförda med PLA. Molboolean gav robust detektion under alla testade förhållanden. Molboolean är framförallt användbart inom forskning, men kan också användas för diagnostik.

I paper 3 undersökte vi hur proteiner från virus binder till värdcellens proteiner via så kallade SLiM-bindningsställen. Vi byggde ett bibliotek av peptider från 229 RNA-virus och använde fagdisplay för att testa om peptiderna kunde binda till 139 olika humana proteiner. Totalt 1712 peptider från olika virus proteiner visade sig binda till humana proteiner. Vi valde 25 av dessa för ytterligare verifieringsstudier och undersökning av hur de binder. En av peptiderna var från Eastern equine encephalitis-viruset, och visade sig binda till clathrin. Med hjälp av *in situ* PLA kunde vi se att virusproteinet stör transport av PDGFR β från cellytan, som clathrin vanligtvis bidrar till. Ett annat virus-peptid var från Hendravirus; detta band till proteinet PABP1. När vi testade att ge peptidet från Hendravirus till celler som var infekterade med andra virus, hämmades infektionen i cellerna. Denna peptid kan därför vara utgångspunkt för vidare utveckling av ett virushämmande läkemedel.

I paper 4 tittade vi på hur PDGFR β klyvs efter att ha aktiverats av signalmolekylen PDGF. Klyvningen av PDGFR β kan vara ett sätt för cellen att reglera signaleringen. Här såg vi att PDGFR β klyvs i två delar och att om transporten av PDGFR β från cellytan in i cellen hämmas, så klyvs inte

PDGFR β . Vi så också att hämning av proteasomen, som är ett stort protein-komplex i cellen där andra proteiner bryts ner, leder till att PDGFR β inte heller klyvs. Hämningen av proteasomen ledde dessutom till uppreglering av mängden av signalmolekylerna PLC γ och STAT3, som PDGFR β vanligtvis aktiverar.

Proteiner som klyver andra proteiner kallas för proteaser. Vi såg att klyvningen av PDGFR β var beroende av calciumjoner och misstänkte därför att proteasen som klyver receptorn är beroende av calciumjoner. För att försöka identifiera proteasen som klyver, testade vi en rad av proteashämmare, men ingen av de vi testade kunde hämma klyvningen. Vi vet därför inte vilken proteas som klyver PDGFR β , men nästa steg i projektet är att försöka identifiera båda det exakta klyvningsstället i PDGFR β och den ansvariga proteas, samt att undersöka om någon av de delarna av PDGFR β kan signalera även om receptorn inte är hel.

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