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Investigations of Protein Tyrosine Phosphatase Functions

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for my parents

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I. Introduction

1. Signal transduction via reversible protein tyrosine phosphorylation in health and disease

There are approximately 100 trillion (10^{14}) cells differentiated in hundreds of different cell types that build the human body (Trosko, 2003). All of these cells need to communicate with each other to ensure the homeostasis of organs and tissues. In general, cellular communication takes place either via direct contact of cells to surrounding cells or to extra cellular matrix and via soluble ligands. Most of the extra cellular signaling molecules bind to and modulate the activity of receptor proteins on the cell surface. Then various intracellular signal transduction pathways are initiated that directly affect different kinds of cytoplasmic machineries or lead to the cell nucleus where gene expression is regulated.

Almost all of these signaling cascades include post-translational modification of its protein components leading to either activation or inhibition of their signal transduction features. Phosphorylation of proteins is the prevalent post-translational modification in these networks and affects an estimated one-third of all proteins in the human genome (Cohen, 2001). Already in 1980, Hunter and co-workers defined the relative amounts of protein-derived phosphoamino acids and found a distribution of 0.05%, 10% and 90% for phosphotyrosine (pTyr), phosphothreonine (pThr) and phosphoserine (pSer) under physiological cell conditions (Hunter and Sefton, 1980). Recently, these observations could be verified in a global phosphoproteomic analysis by Olsen *et al.*, who identified more than 2000 phosphorylated proteins in HeLa cells containing 103 pTyr (1.8%), 670 pThr (11.8%) and 4901 pSer (86.4%) sites (Olsen *et al.*, 2006). Even though tyrosine phosphorylation accounts only for a small part of total protein phosphorylation, it has shown to be a key regulatory mechanism of many different cellular processes such as proliferation, differentiation, control of cell shape and migration in virtually all major organs (Hunter, 1998). In general, tyrosine phosphorylation is extensively utilized only in multicellular eukaryotes and it conducts crucial functions in the organization of higher ordered tissues.

Protein tyrosine phosphorylation is a reversible process which is regulated by the competing actions of two enzyme families: Protein tyrosine kinases (PTKs) and Protein tyrosine phosphatases (PTPs) (Fischer, 1999). The human genome sequencing project identified 90 PTKs (Robinson *et al.*, 2000), (Manning *et al.*, 2002) and 107 PTP genes (Alonso *et al.*, 2004). Both families contain proteins that are diverse in structure and include receptor-like as

well as cytoplasmic enzymes. Protein tyrosine phosphorylation can reversibly modify protein function by increasing or decreasing the biological activity, by facilitating or disrupting protein-protein interactions, by alteration of the subcellular protein localization or by stabilization or targeting proteins for degradation.

Deregulation of protein tyrosine phosphorylation has been identified as the cause of many severe diseases. The first link of alterations in protein tyrosine phosphorylation to cancer was identified by the Nobel laureates Michael Bishop and Harold Varmus, who found that the rous sarcoma virus oncogene product is of cellular origin and speculated that deregulation of this cellular oncogene could lead to cancer (Stehelin et al., 1976). Three years later Toni Hunter made the seminal discovery that phosphorylation takes not only place on Ser/Thr, but also on Tyr residues (Eckhart et al., 1979) and described subsequently that the rous sarcoma oncogene product v-Src is a protein tyrosine kinase (Hunter and Sefton, 1980). Not long after this, sequencing of other human PTKs such as the EGFR revealed functional homology of a human growth factor receptor to the avian oncogene v-erbB (Ullrich et al., 1984), (Downward et al., 1984). Until then, dozens of overexpressed or mutationally activated RTKs have been implicated in human cancers (Blume-Jensen and Hunter, 2001), (Bardelli et al., 2003). The most prominent examples of RTK hyperactivity in human malignancies which have lead to targeted drug developments are activated BCR-ABL in chronic myeloid leukemia (Van Etten, 2004), amplified HER2 in breast cancer (Slamon et al., 1987) and a mutated form of the EGFR in non-small cell lung cancer (Lynch et al., 2004), (Paez et al., 2004). Functional alterations of RTKs have been also connected with other diseases such as developmental dysfunctions (Webster and Donoghue, 1997), (Robertson et al., 2000), diabetes (Malecki, 2005) or immunodeficiencies (Notarangelo et al., 2001).

In comparison to RTKs, only little is known about the regulation of PTPs in health and disease (Li and Dixon, 2000). Nevertheless, aberrant regulation of PTPs is another cause for disturbed cellular phosphotyrosine signaling. Well characterized examples of disease-related PTPs are the tumor-suppressive lipid phosphatase PTEN (Cully et al., 2006) and the oncogenic dual specificity phosphatase cdc25 (Kristjansdottir and Rudolph, 2004). Also pTyr-specific classical PTPs have been linked to human malignancies. In colorectal cancers many receptor-like PTPs such as RPTP ρ have been found to be inactivated by mutation (Wang et al., 2004) and in the same tumor type also PTP DEP1 was found to be frequently deleted (Ruivenkamp et al., 2002). The first oncogenic classical PTP to be identified was SHP2 and its activation by gain-of-function mutations has been associated with the Noonan syndrome and childhood malignancies, like juvenile myelomonocytic leukemia and acute myeloid

leukemia (Niihori et al., 2005). Also phosphotyrosine signaling in diabetes and obesity is affected by PTP activity. Loss-of-function mouse genetics models highlighted the role of PTP1B in energy metabolism by negative regulation of insulin and leptin signaling (Dube and Tremblay, 2005). To date, this prototypic PTP is one of the first members out of this large enzyme family that has been selected for targeted inhibitor development which has now proceeded to several clinical studies.

2. Classical Protein Tyrosine Phosphatases (PTPs) and their regulation by modular domains

In the human genome there are 518 protein kinases (Manning et al., 2002) and about 150 protein phosphatases (Cohen, 2002). Whereas the superfamily of protein kinases derived from a common ancestor (Scheeff and Bourne, 2005), protein phosphatases have evolved in separate families that are mechanistically and structurally distinct. The serine/threonine phosphatase family contains about 40 members and differs from PTPs as they form holoenzyme complexes containing multiple combinations of catalytic and a vast number of regulatory subunits. Also, the catalytic mechanism of serine/threonine phosphatases is consistently different. Serine/threonine phosphatases are metalloenzymes and their active sites contain conserved histidine and aspartic acid residues binding metal ions such as Fe, Co, Zn or Mn. In contrast, the unrelated PTPs usually function as monomeric proteins and are defined by the unique signature motif HC(X)₅R, which contains an invariant catalytically active cysteine residue. Among the 107 human PTP genes, 11 are catalytically inactive, 13 dephosphorylate inositol phospholipids and 81 are active protein phosphatases (Alonso et al., 2004). PTPs are a structurally diverse family of enzymes and can be subdivided into three classes: Low molecular weight PTPs (LMW-PTPs), dual-specific PTPs (DSPs) and classical tyrosine-specific PTPs (Andersen et al., 2001). Only one LMW-PTP is expressed in the human genome, LMPTP, which constitutes with a molecular weight of 18 kDa the smallest PTP and has primary specificity for phosphotyrosine. The DSPs dephosphorylate pTyr residues as well as pSer/pThr residues. Important members of the DSP family are the phosphatidylinositol-specific lipid phosphatase PTEN, the MAP kinase phosphatases (MKPs), or the CDK phosphatases CDC25 and KAP (kinase-associated phosphatase).

This thesis focuses on members of the pTyr-specific classical PTPs. The human genome contains 38 members of this subfamily, which can be further divided into 21 receptor-like

PTPs (RPTPs) and 17 non-transmembrane enzymes (NTPTPs) which localize to the nucleus or cytoplasm (Tonks, 2006) (Figure 1). In addition to the conserved 280 amino acids long catalytic PTPase domain, one of the most striking features of classical PTPs is the occurrence of additional modular domains. These domains regulate their cellular activity for example by targeting them to substrates and subcellular compartments.

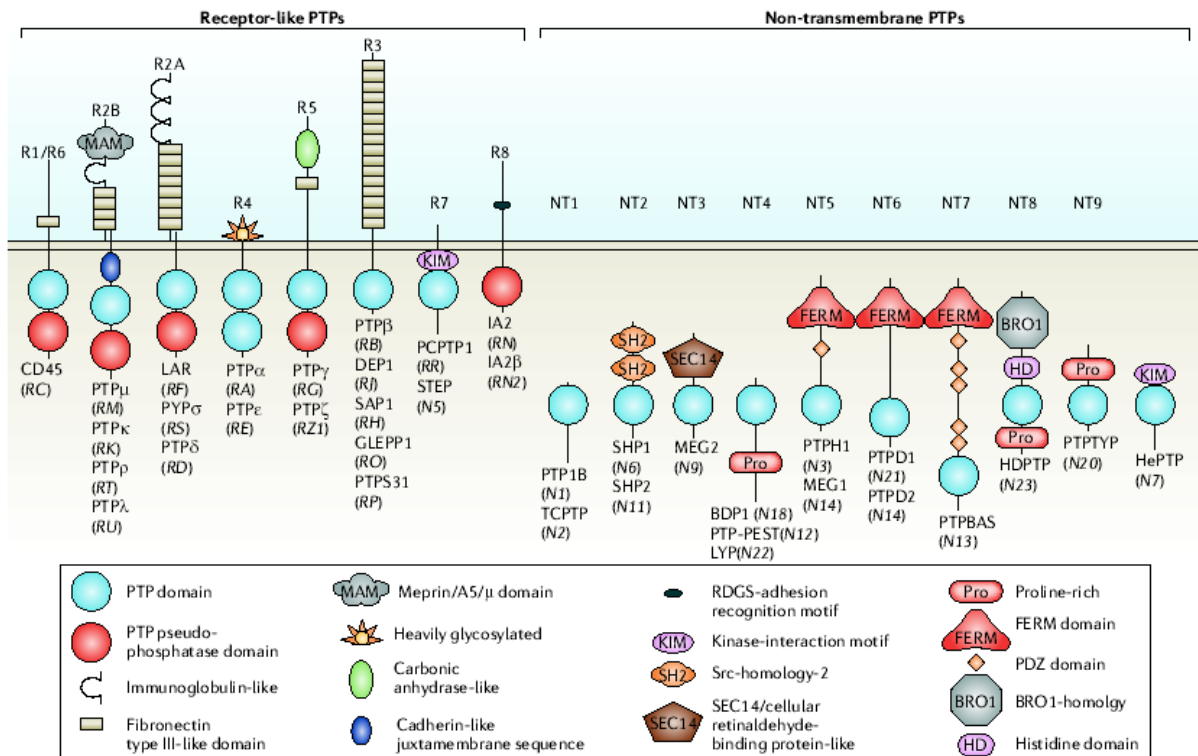


Figure 1: The classical Protein Tyrosine Phosphatase family (Tonks, 2006).

RPTPs consist of one or two PTPase domains in their intracellular segment, a type-I single transmembrane region and structurally diverse extracellular parts. There are 12 human RPTPs containing a tandem arrangement of phosphatase domains. Studies on the enzymatic activity of RPTPs indicate that all of the dephosphorylating activity relies on the membrane-proximal (named D1) PTPase domain. The membrane distal (named D2) PTPase domains are pseudo-phosphatase domains, as mutations in essential catalytic residues render them almost completely inactive (den Hertog, 1999), (Wu et al., 1997). Interestingly, replacement of only two residues in RPTP^α D2 with those that are present in RPTP^α D1 converts this poorly active domain into an active PTPase domain (Lim et al., 1998). Moreover, the D2 domain can function to regulate the catalytic activity of the RPTP D1 domain. For instance, RPTP^α D2 is

important for the regulation of D1 phosphatase activity by dimerization (Blanchetot et al., 2002), (Jiang et al., 2000).

The extracellular parts of RPTPs are variable in size, often glycosylated and contain motifs commonly found in cell adhesion proteins. Domains being identified in these extracellular regions include fibronectin type III (FNIII)-like domains, immunoglobulin (Ig)-like domains, the MAM (Meprin, A5-like, Mu) domain, as well as a carbonic anhydrase-like domain (den Hertog, 1999). Similarly as for RTKs, one function of RPTP extracellular domains is to bind ligands, leading to modulation of their intracellular PTP activity. Another function is the regulation of RPTP dimerization. For instance, it has been reported that CD45 splice variants with shorter extracellular domains homodimerize more easily, than those variants with larger extracellular domains (Xu and Weiss, 2002). The effects of dimerization and ligand binding on RPTP activity will be discussed in the following section in more detail.

Soon after purification and characterization of the first non-transmembrane tyrosine phosphatase PTP1B (Tonks et al., 1988b), CD45 was the first receptor-like protein to be identified that contains PTPase domains (Tonks et al., 1988a). CD45 belongs to the R1/R6 subtype of RPTPs and its extracellular part is composed of a heavily glycosylated FNIII-like domain. It is a positive regulator of antigen- and immunoglobulin-receptor signaling in T-cells and as a consequence, CD45-deficient humans or mice develop a severe combined immunodeficient phenotype (Mustelin et al., 2005). In addition, it has been addressed as a drug target in autoimmune diseases as its inhibition leads to decreased T-cell signaling (Penninger et al., 2001). CD45 acts in a positive manner on T-cell receptor signaling by dephosphorylating the C-terminal negative-regulatory tyrosine residue of SRC-family PTKs, such as LCK, FYN, YES or BLK. This positive modulation of signal transmission is a good example for specificity of PTP action, disproving the hypothesis that PTPs only serve housekeeping functions as negative regulators of cell signaling. As mentioned above, CD45 can undergo dimerization which was shown to inhibit phosphatase activity via insertion of an N-terminal wedge domain of one D1 PTPase domain *in trans* into the D1 domain of the adjacent dimerization partner (Majeti et al., 1998). Interestingly, a loss-of-function mutation in the N-terminal wedge (E613R), causes aberrant CD45 regulation in transgenic mice, accompanied by lymphoproliferation, autoantibody production and death (Majeti et al., 2000). Similarly like CD45, also R4 type RPTPs, such as RPTP α , contain this N-terminal wedge domain in their D1 domain and are inhibited by dimerization. RPTP α is characterized by a short and heavily glycosylated extracellular part, which is in combination with the transmembrane domain, the juxtamembrane region and the D2 domain important for its

dimerization (Jiang et al., 2000). A further commonality to CD45 is the substrate specificity of RPTP α as it as well activates Src family kinases (Zheng et al., 1992).

The R2B subfamily is also named MAM-family of RPTPs because all of its members, such as RPTP μ and RPTP κ , contain the MAM domain. This domain appears to be important for lateral dimerization or oligomerization of receptor molecules as well as their proper folding and transport through the secretory pathway (Tsukuba and Bond, 1998), (Cismasiu et al., 2004). Comparable to MAM-family phosphatases, R2A subfamily RPTPs such as PTP-LAR contain Ig-like domains and multiple FNIII-like repeats. Both domains are also present in cell adhesion molecules like N-CAM or Ng-CAM and permit homo- and heterophilic interactions between receptor molecules. This structural feature suggests a function of respective RPTPs in cell-cell and cell-matrix adhesion (Sallee et al., 2006), (Burrige et al., 2006). Indeed, members of the MAM-family of PTPs are localized at sites of cell-cell contact, whereas PTP-LAR has been observed at points of cell-matrix attachment. Also, R3 type RPTPs whose extracellular domains consist only of FNIII-like domains have been linked with cell adhesion control. For instance, protein expression of DEP-1 increases with cell density proposing a function in contact inhibition of cell growth (Holsinger et al., 2002). Of particular interest is also the carbonic anhydrase-like domain of RPTP ζ , which is enzymatically inactive due to mutation of key histidine residues essential for hydration of CO₂ and forms instead a hydrophobic pocket for ligand binding (Krueger and Saito, 1992). The heparin-binding growth factor pleiotrophin was found to bind to RPTP ζ and to negatively modulate its activity (Meng et al., 2000). Concomitantly, increased tyrosine phosphorylation of its substrate protein β -catenin was observed as a result of pleiotrophin binding. This is so far the only known example of a classical soluble ligand binding to a RPTP.

In analogy to the extracellular domains of RPTPs, also non-transmembrane PTPs contain regulatory sequences that flank the catalytic domain and control intracellular activity (Tonks, 2006). These non-catalytic regions control PTP function for example by regulating subcellular localization. This is the case for NT1 type PTPs, such as PTP1B and TcPTP, which are both localized to the endoplasmatic reticulum (ER) via C-terminal hydrophobic amino acids (Frangioni et al., 1992). As a consequence PTP1B can solely access proteins which colocalize to the ER, such as RTKs on their biosynthetic way from the ER to the plasmamembrane (Lammers et al., 1993), (Boute et al., 2003). Other cellular compartments can be reached only by PTP1B when its C-terminus is cleaved by calpains (Kuchay et al., 2007) or in the case of TcPTP, when the hydrophobic membrane anchor is absent due to alternative splicing (Lorenzen et al., 1995). The regulatory domains of other cytosolic PTPs such as SH2

domains, proline-rich domains, FERM domains and PDZ domains serve a function as protein-protein interaction motifs. In addition, FERM and PDZ domains allow protein binding to phosphatidylinositol-4,5-bisphosphate (PIP₂) in the plasma-membrane. The NT2 members SHP-1 and SHP-2 contain pTyr-specific SH2 domains that are both important for substrate binding and activity regulation (Feng and Pawson, 1994). When these PTPs are not bound to a tyrosine phosphorylated substrate protein, the N-terminal SH2 domain occupies and inactivates the active site. Upon binding of the C-terminal SH2 domain to a pTyr-containing substrate protein, a conformational change abrogates this intramolecular interaction and activates these PTPs (Barford and Neel, 1998), (Hof et al., 1998). Even though both SHP-1 and SHP-2 share 60% sequence identity, their biological functions are totally different. SHP1 acts as negative regulator of several pathways in the hematopoietic system, including growth factor, cytokine, cell adhesion and antigen receptor signaling (Neel and Tonks, 1997). In contrast, SHP2 modulates signaling from particular growth factor receptors, like the EGFR, the FGFR or the PDGFR to the downstream MAPK pathway in a positive way (Yu et al., 1998), (Qu et al., 1999). The proline-rich domain of the NT4 family member PTP-PEST was shown to be important for binding to the SH3 domain of its substrate protein p130cas (Garton et al., 1997). In context with this, fibroblasts being deficient in PTP-PEST show strong motility defects (Sastry et al., 2006). Interestingly, in addition to p130cas, many other proteins involved in cell motility regulation contain SH3 domains, such as cortactin, ZO proteins, RhoGAP proteins or vav-family members. PTPBAS, PTPH1, PTPD1/2 and PTPMEG contain FERM domains (Zhang et al., 1995), (Moller et al., 1994), (Gu and Majerus, 1996). These domains allow targeting of proteins to cytoskeleton-membrane interfaces (Arpin et al., 1994). However, the regulatory function of FERM domains in PTPs is still poorly understood. Since activity of PTPs towards its substrate proteins is in general rather high and needs to be focussed to subcellular regions and protein complexes, the above described regulatory elements serve essential functions in the homeostasis of cellular protein tyrosine phosphorylation.

3. Insights into PTP catalysis

3.1 *The catalytic mechanism of PTPs*

Protein tyrosine phosphatases exert control about their cellular substrates by hydrolytic cleavage of a covalently attached phosphate group on tyrosine residues. Central to this catalytic mechanism is the cysteine residue in the signature motif HC(X)₅R residing at the bottom of the active site cleft (Guan and Dixon, 1991). Structural studies demonstrated that this cleft forms an approximately 9 Å deep phosphotyrosine binding pocket (Figure 2A). The depth of this pocket fits exactly the length of a pTyr residue and allows specificity for pTyr, since shorter pSer/pThr moieties cannot reach to the bottom to be hydrolyzed (Jia et al., 1995). The active site consists of four loops: (1) the pTyr-loop which defines the depth of the cleft and therewith pTyr-specificity, (2) the PTP-loop at the bottom of the cleft positioning the catalytically active cysteine residue, (3) the WPD-loop containing an invariant aspartic acid residue that is involved as a general acid and base in both catalytic steps, and (4) the Q-loop which contributes a glutamine residue to the active center that is important for coordinating a H₂O molecule for hydrolytic cleavage. Binding of pTyr containing substrates to the active center of PTPs is a nice example of Koshland's "induced fit" concept, which describes an induction of conformational changes in enzymes upon interaction with their substrate molecules. In the case of PTPs, the catalytic cleft closes around the substrate's pTyr moiety and the catalytically active residues are positioned properly for the hydrolytic cleavage.

The catalytic mechanism of PTPs can be subdivided into two steps, which involve the formation of a cysteinyl-phosphate intermediate (Figure 2B) (Pannifer et al., 1998). Central to this mechanism is the unprotonated thiolate-anion on the catalytic cysteine residue, which has a pK_a value of 4.5 to 5.5 due to its surrounding amino acids (normal pK_a of Cys ~ 8.5) (Lohse et al., 1997). This thiolate-anion attacks the phosphate group of a substrate pTyr residue in a nucleophilic way to generate a cysteinyl-phosphate intermediate (Step 1). The protonated aspartic acid of the WPD loop supports cleavage of the P-O bond by acting as a general acid catalyst in this process. In the second step, the cysteinyl-phosphate intermediate is then hydrolyzed by a H₂O molecule, which is positioned in the active center by a glutamine residue on the Q-loop. The invariant aspartic acid residue serves in this step a general base function as it abstracts a proton from the H₂O molecule and returns to its original protonated state.

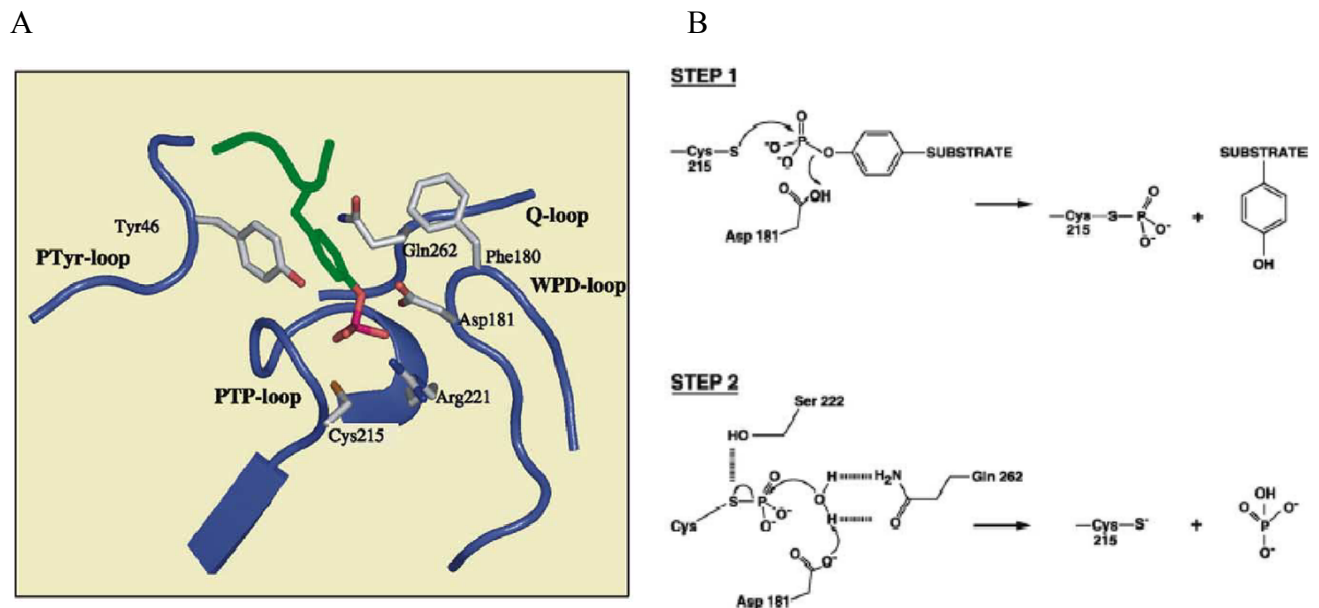


Figure 2: Reaction mechanism catalyzed by PTP1B and its architecture. (A) Structure of the active center of PTP1B in complex with a phosphotyrosine containing substrate peptide (shown in green). (B) Schematic representation of the catalytic mechanism of PTP1B. Step 1: Formation of the cysteinyl-phosphate intermediate. Step 2: Hydrolysis of the cysteinyl-phosphate intermediate (Barford et al., 1998) (Tonks, 2003).

3.2 Catalytically inactive substrate trapping mutants are used to study PTP substrate specificity

Mutational analysis of the catalytic cysteine residue by exchanging it with a Ser or an Ala residue showed an essential function of this amino acid for PTPase activity (Guan and Dixon, 1990). Similarly, substitution of the WPD-loop Asp residue or the Q-loop Gln residue abolished the catalytic activity almost completely. As these inactive phosphatase mutants cannot dephosphorylate their substrate molecules any more, they form stable complexes with tyrosine phosphorylated proteins (Flint et al., 1997), (Xie et al., 2002). These so called “substrate-trapping” mutants have been used widely to study substrate specificity of PTPs (Blanchetot et al., 2005). Three characteristics are important for good substrate trapping-mutants: (1) they need to be inactive or barely active (low k_{cat}), (2) they should have a strong binding affinity to physiological substrates (low K_m), and (3) their structural integrity must be similar to the wild-type conformation. Depending on the PTP of interest, different substrate-trapping mutants containing various combinations of the Cys→Ser (C-S), Asp→Ala (D-A) or Gln→Ala (Q-A) mutations were identified to have suitable PTP-substrate affinities. C-S substrate-trapping mutants have for instance been useful to study substrates of SHP1, SHP2 or

CD45, whereas D-A mutants showed better substrate affinities in case of PTP1B, TcPTP, PTPH1 or DEP1 (Blanchetot et al., 2005). In addition, also combinations of these mutations were generated to improve this approach. Agazie *et al.* used D-A/C-S mutants of SHP2 to identify the EGFR and Gab1 as substrate proteins (Agazie and Hayman, 2003). In this case, the double substrate-trapping mutant was more effective than either mutant alone. This was also found to be true for PTP1B, as its D-A/Q-A substrate trapping mutant is fivefold better in substrate trapping than its single D-A or C-S mutants (Xie et al., 2002).

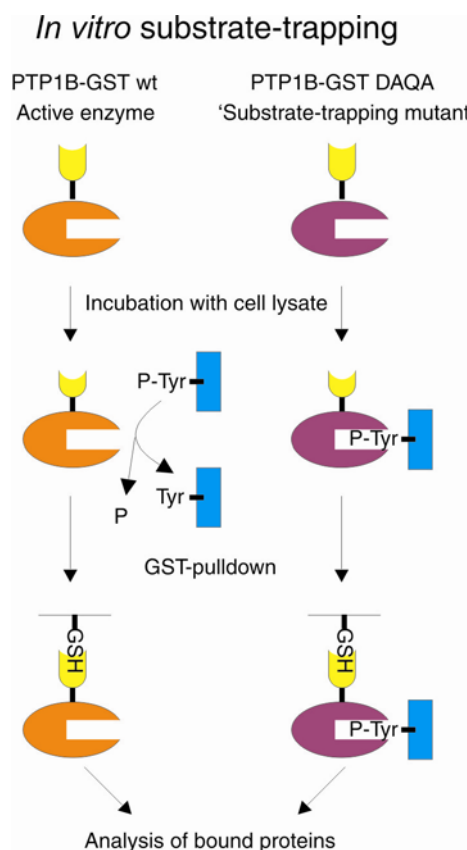


Figure 3: *In vitro* substrate trapping approach to identify PTP substrates. Recombinant PTP1B-GST wild-type and phosphatase inactive (D182A/Q262A) fusion proteins are incubated with cell lysates. Substrate-trapping mutants form stable interactions with substrates via pTyr-dependent binding to the inactive catalytic center.

There are two different methodologies to study PTP substrate proteins using substrate trapping-mutants. First, the *in vitro* binding of substrates out of cellular extracts to recombinant substrate-trapping mutants, and second, the *in vivo* association of substrate proteins to PTPase inactive mutants expressed in living cells (Blanchetot et al., 2005). The *in vivo* substrate-trapping method relies on sufficient expression levels of the inactive phosphatase mutants and is therefore difficult to scale up, but gives important information

about the localization of PTPs in complex with their substrates in living cells (Haj et al., 2002). On the other hand, the *in vitro* substrate-trapping approach allows an easy scale up of experiments, which is often necessary due to the weak binding affinities of PTP substrate-trapping mutants. Figure 3 illustrates an experimental approach using *in vitro* substrate trapping to enrich sufficient amounts of substrates for subsequent analysis by means of Western blotting or mass spectrometry. Together with loss-of-function models and ectopic expression techniques, the substrate-trapping approach is one of most important and commonly used method to elucidate cellular functions of protein tyrosine phosphatases.

3.3 Regulation of PTP activity via oxidation of the active site

Reversible regulation of signal transduction pathways is a prerequisite for the dynamic uptake of external information by cells in higher ordered organisms. Besides reversible phosphorylation, protein components of many signal transduction pathways are also regulated by reversible oxidation (Spickett et al., 2006). Signal transduction via protein phosphorylation is linked to redox signaling, as oxidation constitutes an important reversible mechanism for the regulation of PTP activity. The catalytically active cysteine residue in PTPase domains displays an unusually low pKa and is predominantly present as a thiolate anion at neutral pH. Although, this enhances its function as a nucleophile in catalysis, it also renders PTPs susceptible to inactivation by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (Tonks, 2003). A variety of physiological stimuli induce production of the intracellular messenger H_2O_2 , which reversibly oxidizes the thiolate anion to a singly oxidized sulfenic acid form (Figure 4) (Denu and Tanner, 1998), (Meng et al., 2002). Thereby PTPase activity is inhibited, as no nucleophilic attack on pTyr residues can take place anymore. Further oxidation converts sulfenic acid into the doubly oxidized sulfinic acid and the triply oxidized sulfonic acid. These additional oxidations are irreversible and can be induced by the potent PTP inhibitor pervanadate (Huyer et al., 1997) or for instance by long term incubation of recombinant PTP1B for more than 16 h with physiological concentrations of H_2O_2 (Salmeen et al., 2003). Salmeen *et al.* could demonstrate in a crystallographic study that usually before the irreversible oxidation of PTP1B by H_2O_2 takes place, the sulphenic acid intermediate converts under loss of a water molecule into a sulfenylamide species (Salmeen et al., 2003). This conversion takes place via the formation of a covalent bond between the sulfur atom of the active Cys215 and the main chain nitrogen of the adjacent

Ser216. By this, a novel five-atom ring structure is produced at the active site. The sulfenamide species protects PTP1B from further oxidation and is accompanied by large conformational changes in the catalytic site which prevents substrate binding. In addition, access of reducing agents to the now exposed sulfenamide facilitates reversion to the active state of the enzyme. *In vitro* this can be achieved by incubation with the reducing agent DTT and in living cells with the redox regulator glutathione. This mechanism is an interesting example for the physiologically important reversible regulation of PTP activity by oxidation and reduction. Other PTP family members have also been shown to be reversibly regulated by similar redox mechanism, such as PTEN and Cdc25 (Lee et al., 2002), (Caselli et al., 1998). In these cases, instead of a sulphenamide a disulfide bridge is formed between the active cysteine residue and vicinal cysteines. However, the pTyr-specific classical PTPs do not contain any equivalent thiol residues at their active sites.

The physiological relevance of PTP inactivation by ROS-mediated oxidation has been demonstrated for various signaling mechanisms: ROS-mediated inactivation of PTPs is crucial for (1) phosphorylation and activation of downstream signaling proteins of the EGFR (Bae et al., 1997) and the PDGFR (Sundaresan et al., 1995) upon ligand stimulation, (2) the activation of Lyn kinase and the signal intensity of B-cell antigen receptor signaling (Singh et al., 2005), (3) TNF α -induced cytotoxic JNK activation in the absence of Nf κ B (Kamata et al., 2005), and (4) UV-induced activation of the EGFR in keratinocytes (Xu et al., 2006). Thus, redox-mediated regulation of phosphotyrosine signaling is another example which highlights the importance for a tight control of PTP activity in living cells.

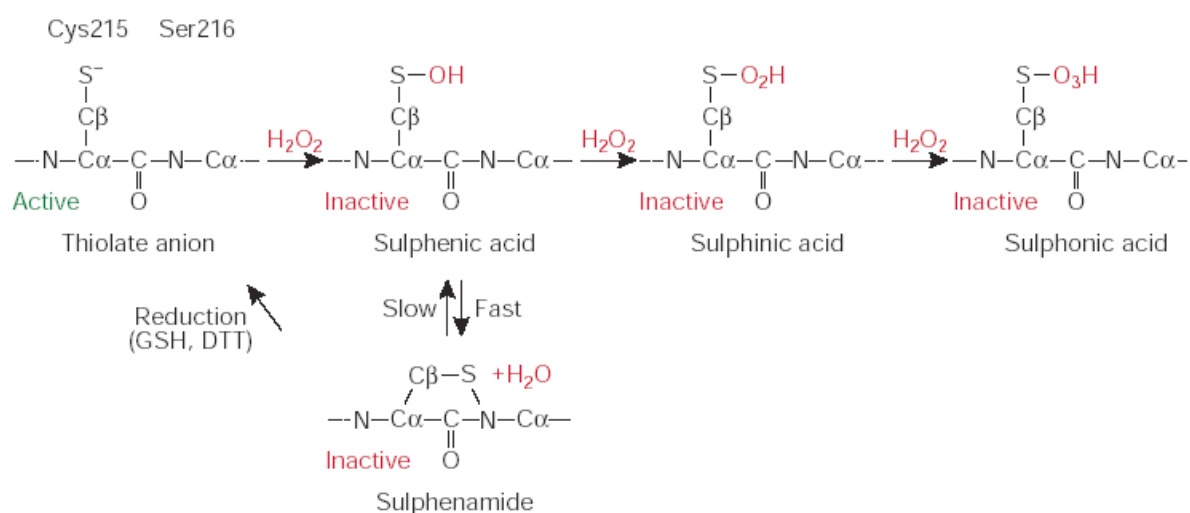


Figure 4: Reversible redox regulation of the catalytically active Cys215 in PTP1B (den Hertog, 2003).

4. Deciphering PTP function from the substrate perspective

4.1 Control of epithelial cell adhesion by MAM-family member RPTP κ

Epithelial tissues are held together by stable intercellular adhesions and cell junctions between interacting cells. Adherens junctions (AJs) and desmosomes allow strong cell-cell adhesion, whereas tight junctions (TJs) form a virtually impermeable barrier to molecules and ions between cell spacings. AJs in epithelia are mainly composed of the adhesion molecule E-cadherin, whose extracellular domains allow homophilic Ca^{2+} -dependent adhesion, and intracellular adaptor proteins, linking sites of cell-cell contact to the actin cytoskeleton, such as α - and β -catenin or p120ctn (Perez-Moreno and Fuchs, 2006). The opposing actions of PTKs and PTPs regulate the integrity of adherens junctions by protein tyrosine phosphorylation. In general, increased tyrosine phosphorylation of AJs components leads to disruption of cell-cell adhesion and disassembly of AJs from the cytoskeleton (Nelson and Nusse, 2004). For example, phosphorylation of β -catenin by Src or the EGFR disrupts its binding to cadherins (Roura et al., 1999), whereas phosphorylation by Fer diminishes binding of β -catenin to α -catenin (Piedra et al., 2003). Piedra *et al.* further showed that phosphorylation of p120ctn by Src results in loss of cadherin complexes from the cell surface. Cytosolic PTPs such as PTP1B or SHP2, as well as RPTPs like RPTP κ , RPTP μ , DEP-1, and VE-PTP have been found to localize and bind to members of the cadherin-catenin complex (Sallee et al., 2006), (Fuchs et al., 1996). These phosphatases are acting against the above mentioned PTKs to stabilize cell adherens junctions.

Cadherin cell adhesion molecules interact homophilically with each other and perform an *in vivo* cell sorting function during embryonic development to separate for instance N-cadherin expressing neural epithelial cells from E-cadherin expressing ectoderm cells (Gumbiner, 2005). Similarly, members of the R2B or MAM-family of RPTPs, like RPTP κ and RPTP μ , exhibit strict homophilic binding properties and cause cell aggregation when expressed on the surface of normally non-adhesive cells (Brady-Kalnay and Tonks, 1994), (Sap et al., 1994). Furthermore, RPTP κ and RPTP μ show complementary but distinct expression patterns during embryonic development, as RPTP κ is mainly expressed in tissues of epithelial origin, whereas RPTP μ predominantly occurs in the vasculature (Fuchs et al., 1998). These studies suggest an *in vivo* cell sorting function for MAM-family RPTPs, similarly to the one observed for cadherins. Homophilic binding of the extracellular domains of MAM-family RPTPs occurs *in trans* (between opposing cells) and *in cis* (on the same cell). Aricescu *et al.* elucidated in two

crystallographic studies the extracellular structure of RPTP μ and characterized high affinity *trans* interactions between the MAM, IgG-like and the first FNIII-domains of opposing RPTP μ extracellular parts (Figure 5A&B) (Aricescu et al., 2006), (Aricescu et al., 2007). Moreover, they hypothesized that further low affinity cis interactions which are thought to be mediated by the second FNIII-like domain are necessary for the cell adhesion function. Interestingly, serial deletion of FNIII-like domains as shown in Figure 5B revealed a novel spacer-clamp function of MAM-family RPTPs, as the plasmamembrane distances of neighboring cells directly correlated with the length of the extracellular domain of RPTP μ (Aricescu et al., 2007).

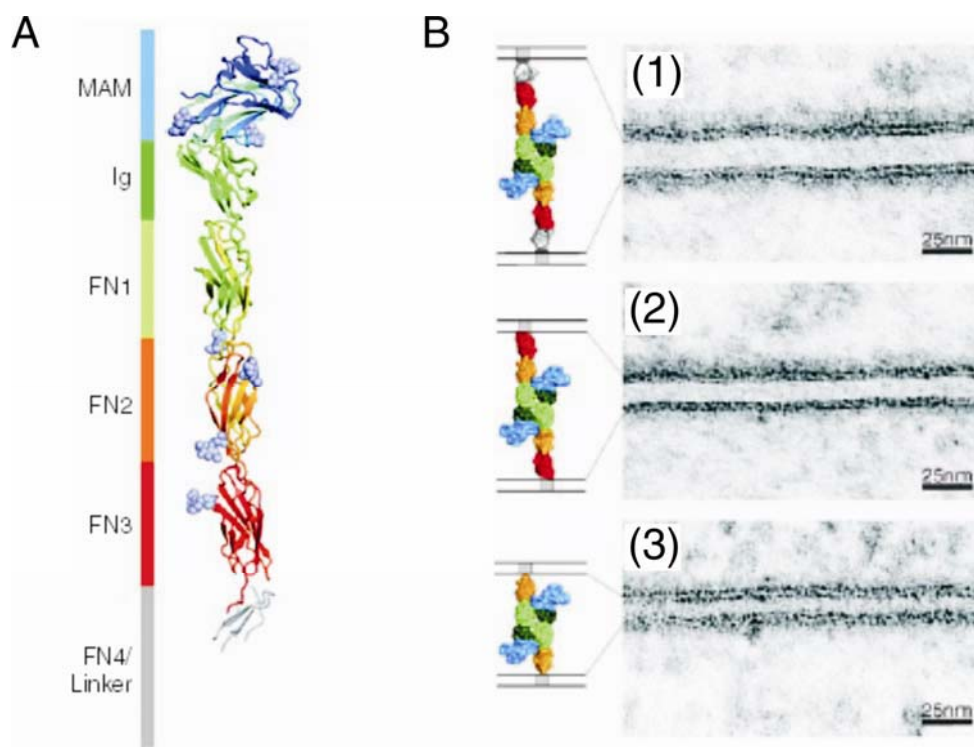


Figure 5: Structure of the extracellular part of the MAM-family member RPTP μ , its homophilic binding and its function as a spacer-clamp. (A) Ribbon diagram depicting the modular extracellular structure of the RPTP κ -related R2B phosphatase RPTP μ . (B) Serial deletion analysis of one (2) and two (3) FNIII-like domains reveals a novel distance gauge mechanism for R2B RPTPs. Note the minimal dimerization unit consisting of the MAM domain, the IgG-like domain and the first FNIII-like domain. (Aricescu et al., 2007)

The proposed function of homophilic binding of these extracellular domains is to position MAM-family RPTPs at cell-cell adhesion sites (Del Vecchio and Tonks, 2005). This allows

for example colocalization and binding of RPTP κ to its putative substrate β -catenin at cell AJs, to stabilize cell-cell adhesion (Fuchs et al., 1996). Concordantly, overexpression of RPTP κ decreased and siRNA-mediated down-regulation increased cell motility in colon cancer cells (Kim et al., 2006). Further support for the hypothesis that MAM-family RPTPs can directly sense cell-cell contacts came from studies, where an increased expression of these phosphatases was detected at high cell densities (Fuchs et al., 1996), (Gebbink et al., 1995). These observations have led to the proposal, that MAM-family RPTPs could mediate contact inhibition of cell growth. In line with this goes the identification of the EGFR as another substrate of RPTP κ (Xu et al., 2005). For instance, negative regulation of this important growth factor receptor by RPTP κ decreases cell proliferation in human keratinocytes.

Homophilic binding of the extracellular domains of MAM-family RPTPs leads to a local increase in phosphatase activity at cell-cell adhesion sites and thereby to stabilization of these intercellular connections. Recently, Aricescu *et al.* speculated that due to the high affinity of these *in trans* interactions, the only way to break up cell-cell adhesion is the proteolytic cleavage and shedding of the extracellular domains of cell adhesion molecules such as MAM-family RPTPs (Aricescu et al., 2007). A mechanism like that would allow cells to switch from a resting state in close cell-cell contact with other cells to a motile state without intercellular connections. In fact, proteolytic cleavage and shedding of the extracellular parts of RPTPs has already been shown for the R2A phosphatases LAR and RPTP σ , leading to their relocalization and internalization away from AJs and desmosomes (Aicher et al., 1997). Later on, the metalloprotease TACE was found to be the responsible sheddase for LAR cleavage (Ruhe et al., 2006). Also MAM-family RPTPs were found to be regulated by metalloproteinase-mediated shedding of their extracellular domains, but the identity of the metalloproteinase and the functional consequences of this proteolytic cleavage remained unclear (Anders, 2004).

In this study, we have characterized the regulation of RPTP κ by limited proteolysis in greater detail. We identified ADAM10 to be responsible for RPTP κ cleavage and found that the phosphatase gets further processed by γ -secretase, leading to liberation of its intracellular part from the plasmamembrane and its translocation to the nucleus. Furthermore, we observed that PTP activity was not altered by these processing steps and consequently we studied the regulation of β -catenin, a direct substrate of RPTP κ , at the plasmamembrane and in the nucleus.

4.2 PTP1B function in metabolism and cancer

Protein tyrosine phosphatase 1B (PTP1B) was the first protein tyrosine phosphatase to be identified (Charbonneau et al., 1989) and is currently one of the best understood members of the classical protein tyrosine phosphatase family. It has received much attention due to its proposed role in type2 diabetes, obesity, immunity and cancer (Dube and Tremblay, 2005), (Tonks, 2003). Fundamental insights into its physiological functions have been achieved by loss-of-function mouse genetics models. PTP1B is not required for embryonic development and PTP1B-deficient mice develop normally. However, when subjected to high fat diet, these mice remain insulin sensitive and are resistant to weight gain, whereas wild-type littermates become insulin resistant and gain weight rapidly (Elchebly et al., 1999), (Klaman et al., 2000). Early studies on the cellular function of PTP1B revealed a negative regulatory function on insulin receptor (IR) phosphorylation (Cicirelli et al., 1990), (Lammers et al., 1993). In addition, direct physical interaction of PTP1B to the IR was detected using the substrate-trapping approach (Seely et al., 1996). As a functional consequence of IR dephosphorylation it was found that overexpression of PTP1B in rat adipose cells reduced GLUT4 translocation to the cell surface and cellular glucose uptake (Chen et al., 1997). Moreover, in a crystallographic study, Salmeen *et al.* identified specificity of PTP1B for the consensus tandem pTyr motif X-pY-pY-X in the activation loop of the IR (sites Tyr-1162/1163) (Salmeen et al., 2000). *In vivo*, PTP1B knock-out mice showed increased phosphorylation levels of the IR in muscle tissues and the liver, but not in adipose tissues (Figure 6, right side) (Elchebly et al., 1999), (Klaman et al., 2000). This increased insulin-sensitivity upon PTP1B-deficiency highlights the important regulatory function of PTP1B in IR signaling and glucose metabolism.

Concordant with the tandem pTyr motif of the IR, also the cytoplasmic tyrosine kinases JAK2 and TYK2 contain an E/D-pY-pY-R/K motif in their activation loops. Based on this information, both kinases were identified to be substrates of PTP1B by means of substrate-trapping experiments and due to increased phosphorylation in PTP1B-deficient fibroblasts (Myers et al., 2001). Janus kinases (JAKs) phosphorylate and activate the transcriptional regulatory STAT proteins. JAKs act downstream of a broad range of receptors specific for interferons, interleukins, prolactin, growth hormone and also the adipokine leptin (Kerr et al., 2003), (Forsyth and Wallis, 2002), (Fruhbeck, 2006). Interestingly, leptin is a key adipokine (adipocyte-secreted hormone) which is responsible for increasing energy expenditure and

limiting food uptake in mammals by acting on the hypothalamus. Studies on leptin signaling in the hypothalamus of PTP1B deficient mice have revealed enhanced leptin sensitivity and

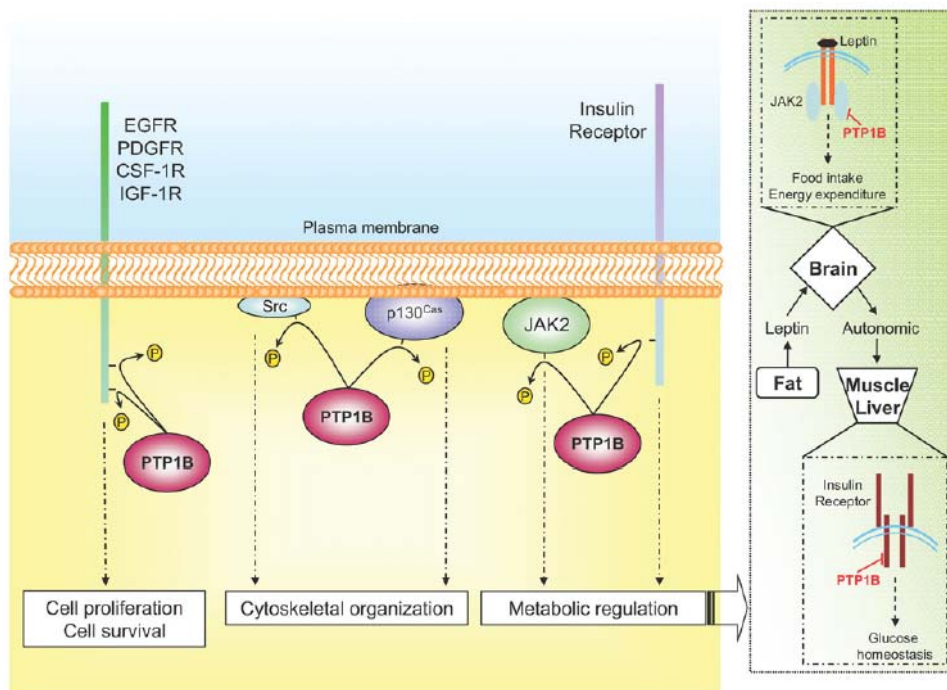


Figure 6: Physiological and cellular functions of PTP1B and its substrates (Tiganis and Bennett, 2007).

increased STAT3 phosphorylation (Cheng et al., 2002), (Zabolotny et al., 2002). Conditional deletion of PTP1B in the brain further supported these observations, as these mice have reduced weight and adiposity (Bence et al., 2006). Thus, neuronal deletion of PTP1B connects its body mass and adiposity regulating function directly to leptin signaling in the hypothalamus due to dephosphorylation of JAK2.

In tumorigenesis, different functions were assigned to PTP1B dependent on its site of action. As a negative regulator of oncogenic PTKs such as the EGFR, the PDGFR (Figure 6, left side) (Haj et al., 2003) or p210 Bcr-Abl (LaMontagne et al., 1998a), PTP1B was thought to act as a tumor suppressor in malignancies. In deed, PTP1B was shown to suppress oncogenic transformation of murine fibroblasts overexpressing p210 Bcr-Abl (LaMontagne et al., 1998b), or other oncogenic PTKs like Neu (Brown-Shimer et al., 1992) or c-Src (Woodford-Thomas et al., 1992). Despite of the potential of enhanced oncogenic signaling in PTP1B-deficient mice, its deletion does not lead to any malignancies or development of spontaneous tumors. To study in more detail what influence PTP1B has on the development of

malignancies in tumor-prone mice, PTP1B knock-out mice were crossed with p53-deficient mice which develop mainly B-cell lymphomas (Dube et al., 2005). Loss of PTP1B was shown to decrease survival rate and increase susceptibility towards development of B-cell lymphomas in p53 null mice. Therefore a tumor-suppressive activity of PTP1B was discussed in B-cell lymphomas, but no specific signaling pathway or substrate could be assigned to this function (Dube et al., 2005). On the other hand, crossing of PTP1B-deficient mice with transgenic mice expressing activated forms of ErbB2 in the breast, revealed an oncogenic function of PTP1B in mammary tumorigenesis (Julien et al., 2007), (Bentires-Alj and Neel, 2007). Julien *et al.* found that loss of PTP1B leads to significantly delayed breast tumor development and also decreased the incidence of lung metastases. Moreover, administration of an orally available small-molecule inhibitor against PTP1B also delayed tumorigenesis (Julien et al., 2007). These effects were correlated to attenuated activation of the Ras/MAPK pathway. In fact, PTP1B was already before linked to positive regulation of Ras signaling, by dephosphorylating p62DOK which binds to and positions p120RasGAP, a Ras negative regulator (Dube et al., 2004). Further support for an oncogenic activity in breast tumors comes from an early study which reported that PTP1B is overexpressed in human breast cancer specimens and that its expression correlates with ErbB2 expression (Wiener et al., 1994). Interestingly, PTP1B was identified to be the primary PTP which dephosphorylates and thereby activates c-Src in several human breast cancer cell lines (Bjorge et al., 2000). However, no change in c-Src activity was observed in the above described animal models of breast cancer.

Activation of c-Src by PTP1B was also found to be important in integrin signaling and fibronectin-induced cell spreading and migration (Figure 6, middle part) (Cheng et al., 2001). Moreover, PTP1B regulates tyrosine phosphorylation of p130Cas, which is an important adaptor protein in integrin mediated motility responses (Kabuyama et al., 2006). In contrast, migration studies using non-coated cell culture dishes revealed a strong increase in cell motility in PTP1B-deficient cells (Buckley et al., 2002). Similarly, ectopic expression of PTP1B in Hela cells has been shown to reduce serum induced cell migration (Yigzaw et al., 2003). So far, the regulation of cell motility processes by PTP1B remains controversial and different cellular functions were observed in diverse cellular systems.

As not all cellular and physiological functions of PTP1B can be explained with the already existing set of known substrates, we used mass spectrometry-based methods to identify so far unknown substrates of this prototypic phosphatase. To this end, we employed quantitative MS analysis to study phosphotyrosine signaling in PTP1B-deficient fibroblasts in this study.

Additional information about the physical interaction of potential substrates to the active site of PTP1B was obtained by using the substrate-trapping approach in a second MS analysis. This combined effort allowed us to propose novel substrates of PTP1B, which correspond to the described phenotype of its knock-out mouse fibroblasts.

II. Specific Aims

Signal transduction via reversible protein tyrosine phosphorylation is crucial for cellular processes such as proliferation, migration and differentiation in health and disease. Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) regulate these processes in an antagonistic way, however the family of PTPs is only poorly characterized compared to PTKs. To elucidate the cellular functions of PTPs in more detail it is necessary to understand the way their enzymatic activity is regulated and to know the substrate proteins which are affected by this activity.

In this thesis, the goal of the first project was to characterize the regulation of the receptor-like RPTP κ by proteolytic cleavage. In addition to identifying the involved proteases, also the functional outcome of this process on subcellular localization of RPTP κ and its activity was investigated.

The objective of the second project was to establish new experimental approaches to study cellular substrate specificity of PTPs in a global and generic way. Mass spectrometry-based methods were applied to identify novel substrates of the prototypic cytosolic PTP1B. To this end experimental strategies were developed which allow a systematic analysis of cellular protein tyrosine phosphorylation signaling and the identification of proteins physically interacting with the active site of PTPs.

III. Materials and Methods

1. Material sources

1.1 Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
Batimastat	British Biotech, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Chloroquin	Biotrend Chemikalien, Köln
Crystal Violet	Sigma, Taufkirchen
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
EGF	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Geneticin (G418, GibCo)	Invitrogen, Eggenstein
HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))	Serva, Heidelberg
IAA (Iodoacetic acid)	Sigma, Steinheim
IPTG (Isopropyl β -D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine (GibCo)	Invitrogen, Eggenstein
Lipofectamine 2000® (GibCo)	Invitrogen, Eggenstein
Lysozyme	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
Trifluoperazine	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg

All other chemicals were purchased in analytical grade from Merck (Darmstadt).

1.2 Chemicals for SILAC and MS-analysis

Acetonitrile for HPLC	Sigma, Taufkirchen
Ammoniumbicarbonate	Sigma, Taufkirchen
Ammonium hydroxide	Merck, Darmstadt
Antioxidance	Invitrogen, Eggenstein
2,5-Dihydroxybenzoic acid	Fluka, Taufkirchen
DTT	Sigma, Taufkirchen
Fetal bovine serum, dialyzed	Gibco, USA
Iodoacetamide	Sigma, Taufkirchen
L-Arginine	Gibco, USA
L-Arginine: HCl, U- ¹³ C ₆ ¹⁴ N ₄	Cambridge Isotope Laboratories, USA
L-Arginine: HCl, U- ¹³ C ₆ ¹⁵ N ₄	Cambridge Isotope Laboratories, USA
L-Glutamine	Gibco, USA
L-Lysine	Gibco, USA
L-Lysine: 2 HCl, ² H ₄	Cambridge Isotope Laboratories, USA
L-Lysine: 2 HCl, U- ¹³ C ₆ ¹⁵ N ₂	Cambridge Isotope Laboratories, USA
Lys-C	WAKO, Neuss
n-octosylglucoside	Roche, Mannheim
Penicillin/Streptomycin, 100x	PAA, Germany
SILAC DMEM	Gibco, USA
Thio urea	Invitrogen, Eggenstein
Trypsin (seq. grade modified)	Promega, USA
Urea	Merck, Darmstadt

1.3 Enzymes

Calf Intestine Alkaline Phosphatase	MBI Fermentas, St. Leon-Rot
DNAse I, RNAse free	Roche, Mannheim
Restriction Endonucleases	NEB, Frankfurt/Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
LA Taq-DNA Polymerase	Takara, Japan
Trypsin (GibCo)	Invitrogen, Eggenstein

1.4 “Kits“ and other materials

Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, UK
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
ECL Kit	PerkinElmer/NEN, Köln
GSTrap TM HP	Amersham Pharmacia, Freiburg
Hyperfilm MP	Amersham Pharmacia, Freiburg
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Amersham Pharmacia, Freiburg
Protein G-Sepharose	Amersham Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden

QIAquick PCR Purification Kit (50)	Qiagen, Hilden
QIAGEN Plasmid Mini Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, Rotenburg/Fulda

1.5 Growth factors and ligands

EGF (human)	Peprotech, USA
PDGF-BB (human)	Peprotech, USA
Insulin	Lilly, Giessen

2. Media

2.1 Bacterial media

LB or 2xYT media were used for cultivation of all *Escherichia coli* strains. If and as required 100 µg/ml Ampicillin or 70 µg/ml Kanamycin were added to media after autoclavation. For the preparation of LB-plates 1.5% Agar was also added.

LB-Medium	1.0 % Tryptone 0.5 % Yeast Extract 1.0 % NaCl pH 7.2
2xYT-Medium	1.6 % Tryptone 1.0 % Yeast Extract 1.0 % NaCl pH 7.2

2.2 Cell culture media

Gibco™ media and additives were obtained from Invitrogen (Eggenstein). Media were supplemented to the requirements of each cell line. Freeze medium contained 95% heat-inactivated FCS and 5% DMSO.

3. Stock solutions and commonly used buffers

BBS (2x)	50 mM 280 mM 1.5 mM pH 6.96	BES NaCl Na ₂ HPO ₄
Collecting gel buffer (4x)	0,5 M 0,4 %	Tris/HCl pH6.8 SDS
HBS (2x)	46 mM 274 mM 1.5 mM pH 7.0	HEPES, pH 7.5 NaCl Na ₂ HPO ₄
HNTG	20.0 mM 150 mM 0.1 % 10.0 % 10.0 mM	HEPES, pH 7.5 NaCl TritonX-100 Glycerol Na ₄ P ₂ O ₇
DNA loading buffer (6x)	0.05 % 0.05 % 30.0 % 100.0 mM	Bromphenol blue Xylencyanol Glycerol EDTA pH 8.0
Laemmli buffer (3x)	100 mM 3.0 % 45.0 % 0.01 % 7.5 %	Tris/HCl pH 6.8 SDS Glycerol Bromphenol blue β-Mercaptoethanol
NET	50.0 mM 5.0 mM 0.05 % 150.0 mM	Tris/HCl pH 7.4 EDTA Triton X-100 NaCl
PBS	137.0 mM 27.0 mM 80.9 mM 1.5 mM	NaCl KCl Na ₂ HPO ₄ KH ₂ PO ₄ pH 7.4
SD-Transblot	50.0 mM 40.0 mM 20.0 % 0.004 %	Tris/HCl pH 7.5 Glycine Methanol SDS
Separating gel buffer (4x)	0,5 M 0,4 %	Tris/HCl pH 8.8 SDS

“Strip” buffer	62.5 mM 2.0 % 100.0 mM	Tris/HCl pH 6.8 SDS β-Mercaptoethanol
TAE	40.0 mM 1.0 mM	Tris/Acetate pH 8.0 EDTA
TE10/0.1	10.0 mM 0.1 mM	Tris/HCl pH 8.0 EDTA pH 8.0
Tris-Glycine-SDS	25.0 mM 200.0 mM 0.1 %	Tris/HCl pH 7.5 Glycine SDS

4. Cells

4.1 Eukaryotic cell lines

Cell Line	Description	Origin/Reference
293-PS1-wt	HEK 293 cells stably expressing wild-type presenilin 1 cDNA	Haass, Munich
293-PS1-D385N	HEK 293 cells stably expressing dominant-negative presenilin 1 (D385N)	Haass, Munich
786-0	Human primary renal cell carcinoma	ATCC, USA
ADAM10 ^{+/+} MEFs	wild-type mouse embryonic fibroblasts	Saftig, Kiel
ADAM10 ^{-/-} MEFs	ADAM10 knock-out mouse embryonic fibroblasts	Saftig, Kiel
ACHN	Human primary renal cell carcinoma	ATCC, USA
Caki-1	Human renal metastatic cell carcinoma	ATCC, USA
COS-7	African green monkey kidney fibroblasts	ATCC, USA
HCT 116	Human colon carcinoma	ATCC, USA
HEK 293T	Human embryonic kidney fibroblasts, transformed with adenovirus Type V DNA	ATCC, USA
K562	Chronic myelogenous leukemia cell line, Bcr-Abl positive	ATCC, USA
NIH3T3	Mouse embryonic fibroblasts	ATCC, USA
PS 1 ^{+/+/2+/+}	wild-type mouse embryonic fibroblasts	De Strooper, Belgium
PS 1 ^{-/-/2^{-/-}}	Presenilin 1/2 double knock-out mouse embryonic fibroblasts	De Strooper, Belgium
PTP1B ^{+/+} MEFs	wild-type mouse embryonic fibroblasts immortalized with SV40 large T antigen	Tremblay, Canada
PTP1B ^{-/-} MEFs	PTP1B knock-out mouse embryonic fibroblasts immortalized with SV40 large T antigen	Tremblay, Canada

ATCC, American Type Culture Collection, Manassas, USA

DKFZ, Deutsches Krebsforschungszentrum, Heidelberg

4.2 *E. coli* strains

<u>E. Coli strain</u>	<u>Genotype Description</u>	<u>Origin/Reference</u>
DH5 α F'	F' endA1 hsd17 (r _k ⁻ m _k ⁺) supE44 recA1 gyrA (Nal) thi-1 Δ (lacZYA-argF196)	Genentech, USA
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacI ^q Z Δ M15 Tn10 (Tet ^r)]	Stratagene, NL
BL21 Rosetta TM (DE3)	F' ompT hsdS(r _B ⁻ m _B ⁻) gal dcm (DE3) pRARE ² (Cam ^r)	Novagen, USA

5. Antibodies and recombinant proteins

Names of people given as reference without further designation were members of this group.

5.1 Primary antibodies

The following antibodies were used for immunoprecipitation or as primary antibodies in immunoblot or immunofluorescence analysis.

<u>Antibody</u>	<u>Description/ Immunogen</u>	<u>Origin/Reference</u>
ADAM10	Rabbit, polyclonal, recognizes amino acids 732-748 of human ADAM10	Chemicon, Hofheim
ADAM15	Rabbit, polyclonal, recognizes amino acids 189-208 of human ADAM15	Stefan Hart
ADAM17/TACE	Rabbit, polyclonal, recognizes amino acids 807-823 of human ADAM17	Chemicon, Hofheim
β -Catenin	Mouse, monoclonal, binds C-terminal part of β -Catenin	Transduction Laboratories, USA
β -Catenin	Rabbit, polyclonal, recognizes amino acids 373-781	Thomas Müller
α -cortactin	Rabbit, polyclonal, directed against the KLH-coupled C-terminal peptide KGRYGLFPAN YVELRQ	Reiner Lammers
HA.11	Mouse, monoclonal, recognizes the influenza hemagglutinin epitope	BAbCo, USA
α -PLC γ	Mouse, monoclonal	Transduction Laboratories, USA

pTyr (4G10)	Mouse, monoclonal, recognizes phosphotyrosine residues	UBI, USA
RPTP α -D2	Rabbit, polyclonal, binds to the second PTPase domain	Reiner Lammers
RPTP μ -JM	Rabbit, polyclonal, binds juxtamembrane fragment of RPTP μ	Markus Schmid
RPTP κ -JM	Rabbit, polyclonal, binds juxtamembrane fragment of RPTP κ	Marta Murgia
RPTP κ -JM	Goat, polyclonal, binds juxtamembrane fragment of RPTP κ	Marta Murgia
α -Tubulin	Mouse, monoclonal, ascites	Sigma, Taufkirchen
α -VSV	Mouse, monoclonal	Boehringer, Mannheim

5.2 Secondary antibodies

For immunoblot analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) were utilized.

<u>Antibody</u>	<u>Dilution</u>	<u>Origin/Reference</u>
Goat anti-mouse-HRP	1 : 10,000	Sigma, Taufkirchen
Goat anti-rabbit-HRP	1 : 50,000	BioRad, München

5.3 Recombinant proteins

<u>Protein</u>	<u>Description/ Immunogen</u>	<u>Origin/Reference</u>
RPTP κ Ec-Fc	RPTP κ extracellular domain (1-640) fused to human IgG1-Fc part	this study
GST-PTP1B-wt	37 kDa (residues 1-321) PTP1B wild-type	Jörg Renkawitz
GST-PTP1B-DA	37 kDa PTP1B, containing D181A mutation	Jörg Renkawitz
GST-PTP1B-DAQA	37 kDa PTP1B, containing D181A and Q262A mutations	Jörg Renkawitz

6. Plasmids and oligonucleotides

6.1 Primary vectors

Vector	Description	Origin/Reference
pcDNA3	Mammalian expression vector, Amp ^r , Neo ^r , CMV promotor, BGH poly A, high copy number plasmid, F1+ origin	Invitrogen, USA
pEGFP-C1	cDNA of the enhanced green fluorescent protein (eGFP)	Clontech, USA
pGEX-5X-1	Prokaryotic expression vector for the generation of glutathione-S-transferase fusion proteins, Amp ^r , IPTG inducible	Amersham Pharmacia, Freiburg
pRK5	Expression vector, Amp ^r , CMV promotor, SV40 poly A	Genentech, USA
pRL-CMV	Expression vector; cDNA of renilla luciferase under CMV promoter	Promega, USA
pSUPER.retro	siRNA expression vector, Amp ^r , Puro ^r , H1 promoter	OligoEngine, USA

6.2 Constructs

Vector	Insert description	Reference
pcDNA3-RPTP _μ -VSV	cDNA of RPTP _μ -VSV	Jens Ruhe
pcDNA3-RPTP _κ Ec-Fc	cDNA of RPTP _κ Ec-Fc (containing a tPA secretion signal)	this study
pEGFP-PIC	cDNA of eGFP N-terminally fused to RPTP _κ -PIC	this study
pGL3-OF	improved pTOPFLASH vector containing luciferase cDNA under control of a mutated, inactive TCF promotor	Vogelstein, USA
pGL3-OT	improved pTOPFLASH vector containing luciferase cDNA under control of an active TCF promotor	Vogelstein, USA
pRK5-RPTP _κ	cDNA of RPTP _κ	Miriam Fuchs
pRK5-RPTP _κ -C/S1-HA	cDNA of RPTP _κ -C/S1-HA	Lars Anders

pRK5-RPTP κ -HA	cDNA of RPTP κ -HA	Miriam Fuchs
pRK5-RPTP κ -P Δ E	cDNA of RPTP κ -P Δ E-HA	Lars Anders
pRK5-RPTP κ -PIC	cDNA of RPTP κ -PIC-HA	this study
pRK5-RPTP κ -PIC-C/S1	cDNA of RPTP κ -PIC-C/S1-HA	this study
pRK5-RPTP κ -PIC- Δ JM	cDNA of RPTP κ -PIC- Δ JM-HA	this study
pSUPER.retro-kscr	scrambled cDNA of RPTP κ (nt: 392-412); 2 substitutions	this study
pSUPER.retro-RPTP κ	cDNA of RPTP κ (nt: 392-412)	Marta Murgia

6.3 Important oligonucleotides

<u>Sequence (description)</u>	<u>Name</u>
5' GATCCCC GGA CTT ATT GCC AAT CCA A TTCAAGAGA T TGG ATT GGC AAT AAG TCC TTTTGGAA 3'	RPTP κ -scr-fwd
5' AGCTTTTCCAAAA AGG ACT ATT GCC AAT CCA A TCTCTTGAA T TGG ATT GGC AAT AAG TCC GGG 3'	RPTP κ -scr-rev
(Cloning of pSUPER.retro-kscr construct)	
5' GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT GCC CGA TTC AGA AGA GGA GCC AGA GCC CAA GGC CAG TTC TCC 3'	PTP κ TPA1-fwd
5' G GAA TTC GCC ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CC 3'	PTP κ TPA2-fwd
5' TC CTC GAG TCC AGA TCC AGA GTG TGG GTG CAG TTC TTC 3'	PTP κ Fu-rev
(Cloning of pcDNA3-RPTP κ Ec-Fc; insertion of tPA secretion signal required two-step PCR)	
5' CCG CTCGAG GT ATT GTA AAA AAG AGC AAA CTT GCT 3'	GFP-PIC-fwd
5' CCC AAGCTT CTA AGA TGA TTC CAG GTA CTC C 3'	GFP-PIC-rev
(Cloning of pEGFP-PIC)	
5' AGAGTGGTGAAAATAGCAGG 3'	RPTP κ -P Δ E-fwd
5' TGCTATTTTGACCACTCTGGATCCCAGGAGAGGCCAAG GAGAGAGGAGCAAG 3'	RPTP κ -P Δ E-rev
(Cloning of pRK5-RPTP κ -P Δ E-HA)	
5' GAA TTC GCC ACC ATG ATT GTA AAA AAG AGC AAA CTT GCT AA 3'	RPTP κ -PIC-fwd
5' GAA TCA TCT TAC CCG TAC GAT GTC CCG GAC	RPTP κ -PIC-rev

TAC GCG TAG CTC GAG 3'
(Cloning of pRK5-RPTP κ -PIC-HA)

5' G GAA TTC GCC ACC ATG GAT CAA AAT AGA GC 3'
5' CT GAA TTC AGA GTC TGA AAT TCA TCC TTG 3'
(Cloning of pRK5-RPTP κ -PIC Δ JM-HA)

RPTP κ -PIC Δ JM-fwd
RPTP κ -PIC Δ JM-rev

6.4 siRNA oligonucleotides

siRNA	Description/Sequence	Reference
gl2	directed against firefly luciferase CGUACGCGGAAUACUUCGAdTdT	Stefan Hart
ADAM10	UGAAGAGGGACACUUCCCUdTdT GUUGCCUCCUCCUAAACCAdTdT	Stefan Hart
ADAM15	CUCCAUCUGUUCUCCUGACdTdT AUUGCCAGCUGCGCCCGUCdTdT	Stefan Hart
ADAM17	AGUUUGCUUGGCACACCUdTdT AGUAAGGCCAGGAGUGUdTdT AGCCCUGUACAGUAGGAUdTdT	Stefan Hart

7. Enzymatic manipulation of DNA

7.1 Plasmid Preparation

Small amounts of plasmid DNA were prepared using the Qiagen Plasmid Mini Kit, larger amounts of DNA were obtained with the Qiagen Plasmid Maxi Kit following the manufacturer's instructions.

7.2 Restriction digestion of DNA

The ratio of Enzyme/DNA, the temperature, the buffer and the time of incubation were adjusted according to manufactures instruction. Usually, incubations for 2 hour at 37°C with a calculated 5-fold over digestion and the buffers as supplied by the manufacturers were chosen.

7.3 Dephosphorylation of DNA 5'-termini

In order to prevent self-ligation of vector termini generated by restriction digest, 5'-termini of vectors were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP). This

phosphatase removes 5'-phosphate residues from DNA as well as RNA. For dephosphorylation, 1 µg of cut vector DNA was incubated with 5 units CIAP in adequate reaction buffer (e.g. 50 mM Tris/HCl pH 8.0, 0.1 mM EDTA pH 8.5) at 37°C for 10 minutes. Either reactions were stopped by heat inactivation at 85°C for 10 minutes or DNA was directly purified using the QIAquick PCR Purification Kit.

7.4 Ligation of vector and insert DNA

Purified, digested and dephosphorylated vector DNA (40 ng), the designated insert DNA, 1 µl 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7.5, 50 mM MgCl₂, 50 mM DTT, 10 mM ATP) and 1 unit T4 DNA Ligase were combined. A molar ratio between insert and vector of 3 to 1 was usually chosen. Reactions were either left on 14°C over night or at 37°C for 2 hours and subsequently transformed into competent bacteria.

7.5 Agarose gel electrophoresis

Depending on the size of the fragments of interest 0.7-2% agarose gels were prepared in horizontal chambers. TAE buffer was used for the electrophoresis. Voltage was usually set to 4-10 V per cm width of the gel. After separation, DNA fragments were stained by gently agitating gels in TAE containing 0.5 µg/ml ethidium bromide and were subsequently viewed under UV light.

7.6 Isolation of DNA fragments from agarose gels

Following gel electrophoresis gel slices bearing DNA fragments of interest were cut out of the gel. Agarose was dissolved and DNA was purified using the QIAquick Gel Extraction Kit following Qiagen's protocol.

7.7 Preparation of competent cells

The preparation of competent cells followed the procedure described by Chung and Miller (Chung and Miller, 1993). Competent cells were shock frozen in liquid nitrogen and stored for up to one year at -70°C. Transformation frequency ranged between 10⁵ and 10⁷ colonies/µg DNA.

7.8 Transformation of competent bacteria

A 50 µl aliquot of competent bacteria was added to a 50 µl mixture of DNA ligation cocktail, 10 µl 5x KCM solution (500 mM KCl, 150 mM CaCl₂, 250 mM MgCl₂) and water. After thoroughly mixing samples were incubated on ice for 20 minutes and 10 minutes at room temperature. Then, 300 µl LB broth were added and samples were incubated at 37°C for 1 hour while constantly shaking. Bacteria were streaked out on appropriate agar plates containing ampicillin for the selection of transformants.

7.9 Enzymatic amplification of DNA by polymerase chain reaction (PCR)

1 µl	template DNA, 1-10 ng
1 µl	"forward" oligonucleotide, 10 pmol/µl
1 µl	"reverse" oligonucleotide, 10 pmol/µl
2.5 µl	10x PCR buffer II containing 20 mM MgCl ₂
2 µl	dNTP-Mix, 2.5 mM each
0.5 µl	Taq DNA Polymerase (5 U/µl)
ad 25 µl	H ₂ O

PCR reactions were carried out using an automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to each specific application:

first denaturation:	3 min 94°C
amplification 25-30 cycles:	1 min 94°C (denaturation)
	1 min 54°C (annealing)
	1 min / kb product 72°C (extension)
last extension:	7 min 72°C

PCR products were either separated by agarose gel electrophoresis, excised and subsequently purified or directly purified with QIAquick Gel Extraction or PCR Purification Kit, respectively.

7.10 DNA sequencing

DNA sequencing was performed according to the “Big Dye Terminator Cycle Sequencing Protocol” (ABI). The following mix was subjected to a sequencing-PCR run:

0.5 µg DNA of interest	
10 pmol oligonucleotide	
4 µL Terminator Ready Reaction Mix	
ad 20 µL H ₂ O	
25 cycles:	30 sec 94°C
	15 sec 45-60°C
	4 min 60°C

The sequencing products were purified by sodium acetate/EtOH precipitation, dissolved in 20 µL HPLC grade ddH₂O and analyzed on a 310-Genetic Analyzer (ABI Prism).

7.11 RT-PCR analysis

Knock-out of PTP1B expression in PTP1B KO MEFs was confirmed by RT-PCR. RNA isolated using RNeasy Mini Kit (Qiagen, Hilden) was reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim). PuReTaq Ready-to-Go PCR beads (Amersham Biociences, Freiburg) were used for PCR amplification. Primers (Sigma, Steinheim) were for PTP1B 5' TCTCACCCAGGGCCCTTACC 3' (PTP1B-fwd-RT) and 5' CAGCAGTACTTTCTTGATGTCCACGG 3' (PTP1B-rev-RT); for GAPDH 5' TTCCAGTATGACTCCACTCACGGC 3' (GAPDH-fwd-RT) and GCAGAAGGGGCGGAGATGATG (GAPDH-rev-RT).

PCR products were subjected to electrophoresis on a 2.5 % agarose gel and DNA was visualized by ethidium bromide staining.

8. Methods in mammalian cell culture

8.1 General cell culture techniques

Cell lines were grown in a humidified 93% air, 7% CO₂ incubator (Heraeus, B5060 Ek/CO₂) at 37°C and routinely assayed for mycoplasma contamination using a bisbenzimidestaining kit (Sigma). Before seeding cells were counted with a Coulter Counter (Coulter Electronics). All of the cell lines (American Type Culture Collection, USA) were routinely grown according to the supplier's instructions.

8.2 Transfection of cells with calcium phosphate

HEK-293 cells in six-well dishes were transfected transiently at about 70% confluence with a total of 2 µg DNA by using a modified calcium phosphate precipitation method as described previously (Chen and Okayama, 1987). In this protocol, a calcium phosphate-DNA complex is formed gradually in the medium during incubation with cells.

The transfection mix of DNA and CaCl₂ in water was prepared as follows:

Dish	6-well	6 cm	10 cm
area	10 cm ²	21 cm ²	57 cm ²
Volume of medium	1 mL	2 mL	4 mL
DNA in H ₂ O bidest	2 µg in 90 µL	5 µg in 180 µL	10 µg in 360 µL
2.5 M CaCl ₂	10 µL	20 µL	40 µL
2 x BBS (pH 6.96)	100 µL	200 µL	400 µL
Total volume	200 µL	400 µL	800 µL

To initiate the precipitation reaction, the adequate volume of 2xBBS was added and mixed by vortexing. The reaction was incubated for 10 min at room temperature before being added to each well. Plates were placed in a humidified incubator at 3% CO₂ overnight. Transfection efficiency of 293 cells was typically about 75% as determined by GFP fluorescence after transfection of a GFP-containing expression plasmid. For transfection of Phoenix A cells 2xHBS was used instead of 2xBBS.

8.3 Transfection of siRNA duplexes

Transfection of 21-nucleotide small interfering RNA (siRNA) duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting endogenous genes in Caki-1 cells was carried out using Lipofectamine 2000 (Invitrogen) and 2.7 µg siRNA duplex per 6cm dish according to the manufacturer's protocol.

Cells were assayed 2 days after transfection. Highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest. Specific silencing of targeted genes was confirmed by Western blotting.

8.4 Retroviral infection of cell lines

For production of retroviral supernatants, Phoenix A cells were transfected with pSUPER.retro constructs using the calcium phosphate method. To enhance efficiency of transfection, chloroquin (50 μ M) was added to the cells. One day after transfection retroviral supernatants were harvested three times with an accumulation time of 3 h. Supernatants were filtered through a 0.45 μ m filter and polybrene (8 μ g/ml) was added. Subsequently, ACHN cells were infected with retroviral supernatants three times in a row and at any one time for 3 h. One day after infection ACHN cells were selected with 1,5 μ g/ml puromycin for 5 days.

9. Protein analytical methods

9.1 Lysis of cells with Triton X-100

Prior to lysis, cells were treated with inhibitors and agonists as indicated in the figure legends. Cells were washed with cold PBS and then lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/mL aprotinin. Lysates were precleared by centrifugation at 12,500 g for 10 min at 4°C.

9.2 Determination of protein concentration in cell lysates

The „Micro BCA Protein Assay Kit” (Pierce, Sankt Augustin) was used according to the manufacturer’s recommendations.

9.3 Immunoprecipitation of proteins

An equal volume of HNTG buffer was added to the precleared cell lysates that had been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20 μ L of protein A- or G-Sepharose for 4 h at 4°C. Precipitates were washed four times with 1 mL of HNTG buffer, suspended in 3 \times Laemmli buffer, boiled for 5 min, and subjected to SDS-PAGE.

9.4 SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook, 1990). The following proteins were used as molecular weight standards:

<u>Protein</u>	<u>MW (kDa)</u>	<u>Protein</u>	<u>MW (kDa)</u>
Myosin	205.0	Ovalbumin	42.7
β -Galaktosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

9.5 Coomassie staining of polyacrylamide gels

Polyacrylamide gels were stained with Coomassie-brilliant-blue G-250 using a colloidal staining method. In brief, gels were fixed in 12% TCA solution for one hour and stained thereafter over night in Coomassie-brilliant-blue solution (0.1% Coomassie-brilliant-blue G-250, 2% H₃PO₄, 10% (NH₄)₂SO₄, 20% methanol). Destaining was performed in 25% methanol for 2h. For drying, gels were fixed between two cellophane foils and dried in a fan heater.

9.6 Transfer of proteins onto nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 2 h at 0.8 mA/cm² using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer, proteins were stained with Ponceau S (2 g/l in 2 % TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

9.7 Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25% gelatin for at least 1 h. The membrane was then probed with primary antibody (typically overnight). Antibodies were diluted 1:500 to 1:2000 in NET, 0.25% gelatin. The membrane was washed 3 times in 1x NET, 0.25% gelatin, incubated for 1 h with secondary antibody and washed again 3x for 20 min. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films or a digital camera unit. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 h at 50°C. Stripped membranes were blocked and re probed with different primary antibody to confirm equal protein loading.

10. Analysis of whole cell-based assays

10.1 Immunofluorescence analysis

NIH-3T3 and COS-7 cells were fixed with 4 % paraformaldehyde for 10 min, incubated with 0.2 % Triton X-100 in PBS for 10 min, blocked with PBG (0.5 % bovine serum albumin and 0.045 % gelatin in PBS) plus 5 % goat serum, and then incubated for 2 h with anti-HA (mouse) and anti- β -catenin (rabbit) antibody. After washing three times with blocking buffer, the cells were incubated for 2 h with AlexaFluor 488-labeled (anti-mouse) and AlexaFluor 546-labeled (anti-rabbit) secondary antibodies (Molecular Probes). Cells were then washed with PBS and mounted with Fluoromount G (Biozol) for observation. A Leica confocal microscope was used to view and analyze the immunofluorescence slides.

10.2 MTT-assays

In a 96-well flat bottom plate (Nunc, USA) approximately 2,000 cells / 100 μ l cell suspension were seeded. PTP1B wild-type and KO cells were allowed to grow for 24, 48 and 72 h and at respective time points, the tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; thiazolyl blue, Sigma, Taufkirchen) was added to each well to a final concentration of 1 mg/ml MTT. Plates were incubated in the presence of MTT for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which was solubilized (DMSO, acidic acid, SDS) and absorbance was read at 570 nm on a micro-plate reader.

10.3 Migration assays

PTP1B wild-type and KO cells were harvested with trypsin, washed twice with serum-free medium and 5×10^4 cells were resuspended in serum-free media and then added to the upper part of the Boyden chamber (Becton Dickinson, France). The lower well (24-well) was filled with 800 μ l of medium containing 10 % FCS as an attractant. Migration was allowed for 24 h at 37°C and 7 % CO₂. Subsequently, migratory cells were fixed with 100 % ice cold methanol and stained with crystal violet (0.5 % (w/v) crystal violet in 20 % methanol). Cells on the upper chamber surface were removed with a cotton swab. Stained cells which have migrated to the bottom part were photographed and counted.

10.4 Wound healing assay

PTP1B wild-type and KO cells were seeded on 6-wells and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and maintained under standard conditions for 20 h. Plates were washed once with fresh medium to remove non-adherent cells and photographed.

11. Gene reporter assays

50×10^3 HCT116 cells were seeded on 24-well tissue culture plates 24 h before transfection. Lipofectamine was used to transfect cells with 2 ng of an internal control (pRL-CMV), 100 ng of a reporter construct (pGL3-OT or pGL3-OF), and different RPTP κ isoform expression vectors. pGL3-OT is an improved pTOPFLASH vector containing an optimized TCF-binding site upstream of a luciferase reporter gene, whereas pGL3-OF contains a mutated site that does not bind TCF. Thirty-six hours after transfection, luciferase activities were measured and normalized for background Renilla luciferase activities (transfection control) by using the dual luciferase reporter assay system (Promega). Moreover, normalized values were corrected for non-specific transcription by subtracting pGL3-OF values.

12. SILAC experiments and MS-analysis

12.1 Cell culture in SILAC medium

PTP1B wild-type and KO cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and antibiotics (5 mg/ml penicillin/streptomycin; Invitrogen). For SILAC experiments cells were grown for seven days in media containing either normal L-arginine and L-lysine (Sigma), L-arginine-U-¹³C₆¹⁴N₄ and L-lysine ²H₄ or L-arginine-U-¹³C₆-¹⁵N₄ and L-lysine-U-¹³C₆-¹⁵N₂ (Cambridge Isotope Laboratories, USA), as well as dialyzed FBS (Gibco).

12.2 Cell lysis and anti-pY immunoprecipitation for MS-analysis

After washing once with ice-cold PBS, SILAC labeled cells were lysed for 20min in ice-cold lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1 % NP40; 0.1 % sodium deoxycholate; 1 mM EDTA; 1 mM sodium orthovanadate; 1 mM PMSF; 0.1 µg/ml aprotinin; 10 mM NaF). Lysates were precleared by centrifugation at 16,500 g for 15 min. The BCA-assay (Pierce) was then used to determine the absolute protein amount. In SILAC double labeling experiments cell lysates were mixed 1:1 and in triple labeling experiments 1:1:1 directly after protein amount determination. For anti-pTyr immunoprecipitation 200µg 4G10 antibody was added together with 40 µl protein A-Sepharose (Amersham Biosciences) to mixed cell lysates containing up to 20 mg total protein and incubated for 4h or over night at 4°C. Precipitates were subsequently washed four times with lysis buffer and precipitated proteins were eluted twice with urea buffer (7 M urea; 2M thiourea; 50 mM HEPES pH 7.5; 1 % n-octosyl glucoside) for 10 min at 37°C.

12.3 In vitro substrate trapping for MS-analysis

The catalytic domain of PTP1B (residues 1-321) was linked to GST-fusion protein and PTP1B substrate trapping mutants D181A and D181A/Q262A were generated as it was reported before (Flint et al., 1997), (Xie et al., 2002). The recombinant PTP1B wild-type and mutant fusion proteins were expressed in E.coli and purified with GST-Sepharose (Amersham). In vitro substrate trapping assays were performed as described (Blanchetot et al., 2005). Briefly, cells were stimulated for 30 min with 100 µM pervanadate and lysed for 20 min in ice cold lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1 % TX-100; 10 % glycerol; 1 mM EDTA; 1 mM PMSF; 0.1 µg/ml aprotinin; 5 mM iodoacetic acid). Iodoacetic acid was inactivated by adding DTT to a final concentration of 10 mM and samples were incubated for 10 min. Lysates were precleared by centrifugation at 16,500 g for 15 min. GSH-beads were loaded with 10 µg of recombinant PTP1B wild-type enzyme or substrate trapping mutant (Amersham) and subsequently incubated for 4 h at 4°C with lysates containing 10 mg total protein. Precipitated proteins were washed 3 times with HNTG buffer (20 mM HEPES, pH 7.5; 150 mM NaCl; 10 % glycerol; 0.1 % Triton X-100) and for subsequent MS-analysis proteins were eluted with urea buffer (7 M urea; 2 M thiourea; 50 mM HEPES pH 7.5; 1 % n-octosyl glucoside) for 10 min at 37°C.

12.4 *In solution protein digestion*

After substrate trapping or anti-pTyr immunoprecipitation, purified proteins were eluted in the above described urea buffer and protein amount was measured using the Bradford-assay. Thiol groups on proteins were reduced by adding 2 mM DTT (final conc.) for 45 min at 25°C and cysteines were then carboxymethylated with 5.5 mM iodoacetamide for 30 min at RT. The endoproteinase Lys-C (Wako) was added in an enzyme/substrate ratio of 1/100 (usually 1-3 µg Lys-C) and the proteins were digested for 4 h at RT. Thereafter, the resulting peptide mixtures were diluted with ddH₂O to achieve a final urea concentration below 2 M. Then, modified trypsin (sequencing grade, Promega) was added in an enzyme/substrate ratio of 1/100 (usually 1-3 µg trypsin) and the digest was incubated at RT over night. In the end, trypsin activity was quenched by adding 1 % trifluoroacetic acid (TFA).

12.5 *Titansphere enrichment of phosphopeptides*

Subsequently to the trypsin digest, phosphopeptides were enriched using Titansphere-chromatography (TiO₂) columns as described (Larsen et al., 2005), (Olsen et al., 2006). Peptide samples were diluted 1:6 with 30 g/L 2,5-dihydroxybenzoic acid (DHB) in 80 % MeCN (acetonitrile) / 0.1 % TFA. 5 µg TiO₂ resin (GL Sciences Inc.) was washed once with elution buffer (NH₃ water in 20 % MeCN, pH 10.5) and equilibrated with washing buffer (50 % MeCN, 0.1 % TFA). The TiO₂ resin was preloaded with DHB by washing with loading buffer (6 g/L DHB in 15 % MeCN). Peptide samples were then loaded onto the TiO₂ resin for 30 min at RT on a spinning wheel. Then, the resin was washed 3 times with washing buffer and bound phosphopeptides were eluted twice for 10 min at RT with elution buffer. The eluted peptides were filtered through home-made C₈ STAGE Tips in 200 µl pipette-tips. 30 µl of 80 % MeCN / 0.5 % acetic acid was applied to the STAGE Tips after filtering and the flow through was combined with the filtered sample. Using TFA the pH value of the sample was adjusted to a value of approximately pH 7 and the eluates were concentrated in a speed-vac. To prepare samples for MS-analysis, 5 % MeCN and 0.1 % TFA (final concentrations) were added to the samples.

12.6 *In-gel protein digestion for protein expression analysis*

Up to 300 µg of mixed total protein extracts from PTP1B wild-type and KO cells were separated by SDS-PAGE, using NuPAGE Novex Bis-Tris gels (Invitrogen) according to the manufacturer's instructions. The colloidal Blue Staining Kit (Invitrogen) was used to stain the gel with Coomassie blue. The lane containing all labeled proteins was cut into 15 slices that were subject to in-gel digestion which was basically performed as described (Shevchenko et al., 1996). Gel slices were cut into small pieces and washed with 50 mM ammonium bicarbonate (ABC) / 50 % ethanol until cubes were fully destained. Gel pieces were dehydrated with ethanol and rehydrated with 50 mM ABC containing 10 mM DTT. Thiol groups on proteins were reduced for 1 h at 56°C. The reduced thiol groups were then alkylated by adding 55 mM iodoacetamide in 50 mM ABC for 1 h at 25° C in the dark. Gel pieces were again washed twice with a 50 mM ABC / 50 % ethanol solution, dehydrated with 100 % ethanol and dried in a speed vac concentrator. Each gel fraction was re-hydrated in 50 mM ABC solution containing 0.4 µg trypsin and samples were digested at 37° C over night. Supernatants were collected in new tubes and residual peptides were extracted out of the gel pieces by double incubation with 30 % MeCN in 3 % TFA and double incubation with 100 % MeCN. All extracts were combined for each gel fraction and MeCN was evaporated in a

speed vac. Then, samples were desalted using home-made RP-C18 STAGE Tip columns (Rappsilber et al., 2003) and the purified peptides were subjected to mass spectrometric analysis.

12.7 NanoLC-MS/MS analysis

All obtained peptide samples were separated by online reverse phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray tandem mass spectrometry (ES MS/MS). Using an Agilent 1100 nanoflow system (Agilent technologies, Palo Alto, USA), samples were injected onto a 15 cm RP, fused-silica capillary column (inner diameter 75 μ m, packed in-house with 3 μ m ReproSil-Pur C18-AQ media Dr. Maisch GmbH, Ammerbuch-Entringen). The LC setup was connected to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen) which was equipped with a nano-electrospray ion source (Proxeon Biosystems, Denmark). After loading, peptides were eluted with 140 min gradients from 5 to 40% MeCN in 0.5% acetic acid. Data-dependent acquisition was performed on the LTQ-Orbitrap in the positive ion mode. During measurement, the instrument was recalibrated in real-time by co-injection of an internal standard from ambient air into the C-trap ("lock mass option") (Olsen et al., 2005). Survey MS-spectra were acquired with a resolution of 60,000 in the orbitrap. Up to five most intense ions per cycle were fragmented and MS/MS-spectra were acquired in the LTQ part of the instrument. To improve phosphopeptide analysis, the neutral loss species at 97.97, 48.99, or 32.66 m/z below the precursor ion were activated for 30ms during fragmentation (pseudo-MS³) (Schroeder et al., 2004).

12.8 Peptide identification using the MASCOT search engine

The MASCOT search engine (Matrix Science, UK) was used to identify peptide sequences by searching all tandem mass spectra against an in-house curated decoy IPI mouse protein database of the International Protein Index (Decoy IPI Version 3.18) containing forward and reversed sequences. Moreover, contaminants such as human keratins, porcine trypsin and endoproteinase Lys-C were included in this database. A concatenated target/decoy database allows to define a cut-off score threshold that permits a false-positive rate of peptide identification of less than one percent ($p < 0.01$) (Elias et al., 2005). Mass accuracy for MS spectra after recalibration was usually better than 1 ppm, therefore no peptides with a mass deviation greater 5 ppm were allowed. Mass tolerances for protein identification on MS peaks were 5 ppm and on MS² peaks were 0.5 Da. Up to three "missed cleavages" were allowed. Carbamidomethylcysteine was set as fixed modification, and oxidized methionine, phosphorylation of Ser/Thr/Tyr, protein N-acetylation, N-pyroglutamate, and the SILAC-labels - Lys-D4, Lys-8, Arg-6 and Arg-10 - were searched as variable modifications. "ESI-Trap" was specified as the instrument setting for all Mascot searches.

12.9 Post-translational modification (PTM) scoring and peptide quantitation using MSQuant

Sequence assignments and spectra obtained from MASCOT were imported into MSQuant (<http://msquant.sourceforge.net>). The PTM scoring algorithm in this program is a probability based scoring system for phosphorylation site assignment within peptide sequences and has been described recently (Olsen et al., 2006). After the localization of phospho groups has been assigned to peptide sequences and PTM scores were determined, phosphopeptide quantitation was performed using the MSQuant software. MSQuant calculated the corresponding XIC

values, for each SILAC doublet or triplet, and all assignments made for quantitation were displayed and manually validated (Schulze and Mann, 2004), (Olsen et al., 2006). The quantitation data of each SILAC experiment was reproduced at least twice and final peptide quantitation ratios were acquired by calculation of the average value of single measurements.

IV. Results

To understand the cellular function of PTPs it is important to elucidate regulatory mechanisms that control their intracellular localization and activity, as well as to identify their cellular substrate specificities. Here, a novel proteolytic regulatory mechanism for a receptor-like PTP will be described, which induces its translocation from the plasmamembrane to the nucleus and thereby alters the transcriptional activity of one of its substrate proteins.

In the second part, new mass spectrometry-based methods are presented that allow the global analysis of cellular PTP function. Changes in the phosphotyrosine proteome of PTP1B-deficient cells have been analyzed and the physical interactions of regulated proteins to PTP1B substrate trapping mutants have been defined.

1. ADAM10- and γ -Secretase-mediated cleavage of RPTP κ and regulation of β -Catenin's transcriptional activity

1.1 ADAM10 mediates shedding of RPTP κ 's extracellular domain

RPTP κ consists of two subunits at the cell surface. Its extracellular E-subunit harbors cell adhesion domains which allow highly specific homophilic binding to other RPTP κ proteins (Sap et al., 1994), whereas its transmembrane P-subunit contains two PTP domains which transmit extracellular cell-cell contact signals into the cell (Figure 7A). The dimeric structure is generated by proteolytic processing (S1 cleavage) of a precursor protein by furin convertase in the Golgi-apparatus (Jiang et al., 1993), (Fuchs et al., 1996). In a previous study, our laboratory demonstrated that RPTP κ expression rises with cell density and reaches its highest level at cell confluence (Fuchs et al., 1996). Furthermore, we observed a cell density dependent cleavage of the dimeric RPTP κ protein, accompanied by shedding of its extracellular E-subunit and accumulation of the transmembrane P Δ E cleavage product (Anders et al., 2006) (Figure 7B). Experiments employing protease inhibitors revealed a sensitivity of this cleavage process towards the metalloprotease inhibitor BB-94. Additionally, the phenothiazine trifluoperazine (TFP), one of the commonly known "shedding inducers", was found to induce the responsible metalloproteinases (Anders et al., 2006).

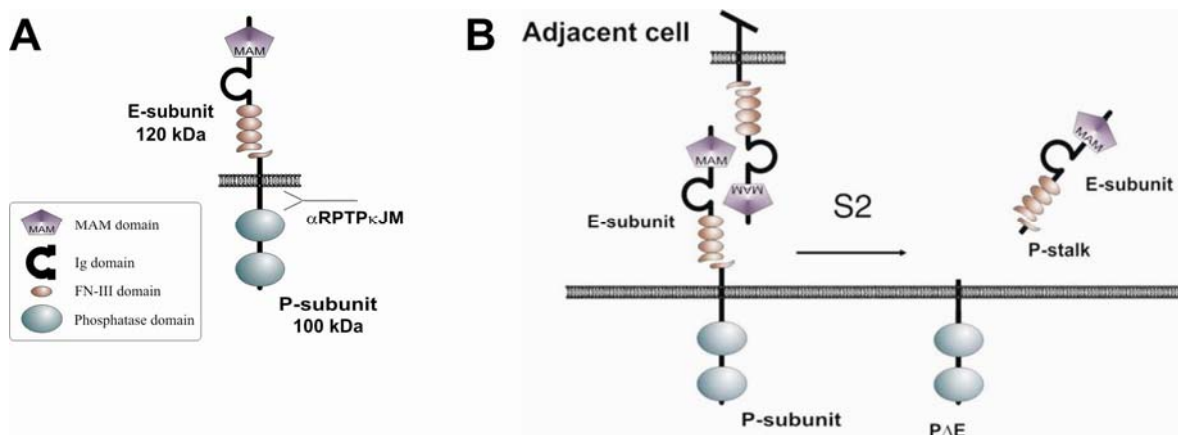


Figure 7: Schematic representation of RPTP κ on the cell surface. (A) Scheme illustrating the two-subunit structure of RPTP κ . The fragment sizes are indicated. Ig, immunoglobulin. (B) Putative model of cell density induced metalloprotease-mediated shedding of RPTP κ 's extracellular domain.

We named this process S2 cleavage, as it occurs at the cell surface after RPTP κ has been cleaved by furin in the Golgi-apparatus. In order to identify the candidate metalloproteinase in this study, we chose to target the ADAM family members ADAM10, -15 and -17 by decreasing their cellular expression levels using the siRNA approach (Figure 8).

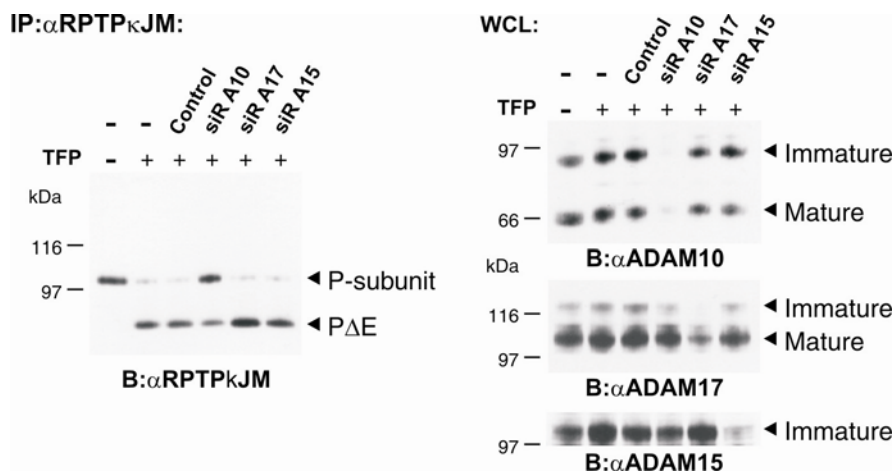


Figure 8: ADAM10 mediates S2 processing of RPTP κ . Trifluoperazine (TFP)-induced shedding is executed by ADAM10. Caki-1 cells were transfected with siRNA (siR) directed against ADAM10 (A10), ADAM15 (A15) and ADAM17 (A17). After 48 h, cells were treated (+) or not treated (-) with 100 μ M TFP for 15 min. Left panel, RPTP κ was immunoprecipitated with anti-RPTP κ -JM antibody and detected by Western blotting. Right panel, specific silencing of ADAM expression was confirmed by immunoblot analyses of whole-cell lysates with antibodies to ADAM10, ADAM15 and ADAM17. Abbreviations: α , anti; B, blotting; IP, immunoprecipitation; WCL, whole cell lysate.

We selected ADAM10 as it was found to control cell adhesion processes, ADAM15 due to its localization at cell adhesion sites, and ADAM17 which is involved in most of the already described shedding events (Seals and Courtneidge, 2003). For all three proteinases an efficient down-regulation was achieved in Caki-1 kidney cancer cells, but only after knock-down of ADAM10 a significant decrease in TFP induced RPTP κ cleavage was observed.

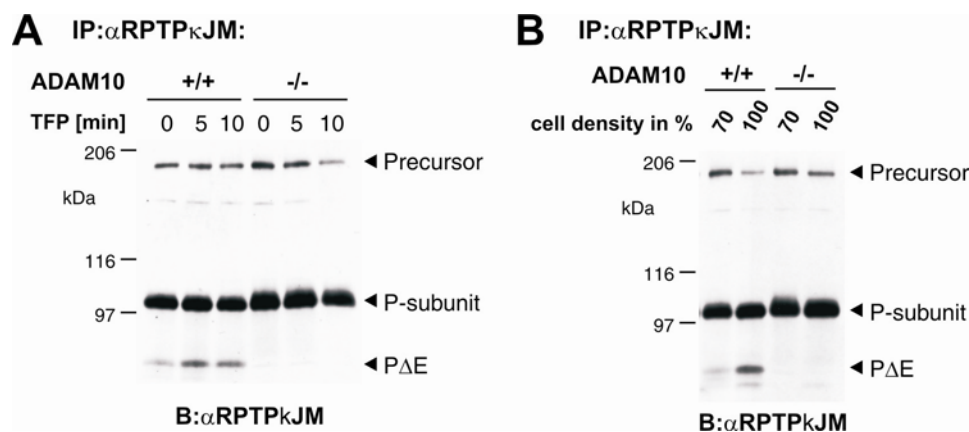


Figure 9: Trifluoperazine- and cell density induced S2 cleavage of RPTP κ is absent in ADAM10-deficient MEFs. (A) ADAM10 +/+ and -/- fibroblasts were stimulated for the indicated times with 100 μ M TFP and RPTP κ processing was analyzed by immunoprecipitation and Western blotting. (B) ADAM 10 +/+ and -/- fibroblasts were grown to different cell densities and RPTP κ cleavage was analyzed as illustrated.

To test the generality of this mechanism we used a different loss-of-function model and changed the cell system. For this purpose, we induced RPTP κ cleavage with TFP in ADAM10-deficient mouse embryonic fibroblasts and their wild-type counterparts (Hartmann et al., 2002). As expected, only in ADAM10 +/+ cells TFP-induced cleavage of RPTP κ was observed, whereas no metalloproteinase cleavage occurs in ADAM10 -/- cells (Figure 9A). This indicates that TFP specifically activates ADAM10 in mouse fibroblasts to induce shedding of RPTP κ . Next, we were interested if ADAM10 also executes RPTP κ shedding under more physiological conditions. To this end, we cultured ADAM10 wild-type and KO MEFs at different cell densities and analyzed RPTP κ S2 cleavage. Interestingly, only in wild-type MEFs the cleavage product P Δ E appeared at high cell density, whereas no cleavage was

induced in KO cells (Figure 9B). This clearly demonstrates that ADAM10 is a RPTP κ sheddase under physiological conditions.

1.2 Intramembrane proteolysis of the MAM-phosphatases RPTP κ and RPTP μ by γ -Secretase

Most of the known proteins being sequentially processed by furin convertase and ADAM family metalloproteases are subsequently cleaved by a third intramembrane γ -secretase-mediated proteolytic step (S3 cleavage) (Kopan and Ilagan, 2004). To test if ADAM10 also triggers γ -secretase mediated cleavage of RPTP κ , we studied its cleavage in mouse embryonic fibroblasts that are deficient in both presenilin 1 and 2 proteins (PS1^{-/-} PS2^{-/-}), which constitute the catalytic subunits of the γ -secretase complex (Herreman et al., 1999). In PS1/PS2-deficient cells we observed a strong accumulation of the P Δ E subunit of RPTP κ due to a non functional γ -secretase complex (Figure 10A). This provides evidence for a presenilin dependent S3 cleavage of RPTP κ as its P Δ E isoform might be a direct substrate for the γ -secretase complex. Next, we were interested if also other receptor-like PTPs are cleaved by presenilin and tested lysates from PS1/PS2 wild-type and KO cells with antibodies against the MAM-family member RPTP μ (Figure 10B) and the more distantly related RPTP α (Figure 10C).

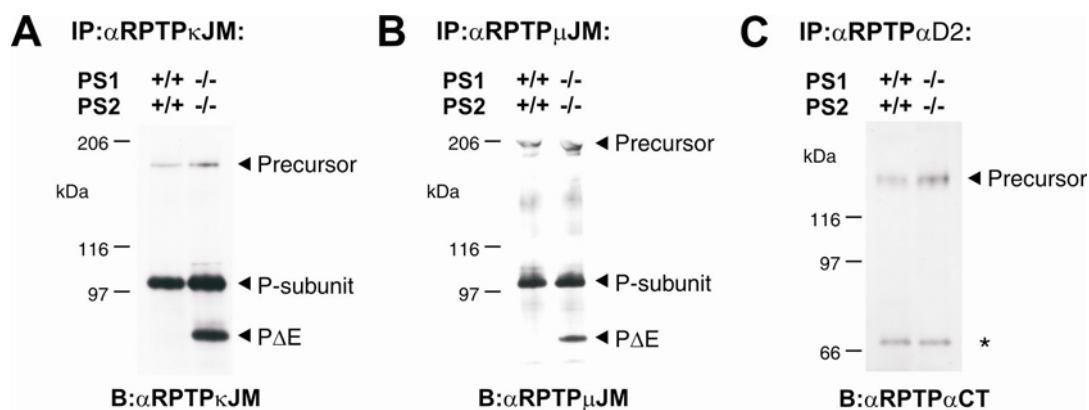


Figure 10: The S2 cleavage product P Δ E of RPTP κ and RPTP μ is subject to γ -secretase-dependent processing. RPTP κ (A), RPTP μ (B) and RPTP α (C) were immunoprecipitated from PS1^{+/+}/PS2^{+/+} or PS1^{-/-}/PS2^{-/-} fibroblasts and occurrence of cleavage products was analyzed with the indicated antibodies. Note the accumulation of P Δ E isoforms in presenilin 1/2-deficient MEFs in (A) and (B). The protein band marked with an asterisk in (C) is probably a calpain-mediated cleavage product of RPTP α .

Similar to the P Δ E isoform of RPTP κ , we detected an accumulation of a RPTP μ P Δ E isoform that migrates at 75kDa upon PS1/PS2-deficiency. In contrast, no increased levels of RPTP α isoforms could be found in the KO cells, indicating that RPTP α is not a substrate of the γ -secretase complex.

Moreover, we used an alternative approach to prove presenilin-mediated cleavage of RPTP κ and RPTP μ by transfecting both full-length enzymes into HEK293 cells stably expressing wild-type PS1 or the dominant-negative mutant PS1 D385N (Lammich et al., 2002). As expected, we identified increased P Δ E levels for RPTP κ and RPTP μ in cells expressing dominant-negative PS1 (Figure 11A, 11B). The same was true when presenilin function was blocked by using the γ -secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) in PS1 wild-type expressing HEK293 cells.

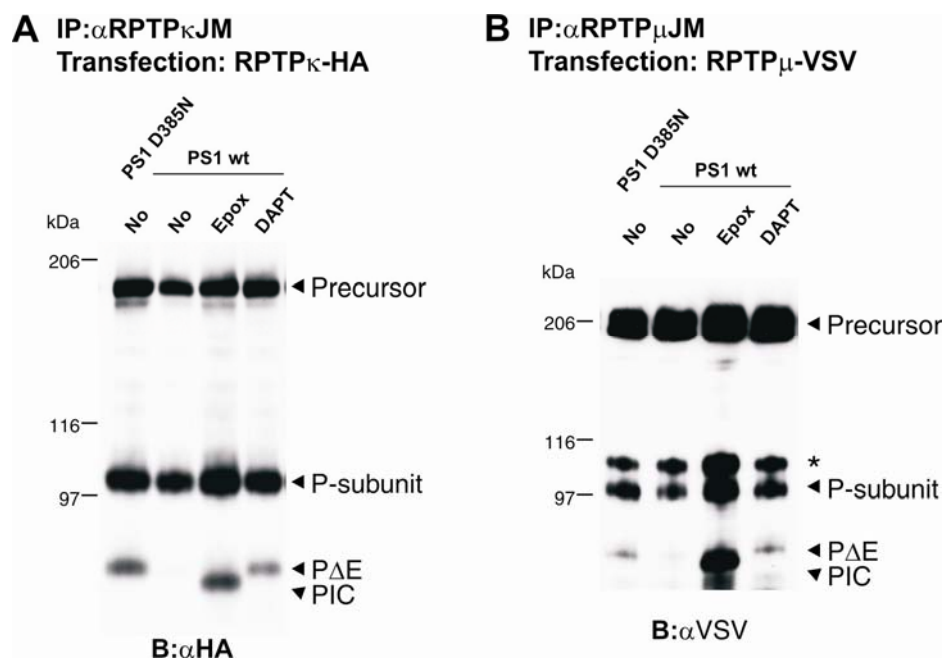


Figure 11: The S2-cleavage product P Δ E of RPTP κ and RPTP μ accumulates in the presence of dominant negative presenilin 1 or presenilin inhibitor DAPT. HEK293 cells expressing wild-type PS1 or PS1 D385N were transiently transfected with HA-tagged RPTP κ (A) or VSV-tagged RPTP μ (B). PS1 wild-type cells were incubated with the γ -secretase inhibitor DAPT (2 μ M) for 8 h or the proteasomal inhibitor Epoxomycin (5 μ M; Epox) for 12 h as indicated. Note the stabilization of γ -secretase cleavage product PIC after proteasomal inhibition. RPTPs were immunoprecipitated and detected by blotting with the described antibodies. The asterisk indicates a differentially glycosylated form of the RPTP μ P-subunit.

Constitutive γ -secretase activity leads to clearance of P Δ E levels on the cell surface and raises the question whether a S3 cleavage product can be detected under normal conditions or if this also gets degraded in turn. Studies on other γ -secretase substrates like the transmembrane protein notch revealed a quite labile S3 cleavage product that is hardly detectable from living tissues in Western blots (Kopan and Ilagan, 2004). For this reason, we treated HEK293 cells expressing wild-type presenilin with the proteasomal inhibitor Epoxomycin. After this treatment we observed a strong accumulation of a RPTP κ isoform about 5 kDa smaller than P Δ E, whereas there was no increase in P Δ E levels itself. Importantly, this isoform was not detectable in Epoxomycin treated cells expressing a non functional γ -secretase complex (Anders et al., 2006). We named this novel RPTP κ isoform PIC (phosphatase intracellular portion) as we could further demonstrate the liberation of the intracellular RPTP κ domains from the plasmamembrane (Anders et al., 2006). Similar to the results obtained for RPTP κ , we also found a RPTP μ PIC isoform after proteasomal inhibition with Epoxomycin (Figure 11B). These results highlight the MAM-family members RPTP κ and RPTP μ as novel substrates of the presenilin dependent γ -secretase complex.

1.3 The intracellular RPTP κ PIC isoform localizes to the nucleus

The presenilin dependent γ -secretase complex converts RPTP κ P Δ E to the soluble intracellular PIC isoform. Consequently, RPTP κ PIC is not restrained to the plasmamembrane anymore and can translocate to other cellular compartments. To test this, we transfected Cos7 cells with HA-tagged RPTP κ full-length, P Δ E and PIC isoforms and analyzed their subcellular localization by confocal microscopy. The recombinant P Δ E protein contained the cytoplasmic and transmembrane part plus four amino acids from the extracellular region, whereas the PIC protein consisted of the cytoplasmic RPTP κ domain plus two N-terminal residues (773 and 774) out of the transmembrane region. As no sequence information about the S2 and S3 cleavage sites was available, the N-terminal residues for both P Δ E and PIC were selected in a way to produce recombinant proteins with the same molecular weights as the endogenous RPTP κ cleavage isoforms. The full-length isoform of RPTP κ localized to the endoplasmatic reticulum and the plasmamembrane (Figure 12A). Interestingly, a co-localization to the cell adhesion complex component β -catenin was detected at some parts of the plasmamembrane. This correlates to earlier observations made from our group that β -

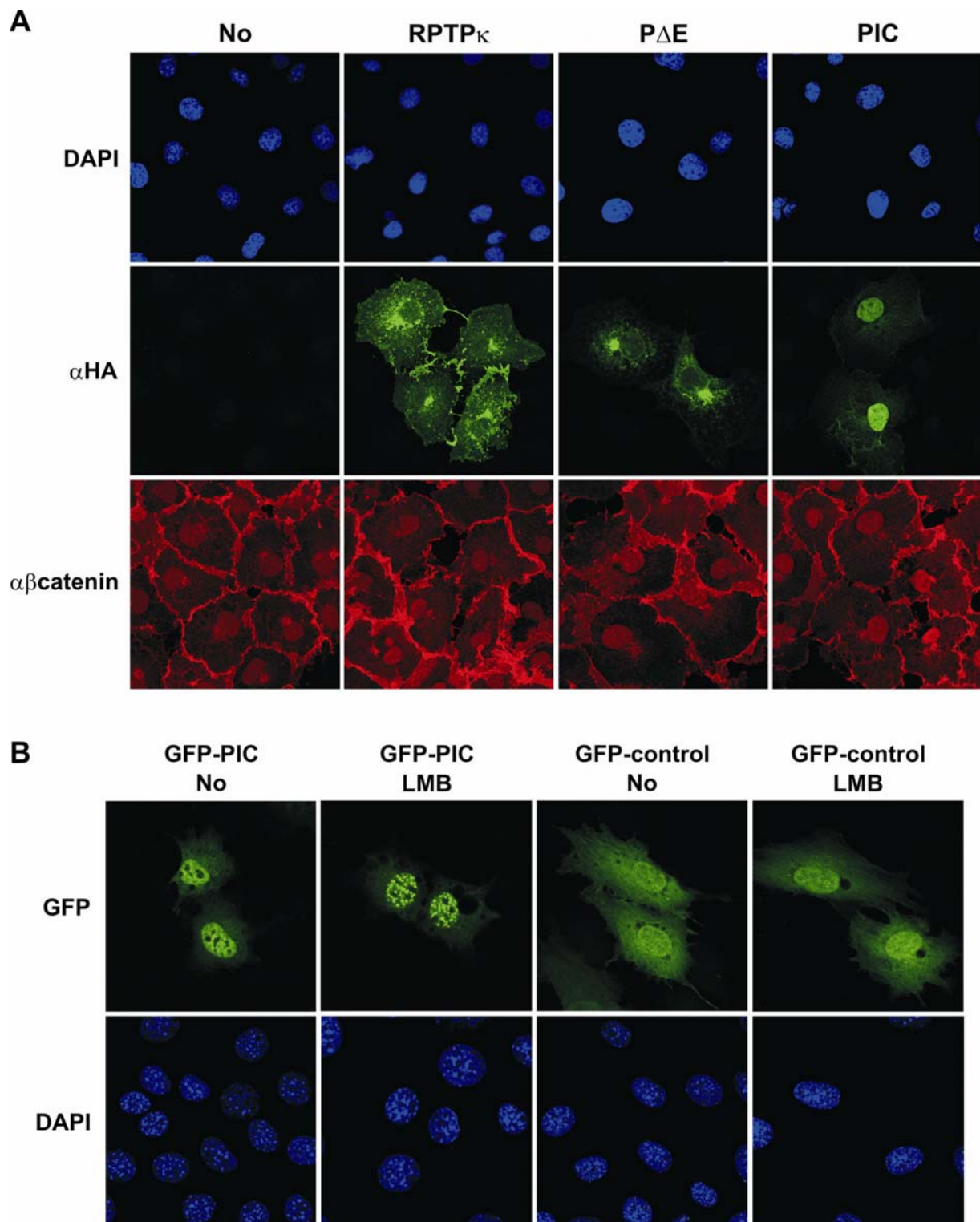


Figure 12: Ectopically expressed RPTP κ PIC localizes to the nucleus. (A) The indicated HA-tagged RPTP κ isoforms were transfected into Cos-7 cells. After 24 h, cells were fixed and immunostained by using anti-HA antibody and AlexaFluor 488-labeled secondary antibodies (green). Endogenous β -catenin was detected with a polyclonal antibody to β -catenin and AlexaFluor 546-labeled secondary antibody (red). Nuclear staining with DAPI is shown in the upper panels. Note the detection of PIC in the nucleus by confocal microscopy. (B) Inhibition of CRM1-dependent nuclear export leads to accumulation of PIC in nuclear bodies. NIH-3T3 cells were transfected with either GFP-PIC (left panels) or GFP control vector (right panels) and were treated or not treated with leptomycin B (LMB) at 25 ng/ml for 3 h. After fixation, cells were stained with DAPI and observed with a Leica confocal

microscope. Note the specific accumulation of GFP-PIC in nuclear bodies upon LMB treatment.

catenin binds to the cytosolic part of RPTP κ (Fuchs et al., 1996). β -catenin itself mainly localized to the plasmamembrane and in minor amounts also to the nucleus. Ectopic expression of RPTP κ full-length, P Δ E and PIC isoforms did not substantially alter the subcellular localization of β -catenin. Whereas recombinant P Δ E mainly localized to the ER and Golgi, the intracellular PIC isoform surprisingly localized to the nucleus. We further investigated this nuclear localization of PIC by linking GFP to the N-terminus of PIC (residue 773) and expressed the fusion protein in NIH-3T3 fibroblasts. Likewise, in this cell system RPTP κ PIC localized to the nucleus whereas the GFP control was found also in the cytoplasm. In addition, when cells were treated with the CRM1-dependent nuclear export inhibitor Leptomycin B (Zheng et al., 2001), the nuclear localization of PIC increased further and accumulated in small granular speckles (Figure 12B), due to inhibition of its nuclear export. This implies that RPTP κ PIC has a leptomycin B-sensitive nuclear export signal and is actively transported in and out of the nucleus. These results demonstrate for the first time the localization of a receptor-like PTP to the nucleus.

1.4 β -catenin is a cellular substrate of RPTP κ

Existing data about the binding of β -catenin to RPTP κ (Fuchs et al., 1996) and its co-localization in our immunofluorescence analysis (Figure 12A) prompted us to elucidate if β -catenin is a cellular substrate of RPTP κ . In first place, we confirmed that β -catenin can be co-precipitated with endogenous full-length RPTP κ (Figure 13A). To analyze the enzyme-substrate relationship of RPTP κ and β -catenin in living cells, we generated ACHN kidney cancer cell lines stably expressing siRNAs against RPTP κ . These cell lines exhibited a decrease of RPTP κ expression of approximately 80% and the down-regulation of the phosphatase directly correlated with increased β -catenin tyrosine phosphorylation after EGF stimulation (200 ng/ml; 5 min) (Figure 13B). Thus, our data suggest a direct dephosphorylation of β -catenin by RPTP κ in living cells.

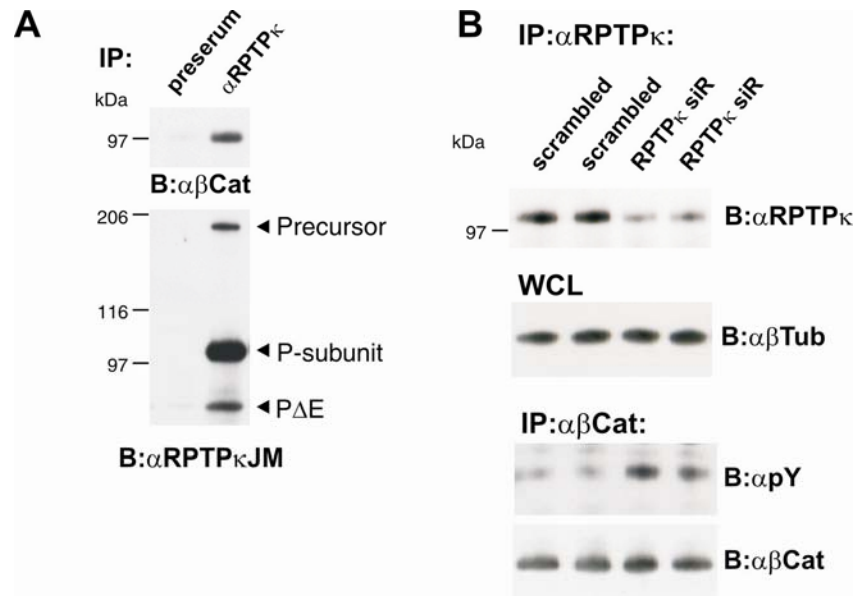


Figure 13: RPTP κ dephosphorylates β -catenin in living cells. (A) Co-immunoprecipitation of β -catenin with RPTP κ in 786-O cell lysates. Upper panel, detection of β -catenin in anti-RPTP κ -JM antibody immunoprecipitates. Preserum was used as a negative control. Lower panel, the blot was analyzed again with anti-RPTP κ -JM antibody. (B) Short interfering RNA (siR)-mediated knock-down of RPTP κ increases β -catenin tyrosine phosphorylation. Stably transfected ACHN cells were analyzed for RPTP κ expression and β -catenin tyrosine phosphorylation. RPTP κ (top panel) and β -catenin (bottom panel) were immunoprecipitated and probed with the indicated antibodies. Tubulin was used as a loading control (middle panel). WCL, whole cell lysate.

1.5 Proteolytic processing of RPTP κ does not affect its catalytic activity or its binding to β -catenin

Next, we wanted to investigate whether proteolytic processing of RPTP κ by ADAM10 or γ -secretase affects its catalytic activity. To this end, we used the direct RPTP κ substrate β -catenin as an indicator of its dephosphorylation activity in HEK293 cells. We transfected these cells with a constitutive active mutant of Src (Y530F) to induce phosphorylation of β -catenin. Catalytic activity of RPTP κ isoforms was then studied by expression of the full-length, P Δ E, PIC, PIC-C1082S or PIC- Δ JM isoforms and by analysis of their impact on cellular β -catenin tyrosine phosphorylation. PIC-C1082S is a catalytic inactive mutant of the intracellular phosphatase part and PIC- Δ JM corresponds to the intracellular part of RPTP κ

lacking the juxtamembrane sequence. As expected, full-length RPTP κ dephosphorylated β -catenin in HEK293 cells (Figure 14A). Also the P Δ E and PIC isoform were catalytically active in this experiment and dephosphorylated β -catenin. This shows that proteolytic processing of RPTP κ by ADAM10 and γ -secretase does not affect the catalytic activity of RPTP κ . As expected, mutation of the conserved cysteine residue 1082 to serine in RPTP κ PIC diminished its dephosphorylating activity. Interestingly, also PIC- Δ JM showed catalytic activity against β -catenin. In addition, direct physical interaction of the above described RPTP κ isoforms could be demonstrated as they co-precipitated with β -catenin (Figure 14B). Binding of PIC- Δ JM to β -catenin is surprising, as the juxtamembrane region was described to be a β -catenin binding site (Fuchs et al., 1996). This suggests that binding of RPTP κ to β -catenin relies also on other parts of the intracellular domains and not only on its juxtamembrane sequence.

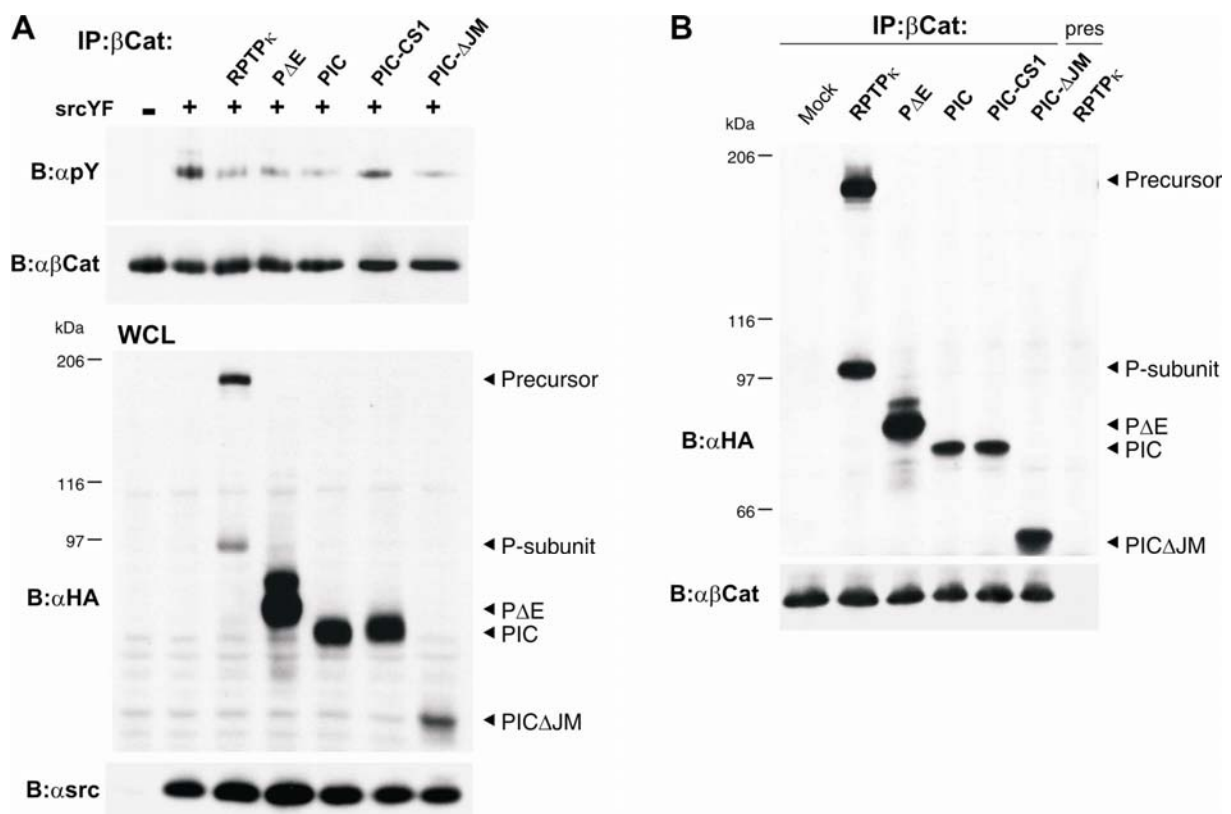


Figure 14: RPTP κ processing does not affect its catalytic activity or its binding to β -catenin. (A) PIC is an active phosphatase and dephosphorylates β -catenin. HEK293 cells were transfected with empty vector (-) or constitutive active Src kinase (Y530F) (+) to induce tyrosine phosphorylation of β -catenin. RPTP κ , P Δ E, PIC, PIC-CS1 and PIC Δ JM were co-transfected, and tyrosine phosphorylation of β -catenin was analyzed (upper panel). All

RPTP κ constructs shown here contain a HA-tag at the C-terminus. Transfection controls are shown below. PIC-CS1 is a catalytically inactive mutant; PIC Δ JM is devoid of the juxtamembrane sequence. (B) β -catenin co-precipitates with PIC and PIC Δ JM. HEK293 cells were transfected with empty vector (mock) or the indicated HA-tagged constructs, and β -catenin was immunoprecipitated. Preserum was used as a control (right lane). RPTP κ isoforms were detected with anti-HA antibody.

1.6 RPTP κ PIC enhances transcriptional activation of β -catenin, whereas full-length RPTP κ suppresses it

Besides its function in E-cadherin mediated cell adhesion complexes, β -catenin can also translocate to the nucleus and activate TCF/LEF transcription factors (Logan and Nusse, 2004). Both its adaptor function at cell adhesion sites, as well as its transcriptional coactivator function in the nucleus are modulated by tyrosine phosphorylation (Piedra et al., 2001), but the underlying regulatory mechanisms are poorly defined. This prompted us to study the regulatory function of RPTP κ and its cleavage isoforms on β -catenin's transcriptional activity. To this end, luciferase gene reporter experiments under the control of a β -catenin/TCF promotor or a mutated version thereof as a negative control were performed (Korinek et al., 1997). For these experiments, HCT116 colon cancer cells were chosen as they contain stabilizing mutations in the β -catenin gene leading to a constitutive oncogenic transcriptional activity in the nucleus (Morin et al., 1997). Full-length RPTP κ or its cleavage isoforms P Δ E and PIC were transfected together with the β -catenin/TCF gene reporter constructs into HCT116 cells and luciferase activity was measured. In addition, catalytically inactive PIC-C1082S and the juxtamembrane deletion mutant PIC- Δ JM were tested to study the influence of PIC's catalytic activity on β -catenin-mediated TCF activation and to analyze the impact of the juxtamembrane region on this process. As expected, RPTP κ full-length isoform decreased β -catenin's transcriptional activity, probably by stabilizing its association to E-cadherin and thereby decreasing its translocation to the nucleus (Figure 15).

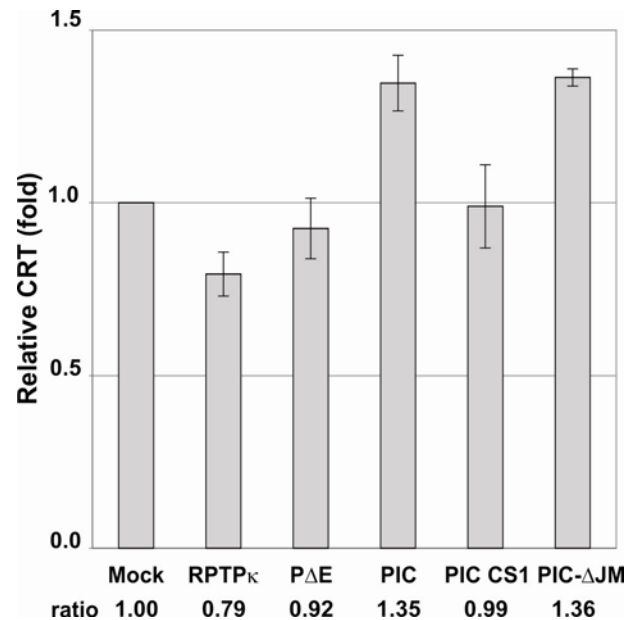


Figure 15: PIC increases transcriptional activation of β -catenin, whereas full-length RPTP κ suppresses it. HCT116 cells were co-transfected with β -catenin/TCF reporter constructs and one of the RPTP κ isoforms or empty vector (Mock), as indicated. Luciferase activity was determined using the dual luciferase kit (Promega), and the data were normalized for transfection differences and non-specific transcription was subtracted. Error bars represent the standard deviations of triplicate assays.

The ER- and Golgi-localized P Δ E isoform had almost no regulatory effect due to localization to a cellular compartment that does not contain β -catenin. Surprisingly, nuclear PIC modulated β -catenin-mediated transcription in a positive manner. This activation process was dependent on the catalytic activity of PIC, as transfection of PIC-C1082S resulted in no increase, and it did not change when the juxtamembrane region of PIC was deleted. Although RPTP κ , P Δ E and PIC can dephosphorylate β -catenin, they have different effects on β -catenin mediated transcription due to their localization to different cellular compartments.

1.7 Homophilic binding of RPTP κ Ec-Fc fusion proteins to RPTP κ does not induce its shedding

To investigate the physiological function of RPTP κ processing in more detail it is necessary to specifically activate this proteolytic cascade. As RPTP κ S2 shedding triggers the whole mechanism and is itself dependent on high cell density, we thought that maybe homophilic interactions of RPTP κ 's extracellular domains, which take place at cell confluence, could activate the responsible metalloproteinase. This has been shown to be true for ADAM10-mediated ephrin-A2 cleavage after heterophilic Eph receptor binding in neuronal axons (Hattori et al., 2000). For this reason, the extracellular domain of RPTP κ was fused C-terminally (residue 640) to two heavy chain human IgG domains, which still allowed dimerization (Figure 16A). The recombinant RPTP κ Ec-Fc fusion proteins were then used to stimulate Cos7 cells transfected with RPTP κ -HA (Figure 16B). TFP stimulation was performed as a positive control to ensure that ADAM10 can be activated to cleave RPTP κ in this cell system. Both stimulation for 10 min as well as 60 min with RPTP κ Ec-Fc did not induce any shedding. As preclustering of ephrin receptor fusion proteins was described to be important for ADAM10-mediated ephrin-A2 cleavage (Hattori et al., 2000), we also used preclustered RPTP κ Ec-Fc fusion proteins in our experiment, but no S2-cleavage was induced. Increasing the concentration of RPTP κ Ec-Fc up to 50 μ g/ml did not result in RPTP κ shedding either (data not shown). This lack of RPTP κ -cleavage upon homophilic binding with RPTP κ Ec-Fc proteins might be explained with findings from Janes *et al.*, who observed that ADAM10 cleaves its substrate ephrinA5 after heterophilic binding to the EphA3 receptor *in trans*, i.e. ADAM10 cleaves some of its substrates on the cell surface of adjacent cells (Janes et al., 2005). In our experiment this would lead to cleavage of the RPTP κ Ec-Fc fusion protein instead of the RPTP κ protein on the plasmamembrane.

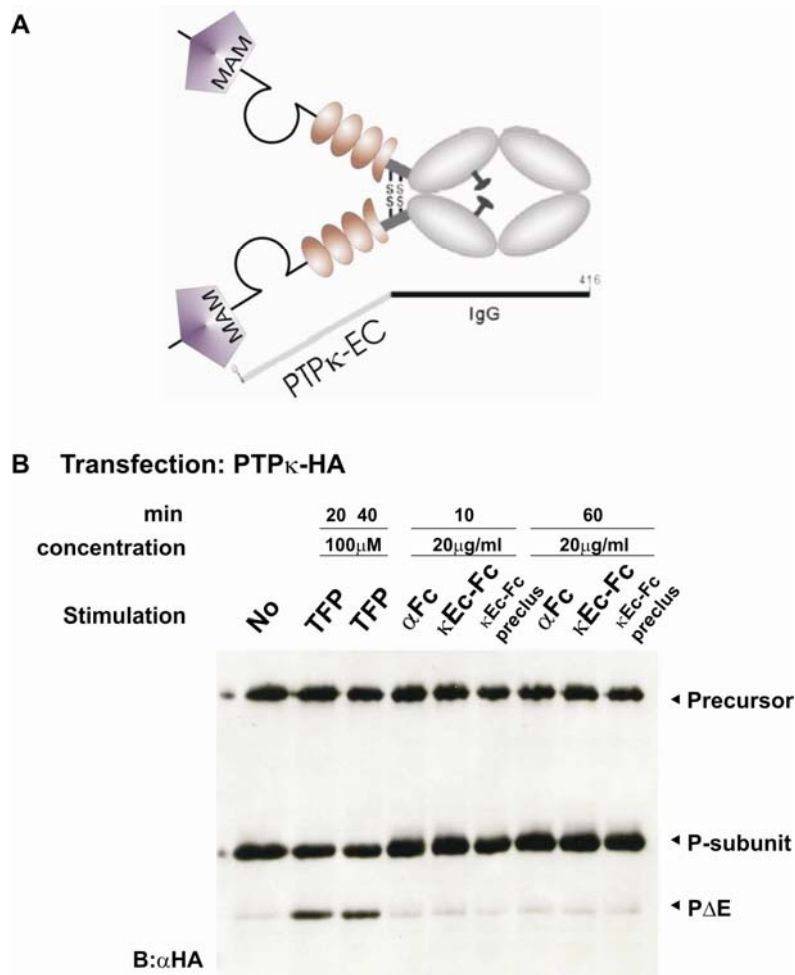


Figure 16: Homophilic binding of RPTP κ Ec-Fc fusion proteins to RPTP κ does not induce its shedding. (A) Scheme illustrating RPTP κ EcFc fusion proteins, which form dimers via disulfide bridges between their Immunoglobulin (IgG) domains. (B) RPTP κ EcFc fusion proteins do not induce RPTP κ S2-cleavage. HA-tagged RPTP κ was ectopically expressed in Cos-7 cells and cells were stimulated with TFP, RPTP κ Ec-Fc fusion proteins (κ EC-Fc) and preclustered RPTP κ Ec-Fc fusion proteins for the indicated times and concentrations. Preclustering of RPTP κ Ec-Fc was achieved via binding of anti-IgG antibodies to the Fc-part of the fusion proteins.

2. Proteomics based identification of new potential PTP1B substrates

2.1 PTP1B-deficient mouse fibroblasts as a model system to study PTP substrate specificity

PTP1B deficient mouse embryonic fibroblasts (MEFs) are a well established model system to study substrate specificity for this prototypic PTP family member. Due to loss of PTP1B expression (Figure 17), tyrosine phosphorylation of its substrate proteins should be increased in KO cells compared to wild-type cells. This has been shown to be the case for a number of protein tyrosine kinases, like the EGFR and the PDGFR (Haj et al., 2003), the Insulin receptor (Galic et al., 2005) and the cytosolic kinases JAK2 and TYK2 (Myers et al., 2001).

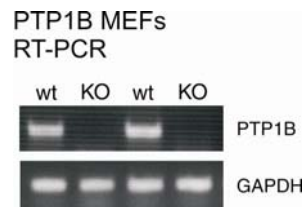


Figure 17: Verification of PTP1B knock-out in immortalized mouse embryonic fibroblasts. Gene expression of PTP1B in +/+ and -/- MEFs was analyzed by RT-PCR.

To analyze how overall cellular tyrosine phosphorylation is affected by PTP1B-deficiency, we performed a phosphotyrosine specific Western blot analysis of wild-type and KO cells under unstimulated and EGF- or PDGF-stimulated conditions (Figure 18). A number of proteins exhibited increased tyrosine phosphorylation levels in KO cells compared to wild-type cells under each condition. However, identification of PTP1B-regulated proteins by molecular weight only is difficult. Therefore, we used quantitative mass spectrometry (MS) analysis to investigate which proteins are regulated by PTP1B in this cellular system (chapters 2.2 & 2.3).

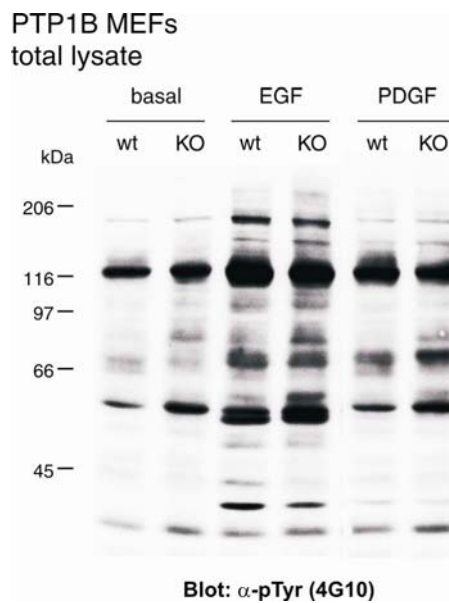


Figure 18: PTP1B-deficiency leads to alterations in cellular tyrosine phosphorylation under basal and EGF- or PDGF-stimulated conditions. Western blot analysis of tyrosine phosphorylated proteins in wild-type and KO cells under basal and EGF- (50 ng/ml, 5 min) or PDGF-stimulated (20 ng/ml, 5 min) conditions. Cells were starved for 3 h with medium containing no FCS before stimulation.

Interestingly, loss of PTP1B function does not only lead to changes in cellular tyrosine phosphorylation, but also to cell physiological alterations. In previous studies it was demonstrated that PTP1B-deficiency leads to a significant reduction in proliferation of mouse fibroblasts (Dube et al., 2004), whereas the migration rate of these cells was found to be strongly increased (Buckley et al., 2002). We could verify both observations using an MTT-assay for proliferation analysis (Figure 19A) and wounding assays or Boyden chamber assays for migration analysis of PTP1B MEFs (Figure 19B). Taking this into consideration, our PTP1B substrate identification analysis should reveal proteins being regulated by PTP1B that control proliferation and motility of mouse fibroblasts. The next chapters show that this assumption proves to be true for our MS-based PTP1B substrate analysis.

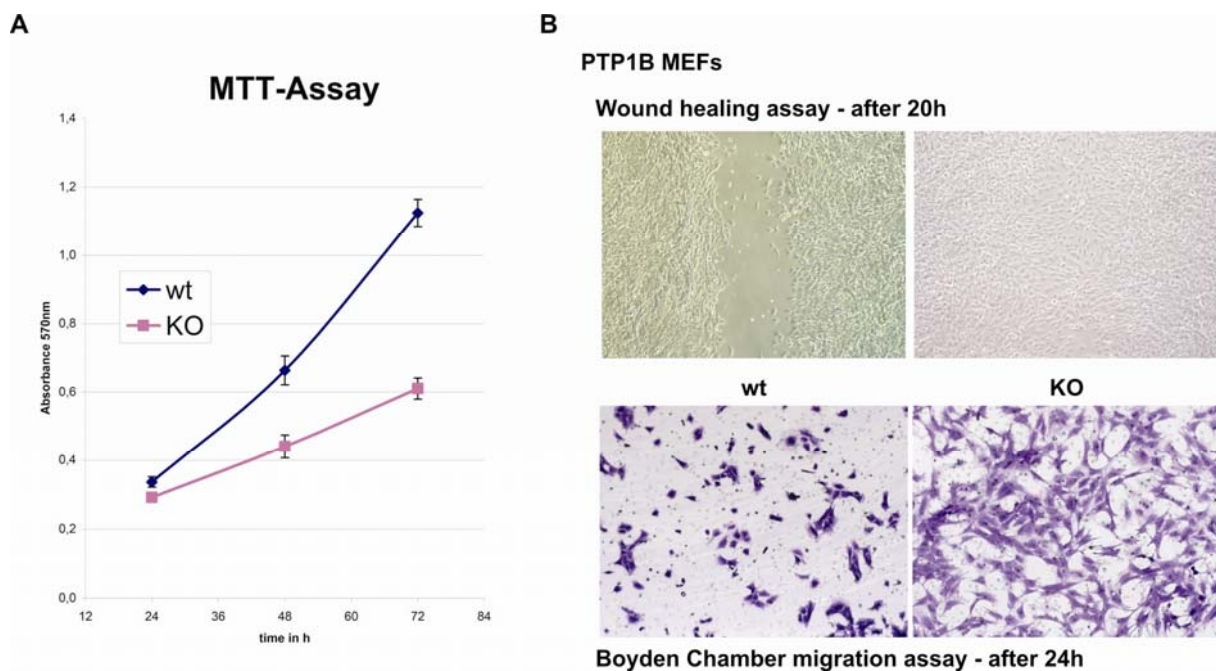


Figure 19: PTP1B KO MEFs show decreased proliferation and increased migration compared to wild-type cells. (A) MTT-assays were used to analyze the proliferation rate of wild-type and KO cells. (B) Upper panel: Within 20 h KO cells close a wound scratched into the confluent cell layer, whereas wild-type cells do not. Lower panel: The migration rate of PTP1B KO cells in a Boyden chamber assay is threefold higher compared to wild-type cells with FCS as an attractant.

2.2 Loss of PTP1B leads to alterations in the phosphotyrosine proteome of mouse embryonic fibroblasts

To study alterations in phosphotyrosine signaling of PTP1B deficient cells in an unbiased manner we used quantitative high resolution LC-MS/MS. For this purpose, wild-type and KO fibroblasts were labeled with SILAC media containing either normal arginine and lysine amino acids or heavy Arg-10 and Lys-8 amino acids (Ong et al., 2002) (Figure 20). After cell lysis, samples were mixed 1:1 and tyrosine phosphorylated proteins were immunoprecipitated by saturating amounts of anti-phosphotyrosine antibodies. Subsequently, enriched proteins were eluted with urea and digested enzymatically in solution. The resulting peptide mixture was then enriched for phosphopeptides with a TiO_2 resin (Larsen et al., 2005) and analyzed by online LC-MS/MS on a linear ion trap/orbitrap mass spectrometer (LTQ-Orbitrap) (Makarov et al., 2006). For sequencing, phosphopeptides were fragmented by tandem MS or, in case of

neutral loss events of pSer or pThr residues, by multistage activation (“pseudo-MS3”) (Schroeder et al., 2004). The phosphorylation levels of phosphosites on proteins in wild-type and KO cells correspond to the intensities of respective phosphopeptides. All labeled peptides co-elute and appear in the MS spectra as characteristic doublets, since stable isotopic labeling with Arg-10 and Lys-8 does not change the elution properties of peptides from C18 columns.

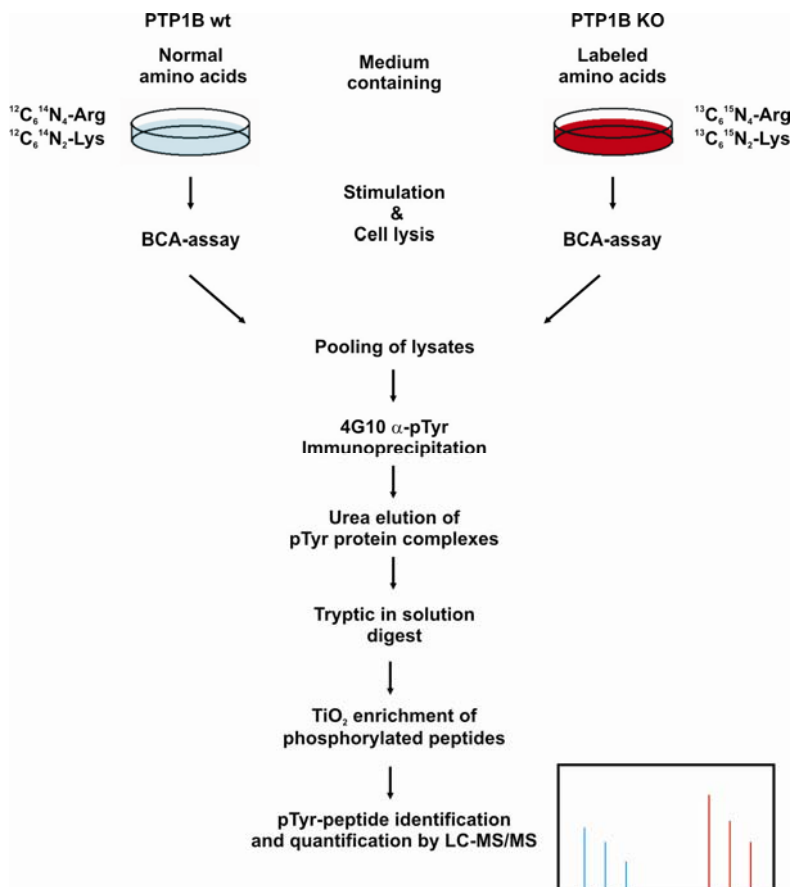


Figure 20: Experimental working scheme for mass spectrometric analysis of tyrosine phosphorylation differences between PTP1B +/+ and -/- MEFs. Note the differential labeling with light and heavy SILAC amino acids.

MS- and corresponding MS/MS-spectra were converted into peak-list files and searched against mouse IPI databases using the MASCOT search engine. A concatenated target/decoy database (Elias et al., 2005) allowed to define cutoff score thresholds for a false-positive rate of less than one percent ($p < 0.01$) for peptide identification.

Peptide quantitation was performed by MSQuant software, whereby all peptides were checked manually (Schulze and Mann, 2004). Post-translational modification (PTM) scoring was used to further evaluate the position of each phosphosite as reported by Olsen *et al.* (Olsen *et al.*, 2006). Most of the analyzed phosphotyrosine peptides had a localization probability for their phospho-groups of 1 and in rare cases of 0.5, i.e. one phospho-group could be assigned to two residues with the same probability.

Additional information about expression levels of the identified tyrosine phosphorylated proteins was obtained by mixing whole protein extracts of SILAC labeled wild-type and KO cells in equal amounts and subsequent MS analysis. To this end, protein samples were loaded onto a 4-12% gradient polyacrylamide gel, the lanes were cut into 15 pieces and an in-gel digest was performed. Next, the 15 peptide samples were analyzed by LC-MS/MS on an orbitrap mass spectrometer and measured data was evaluated using MASCOT and MSQuant. In total, we acquired relative protein expression data of KO and wild-type cells for 56 proteins that were found to be tyrosine phosphorylated in this study. For this reason, quantitation was performed only with non-phosphorylated peptides of these proteins. Importantly, most of the analyzed protein expression ratios were close to one, indicating that protein expression of tyrosine phosphorylated proteins does not change markedly due to PTP1B-deficiency.

Altogether, 80 tyrosine phosphorylated proteins containing 168 phosphotyrosine sites could be quantified in PTP1B wild-type and KO MEFs under basal conditions using our quantitative phosphopeptide analysis. As an increased tyrosine phosphorylation of putative substrates is expected in PTP1B deficient cells, phosphotyrosine peptides were rated as positive hits, that exhibited an at least twofold stronger phosphorylation in KO cells. For certain phosphopeptides solely the heavy labeled stronger phosphorylated peptide could be detected, therefore quantitation was performed over background and estimations of the minimum KO/wild-type ratio were made.

Using this approach we found 20 proteins hyperphosphorylated at 33 phosphotyrosine sites in PTP1B KO cells under standard cell culture conditions. In Table 1, all corresponding KO/wild-type peptide abundance ratios as well as respective pTyr sites of these proteins are listed. In addition, relative whole protein expression data is provided and the main cellular function of these proteins is described in the left column. Three of these proteins have previously been reported to be higher tyrosine phosphorylated in PTP1B KO cells and to be substrates of PTP1B: p62DOK, the PDGFR and the Insulin receptor/IGF1R (Dube *et al.*, 2004), (Liu and Chernoff, 1997), (Seely *et al.*, 1996), (Elchebly *et al.*, 1999). The 8.75-fold higher tyrosine phosphorylation of the Insulin receptor/IGF1R peptide could not be directly

linked to one of the proteins, since no unique peptides of these kinases could be identified. These data confirm not only results obtained by other groups, but provide also evidence of the validity of our measurements.

Table 1: Hyperphosphorylation of 20 proteins in PTP1B-deficient fibroblasts under basal conditions.

function in	protein	phosphopeptide sequence	pY site	pY site	
				KO/wt ratio	whole protein KO/wt ratio
Cell shape and motility	Lipoma-preferred partner homolog (LPP)	SAQSPHYMAGPSSGQlpYGPGR	245	4.19	1.56
		SEGDTApYQQVQPNTWK	318	3.44	1.56
		YYEPYpYAAAGPSYGG	302	2.49	1.56
Cell motility	Vav-3	TPIALATGIRPFTEESINDEDlpYK	141	>10	
	Tks5 / Fish protein	VGESSEDALEEETlpYENEGFRPYTEDLSAR	639	4.65	
		VKYEPEpYDVPAFGFDpSEPEMNEEPSGDR	557	3.42	
	Fer	QEDGGVpYSSSLK	714	3.88	1.36
		VQENDGKEPPVpVpYEEEDAR	402	2.83	1.36
RhoGAP-12	ApT(0.5)pT(0.5)PPNQGRPDpSPVpYANLQELK	241	3.22		
Cell motility / vesicular transport	Cortactin	NASTFEEVQVPSApYQK	334	5.58	1.03
		KQpTPPpSPpSPQPIEDRPPpS(0.5)pS(0.5)PlpYEDAAPFK	421	3.50	1.03
	Cas-L	DVYDVPSSHSTQGVpYDIPSSVK	188	2.45	
		EpYDFPPMK	240	2.15	
		HQSFSLHAPSQLGQSGDTQSDApYDVPR	316	2.03	
Vesicular transport	Isoform 2 of Intersectin-2	GEPEALpYAAVTK	921	2.14	0.53
Cell adhesion	Catenin delta-2	DpYETYQFPFNSTR	1176	7.73	
		QDVPYGPQPV	257	3.84	0.93
	p120ctn	FHPEPpYGLEDDQR	280	3.07	0.93
		SMGYDDLDYGMMSDpYGTAR	302	2.56	0.93
		LNGPQDNHLLpYSTIPR	96	2.29	0.93
Tight junctions assembly	ZO-1	YRPEAQpYSSSTGPK	1177	>10	1.00
		HEEQPAPpYEVHNR	1164	4.47	1.00
		VQIPVSHPDPEPpSDNEDDpYDEEVHDPR	132	2.46	1.00
IP ₃ and DAG signaling	PLC γ 1	IGTAEPDpYGALYEGR	771	2.50	0.98
Phagocytosis	MEGF10 protein	DSPpYAEINNSTPANR	1061	>10	
Proliferation	p62DOK	FSALEMLENSlpYSPTWEGSQFWTSQK	146	2.45	1.21
		GLpYDLPQEPR	376	2.11	1.21
Proliferation / migration	Eph receptor A3/4/5	VLEDDPEApYTTR	779	3.85	
	Platelet-derived Growth Factor Receptor β	DESIDpYVPLDMK	751	2.04	1.72
Cell growth / metabolism	Insulin-like Growth Factor 1 Receptor / Insulin Receptor similar to Oligophrenin 1	DlpYETDpYYRK	1193/1197	8.75	
Unknown	Calcium-independent alpha-latrotoxin receptor homolog 2	LWLEAMDGKEPlpYTLPAISK	371	3.70	
		THpSLlpYQPK	689	2.79	

Most of the other 17 proteins, which have not been described to be regulated by PTP1B so far, control cell adhesion and motility. For example, adapter proteins that are involved in actin cytoskeleton regulation at sites of cell/matrix and cell/cell adhesion, like Cortactin, Lipoma-preferred partner homolog (LPP), Cas-L, p120ctn and ZO-1 are stronger phosphorylated in KO cells. Interestingly, tyrosine phosphorylation of many of these proteins has been linked to increased cell motility, like for Cortactin in transformed endothelial cells (Huang et al., 1998) and for Cas-L in T-cell migration (Ohashi et al., 1999). In line with this, reduced cell adhesion has been connected to tyrosine phosphorylation of p120ctn (Ozawa and Ohkubo, 2001) and ZO-1 (Takeda et al., 1995). These results correspond to the already described increased migration rate of PTP1B KO cells compared to their wild-type counterparts (Buckley et al., 2002). Increased tyrosine phosphorylation was also measured for other cell motility regulators, such as the Rho regulators Vav-3 and Rho-GAP-12, Fer kinase which localizes to

N-cadherin based adherens junctions (Greer, 2002), the podosome regulator Tks5/Fish (Seals et al., 2005) and the actin polymerization regulator Nck2 (Rivera et al., 2006).

Apart from the PDGFR, a further tyrosine kinase family involved in migration and proliferation control was activated in PTP1B KO cells. A common pTyr peptide in Ephrin receptors A3, A4 as well as A5 was up-regulated, but as no unique peptide could be identified the identity remains elusive.

All identified protein expression differences between PTP1B wild-type and KO cells in Table 1 are less than twofold, indicating that most of the observed alterations in phosphotyrosine signaling do not result from protein expression differences.

Figure 21A shows three examples of how pairs of labeled phosphotyrosine peptides were quantified with the MSQuant software. The lower mass isotope clusters represent peptides derived from the normal Arg-0 and Lys-0 labeled PTP1B wild-type cells, whereas the higher mass isotope clusters derive from the heavy isotope labeled (Arg-10/Lys-8) KO cells. Only the monoisotopic peaks were used for quantitation. As denoted, phosphotyrosine peptide MS-spectra of PLC γ 1, Cortactin and tyrosine kinase Fer are illustrated with corresponding phosphopeptide sequences and KO/wt ratios.

To test if our mass spectrometry data can be reproduced with phosphotyrosine specific Western blotting, we performed a tyrosine phosphorylation analysis of PLC γ 1, Cortactin and tyrosine kinase Fer (Figure 21B). Expression and phosphorylation levels of all three proteins analyzed with immunoblotting match perfectly to the data we obtained from our MS analyses. Furthermore, two exemplary fragmentation MS/MS spectra used to identify the peptide sequence and the localization of respective phosphogroups are illustrated in Figure 22. In contrast to pSer and pThr, no neutral loss of the phosphoric acid occurred on pTyr residues during collision in the ion trap and a shift of 79.97 m/z units can be seen on the pTyr containing fragments in the singly charged y-ion series.

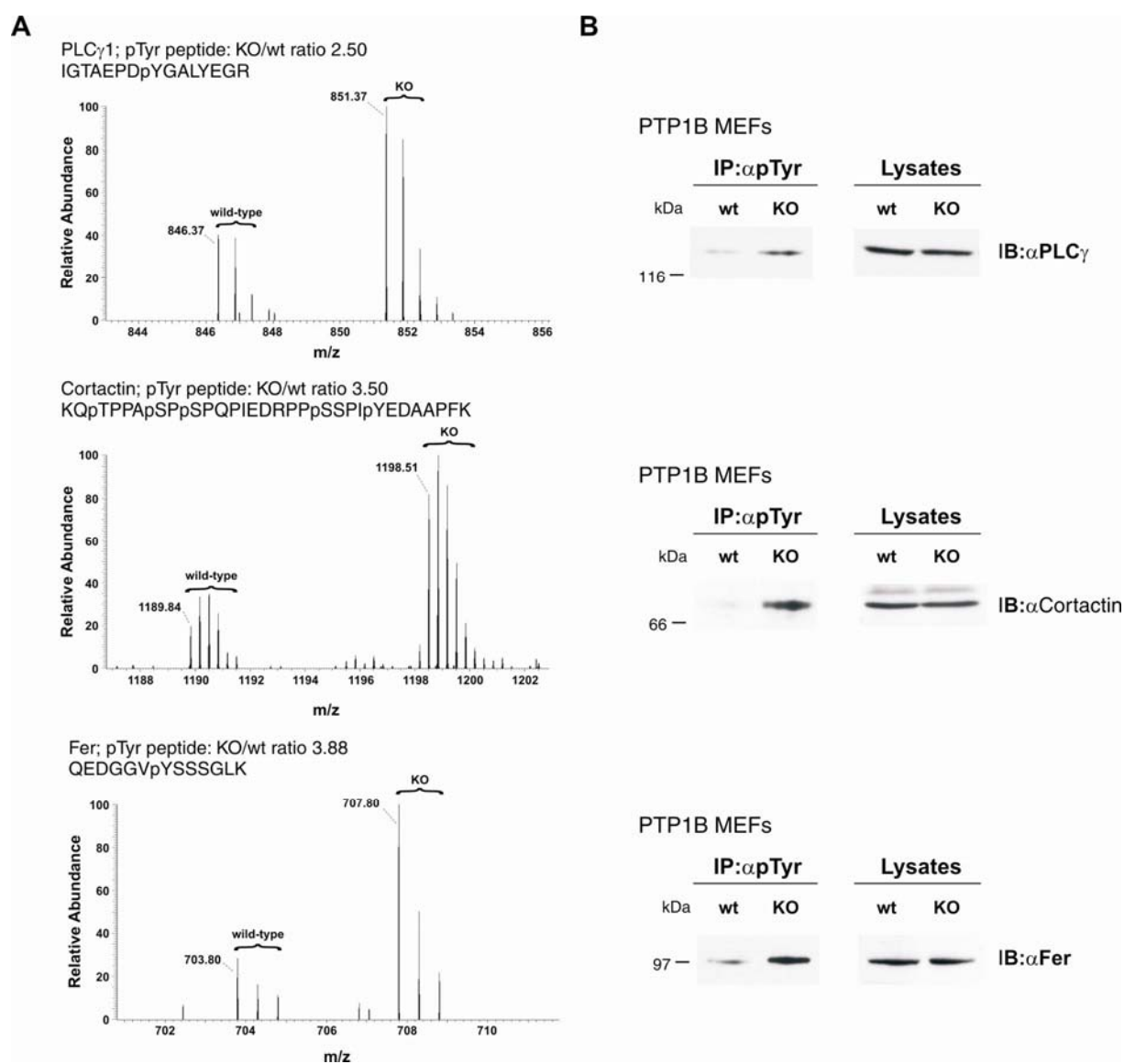


Figure 21: Comparison of Quantitative MS and Western blotting to study phosphorylation differences between PTP1B wild-type and KO cells. (A) Selected MS spectra of pTyr peptides listed in Table 1 are illustrated here. The MSQuant software was used to evaluate all of those MS-spectra. Isotope clusters of pTyr peptides derived from wild-type and KO cells are indicated by brackets. Only monoisotopic peaks were used for quantitation which are marked with corresponding m/z values. (B) Using anti-pTyr antibodies, tyrosine phosphorylated proteins of unstimulated cells were immunoprecipitated. Then, immunoprecipitated proteins were separated by SDS-PAGE and probed with the indicated antibodies. Moreover, cellular protein expression levels were analyzed by loading whole cell extracts.

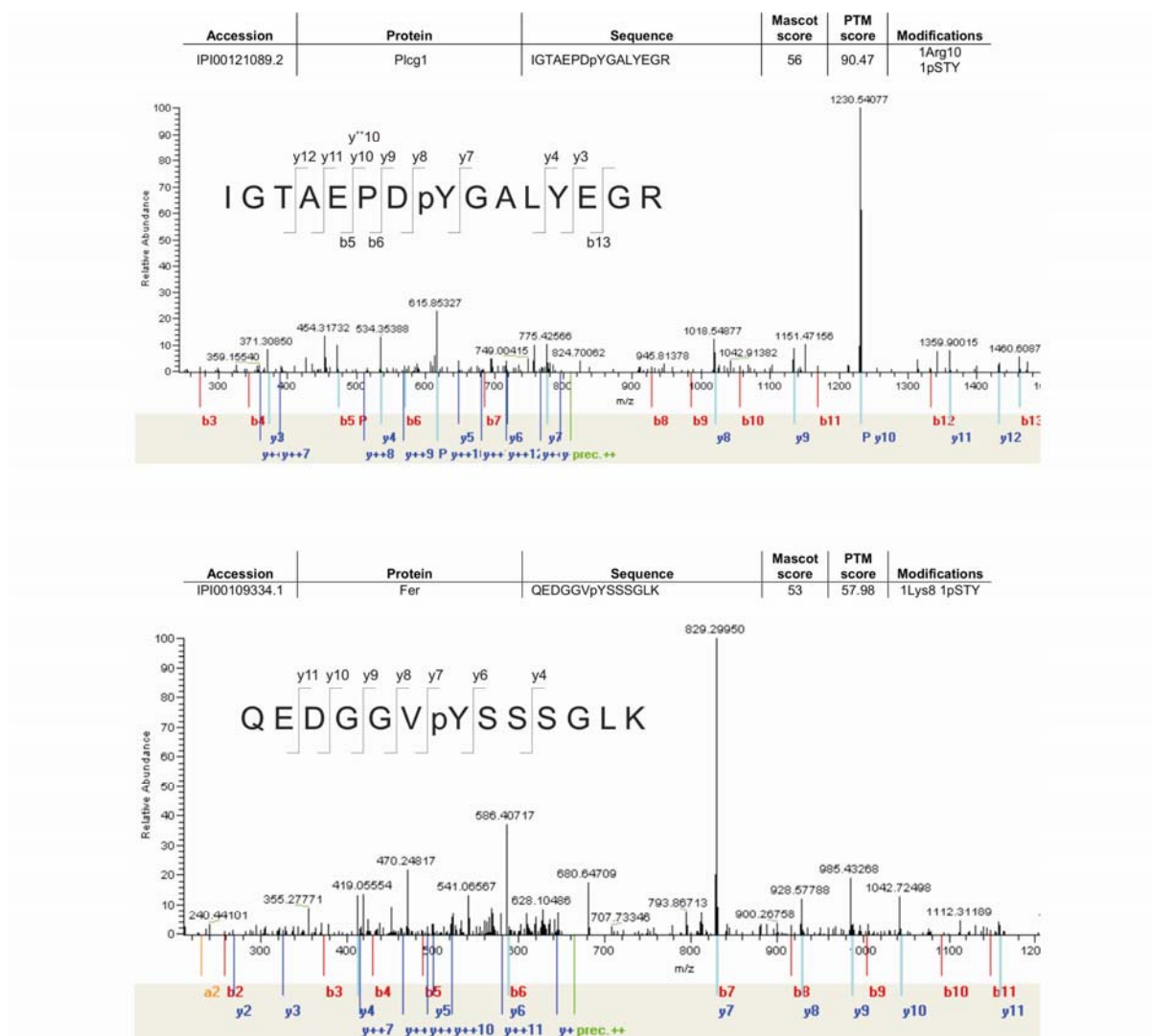


Figure 22: MS/MS spectra of selected tyrosine phosphorylated peptides. Precursor ion masses were measured in the orbitrap mass spectrometer, and MS/MS spectra were acquired in the LTQ mass spectrometer of the LTQ-Orbitrap instrument. MS/MS peaks highlighted in blue were identified by MSQuant software. Respective b- and y-ion series are indicated below and above the phosphopeptide sequence.

2.3 Analysis of PTP1B function in EGFR- and PDGFR-signaling

PTP1B has been shown to negatively regulate both EGFR- and PDGFR-signaling (Lammers et al., 1993), (Haj et al., 2003), (Haj et al., 2002). As both pathways have important functions in growth and motility control of mouse embryonic fibroblasts, we decided to extend our analysis on these growth factor receptors. Figure 23 illustrates our strategy to study phosphorylation differences between wild-type and KO cells in three parallel experiments under basal, EGF and PDGF stimulated conditions using SILAC double labeling and quantitative MS. In a fourth experiment, we used SILAC triple labeling to detect alterations in phosphorylation in PTP1B KO cells resulting from growth factor stimulation compared to unstimulated cells. This provides additional information about proteins being regulated by the respective growth factors.

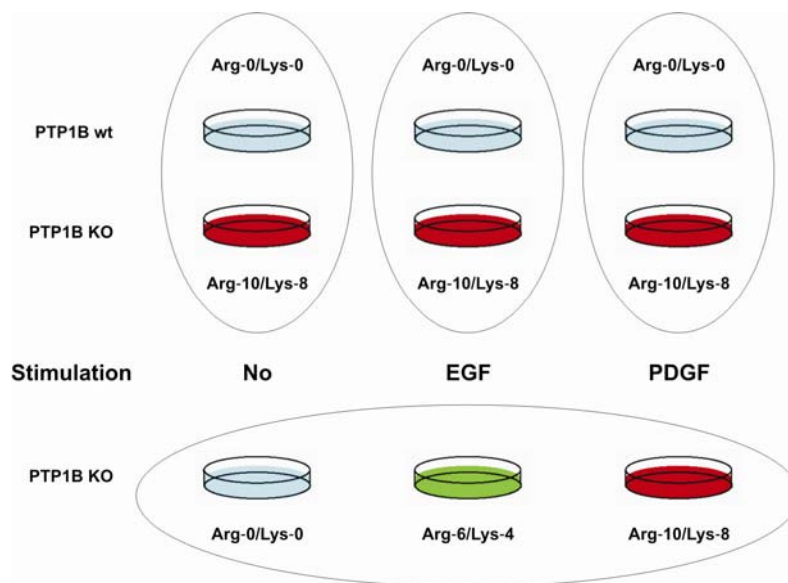


Figure 23: Experimental setup to identify proteins regulated by PTP1B in EGFR- and PDGFR-signaling. Scheme depicting the strategy to analyze phosphorylation differences in PTP1B wild-type and KO cells upon EGF (50 ng/ml, 5min) and PDGF (20 ng/ml, 5min) stimulation. Cells were starved for 3 h. Each independent SILAC experiment is represented by an oval.

Using our quantitative mass spectrometry based phosphotyrosine peptide analysis we could quantitate 53 phosphotyrosine sites of 26 proteins that were induced at least two-fold by EGF and 49 phosphotyrosine sites of 23 proteins that were induced at least two-fold by PDGF. As

PTP1B substrates are expected to be hyperphosphorylated in PTP1B KO cells also in this set-up, we defined all proteins as positive hits which exhibited at least two fold stronger phosphorylation on one of their tyrosine sites in KO cells than in wild-type cells.

By using these selection criteria for our EGF data set, we identified 10 proteins that were not only at least twofold stronger phosphorylated upon EGF stimulation, but also more than twofold stronger phosphorylated in KO cells than in wild-type cells (Table 2A).

Unexpectedly, all seven quantitated EGFR phosphotyrosine peptides showed a KO/wt ratio of approximately one and also the relative protein levels were close to one. As expected, the EGFR was 30-50 fold stronger tyrosine phosphorylated after EGF stimulation. Similar activation of the EGFR in wild-type and KO cells should lead to an equal phosphorylation of down-stream target proteins. Nevertheless, ten proteins were found to be at least two-fold stronger phosphorylated in KO than in wild-type cells. Among them were negative regulators of proliferation like p62DOK (Zhao et al., 2001), Sprouty 1 (Lo et al., 2004) and CYLD (Massoumi et al., 2006). Strongly induced by EGF were also the phosphoinositide regulators PLC γ 1 and SHIP2, which exhibited in case of PLC γ 1 pTyr-771 a 6-fold increase and in case of SHIP2 pTyr-1136 a 4-fold increase in phosphorylation upon PTP1B deficiency. Again, also after EGF treatment the Rho regulator Vav-3 was 10-fold hyperphosphorylated in KO cells and 23.6-fold induced by EGF.

After PDGF stimulation 15 proteins were detected having a KO/wild-type phosphorylation ratio greater than two and being implicated in PDGF signaling (Table 2B). Starting with PDGFR which is about 2.5-fold higher phosphorylated in KO cells, also the downstream kinases Fyn, Fer and PI3K were found to be upregulated even stronger. Similar to the EGF data set, also after PDGF stimulation a number of RTK downstream signaling modulators emerged from our analysis. Among the RTK negative regulators were the ubiquitin ligases CBL and CBL-B (Reddi et al., 2007), which control internalization, and the Ras signaling negative regulators sprouty1 (Hanafusa et al., 2002), p62DOK and p120RasGAP (Dube et al., 2004). On the other hand, also positive regulators of RTK signaling were hyperphosphorylated in PTP1B KO cells such as SHP2 (Neel et al., 2003) and Gab2. Of particular interest were also PLC γ 1 and the diacylglycerol binding kinase PKC δ , which were affected in the same manner due to PTP1B-deficiency.

Table 2: Analysis of PTP1B function in EGF and PDGF signaling

A: EGF stimulation leads to hyperphosphorylation of 10 proteins in KO cells.

function in	name	phosphopeptide sequence	pY site	pY site		stimulation ratio
				KO/wt ratio	whole protein KO/wt ratio	
Cell motility	Vav-3	TPIALATGIRPPFTEESINDEDpYK	141	10.58		23.60
IP ₃ and DAG signaling	PLC _γ 1	IGTAEPDpYGALYEGR	771	5.94	0.98	12.87
		pYQQPFEDFR	1253	3.18	0.98	11.13
		NPGFpYVEANPMPTFK	783	2.76	0.98	8.27
Phosphoinositide signaling	SHIP2	TLSEVDpYAPGGR	1136	4.06	0.65	48.61
		NSFNNApY(0.5)pY(0.5)VLEGVPHQLLPLEPPSLAR	987	2.86	0.65	16.18
Adaptor in RTK signaling	Gab2	SSLTGSETDNEDVpYTFK	290	3.60		3.42
		VDpYVQVDK	633	3.06		9.14
RTK negative regulation	Sprouty-1	GSNEpYTEGPSVAR	53	3.72		6.69
Proliferation	p62DOK	GFSSDTALpYSQVQK	450	3.04	1.21	2.38
		TKLTDSKEDIpYDEPEGLAPAPPR	361	2.63	1.21	3.28
		ubiquitin carboxyl-terminal hydrolase CYLD	VTSPpYWEER	15	2.18	
Unknown	FAM59A	IDGAEEDPTAGSLDLEDQpYFVRK	767	3.96		>10.00
		TEVPpYEELWLEEGKPSRQPLTR	453	3.16		>10.00
	Sugen Kinase 269	pYQEVWTSSTSPR	528	2.93	0.58	3.19
		TTSVISHTpYEEIETESK	662	2.11	0.58	2.21
Ankyrin repeat domain-containing protein 13	SQDLSPASNGGVSHTHSYEAQpYER	522	2.11		>10.00	
Proliferation / migration	Epidermal growth factor receptor	pYSSDPTGAVTEDNIDDAFLPVEpYVNSQVPK	1069/1092	1.31	0.78	45.04
		MHLPSPTDSNFpYR	1000	1.20	0.78	>50.00
		RPAGSVQNPVpYHNQPLHPAPGR	1110	1.09	0.78	>50.00
		ALMDEEDMEDVDADEpYLIPQGGFFNSPSTSR	1018	1.07	0.78	>50.00
		pYSSDPTGAVTEDNIDDAFLPVEpYVNSQVPK	1069	1.06	0.78	39.02
		GSHQMSLDNPDpYQQDFFPK	1172	1.05	0.78	37.97
		GPTAENAEpYLR	1197	1.02	0.78	53.28

B: PDGF stimulation leads to increased phosphorylation of 15 proteins in PTP1B KO cells.

function in	name	phosphopeptide sequence	pY site	pY site		stimulation ratio
				KO/wt ratio	whole protein KO/wt ratio	
Cell motility	Fer	VQENDGKPPPVVNpYEEDAR	402	4.26	1.36	3.92
IP ₃ and DAG signaling	PLC _γ 1	IGTAEPDpYGALpYEGRNPGFpYVEANPMPTFK	771/775/783	6.11	0.98	28.47
		IGTAEPDpYGALYEGR	771	3.96	0.98	23.29
		pYQQPFEDFR	1253	2.40	0.98	24.41
Ras negative regulation	p120RasGAP	EIpYNTIR	451	6.66	1.51	
Ras positive regulation	SHP2	IQNTGDpY(0.5)pY(0.5)DLYGGEK	62 or 63	4.77	1.24	>20
		VpYENVGLMQQR	584	2.97	1.24	16.65
		GHEpYTNIK	546	2.69	1.24	18.88
RTK internalization	E3 ubiquitin-protein ligase CBL	LPPGEGESEEDTEpYMTPTSRPVGQKPEPK	698	3.61		7.80
		SNIPDLGpYLK	763	2.19		6.97
RTK negative regulation	Sprouty-1	GSNEpYTEGPSVAR	53	3.41		12.87
Adaptor in RTK signaling	Gab2	SSLTGSETDNEDVpYTFK	290	3.15		2.85
Proliferation	p62DOK	IPPGSQDSVpYSDPLGSPAGAGEGVHSHK	314	2.92	1.21	
		TVPPPVPQDPLGSPALpYAEPLDSLRL	295	2.68	1.21	
Proliferation / migration	PKC δ	KLDTTESVGIpYQGFEK	311	7.23	0.91	
		Phosphatidylinositol 3-kinase regulatory β subunit	NETEDQpY(0.5)pS(0.5)LMEDEDALPHHEER	599	4.26	
	Fyn	DGSLNQSSGpYR	27	3.94		>20
		LIEDNEpYTAR	423	3.32		3.02
		Platelet-derived Growth Factor Receptor β	YADIESPSYMAPYDNpYVPSAPER	778	2.63	1.72
		DESIDpYVPLMDMK	751	2.38	1.72	>50
		DIMRDSNpYISK	857	2.18	1.72	21.75
	pYQQVDDEEFLR	974	2.00	1.72	73.45	
ER-Golgi transport	Rab-1A	FADDTYTESpYISTIGVDFK	39	2.82	0.92	20.88
Phagocytosis	MEGF10 protein	DSPpYAEINNSTPANR	1061	>10.00		2.14

2.4 Physical interaction of newly characterized potential substrate proteins to PTP1B substrate trapping mutants

As an upregulation of tyrosine phosphorylation in PTP1B deficient cells indicates a functional association, but does not necessarily prove direct binding to and dephosphorylation by PTP1B, we used a parallel approach to identify proteins that are presumably directly regulated by PTP1B. A well-established strategy to discover direct substrates for protein tyrosine phosphatases is the *in vitro* substrate trapping method. This method employs recombinant phosphatase inactive mutants with substitutions in their essential catalytic residues, which for PTP1B are D182A and Q262A (Flint et al., 1997) (Xie et al., 2002). These mutants are incapable of dephosphorylating substrate proteins and instead form stable complexes with their substrates.

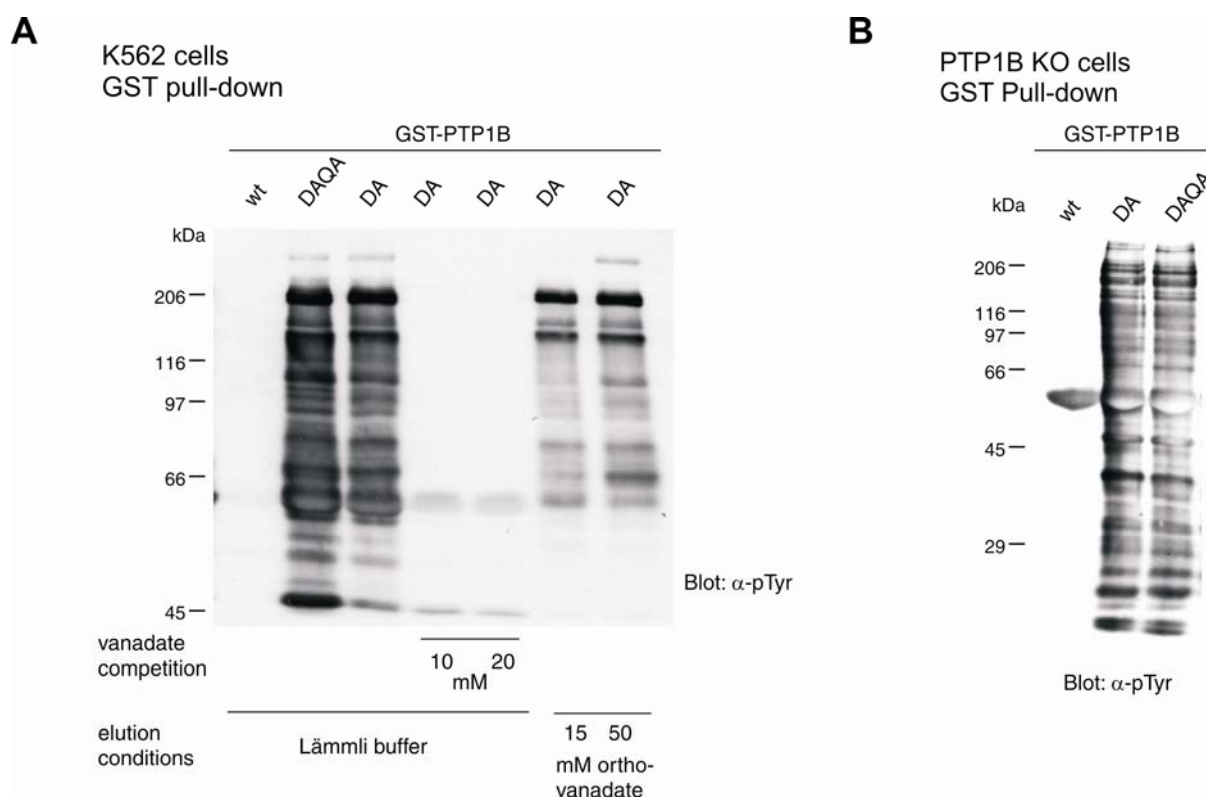


Figure 24: *In vitro* substrate trapping as an alternative strategy to identify PTP substrates. Recombinant PTP1B-GST wild-type and phosphatase-dead (D182A&Q262A) fusion proteins were incubated with cell lysates of pervanadate treated K562 cells (A) or PTP1B KO cells (B). Substrate-trapping mutants form stable interactions with potential substrates via phosphotyrosine-dependent binding to the inactive catalytic center. This binding can be competitively inhibited by adding 10 or 20 mM ortho-vanadate. Moreover, tyrosine phosphorylated proteins can be eluted from substrate trapping mutants by incubation with ortho-vanadate (right lanes in (A)). Binding of tyrosine phosphorylated proteins to

PTP1B-GST wild-type and substrate-trapping mutants was detected by anti-pTyr (4G10) Western blotting.

On the other hand, no tyrosine phosphorylated proteins are pulled down with the wild-type enzyme as all interacting proteins are dephosphorylated. To survey the amount of tyrosine phosphorylated proteins interacting with recombinant PTP1B substrate trapping mutants, a Western blot analysis was performed with cellular extracts from pervanadate treated K562 cells (Figure 24A) and PTP1B KO cells (Figure 24B). As expected, no tyrosine phosphorylated proteins were precipitated with the wild-type enzyme, whereas a large number interacted similarly with PTP1B D182A and D182A&D262A substrate trapping mutants. Moreover, addition of the phosphatase inhibitor ortho-vanadate, which binds to the active center of PTPs, competitively inhibited binding of tyrosine phosphorylated proteins. Ortho-vanadate also induced elution of tyrosine phosphorylated proteins from the substrate trapping mutants. This competitive inhibition of binding by ortho-vanadate further indicates that the detected tyrosine phosphorylated proteins directly bind to the active center of PTP1B substrate trapping mutants.

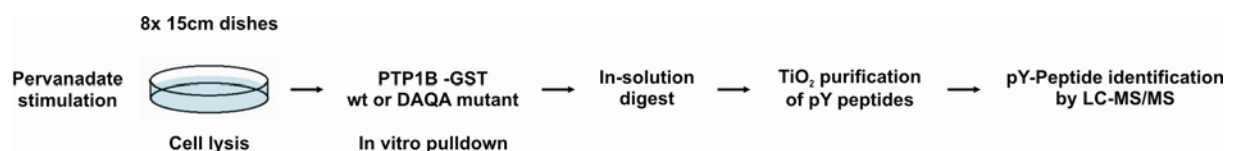


Figure 25: Working scheme for MS sample preparation to qualitatively analyze proteins interacting with PTP1B substrate-trapping mutants.

Our experimental approach in this study combines substrate trapping with mass spectrometric analysis. After regular substrate trapping, bound proteins were eluted with urea, digested in solution and phosphotyrosine peptides were further enriched by means of TiO₂ purification (Figure 25). Subsequently, phosphopeptides were analyzed by LC-MS/MS in a LTQ-Orbitrap mass spectrometer. Qualitative mass spectrometric analysis was sufficient as almost no tyrosine phosphorylated proteins were enriched with the recombinant wild-type enzyme. All phosphopeptides detected after pull-down with the substrate trapping mutant but not with the wild-type enzyme were rated as positive hits. We stimulated PTP1B KO cells with pervanadate to provide a sufficient amount of tyrosine phosphorylated proteins for substrate

trapping and MS analysis. Substrate trapping experiments with unstimulated samples or after stimulation with EGF or PDGF did not result in any detectable phosphotyrosine peptides (data not shown), as the enrichment factor and binding affinity of the used substrate trapping mutants in these cases was not sufficient to enrich phosphoproteins out of up to 10 mg protein sample for phosphopeptide MS analysis. However, pervanadate treated samples contained sufficient amounts of phosphotyrosine proteins for this approach. In total, 53 tyrosine phosphorylated proteins bound to the PTP1B D182A&Q262A substrate trapping mutant, but not to the wild-type enzyme.

Table 3: *In vitro* substrate trapping identifies 11 proteins that both bind to the active site of PTP1B and were found to be hyperphosphorylated at pTyr sites in PTP1B-deficient cells.

name	pY site	phosphopeptide sequence	positive in				already described
			substrate trap	basal	EGF	PDGF	
p120ctn	280	FHPEppYGLEDDQR	X	X			
	257	QDVpYGPQPQVR	X	X			
	302	SMGYDDLIDYGMMSDpYGTAR	X	X			
p62DOK	376	GLpYDLPQEPR	X	X			(Dube et al., 2004)
Cas-L	176/188	DVpYDVPPSHSTQGVPYDIPPSSVK	X	X			
Lipoma-preferred partner homolog (LPP)	245	SAQPSPHYMAGPSSGQlpYGGPGR	X	X			
	318	SEGDTApYGGQVQPNTWK	X	X			
	301/302	YYEPpYpYAAGPSYGGRR	X	X			
Cortactin	421	KQpTPPApSPSPQPIEDRPPpS(0.5)pS(0.5)PlpYEDAAPFK	X	X			
	334	NASTFEVQVPSApYQK	X	X			
ZO-1	1164	TSTLRHEEQPAPApYEVHNR	X	X			
Platelet-derived Growth Factor Receptor β	751	DESIDpYVPLDMK	X	X		X	(Haj et al., 2003)
PLC γ 1	771	IGTAEPDpYGALYEGR	X	X	X	X	
	1253	pYQQPFEDF	X		X	X	
SHIP2	1136	TLSEVDpYAPGPGR	X		X		
p62DOK	450	GFSSDTALpYSQVQK	X		X		(Dube et al., 2004)
Epidermal Growth Factor Receptor	1197	GPTAENAepYLR	X				(Haj et al., 2003)
	1172	GSHQMSLDNPDpYQQDFFPK	X				(Haj et al., 2003)
	1000	MHLPSPTDSNFpYR	X				(Haj et al., 2003)
	1069/1092	pYSSDPTGAVTEDNIDDAFLPVEpYVNNQSVPK	X				(Haj et al., 2003)
p120RasGAP	451	ElpYNTIR	X			X	
p62DOK	295	TVPPPVPQDPLGpSPPALpYAEPLDSLRL	X			X	(Dube et al., 2004)
SHP2	584	VpYENVGLMQQR	X			X	
Platelet-derived Growth Factor Receptor β	763/778	pYADIESPSYMAPYDnpYVPSAPER	X			X	(Haj et al., 2003)

A subset of eleven proteins exhibited the same phosphorylation status as in our preceding analysis for hyperphosphorylated proteins in PTP1B KO cells and could be identified as potential PTP1B substrates (Table 3). Three of the proteins listed in Table 3, the EGFR, the PDGFR and p62DOK have already been reported to bind to PTP1B substrate trapping mutants and thus validate the reliability of this method (Blanchetot et al., 2005). The functional association of PTP1B to nine potential new substrate proteins was strengthened as presumably a direct site specific interaction of these proteins is taking place with the active site cleft of PTP1B. Among these proteins are mainly regulators of motility like Cas-L, LPP or Cortactin and regulators of cell adhesion such as p120ctn or ZO-1. Tyrosine phosphorylation of the identified sites correlates well with the increased cellular motility of

PTP1B deficient MEFs. Moreover, we detected two key-regulators of Ras-signaling in our combined substrate screening: p120RasGAP and SHP2. Furthermore, we could establish a novel link of PTP1B to phosphoinositide signaling via the lipid hydrolase PLC γ 1 and the lipid phosphatase SHIP2.

All of the newly characterized potential PTP1B substrate proteins shown in Table 3 exhibit regulatory functions in cell physiological processes like cell adhesion, cell motility or proliferation. Therefore, our results help to explain the cellular alterations observed in mouse fibroblasts due to PTP1B-deficiency.

V. Discussion

1. Proteolytic processing of RPTP κ and its implications on β -catenin signaling

1.1. β -catenin is a cellular substrate of RPTP κ

PTPs perform their cellular functions by dephosphorylation of substrate proteins in a specific manner. The receptor-like RPTP κ has been shown to negatively regulate cellular processes like proliferation, for example by dephosphorylation of the EGFR in keratinocytes (Xu et al., 2005), whereas its actions on motility control depend on the cellular system. In HER2 transformed MCF10a breast cells RPTP κ was found to promote TGF β -induced cell motility by activation of Src kinases (Wang et al., 2005). In contrast, in WiDr colon cancer cells overexpression of RPTP κ decreased and siRNA-mediated down-regulation increased cell motility (Kim et al., 2006). This negative effect on cell motility was linked to previous findings of our group that RPTP κ co-localized with β -catenin at adherens junctions and that both proteins form a physical interaction (Fuchs et al., 1996). In this study, we demonstrate a cellular enzyme-substrate relationship between RPTP κ and β -catenin, as siRNA-mediated down-regulation of RPTP κ leads to an increase and overexpression of the phosphatase to a decrease in β -catenin tyrosine phosphorylation. Furthermore, we verified that RPTP κ and β -catenin form a physical interaction and co-localize at cell adhesion sites. Together with α -catenin and E-cadherin, β -catenin is a molecular component of cell adherens junctions, which are important cell-cell contact sites in cells of epithelial origin. Müller *et al.* demonstrated a modulatory function of tyrosine phosphorylation on adherens junction assembly and could show that overexpression of the receptor-like RPTP-LAR leads to dephosphorylation of β -catenin and thereby to inhibition of cell motility, probably via stabilization of these cell-cell contacts (Muller et al., 1999). Also other PTPs have been described to dephosphorylate β -catenin and thereby negatively modulate cell migration and motility: PTP1B (Xu et al., 2002), PTP β/ζ (Meng et al., 2000), PCP-2 (Yan et al., 2002) and PTP-Pez (Wadham et al., 2003). This redundancy in PTP function might explain why no obvious phenotype results from RPTP κ deletion in mice (Skarnes et al., 1995). Regulation of β -catenin tyrosine phosphorylation is important in the homeostasis of epithelial cells, as β -catenin does not only control cadherin mediated cell adhesion, but also wnt target gene expression upon

translocation to the nucleus (Nelson and Nusse, 2004). Tyrosine phosphorylation of β -catenin leads to disassembly of cell adherens junctions and to its localization to the cytoplasm. Non-junctional β -catenin is rapidly degraded in the cytoplasm by a complex including adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase (GSK), which phosphorylates β -catenin on Ser-33, Ser-37, and Thr-41 and directs it to degradation by the ubiquitin/proteasome system (Conacci-Sorrell et al., 2002). Inhibition of β -catenin degradation via mutation of APC or β catenin in cancer cells (Morin et al., 1997) or via activation of the wnt pathway (Nelson and Nusse, 2004) results in its accumulation in the nucleus, where it forms a complex with T-cell factor (TCF) and transactivates oncogenic target genes such as Cyclin D1 (Tetsu and McCormick, 1999) or c-Myc (He et al., 1998). Thus, transcriptional activity of β -catenin in the nucleus leads to oncogenic transformation of epithelial cells. Therefore, a tumor-suppressive function has been assigned to PTPs such as RPTP κ which stabilize localization of β -catenin at cell adherens junctions.

1.2 ADAM10 is a RPTP κ sheddase

The extra-cellular part of MAM family RPTPs, like RPTP κ and RPTP μ , consists of cell adhesion domains. MAM-family RPTPs are highly specific homophilic adhesion receptors (Sap et al., 1994) and are characterized by distinct and complementary expression patterns during late embryogenesis (Fuchs et al., 1998). RPTP κ expression predominantly includes tissues of epithelial origin, whereas RPTP μ expression is restricted to blood vessels of virtually all major organs. Therefore, MAM-family RPTPs have been discussed to be morphoregulatory molecules promoting selective adhesion between cells of different tissues. Trans-cellular interactions of extracellular domains of MAM-family RPTPs are of high affinity and recently it has been proposed that the balance between cell adhesion and mobility can only be shifted by proteolytic cleavage of these extracellular domains (Aricescu et al., 2007). In this study, we present evidence that the responsible metalloproteinase for such a shedding process is ADAM10 (illustrated in Figure 26). Activation of ADAM10 takes place upon releasing it from its negative regulator calmodulin (CaM) with the CaM-inhibitor TFP (Nagano et al., 2004) or under the physiologically more important condition of high cell density. Interestingly, Ca²⁺ induced activation of ADAM10 might take place at high cell confluence, as fibroblasts keep (Timpe et al., 1978) moving even under high cell density and

simultaneous cell stretching could open intracellular stretch-activated Ca^{2+} channels (Lee et al., 1999).

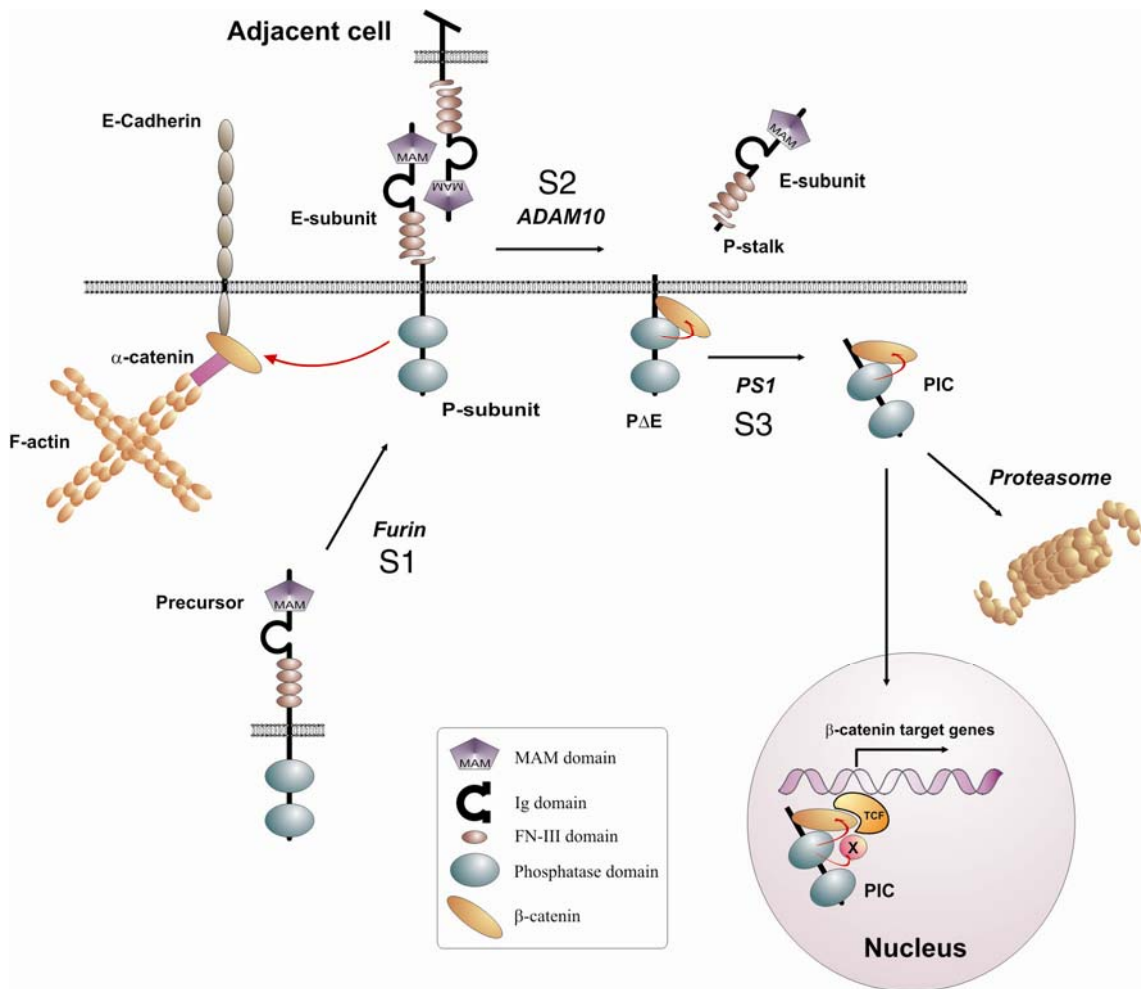


Figure 26: Scheme illustrating RPTPκ regulation by limited proteolysis. Furin-mediated cleavage in the Golgi-apparatus yields two subunit RPTPκ proteins at the cell surface. At high cell densities, homophilic binding between RPTPκ proteins may induce S2 cleavage by ADAM10, resulting in the release of the extracellular part in the cell supernatant. The remaining part, PΔE, is subject to Presenilin 1-dependent intramembrane proteolysis which allows dissociation of catalytically active PIC from the plasmamembrane. In the cytosol PIC gets degraded by the proteasome or when stabilized it translocates to the nucleus. There, PIC interacts with β-catenin and, unlike RPTPκ, increases TCF-mediated transcription. Note that PIC may additionally dephosphorylate other transcriptional regulatory proteins associated with the β-catenin/TCF complex.

Movement of cells which are attached to other cells puts mechanical force on the cytoskeleton. This cell stretching could be reduced when ADAM10 is activated at the rear end via intracellular Ca^{2+} and removes cell adhesion regulators like RPTP κ by shedding from the cell surface. A similar mechanism has already been discussed for the cell-matrix adhesion protein CD44 (Nagano and Saya, 2004). Furthermore, the function of ADAM10 in abolishing cell adhesion is supported by the findings that it contains a basolateral sorting signal which directs it to adherens junctions (Wild-Bode et al., 2006) and that both E-cadherin (Maretzky et al., 2005) and N-cadherin (Reiss et al., 2005) are its substrates. The mechanistic details how ADAM10 cleaves RPTP κ - *in cis* on the same cell surface, or *in trans* on opposing cells – still have to be defined. However, our finding that RPTP κ EcFc fusion proteins do not induce RPTP κ shedding argues for the *in trans* model which has been described for ADAM10-mediated ephrinA5 cleavage after heterophilic binding to the EphA3 receptor by Janes *et al.* (Janes et al., 2005). The function of the shed RPTP κ Ec domain itself remains unclear, but the resulting intracellular P Δ E fragment led us to the discovery of a subsequent RPTP κ cleavage step mediated by γ -secretase, as protein isoforms containing similar short transmembrane domains have been described to be presenilin substrates (Kopan and Ilagan, 2004). The following part shows that RPTP κ S2 cleavage is not only part of a degradation pathway, but also a signaling pathway.

1.3 γ -secretase cleavage of RPTP κ leads to translocation of its intracellular domain to the nucleus and modulates β -catenin's transcriptional activity

To date, a steadily increasing number of type-I transmembrane proteins has been identified as substrates for γ -secretase-dependent intramembrane cleavage after ectodomain shedding. The two most prominent examples are APP, whose extracellular cleavage product A β causes Alzheimer's disease, and Notch family receptor proteins, whose intracellular cleavage fragments (NICD) translocate to the nucleus and alter gene transcription involved in cell fate determination and oncogenesis (Kopan and Ilagan, 2004). In addition, proteins involved in cell adhesion control have been observed to be cleaved by presenilin, such as E-cadherin (Marambaud et al., 2002), N-cadherin (Marambaud et al., 2003) and CD44 (Okamoto et al., 2001), (Lammich et al., 2002). The only γ -secretase substrate described so far which contains an intracellular domain with enzymatic activity is ErbB4 (Ni et al., 2001). The catalytic

activity of the intracellular ErbB4 fragment has been shown to be important for its transforming activity in breast cancer (Maatta et al., 2006). In this study we demonstrated for the first time a γ -secretase-mediated cleavage mechanism for two receptor-like PTPs, RPTP κ and RPTP μ (Anders et al., 2006). We furthermore showed that cleavage mediated by ADAM10 and presenilin does not change the catalytic activity of RPTP κ . This proves the existence of two additional γ -secretase cleavage products containing an enzymatic activity. We did not find RPTP α to be processed by presenilin, but Haapasalo *et al.* provided additional data on intramembrane cleavage of RPTPs by identifying RPTP-LAR as a further γ -secretase substrate in this enzyme family (Haapasalo et al., 2007). Cleavage by γ -secretase can lead to two different functional outcomes for the generated intracellular domains: On the one side, proteolytic degradation by the ubiquitin/proteasome system, and on the other side, release of the intracellular domain from the plasma membrane to the cytosol or nucleus, where binding and regulation of other protein complexes is possible (Kopan and Ilagan, 2004). Proteasomal inhibition leads to a strong increase in the PIC isoform levels of RPTP κ and RPTP μ , indicating a massive turn-over of both proteins in HEK293 cells by γ -secretase and subsequent proteasomal destruction. This linkage of intramembrane cleavage to the ubiquitin/proteasome system can be probably explained with the N-end rule pathway (Varshavsky, 1996). This pathway defines stabilizing and destabilizing N-terminal amino acids. Among the stabilizing residues are Gly, Val, Ser and Met, whereas basic (Arg, Lys, His) or bulky/hydrophobic (Phe, Trp, Tyr, Leu, Ile, Ala) N-terminal amino acids are specifically recognized by the ubiquitin E3 ligase N-recogin. This leads to poly-ubiquitinylation of proteins containing destabilizing N-terminal residues and subsequent proteasomal degradation. The N-terminal amino acid of RPTP κ PIC isoform has not been determined so far, but the C-terminal eleven residues in its transmembrane region, where S3 cleavage should take place, constitute both stabilizing Val residues as well as destabilizing Ile or Leu residues. In some experiments we found that RPTP κ PIC levels were detectable without proteasomal inhibition in HEK293 cells (data not shown), but we could not identify the reason for its stabilization. A possible explanation why the intracellular domains of γ -secretase substrates appear with different stabilities could be due to the fact that the γ -secretase cleavage site specificity can be modified by conformational changes in the proteolytic protein complex (Behr et al., 2004). As soon as PIC gets stabilized in the cytosol it translocates to the nucleus. We demonstrated this by taking advantage of recombinant RPTP κ PIC expression and were able to show the first example for the nuclear localization of a RPTP isoform. Nuclear transport of PIC is characterized by active import and export

processes, as inhibition of CRM1-dependent export leads to an even stronger enrichment in the nucleus and to accumulation in small granular speckles. Before, only non-receptor like PTPs like TcPTP or SHP-1 have been shown to localize to the nucleus, where both of them modulate gene expression (ten Hoeve et al., 2002) ,(Duchesne et al., 2003). Similarly, RPTP κ PIC executes a modulatory function on transcription, as it not only co-localizes with β -catenin in the nucleus, but also increases its co-activator function on the TCF promoter. This effect on β -catenin-mediated transcription is dependent on PIC's phosphatase activity. In contrast, full length RPTP κ decreased β -catenin-mediated transcription, probably via stabilization of β -catenin at adherens junctions and thereby reducing the β -catenin levels available for transcriptional regulation in the nucleus. In previous studies, β -catenin tyrosine phosphorylation has been linked to β -catenin/TCF-mediated gene regulation. Tyrosine phosphorylation of β -catenin on Tyr-654 leads to an increased association with the basic transcription factor TBP and activation of TCF4-mediated transcription (Piedra et al., 2001). Accordingly, nuclear PTPs which co-associate with and dephosphorylate β -catenin, such as SHP-1 (Duchesne et al., 2003) or the intracellular domain of RPTP-LAR (Haapasalo et al., 2007), reduce expression of the β -catenin target gene Cyclin D1. Although both RPTP κ full-length and PIC isoforms dephosphorylate β -catenin with comparable efficiencies, they regulate β -catenin-mediated transcription differentially. A reason for this could be their different sites of cellular localization and concomitant association with distinct protein complexes. Our data do not exclude the possibility that PIC dephosphorylates additional transcriptional regulators which are associated with the β -catenin/TCF complex. For example, β -catenin recruits many other regulatory proteins in the nucleus, such as p300/CBP, Brg-1, pygopus, TIP49 or TBP, which then allow the assembly of the basal transcription machinery (Hurlstone and Clevers, 2002). The observation that RPTP κ full-length and PIC isoforms have acquired opposing roles in the regulation of β -catenin signaling awaits further validation. Of particular interest will be the functional analysis of the contribution of MAM-family RPTPs to ADAM- and γ -secretase-regulated physiological processes ranging from tumor growth to neurodegeneration.

2. Investigation of PTP1B Function by Quantitative Proteomics

2.1 Quantitative proteomics allows systematic analysis of cellular phosphotyrosine signaling in PTP1B-deficient MEFs

PTP1B controls various tyrosine signaling processes that regulate metabolic, oncogenic and immune functions. To date, eleven PTP1B substrates have been described in biochemical studies (Dube and Tremblay, 2005). Many of these studies took advantage of PTP1B deficient cells, where a dysregulation in the phosphotyrosine levels of potential substrates could be expected. Substrates like the EGFR, the PDGFR, the insulin receptor, the IGF1R, JAK2, Tyk2 and p62dok have been identified using PTP1B deficient fibroblasts under respective cytokine and growth factor treatments. In this study, our quantitative mass spectrometric phosphopeptide analysis allowed us to analyze the relative differences in the tyrosine phosphorylation state of 122 proteins containing 293 phosphotyrosine sites between PTP1B KO cells and their wild-type counterparts under basal and EGF- or PDGF-stimulated conditions. In addition, these data give a broad overview about tyrosine phosphorylation processes in this widely used model system. To 36 of these proteins a functional association could be drawn to PTP1B as its deficiency leads to an at least twofold increase in tyrosine phosphorylation on one or multiple of their pTyr sites. Hyperphosphorylation of proteins in PTP1B KO cells does not necessarily mean a direct enzyme-substrate relationship, but indicates that the identified protein is very likely functionally associated with PTP1B. An elegant way to test if such a functional link could be an enzyme substrate relationship is to study binding of the respective tyrosine phosphorylated proteins to catalytically inactive substrate trapping mutants. All of the already known substrate proteins mentioned above have been shown to directly bind to the active center of PTP1B (Blanchetot et al., 2005). Using this substrate-trapping approach allowed us to demonstrate binding of specifically tyrosine phosphorylated proteins to the enzymatically inactive mutant of this phosphatase. In total, we could identify nine so far undescribed proteins that were hyperphosphorylated on specific pTyr sites in PTP1B deficient cells and which bound to PTP1B substrate trapping mutants via these sites. According to these selection criteria we identified the following proteins as putative new substrates of PTP1B: Cortactin, Cas-L, LPP, p120ctn, ZO-1, SHP2, p120rasGAP, PLC γ 1 and SHIP2. Figure 27 gives an overview of the newly identified putative substrates and puts these proteins in a functional context. In addition, also those proteins functionally associated with PTP1B are illustrated, whose tyrosine phosphorylation and

function could be connected to the increased motility and decreased proliferation in PTP1B KO cells. Most of the depicted phosphotyrosine sites have already been described in other studies and can be found in the PhosphoSite database (www.phosphosite.org). However, it should be noted that all pTyr sites described in Figure 27 are detected under cell physiological conditions, whereas many of the sites described in the PhosphoSite database are detected only after massive phosphatase inhibition using pervanadate. The phosphotyrosine residues pTyr-15 of CYLD, pTyr-639 of Tks5/Fish and pTyr-141 of Vav3 are described here for the first time and could not be found in any of the following databases: Phosphosite, Phosida (www.phosida.com) and Swissprot (www.expasy.ch).

All of the proteins identified here as potential new substrates of PTP1B (see Table 3) can be subdivided into three functional classes: First, regulators of cell migration and adhesion; second, regulators of proliferation; and third, regulators of phosphoinositide signaling.

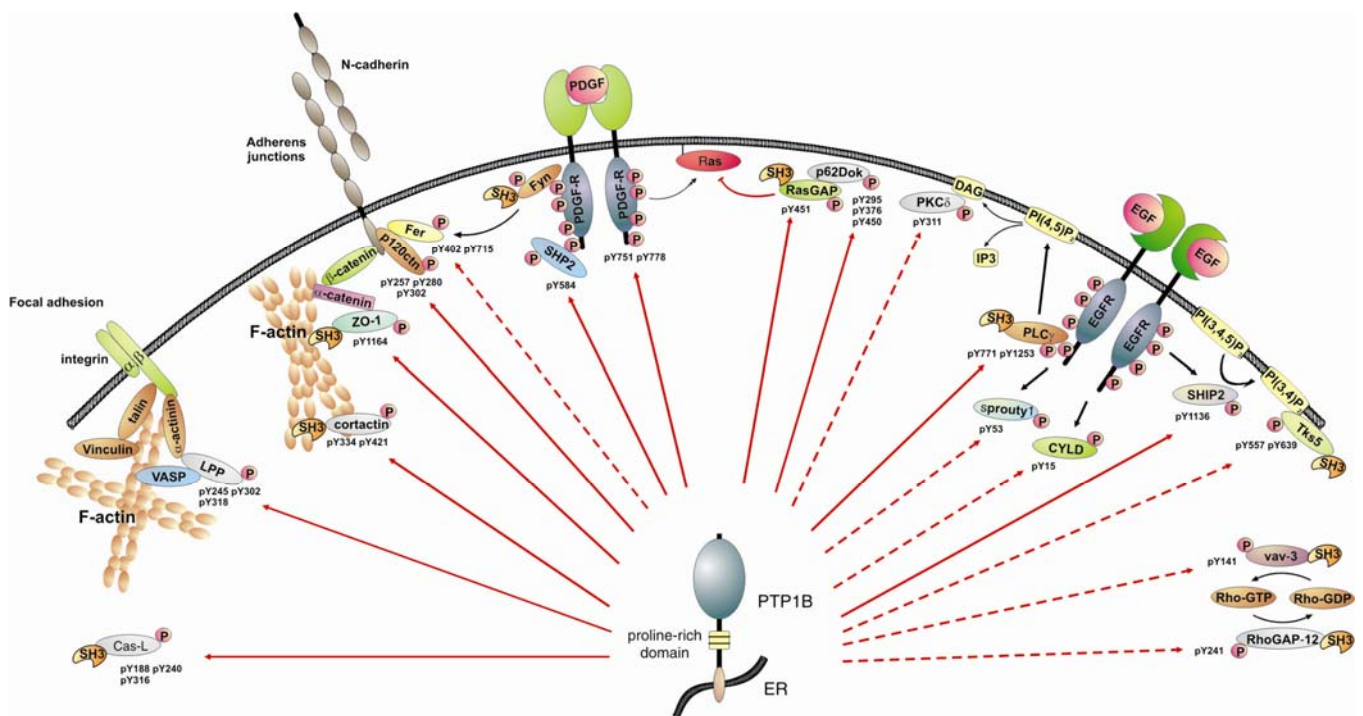


Figure 27: Schematic drawing illustrating newly identified potential PTP1B substrates in a functional context. PTP1B localizes to the ER and contains a proline-rich domain that allows interaction with substrate proteins containing SH3 domains. Red arrows indicate a direct interaction and dephosphorylation of putative substrates, whereas dashed red arrows specify a direct or indirect functional association evident from increased tyrosine phosphorylation upon PTP1B deficiency. All depicted pTyr sites are upregulated in PTP1B-deficient cells. Moreover, pTyr sites of potential substrates were identified as putative interaction sites in the substrate-trapping assay. This scheme was designed on the basis of the following references: Focal adhesion site (Calderwood et al., 2000); PDGFR β mediated

regulation of N-cadherin cell-cell adhesion (Greer, 2002); p62DOK and p120RasGAP regulation of Ras signaling (Dube et al., 2004).

2.2 Novel potential PTP1B substrates control migration of mouse fibroblasts

We could demonstrate that PTP1B deficient cells show a strong increase in cell motility as described by Buckley et al. (Buckley et al., 2002). Consistently, ectopic expression of PTP1B in Hela cells has been shown to decrease serum induced cell migration (Yigzaw et al., 2003). In this study we identified five potential PTP1B substrates that are modulators of cellular migration and adhesion: Cortactin, Cas-L, LPP, p120ctn and ZO-1. Tyrosine phosphorylation of Cortactin promotes cell motility in endothelial cells and leads to reduced actin cross-linking (Huang et al., 1998). Using pTyr-421 site specific antibodies, Head *et al.* could show that Cortactin that is phosphorylated on pTyr-421 localized to podosomes and lamellipodia (Head et al., 2003), whereas the function of pTyr-334 phosphorylation remains unclear. The tyrosine phosphorylation of Cas-L has been found to be essential for T-cell migration (Ohashi et al., 1999), but its single pTyr sites haven't been studied in detail, so far. Cellular regulation of Cas-L by PTP1B is not surprising as its close homolog p130cas has already been described to bind to PTP1B substrate trapping mutants and can be dephosphorylated by PTP1B *in vitro* (Garton et al., 1996). Until now, tyrosine phosphorylation of LPP has not been characterized so far, but it was shown to bind VASP and α -actinin and to localize to focal adhesion junctions (Petit et al., 2003).

PTP1B KO fibroblasts exhibit weaker cell-cell adhesion contacts than wild-type cells (data not shown). This correlates well with tyrosine phosphorylation of p120ctn, as for example p120ctn phosphorylation by Src kinase leads to loss of E-cadherin function (Ozawa and Ohkubo, 2001). Quite the opposite is shown by other studies which state that tyrosine phosphorylation of p120ctn is dispensable for the modulation of cadherin based cell adhesions (Cozzolino et al., 2000). To unravel this discrepancy it is important to obtain site specific information about the regulation of p120ctn by tyrosine kinases and phosphatases (Alema and Salvatore, 2007). Here, we observed that p120ctn is probably regulated by PTP1B at pTyr-257, pTyr-280 and pTyr-302 but not at pTyr-228 and pTyr-904. As PTP1B deficient cells show reduced cell adhesion and enhanced migration and the regulated pTyr sites can be linked to these cellular processes.

A reduction of cell adhesion was furthermore linked to tyrosine phosphorylation of ZO-1, but the hyperphosphorylated pTyr-1164 has not been analyzed so far (Takeda et al., 1995). As

fibroblasts do not contain any tight junctions, ZO-1 presumably localizes to cell adherens junctions (Itoh et al., 1993).

It should be mentioned, that β -catenin was not found to be tyrosine phosphorylated in our study and therefore, the phosphotyrosine mediated regulation of cell adherens junctions disassembly is likely to be accomplished via other proteins like p120ctn or ZO-1.

A functional link of PTP1B could also be drawn to the Rho signaling regulators Vav3 and RhoGAP12. Whether these proteins are direct substrates cannot be defined in this study as no direct binding to PTP1B was shown by our substrate trapping assay. Moreover, Vav3 pTyr-141 is a novel site and RhoGAP12 pTyr-241 has been described but not characterized, so far. Therefore at this point no direct conclusion can be drawn how PTP1B affects rho signaling in mouse fibroblasts. Recently, PTP1B was described to act upstream (Dadke and Chernoff, 2003) and downstream (Kabuyama et al., 2006) of RhoA. Further analysis of phosphotyrosine regulation of these GTPase regulators will improve the understanding of these cross-regulatory interactions.

Another important fibroblast motility regulator was identified as a substrate for PTP1B in our study – the PDGFR β . This has already been demonstrated by others, but no site specific information was available so far (Haj et al., 2003). We found that specifically pTyr-751, pTyr-763 and pTyr-778 are regulated by PTP1B. pTyr-751 is an autophosphorylation site and a binding site for PI3K (Heldin et al., 1998). Interestingly, PI3K was hyperphosphorylated in PTP1B deficient cells after PDGF stimulation. Together with PDGFR β pTyr-740, pTyr-751 has an important function in mediating PDGFR β driven motility responses (Heldin et al., 1998). pTyr-778 was implicated in the regulation of the actin cytoskeleton but no interaction partners have been identified until now (Ruusala et al., 1998). In addition, the SHP2 binding site pTyr-763 (Ronnstrand et al., 1999) was also stronger phosphorylated in KO cells and correlates to the increased tyrosine phosphorylation of SHP2 itself. Phosphorylation of PDGFR β pTyr-763 was directly linked to PDGF β induced motility, but not to any mitogenic effects (Ronnstrand et al., 1999). This strongly resembles the cellular responses we observe in PTP1B-deficient fibroblasts.

2.3 PTP1B regulates proliferation of fibroblasts via newly characterized putative substrates

PTP1B-deficiency leads to a strong decrease in proliferation in mouse embryonic fibroblasts. Dube *et al.* found a correlation of this reduced proliferation with reduced ras activity and increased phosphorylation of its negative regulator p62DOK (Dube *et al.*, 2004). Our quantitative MS analysis allowed us to verify this up-regulated tyrosine phosphorylation in PTP1B KO cells and to detect also hyperphosphorylation of p120RasGAP after PDGF stimulation. p120RasGAP is a p62DOK binding protein which negatively regulates ras activity by increasing its intrinsic GTPase activity (Donovan *et al.*, 2002). In addition, both proteins were identified in our PTP1B substrate trapping assay, providing further evidence for a direct regulation by PTP1B. Another interesting connection of PTP1B to proliferation control of fibroblast cells was manifested in the upregulation of Sprouty1 and CYLD tyrosine phosphorylation. PTP1B has already been shown to mediate the anti-migratory effects of Sprouty in Hela cells (Yigzaw *et al.*, 2003). Lack of PTP1B therefore abolishes this anti-migratory effect. Tyrosine phosphorylation on Tyr-53 of Sprouty1 is indispensable for the negative feedback activity of Sprouty1 on the mitogen-activated protein kinase (MAPK) pathway (Hanafusa *et al.*, 2002). Although, the anti-proliferative effect was linked only to MAPK activation, we see no difference in MAPK activation between wild-type and KO cells after EGF stimulation, whereas Sprouty is 3.7-fold induced. Therefore other molecular mechanism could account for a negative regulatory effect of sprouty on proliferation in KO cells. Phosphorylation of pTyr-15 of CYLD has not been published so far. CYLD is a negative regulator of proliferation, as it decreases growth and survival mediated by NfκB (Massoumi *et al.*, 2006).

2.4 PTP1B interacts with phosphoinositide-modifying enzymes

In our substrate analysis we could also characterize two phosphoinositide modifying enzymes as potential substrates of PTP1B: PLCγ1, which converts PI(4,5)P₂ into IP₃/DAG and SHIP2, which dephosphorylates PI(3,4,5)P₃ into PI(3,4)P₂. Binding of PLCγ1 to PTP1B has already been mapped to the SH3 domain of PLCγ1 (Choi *et al.*, 2006), but a direct dephosphorylation has not been shown so far. We hypothesize that PTP1B dephosphorylates PLCγ1 on pTyr-771 and pTyr-1253, which unlike pTyr-783 are dispensable for its phospholipase activity and might be responsible for other PLCγ1 functions (Sekiya *et al.*, 2004). In our analysis, PDGF

stimulation leads to a two-fold stronger phosphorylation of PLC γ 1 than does EGF stimulation. In addition, only PDGF but not EGF was described to trigger phospholipase activation in mouse fibroblasts (Sekiya et al., 2004). Concomitantly, we found an increase in PKC δ phosphorylation on pTyr-311 only after PDGF stimulation. DAG positively modulates PKC δ tyrosine phosphorylation and thereby increases its activity, which in turn leads to elevated motility of mouse fibroblasts (Iwabu et al., 2004). Furthermore, PTP1B can dephosphorylate PKC δ *in vitro* (Benes and Soltoff, 2001), indicating a functional association of both proteins. Interestingly, also another phosphoinositide modifying enzyme, SHIP2, exhibited increased tyrosine phosphorylation after EGF-stimulation. Tyrosine phosphorylation of SHIP2 on pTyr-986 or pTyr-987 has been shown to be important for its function in lamellipodia formation and actin cytoskeleton regulation (Prasad et al., 2002) and probably regulates its activity. Here, SHIP2 was not only strongly tyrosine phosphorylated after EGF stimulation, but also hyperphosphorylated in KO cells. As binding to PTP1B substrate trapping mutants could be shown as well, we propose that SHIP2 is a direct substrate of PTP1B. Under basal conditions we also found Tks5/Fish to be stronger phosphorylated in PTP1B KO cells. Tks5/Fish contains a PX domain which binds *in vitro* to PI(3,4)P2 (Seals et al., 2005), a product of SHIP2 activity. Also Tks5/Fish was linked to cell motility as shRNA mediated knockdown suppressed the formation of podosome structures in v-Src-transformed fibroblasts and correlated with a severe decrease in Matrigel invasion activity (Seals et al., 2005). As PTP1B did not bind to Tks5/Fish in our substrate trapping assay it remains unclear if Tks5/Fish is a direct substrate of PTP1B.

2.5 Regulation of PTP1B substrate accessibility via SH3 domain interactions but not via common linear phosphotyrosine signature motifs

Linear sequence motif analyses of the phosphorylation sites given in Table 3 using the programs MotifX (motif-x.med.harvard.edu) and Dilimot (dilimot.embl.de) did not reveal any common motif (data not shown). This suggests that the substrate specificity of PTP1B is rather controlled by its subcellular localization and organization into protein complexes than by its binding affinity to linear sequence motifs. An exception are tandem pTyr/pTyr sites of the insulin receptor (Salmeen et al., 2000), Tyk2 and Jak2 (Myers et al., 2001). PTP1B was found to have a high intrinsic affinity to bind peptides containing tandem pTyr sites and to possess an unique additional pTyr binding pocket that accommodates the second pTyr site in

tandem motifs. So far, only the homologous tyrosine phosphatase TCPTP was shown to have a similar pocket. This model is supported by our finding that zyxin family member Lipoma-preferred partner homolog (LPP) is probably regulated by PTP1B at its tandem pTyr residues 301/302.

Another possibility how PTP1B can interact with its substrates is via binding of its proline-rich domain to SH3 domains, as it was demonstrated for example for the SH3 domain of p130cas (Liu et al., 1996). Several of the potential substrate proteins we describe here contain SH3 domains, such as PLC γ 1, Cortactin, Cas-L, ZO-1 and p120rasGAP (Figure 27). Also proteins to which a functional association could be drawn contain SH3 domains, like Tks5/Fish protein, Vav3 and RhoGAP12. Even though no direct binding to the catalytic domain of PTP1B could be demonstrated for these proteins, it might well be possible that PTP1B interacts in a rather transient fashion via its proline-rich domain with these proteins in a cellular context.

Under normal cellular conditions, the full-length PTP1B protein localizes to the endoplasmatic reticulum (ER) due to a C-terminal hydrophobic stretch which anchors it inside the ER-membrane (Frangioni et al., 1992). This raises the question how PTP1B can act on substrate proteins localized at the plasma membrane and cytosol.

PTP1B's cellular functions can be classified by the localization of its substrates. It was reported that PTP1B dephosphorylates receptor proteins like the insulin receptor on its biosynthetic way from the ER to the plasmamembrane (Lammers et al., 1993), (Boute et al., 2003). Fascinatingly, Haj *et al.* could further show that PTP1B targets receptor tyrosine kinases like the EGFR and the PDGFR after ligand stimulation, when the receptors are internalized and endosomes come in close contact to the ER (Haj et al., 2002). A direct contact of ER-bound PTP1B to substrates at the plasma membrane was discussed as well, but remains controversial (Anderie et al., 2007). Similarly to RPTP κ , also PTP1B can be released from its membrane anchor by proteolytic cleavage. Calpain was demonstrated to liberate PTP1B from the ER-membrane in platelets, which leads to its translocation to the cytosol (Frangioni et al., 1992), (Kuchay et al., 2007).

At which cellular location PTP1B dephosphorylates the new potential substrates we have identified in this study will be an interesting issue for further investigations.

VI. Summary

In this thesis the regulation of receptor-like protein tyrosine phosphatases by proteolytic processing was characterized and mass spectrometry-based methods were established to identify novel PTP substrates in a systematic and generic way.

In the first part, proteolytic processing of RPTP κ and shedding of its extracellular domain by the metalloproteinase ADAM10 was demonstrated which is inducible by cell density and trifluoperazine treatment. Subsequently, the remaining membrane-bound RPTP κ P Δ E isoform was found to be further processed by presenilin 1 in the γ -secretase complex. This intramembrane cleavage leads to liberation of the intracellular RPTP κ part termed PIC from the plasmamembrane which in turn gets degraded in the proteasome or when stabilized translocates to the nucleus. Interestingly, no change in PTP activity was observed due to this cleavage cascade, as RPTP κ PIC was similarly active compared to the full-length protein in dephosphorylating the substrate protein β -catenin. Moreover, as RPTP κ PIC and β -catenin both localized to the nucleus, its modulating activity on β -catenin's transcriptional activity was investigated. Surprisingly, RPTP κ PIC enhanced β -catenin mediated transcription whereas the full-length enzyme suppressed it. This study shows for the first time a regulatory mechanism which allows translocation of a RPTP to the nucleus and gives insights into its consequences on gene regulation.

In the second part, mass spectrometry-based experimental strategies are presented which are entirely generic and can be used to address the poorly understood aspects of cellular PTP function. To gain better insights into the cellular activity of the prototypic cytosolic PTP1B, quantitative mass spectrometry was used to monitor alterations in the global tyrosine phosphorylation of PTP1B-deficient mouse embryonic fibroblasts in comparison to their wild-type counterparts. In total, 122 proteins containing 293 phosphotyrosine sites could be quantified under basal, EGF- or PDGF-stimulated conditions. Loss of PTP1B function in these cells leads to increased phosphorylation of 36 proteins which could be functionally linked to PTP1B. Among these proteins, regulators of cell adhesion and motility were overrepresented such as ZO-1, p120ctn, cortactin, LPP or Cas-L. Also regulators of cell proliferation like p62DOK or p120RasGAP showed increased phosphorylation. Moreover, in a parallel MS-based analysis physical interaction of these proteins to phosphatase inactive PTP1B substrate trapping mutants could be demonstrated. The obtained results correlate well with the described phenotype of PTP1B-deficient fibroblasts and explain the increase in motility and decrease in proliferation observed in these cells.

VI. Zusammenfassung

In dieser Doktorarbeit wurde die Regulation von Rezeptor Protein Tyrosin Phosphatasen (RPTPs) durch proteolytische Prozessierung charakterisiert. Des Weiteren wurden massenspektroskopische Methoden etabliert, welche die Identifikation neuer Protein Tyrosin Phosphatase (PTP) Substrate ermöglichen.

Im ersten Teil dieser Arbeit konnte gezeigt werden, dass die rezeptorartige RPTP κ durch die Metalloprotease ADAM10 gespalten wird, wodurch deren extrazelluläre Domäne von der Zelloberfläche entfernt wird. Diese Spaltung kann durch hohe Zelldichte oder Trifluoperazin ausgelöst werden. Im Anschluss daran wird die neu entstandene RPTP κ P Δ E Isoform weiter durch Presenilin 1 und dem γ -Sekretase Komplex prozessiert, mit der Folge, dass der intrazelluläre RPTP κ Teil, welcher PIC genannt wird, von der Plasmamembran befreit wird. Die PIC Isoform wird daraufhin durch das Proteasom degradiert oder wandert, sobald sie stabilisiert wird, zum Zellkern. Interessanterweise wurde keine Veränderung in der Phosphatase Aktivität durch diese Proteolyseschritte beobachtet, denn die RPTP κ PIC Isoform dephosphorylierte das Substratprotein β -catenin genauso effizient wie die ungespaltene RPTP κ Isoform. Sowohl RPTP κ PIC als auch β -catenin befinden sich im Zellkern. Deshalb wurde der Einfluss dieser nukleären Phosphatase auf β -catenin vermittelte Transkription untersucht. Überraschend dabei war, dass RPTP κ PIC die transkriptionelle Aktivität von β -catenin verstärkte, während die ungespaltene RPTP κ Isoform diese verringerte. Die vorliegende Studie zeigt einen neuen regulatorischen Mechanismus für RPTPs, welcher diesen erlaubt von der Plasmamembran zum Zellkern zu translozieren. Ausserdem werden die Auswirkungen dieses Prozesses auf Genregulation beschrieben.

Im zweiten Teil werden experimentelle Strategien präsentiert, welche es mittels Massenspektroskopie erlauben die nur unvollständig verstandenen Aspekte zellulärer PTP Funktionen auf systematische Art und Weise zu untersuchen. Um die zelluläre Aktivität der prototypischen zytosolischen PTP1B besser zu verstehen, wurde quantitative Massenspektroskopie verwendet, mit welcher Veränderungen in der globalen Tyrosinphosphorylierung von PTP1B-defizienten Mausembryofibroblasten und wildtyp Kontrollzellen gemessen wurden. Insgesamt konnten 122 Proteine, welche 293 Phosphotyrosinreste enthielten, unter basalen und EGF- oder PDGF-stimulierten Bedingungen quantifiziert werden. Das Fehlen von PTP1B in diesen Zellen führte zu einer erhöhten Phosphorylierung von 36 Proteinen, welche funktionell mit PTP1B in Zusammenhang gebracht werden konnten. Unter diesen Proteinen waren Regulatoren von

Zelladhäsion und Zellwanderung überrepräsentiert, wie z.B. ZO-1, p120ctn, Cortactin, LPP oder Cas-L. Ebenso Zellproliferationsregulatoren, wie p62DOK oder p120RasGAP zeigten eine verstärkte Phosphorylierung. In einer parallelen MS-Analyse konnten zudem direkte Interaktionen dieser Proteine mit phosphataseinaktiven PTP1B Substratfängermutanten gezeigt werden. Die daraus resultierenden Ergebnisse korrelieren hervorragend mit dem bereits beschriebenen Phänotyp von PTP1B-defizienten Zellen und erklären deren erhöhte Migrationsfähigkeit und deren vermindertes Proliferationspotenzial.

VII. References

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VIII. Appendix

Abbreviations

ADAM	A disintegrin and metalloprotease domain
Amp ^r	Ampicilline resistance
APC	Adenomatous polyposis coli gene
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
Ca ²⁺	Calcium Ions
CD45	Cluster of Differentiation 45
cDNA	Complementary DNA
DEP-1	Density-enhanced phosphatase 1
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin
HEPES	N-(2-Hydroxyethyl)-piperazin-N'-2-Ethansulfonic acid
IP	Immunoprecipitation
IPI	International Protein Index
kb	Kilobase
kDa	Kilodalton
KO	Knock-out
μ	Micro
LAR	Leucocyte antigen-related phosphatase
LC	liquid chromatography
LMW-PTP	Low molecular weight PTP
m	Milli
M	Molar
MAM	Meprin, A5 glycoprotein, RPTPm
MAP	Mitogen-activated protein
MAPK	MAP kinase
MEFs	Mouse embryonic fibroblasts
min	Minute

MMP	Matrix metalloprotease
MS	Mass spectrometry
MS ²	tandem MS or MS/MS
NCAM	Neural cell adhesion molecule
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC	Protein convertase
PCP-2	Pancreatic carcinoma-derived phosphatase 2
PCR	Polymerase chain reaction
PEG	Polyethyleneglycole
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PKC δ	Protein kinase C δ
PLC γ 1	Phospholipase C γ 1
PMA	12-O-Tetradecanoyl-phorbol-13-acetate
pNPP	p-Nitrophenyle-phosphate
pRS	pRetroSUPER vector
PS1	Presenilin 1
pSer	Phosphoserine
pThr	Phosphothreonine
PTP	Phosphotyrosine-specific phosphatase
PTP-MEG	Megakariocyte PTP
PTP-Pez	Band 4.1/ezrin-related protein tyrosine phosphatase
pTyr	Phosphotyrosine
pY	Phosphotyrosine
RNA	Ribonucleic acid
RPTP	Receptor-like protein tyrosine phosphatase
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SH	Src homology
SHP-1	SH2-containing PTP-1
SHP-2	SH2-containing PTP-2
siRNA	Short interfering RNA
Src	Homologue to v-src (sarcoma viral oncogene)
Tc-PTP	T-cell phosphatase
Tris	Tris(hydroxymethyl)aminomethan
o.n.	Overnight
UV	Ultraviolet
V	Volt
VSV	Vesicular stomatitis virus glycoprotein VSV-G
Vol	Volume
WB	Western Blot
WT	Wild type
ZO-1	Zonula occludens 1 protein

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